Identification and Characterization of the Signaling Mechanisms Downstream of Dok-R that Mediate MAPK Attenuation

by

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Abstract

Part 1
Angiopathies are one of the leading underlying causes of morbidity in diabetic patients. Poorly managed blood glucose levels contribute to vascular defects which manifest themselves in numerous different clinical conditions including diabetic retinopathy, nephropathy, peripheral artery disease and compromised wound healing. The Angiopoietin family (Angs 1-4) have been shown to play a critical role in the growth and maintenance of vasculature. Here we show (chapter 2) that a short angiopoietin peptide-mimetic can increase the production of vessels in a diabetic wound leading to accelerated wound closure. We found that when a short peptide known to bind to Tie2 was tetramerized it led to the activation of Tie2 and its associated signaling pathways. This activation was sufficient to produce both *in vitro* and *in vivo* angiogenic responses. Our results demonstrate that activation of the pleiotropic Tie2 signaling axis by a single compound can produce blood vessels that are highly organized and well supported by myogenic support cells. Although the potential of this novel proangiogenic compound in treating microvascular perturbations is not strictly limited to topical administration, we provide evidence as a proof of principle in support of its efficacious use in diabetic wound healing.

Part 2

Dok-R has previously been shown to associate with the Epidermal Growth Factor Receptor (EGFR), become tyrosine phosphorylated, and attenuate MAPK activation in response to EGF stimulation. Herein (chapter 3), we have defined the structural determinants within Dok-R that are required for its ability to attenuate EGF-dependent MAPK activation. We describe a novel mechanism whereby Dok-R forms a complex with epidermal growth factor receptor (EGFR), Src and Csk. Formation of this complex, results in potent inhibition of Src kinase activity and a concomitant decrease in MAPK/Akt activation.

In addition to the role Dok-R plays in mitigating signals emanating from the EGFR we utilized a transgenic mouse model (chapter 4) to examine tissue specific overexpression of a carboxy terminal truncation mutant of Dok-R (Dok-R ΔPRR). We find that, when expressed under the control of the Tie2 promoter, Dok-R ΔPRR results in reproducible defects in the kidney, liver and hematopoietic system. Further examination of Dok-ΔPRR revealed a novel cytoplasmic/nuclear shuttling function, the significance of which remains unknown.

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Chapter 1

Introduction

Part 1

Extracellular Signaling:

The ability of a cell to sense its complex surroundings and respond appropriately is a feat that co-evolved with the origin of cells themselves. For this to occur there must be machinery in place that receives cues from external sources, and mechanisms in some way connected, to respond by way of executing change. Monitoring of the external environment is accomplished through the coordinated action of several different classes of extracellular and intracellular proteins such as peptide based growth factors, cytokines, integral transmembrane receptors, G protein-coupled receptors, integrins, and non-receptor protein tyrosine kinases and phosphatases. Together, these proteins act in conjunction to initiate, receive, and transduce signals which, when perceived at the nuclear level, mediate changes in the transcription and translation of appropriate genes and proteins. Regulated temporal and spatial control of signal transduction is imperative and is highlighted by countless severe defects that arise from even minor perturbations in a single protein.

Assembly of Signaling Cascades and Modular Domains

Reception of an extracellular signal often occurs through growth factor-dependent clustering of receptor tyrosine kinases (RTKs). Once clustered, RTKs typically transphosphorylate adjacent tyrosine residues located within the C-terminus of the receptor. Phosphotyrosines serve as high affinity binding sites for numerous intracellular proteins. Once bound to the activated receptor, these proteins may themselves become phosphorylated, either through receptor-dependent kinase activity, or in the case of some

protein tyrosine kinases (PTKs), through a conformational change which leads to autophosphorylation of the protein. carboxy-terminal phosphorylation of RTKs, and the subsequent recruitment and activation of an entire suite of enzyme-containing proteins serves to amplify the initial stimulus. Proteins deficient in enzymatic activity may also be recruited to sites of activation. These so called adapters, scaffolds, or docking proteins may transport a preassembled, multi-protein, signaling complex which serves a defined role in transducing a given signal. Key to understanding the correspondence that exists between proteins participating in a given signal cascade is the conserved nature and function of modular protein domains. Domains that fold and function autonomously and also convey their inherent functions to other proteins are referred to as protein modules (Gimona, 2006). Protein-protein or protein-lipid interaction domains and the exquisite specificity they provide offer a unifying mechanism by which complex signaling pathways assemble (Pawson, 2004). The inducible nature of many of these interactions serves as a molecular switch, capable of coordinating a protein's location and response to a given stimulus. Constitutive interaction domains often serve to group proteins together into discreet functional clusters, in so doing, a degree of efficiency is created which abolishes the time required for each protein to locate associated proteins in a signaling cascade.

Modular Protein Interaction Domains

SH2 Domain

The Src-homology 2 (SH2) domain was the first modular phosphotyrosine interacting domain to be described and is still thought to be the predominant domain mediating

phosphotyrosine binding. Its presence in a protein is highly suggestive of a role in tyrosine kinase signaling and many proteins that participate in these pathways contain SH2 domains. SH2 domains serve as a prototype protein domain for a diverse collection of protein interaction domains that recognize phosphotyrosine or other ligands such as proline rich sequences or phospholipids. Its discovery, in the $p130^{gag\text{-}fps}$ tyrosine kinase fusion protein of the Fuginami sarcoma virus was instrumental to the current understanding of signal transduction and assembly of multi protein complexes (Sadowski et al., 1986). The domain is made up of approximately 100 amino acids and contains a conserved pocket that recognizes pY and a variable region that interacts with amino acids 3-6 residues C-terminal to the pY which confers specificity(Yaffe, 2002;Schlessinger and Lemmon, 2003). The pY sits in a deep pocket and each phosphate oxygen participates in an elaborate combination of hydrogen bonds and electrostatic interactions. Vital to these interactions are two key invariant arginine residues located within the binding pocket of the SH2 domain. Although much of the binding affinity of an SH2 domain for its target ligand derives from the pY, two SH2 domains, that of SAP and Grb10, have been shown to interact with their targets in a phospho-independent manner (Sayos et al., 1998;Morrione et al., 1999).

PTB Domain

Phosphotyrosine binding domains (PTB) are 100-150 residue protein modules. Initial studies of adapter proteins Shc and IRS1 revealed that PTBs function by binding phosphorylated tyrosines typically arranged as an NPXpY consensus (O'Bryan et al., 1996;Dente et al., 1997;Schlessinger *et al.*, 2003;Uhlik et al., 2005). Amino acids N-

terminal to the phosphorylated tyrosine residue confer specificity and increase the affinity of the PTB domain for its target motif. The PTB domains of proteins such as X11, Dab, Fe56 and Numb recognize NPXY motifs independent of tyrosine phosphorylation (Zwahlen et al., 2000;Stolt et al., 2004;Uhlik *et al.*, 2005). Structurally, PTB domains resemble PH domains, containing a β-sandwich of two anti parallel β-sheets capped at one end by an α-helix. Ligand binding is situated between the α-helix and the two anti parallel β-sheets (Eck et al., 1996). PTB-containing proteins tend to function as receptor-linking adapter proteins by virtue of the fact most of their consensus motifs are located in cytoplasmic portions of transmembrane receptors (Uhlik *et al.*, 2005). However, recently it was found that Grb14 contains a ligand sequence for the PTB of IRS1, suggesting PTBs may serve as yet undetermined roles in non-receptor mediated events (Rajala and Chan, 2005).

SH3 Domain

Unlike the binding of SH2 and PTB domains to their target sequences, SH3-dependent interactions do not depend on post translational modifications. SH3 domains are approximately 60 amino acids in length and bind to proline rich peptides that form a left-handed poly proline type II helix. The minimal sequence recognition consensus consists of PXXP and is usually preceded by an aliphatic residue (Mayer and Eck, 1995). Further differentiating SH3 domains from that of a SH2 and PTB domain, is the fact that SH3 domains are capable of engaging their target ligand in a N-terminal to C-terminal orientation or in the opposite direction, leaving amino acids upstream or downstream of the core PXXP motif to define the binding orientation (Mayer *et al.*, 1995). Promiscuity

of SH3 domains for non-consensus PXXP motifs is now well documented in the literature. This divergence includes the binding of the Gads SH3 to an RXXK motif and high proline content polypeptides not arranged in the typical PXXP consensus, such as those bound by the SH3 domain containing protein Eps8 (Mongiovi et al., 1999;Berry et al., 2002). Although SH3 mediated interactions are considered constitutive, and as such are thought to primarily contribute to protein complex assembly, several examples exist wherein binding of an SH3 domain to a target motif modulates enzymatic activity. For instance, Src family non-receptor tyrosine kinases are held together in a catalytically inactive conformation by an intramolecular SH3 interaction. Destabilization of this interaction, through engagement or substitution of higher affinity ligands is sufficient to induce a conformational change that leads to activation of the kinase domain (Eck et al., 1994). Other examples of SH3 binding-induced changes include increases in GTPase activity of dynamin upon binding SH3-containing proteins and activation of PI3 kinase activity upon binding of its SH3 domain to the p85 subunit (Gout et al., 1993;Pleiman et al., 1994).

PH Domain

Pleckstrin homology (PH) domains are typically found in proteins that associate with the plasma membrane. They consist of a module approximately 100 amino acids in length that closely resembles the structure of PTB domains. PH domains bind with high affinity (low μM or nM K_d) to specific phosphoinositides (PIs) such as PI-4,5-P_2, PI-3,4-P_2 and PI-3,4,5-P_3. As such, binding to PIs allows proteins containing PH domains to respond to the generation of lipid second messengers by inducibly relocating to the plasma

membrane. Often such an event facilitates the juxtaposition of PH-containing proteins next to activated transmembrane receptors (Cozier et al., 2004). Alternate roles for PH domains have also been demonstrated and include the nuclear localization of insulin receptor substrate 3 (IRS3) and direct binding to proteins such as heterotrimeric G proteins and filamentous actin (Touhara et al., 1994;Yao et al., 1999;Maffucci et al., 2003).

EGFR family of RTKs

The epidermal growth factor receptor (EGFR) family of RTKs has for many years served as a prototype family for the study of RTK structure and function. The family and signaling pathways activated by these receptors have been implicated in the genesis and progression of several different types of cancer. The family consists of four members EGFR-1 (HER1), EGFR-2 (HER2), EGFR-3 (HER3), and EGFR-4 (HER4). Genes encoding the EGFRs are homologous to the avian retroviral gene v-erythroblatosis-B, (v-*erb*B). As such, the nomenclature given to the cellular genes that code for Hers 1-4 is *erb*B1-4 respectively. Her2/*erb*B2 is also sometimes referred to as *neu*, as a consequence of the role that it was found to play in promoting neuroblastoma development in rats (Yarden and Weinberg, 1989;Bacus et al., 1990). These receptors are anchored in the cytoplasmic membrane and share similar structural characteristics which include an extracellular ligand binding domain, a hydrophobic transmembrane region, and an intracytoplasmic tyrosine kinase domain (Yarden and Sliwkowski, 2001;Hynes and Lane, 2005). Ligands that bind the EGFR family of receptors are subdivided into two classes. The first class contains EGF-like ligands which include EGF, heparin binding EGF,

transforming growth factor alpha (TGF α), betacellulin, amphiregulin, and epiregulin, while the second class includes neuroregulins, NRG1α, NRG1β, NRG2α, NRG2 β, NRG3, and NRG4 (Beerli and Hynes, 1996;Normanno et al., 2001). Consistent with the accepted paradigm of RTK activation these ligands bind monomeric receptor units and promote receptor dimerization or oligomerization. Ligand-receptor engagement promotes a conformational change of the receptor ectodomain that allows receptor dimerization and transphosphorylation of several COOH-terminal tyrosine residues to occur (Burgess et al., 2003;Hubbard, 2006). Cross specificity of each of the EGFRs for multiple ligands results in a situation in which receptors may homodimerize or heterodimerize with other family members (Pinkas-Kramarski et al., 1998a;Pinkas-Kramarski et al., 1998b). Heterodimerization is particularly critical to the proper functioning of HER3 as it lacks intrinsic kinase activity and thus relies upon transphosphorylation by other EGFR-family members (Guy et al., 1994;Sierke et al., 1997). EGFRs may also become activated in a ligand-independent manner as is seen in many cancers upon receptor overexpression, deletion of the extracellular domain, or in response to radiation exposure which silences phosphatases that normally counteract EGFR activity (Frederick et al., 2000;Fischer et al., 2003;Anido et al., 2006).

EGFR/HER1

Once activated, EGFR/HER1 has been shown to promote downstream assembly and activation of a complex array of signaling cascades that play central roles in such diverse physiological events as cell proliferation, survival, growth, invasion, and migration.

Ras/Raf/MAPK

In large part the pro-proliferative effects of EGFR activation can be accounted for by activation of the Ras/Raf/MAPK pathway. Following EGFR phosphorylation the complex formed by Grb2/Sos may bind directly, or through association with Shc, to target phosphorylation sites on the receptor (Lowenstein et al., 1992;Batzer et al., 1994;Schulze et al., 2005). Recruitment of Sos, a guanine nucleotide exchange factor, brings it into close proximity with p21 Ras, a guanine nucleotide binding protein which is anchored to the cytoplasmic face of the plasma membrane via farnesylation (Kato et al., 1992). The activation status of Ras is controlled through its binding and exchange of GDP or GTP. Ras is activated through the ability of Sos to exchange GDP-bound Ras with GTP (Hallberg et al., 1994;Liebmann, 2001). Once activated, Ras is capable of binding and activating members of the Raf family which include A-Raf, B-Raf and Raf-1. Activated Raf family members bind to, and stimulate members of the Mek family which include Mek 1 and Mek 2. Activated Mek isoforms stimulate extracellular signal regulated kinases 1 and 2 (Erk 1&2) by phosphorylating regulatory residues on these kinases. Erk 1&2, also know as p44 MAPK and p42 MAPK respectively, translocate to the nucleus where they activate specific transcription factors, such as Rsk 1-3, Rel, Elk1, Myc, and Fos, all of which are involved in EGFR-dependent cell proliferation (Spanakis and Brouty-Boye, 1995;Gavin and Nebreda, 1999;Jun et al., 1999;Aplin et al., 2001;Murphy et al., 2002;Moelling et al., 2002;Dunn et al., 2005).

PI3-Kinase/AKT

In addition to activation of the Ras/Raf/MAPK pathway the EGFR has been shown to activate the PI3-kinase/Akt pathway. Activation of PI3-kinase occurs through at least three distinct mechanisms. PI3 kinase is made up of two subunits, the p85 regulatory unit and the p110 catalytic unit. Binding of the p85 subunit to a phosphorylated tyrosine on EGFR-bound Gab1 confers a conformation change necessary to activate the p110 catalytic domain (Mattoon et al., 2004). Alternatively, PI3-kinase may interact directly with GTP-bound Ras at the membrane, and become activated (Walker et al., 1999;Pacold et al., 2000;Chan et al., 2002). Finally, EGFR-dependent activation of PI3-kinase may also occur through heterodimerization with family member HER3, which when phosphorylated on tyrosine contains several p85 binding motifs (Carpenter et al., 1993;Yarden *et al.*, 2001). In any event, interaction with EGFR or GTP-bound Ras places PI3-kinase in a position proximal to the cytoplasmic membrane where it functions to phosphorylate phosphatidylinositol 4-phosphate [PI(4)P] and phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] to yield phosphatidylinositol 3,4-bisphosphate [$PI(3,4)P_2$] and phosphatidylinositol 3,4,5-trisphosphate [$PI(3,4,5)P_3$], respectively. Generation of $PI(3,4)P_2$ and $PI(2,3,4)P_3$ second messengers serve as binding intermediaries for the pleckstrin homology domains of PDK1 and Akt (also known as PKB) (Frech et al., 1995;Frech et al., 1997). Following plasma membrane colocalization PDK1 phosphorylates Akt on a key threonine residue and through mechanisms which are not known Akt also becomes phosphorylated on serine, perhaps through an autocatalytic event initiated by PDK1-dependent threonine phosphorylation (Toker and Newton, 2000). Activation of the PI3-K/PDK1/Akt signaling axis may result in increased cell

proliferation and protection from apoptosis. For instance, activation of NF-κB by Akt promotes cyclin D1 transcription and a consequential increase in cell cycle progression (Ibarra-Sanchez et al., 2001;Conejo et al., 2002). In addition Akt-dependent phosphorylation of GSK3β prevents GSK3β from mediating ubiquitin-dependent degradation of cyclin D1 (Thomas et al., 1999). The antiapoptotic effects of Akt are well documented and stem from a diverse modulation of cellular proteins. Akt has been shown to directly phosphorylate caspase 9, which inactivates its protease activity (Cardone et al., 1998). Inactivation of caspase 9 prevents activation of caspase 3. Caspases play a central role in apoptosis by proteolytically degrading structural proteins such as Gas2, gelsolin, and nuclear laminins, while also degrading antiapoptotic members Bcl-2 and Bcl-X_L. Akt has also been shown to phosphorylate members of the Forkhead Box-O (FOXO) class of transcription factors. Akt-dependent phosphorylation of FOXO proteins prevents their entry to the nucleus where they would normally promote apoptosis and protection from DNA and oxidative stress damage (Huang and Tindall, 2006).

PLCγ

EGFR-dependent activation of PLCγ has been shown to result in Ras-independent activation of the Raf/MEK/ERK pathway. Phosphorylation of inositols at the plasma membrane promotes the translocation of PLCγ into close proximity of the EGFR. Localization of PLCγ to the plasma membrane facilitates direct binding of one or both of the PLCγ SH2 domains to the EGFR and results in activation of lipase activity

(Chattopadhyay et al., 1999). Alternatively, PLCγ SH2 domains bound directly $PI(3,4,5)P_3$, can provide the signal necessary to promote lipase activation (Bae et al., 1998;Rameh et al., 1998). PLCγ functions to convert $PI(4,5)P_2$ to inositol 1,4,5-triphosphate, which promotes intracellular calcium release, and diacylglycerol (DAG) (Patterson et al., 2005). DAG produced by PLCγ activates protein kinase C (PKC) which in turn activates Raf by direct phosphorylation and the downstream pathway consisting of MEK and ERK (Kolch et al., 1993;Cai et al., 1997). As such, activation of the PLCγ/PKC pathway represents an alternate Ras/Sos-independent mechanism through which EGFR is able to activate the RAF/MEK/ERK pathway.

Src Kinase

Clinically, EGFR and Src have been shown to be co-overexpressed with high frequency in breast cancer samples, suggesting a functional role for these two kinases in breast tumorigenesis (Biscardi et al., 2000). Several distinct mechanisms have been described in the literature which helps to explain the collaborative effects of Src and EGFR signaling. For instance, in response to EGF dependent activation of Src, Cbl has been shown to undergo tyrosine phosphorylation, ubiquitination and proteasomal degradation (Bao et al., 2003). Because Cbl is thought to be the primary E3 involved in targeting the EGFR for degradation, these results offer an explanation for the high incidence of Src and EGFR co-overexpression in cancer and provides a novel mechanism whereby Src increases the total number of EGFRs at the plasma membrane, and thus its sensitivity to ligand or autoactivation (Bao *et al.*, 2003) Maa M, *et al.*, have additionally demonstrated a direct physical interaction involving the SH2 domain of Src and tyrosine phosphorylated EGFR

(Maa et al., 1995), whereupon Src becomes transiently activated and phosphorylates downstream targets, including multiple sites on EGFR itself (Muthuswamy et al., 1994;Osherov and Levitzki, 1994;Biscardi *et al.*, 2000). One site in particular, pY845, located within the activation loop of the catalytic domain of the EGFR, has been shown to be critical for EGF-induced mitogenesis. Wu W, *et al.*, reported that Src-dependent phosphorylation of EGFR, Y845 is necessary for Zn^{2+} -induced activation of Ras (Wu et al., 2002), while others have reported roles for pY845 which include activation of mitogenesis through a Stat5b mechanism (Kloth et al., 2003) and inhibition of apoptosis through regulation of cytochrome c oxidase II (CoxII) (Boerner et al., 2004). Finally, Kong M, *et al.*, defined a role for Src activation in EGF-induced hepatocyte mitogenesis through the phosphorylation of Gab2 and the activation of the PI3-kinase/Akt pathway (Kong et al., 2003).

Negative Regulation of EGFR/HER1

Although much is known about the signaling pathways that are activated downstream of the EGFR, comparatively little is know about pathways which naturally antagonize them. Data to suggest that exquisite control of EGFR activity is absolutely critical are numerous and these examples are highlighted by a high incidence of these pathways being implicated in tumorigenesis or cancer progression.

EGFR Degradation

Overexpression of the EGFR is known to occur in several different types of cancer suggesting potential defects in regulation of its turnover (Holbro et al., 2003). EGFR

signaling is negatively regulated at many different levels. Perhaps the best described mechanism involves regulation of EGFR turnover. Once activated the EGFR is ubiquitinated by the E3 ubiquitin ligase Cbl (Shtiegman and Yarden, 2003). Recruitment of Cbl to the EGFR occurs through direct binding of the Cbl phospho-tyrosine domain (PTB) and EGFR pY 1045, or indirectly through EGFR-bound Grb2 (Levkowitz et al., 1996;Waterman et al., 2002). EGFR kinase activity is required to phosphorylate and activate Cbl ubiquitin ligase activity. Once activated, Cbl transfers multiple mono-ubiquitins to the EGFR, targeting the receptor for internalization and lysosomal degradation (Haglund et al., 2003). While this mechanism is generally accepted, some controversy stems from the fact that in studies using cells derived from Cbl knock out mice Cbl-mediated ubiquitination was found to be dispensable for EGF-induced internalization of the receptor (Duan et al., 2003). These data suggest a complex regulatory system in which several layers of EGFR control are necessary and present. Overall regulation of non-ligand stimulated EGFR level is also thought to be maintained at a steady state by novel ubiquitin ligases. These actions are thought to dynamically control the total number of EGFR's that are routed towards the endosome and back to the cell surface or those which are degraded in the lysosome.

EGFR Negative Modulatory Proteins

Numerous studies have demonstrated that EGFR signal strength and duration are critical in eliciting specific and intended cellular responses. Mechanisms not directly involving EGFR degradation have evolved as a means of fine tuning signaling events that emanate from its activation. Negative regulation of EGFR can be subdivided into reversible and

irreversible inhibition. Irreversible inhibition primarily involves receptor degradation. Reversible events include the actions of proteins like Herstatin. Herstatin, a naturally occurring secreted extracellular fragment of HER2, is capable of binding to, and blocking, HER1,2,4 receptor dimerization and activation without blocking ligand binding (Azios et al., 2001;Justman and Clinton, 2002;Jhabvala-Romero et al., 2003). Because Herstatin blocks both ligand dependent activation of EGFR, and activation brought about by overexpression, it has garnered great interest as a therapeutic pan inhibitor of the HER family. Temporal regulation of the EGFR signal is controlled by receptor-associated late transducer (RALT). RALT is pan HER inhibitor that is transcriptionally induced upon HER-dependent activation of the Ras pathway (Fiorentino et al., 2000;Anastasi et al., 2003). A mechanism such as this, which allows a signal to be appropriately propagated and then terminated, serves as a classic negative feedback loop typical of RTK mediated extracellular signaling. Loss of RALT expression, a common event in breast cancer, sensitizes cells to sub-optimal concentrations of growth factors and increases mitogenesis (Anastasi *et al.*, 2003;Anastasi et al., 2005). Members of the suppressor of cytokine signaling (SOCS) family have also recently been implicated in the negative regulation of EGFR. Similar to RALT, SOCS4 and 5 are transcriptionally regulated by EGFR activation. Increases in SOCS4 and 5 have been shown to increase the turnover of non-activated EGFR in a Cbl-independent manner (Kario et al., 2005), while overexpression of SOCS2, through some unknown mechanism, has been shown to dramatically decrease total EGFR phosphorylation (Goldshmit et al., 2004). These examples, although not exhaustive, serve to illuminate the degree and complexity of direct negative regulation of

EGFR signaling. They do not however, touch on any of the multitude of highly evolved stop-checks present in pathways immediately downstream of EGFR activation.

Downstream of Kinase (Dok) Proteins

Work conducted as early as 1990 reported the existence of a RasGAP-bound 62 kDa protein that, in response to EGFR stimulation, became heavily phosphorylated on tyrosine residues (Ellis et al., 1990;Bouton et al., 1991a;Bouton et al., 1991b). Because of these observations, speculation that this unknown 62 kDa protein, in conjunction with RasGAP, was responsible for attenuating Ras transduced signals from activated EGFR (Park and Jove, 1993). In addition to responding to EGFR stimulation, p62 was also found to be phosphorylated upon overexpression of v-Src, v-Fps, v-Fms and v-Abl, placing p62 at the crossroads of a diverse range of tyrosine kinase signaling pathways (Ellis *et al.*, 1990;Bouton *et al.*, 1991b). Utilizing its high phosphotyrosine content in an enrichment scheme, Yamanashi Y., and Baltimore D., (1997), were able to purify enough of the unknown 62 kDa protein to sequence and clone what they called p62Dok, for downstream of kinase (Yamanashi and Baltimore, 1997). Since then six additional family members have been cloned with high sequence and structural homology to p62Dok (also called Dok 1). Members of this family, which include Dok 1, Dok 2/Dok-R/FRIP, Dok 3/DokL, Dok 4/IRS5, Dok 5/IRS6, Dok 6 and Dok 7 are comprised of two domains, an amino terminal pleckstrin homology (PH) domain, and a central phosphotyrosine binding (PTB) domain. The carboxy terminal region of each Dok member (also called the PRR, for proline rich region) is rich in proline and tyrosine residues (fig 1.1) which have been shown to mediate the recruitment of SH3 and SH2-containing proteins. Phylogenetically,

Doks 4-7 form a distinct subgroup from Doks 1-3, characterized by differing intron/exon structure and less sequence homology in the proteins carboxy termini (Favre et al., 2003).

Figure 1.1: Dok family member schematic. PH, PTB and Y represent pleckstrin homology, phosphotyrosine binding domain and putative or confirmed SH2 binding motifs. Adapted from (Okada et al., 2006)

Doks 4 and 5, also bear the name IRS5 and IRS6 for their high similarity to the insulin receptor substrate (IRS) family of proteins. However, all of the Doks are closely related to the IRS family in both structural topology and sequence similarity. Sequence similarity between the Dok family members is highest within the tandem PH and PTB domains, while within the carboxy terminus the sequences are more divergent. Evidence has demonstrated that Dok PH domains are functional and are responsible for plasma membrane localization, while the PTB domain has been shown to bind phosphotyrosyl residues on the cytoplasmic region of several RTKs and cytokine receptors (Songyang et al., 1993;Zhao et al., 2001;Liang et al., 2002;Jones et al., 2003;Bedirian et al., 2004). Preferred consensus binding motifs have been determined for Dok 1 and Dok-R and

consist of Y/MXXNXLpY (Songyang et al., 2001) and LpY (Jones *et al.*, 2003) respectively. In addition to the PTB associating with activated kinases, the PTB domain of Dok 1 and Dok-R have been shown to mediate their own intermolecular homo or heterodimerization, a property which has been shown to be indispensable to their ability to attenuate MAPK activation (Songyang *et al.*, 2001;Boulay et al., 2005). These findings also highlight the potential for establishing very high local concentrations of Dok 1 and Dok-R at signaling complexes should their oligomerization facilitate end to end concatamerization. Because the Doks lack intrinsic enzymatic activity it is believed that collectively, these proteins function by recruiting or clustering other proteins and locally enriching the signaling environment surrounding activated receptors and non receptor kinases.

Expression Profiles

Expression of Doks 1-3 is primarily restricted to hematopoietic cells and hematopoietic precursors (Carpino et al., 1997;Nelms et al., 1998;Lemay et al., 2000). Dok-R expression has also been reported in endothelial cells, possibly due to the shared ancestral origins of endothelial cells and hematopoietic cells, while high level Dok 1 expression has been reported in the neurons of the prefrontal cortex (Master et al., 2001;Master et al., 2003;Smith et al., 2004). Grimm J., *et al.*, and Crowder R *et al.*, report the endogenous association of Doks 4-6 with the c-Ret receptor and show a pattern of expression that co-localizes with c-Ret primarily in neuronal tissue (Grimm et al., 2001;Crowder et al., 2004). Dok 4 is also expressed in endothelial cells and upon activation of Tie 2, the PTB domain of Dok 4, like Dok-R, is able to bind a carboxy terminal phosphorylated tyrosine

of Tie 2, suggesting a high degree of functional conservation in the PTB domains of both family members (Grimm *et al.*, 2001). Moreover, pulldown assays demonstrate Doks 1-6 all associate with c-Ret *in vitro*, but are not necessarily co-expressed within the same cell type, suggesting that differential regulation of expression is necessary to prevent inappropriate signaling events (Grimm *et al.*, 2001;Crowder *et al.*, 2004). Recently the seventh member of the Dok family was described (Okada *et al.*, 2006). Dok 7 mRNA and protein analysis demonstrate high expression in post synaptic neuromuscular junctions (Okada *et al.*, 2006). Consistent with these findings, genetic loss of Dok 7 results in defective neuromuscular synaptogenesis (Okada *et al.*, 2006).

Dok 1 and Dok-R Subfamily

Based on sequence homology, gene arrangement and expression profile Dok 1 and Dok-R are most closely related. Functionally, these two family members have been shown to similarly attenuate signals emanating from a distinct and overlapping repertoire of RTKs and PTKs. Because of this fact, studying the unique properties of either family member has proven difficult due to the confounding issue of complimentarity. Genetic ablation of the mouse Dok 1 gene proved to be non-lethal and resulted in no overt phenotype after one year (Yamanashi et al., 2000). However, B cells removed from Dok $1^{-/-}$ mice display increased proliferation *in vitro* upon co-crosslinking of the BCR and FcγRIIB with rabbit IgG, an effect attributed to disregulation of the Raf/Raf/MAPK pathway (Yamanashi *et al.*, 2000). It was reasoned that B cells in particular were sensitive to the effects of Dok 1 ablation due to the fact that they are also naturally deficient in Dok-R expression (Nelms *et al.*, 1998).

Although the mouse line exists, thus far no thorough examination of Dok-R$^{-/-}$ mice has been reported (Niki et al., 2004;Yasuda et al., 2004). Rather the effects of Dok-R ablation have been reported in the context of simultaneous Dok 1 loss (Niki *et al.*, 2004;Yasuda *et al.*, 2004). Concomitant loss of mouse Dok 1 and Dok-R results in severe myeloproliferative disease resembling CML, that correlates with increases in Ras/MAPK and Akt activation, cellular proliferation, and a reduction in apoptosis (Niki *et al.*, 2004;Yasuda *et al.*, 2004). Bone marrow transplants to WT recipient mice demonstrated a cell-autonomous role for Dok 1/Dok-R loss in these events (Niki *et al.*, 2004). Furthermore, triple mutant mouse lines consisting of Dok 1$^{-/-}$: Dok-R$^{-/-}$ and the oncogenic protein Tec-p210$^{Bcr/Abl}$ displayed an accelerated leukemogenesis profile and shortened time span preceding blast crisis (Niki *et al.*, 2004;Yasuda *et al.*, 2004). Although the results of the knock out studies outlined in the works of Yasuda T., *et al.*, and Niki M., *et al.*, highlight a role for Dok 1 and Dok-R in the regulation of Ras/MAPK and Akt, more recently it has been shown that Dok 1 and Dok-R also function to regulate Src kinase activation downstream of platelet derived growth factor receptor (PDGFR) and EGFR (Van Slyke et al., 2005;Zhao et al., 2006). The precedence for Src family kinase (SFK) disregulation in leukemogenesis is well documented (Geahlen et al., 2004). For instance, it has been shown in Philadelphia chromosome positive (Ph^{+}) CML that Bcr-Abl activates a suite of SFK members which include Lyn, Hck, Blk, Lck, Fgr, and Src (Hu et al., 2004). Moreover, Hu Y, *et al.*, demonstrated that treatment with CGP76030, a SFK inhibitor, could inhibit growth and survival of pre-B lymphoid leukemia cells engineered to express Bcr-Abl T315I mutant, which is resistant to inhibition by both imantinib and CGP76030 (Gorre et al., 2001;Warmuth et al., 2003;Hu *et al.*, 2004). In

light of these findings, it would be interesting to specifically assess the activity levels of SFKs in hematopoietic cells of the Dok 1/Dok-R knock out mice. In addition, genetically crossing the Dok 1/Dok-R mice to the Bcr-Abl T315I mouse line and exposing the leukemic cells to CGP76030 and/or virally reconstituting the cells with Dok 1/Dok-R would provide an excellent model in which to assess relative importance of Dok-dependent SFK inhibition. Based on the knock out studies it has been proposed that Dok 1 and Dok-R function as tumor suppressors. Although attributed to a RasGAP-dependent inhibition of Ras, the importance of Dok 1 and Dok-R in SFK regulation and any other, as yet undefined roles remains to be determined.

Part 2

Vasculogenesis:

Although the cellular composition of tissue in the body varies dramatically based on function, the presence of vasculature within these tissues is almost completely universal. As such, assembly of a functional vascular system occurs early in fetal development, proceeds in a coordinated manner during organogenesis, and is necessary to support normal physiology. Early in embryonic development blood vessels form exclusively through a process termed Vasculogenesis. Angioblasts, precursors of endothelial cells, migrate and differentiate into a vascular plexus by interpreting environmental cues such as growth factor secretion and extracellular matrix deposition. This process of *de novo* blood vessel formation occurs first in the yolk sac and then proceeds in the embryo proper. In the yolk sac, the early vasculogenic process is marked by the formation of blood islands within the mesodermal layer. Blood islands, which consist of angioblasts, eventually coalesce into the vitelline circulation which serves to supply nutrients to the embryo proper from the yolk sac. Extraembryonically, vasculogenesis also occurs within the allontois, a structure responsible for the induction of the placenta (Ferguson, III et al., 2005). Within the embryo proper the endocardium and great vessels are the first endothelium-based structures to form (Drake and Fleming, 2000). Angioblasts located in the presomatic cranial mesoderm enter the pericardial region adjacent to the myocardium and give rise to a vascular plexus which will become the endocardial tube (Drake *et al.*, 2000). Coincident with this, angioblasts positioned just lateral of the midline give rise to the aorta and cardinal veins. This region, which is termed the para-aortic splanchnopleure and then the aorta-gonad-mesonephros, continues to be an important contributor to

undifferentiated hemato-endothelial cell precursors during development of the embryo (de Bruijn et al., 2002). Although primarily associated with embryonic development, recent improvements in cell marker tracking have highlighted a potential role for vasculogenesis in adults. Mobilization of circulating endothelial cells (CECs) or endothelial progenitor cells (EPCs) has been implicated in wound healing, tumorigenesis, and the female reproductive system (Hunting et al., 2005). Furthermore, researchers have explored the possibility of promoting therapeutic vasculogenesis through the use of adult hematopoietic stem cells (Asahara and Kawamoto, 2004).

Angiogenesis:

Subsequent to the formation of the primary embryonic vascular plexus, and in conjunction with the growth of the embryo, increased nutrient and waste exchange necessitate a more fully elaborated vascular network. Fulfillment of this increased metabolic need is accomplished through the process of angiogenesis. Angiogenesis, the formation of nascent blood vessels from preexisting larger vessels, can be subdivided into three distinct forms-sprouting, intercalated and intussusceptive angiogenesis. The formation of new blood vessels by sprouting angiogenesis follows a well defined cellular program. First, endothelial cells must receive a signal to sprout, often in the form of a peptide based growth factor. Subsequent to that initiating event the tip cell (endothelial cell at the leading edge) will begin to secrete extracellular matrix degrading enzymes such as matrix metalloproteinases (MMP) 2 and 9, which are necessary to facilitate endothelial cell proliferation and migration. The emerging endothelial cells form a solid sprouting structure and migration will occur towards the source of the initiating signal in

a concentration dependent manner. When the invading sprout intercepts a nearby vessel, vascular remodeling occurs, and the once solid sprout will form a functional lumen which becomes integrated into the vascular network. Sprouting angiogenesis is the primary form of angiogenesis that contributes to developmental vascularization of the brain, kidneys and intersomitic arteries. It is also of importance to such physiological events as wound healing, and female menstruation. Misregulated sprouting angiogenesis is well known for its contribution to pathological conditions which include cancer angiogenesis and diseases of the eye and psoriasis. Once a sprout is fully elaborated, the process of intercalated angiogenesis may serve to increase vessel circumference through endothelial cell proliferation, or incorporation of CEPs/CECs. Discovery of this form of angiogenesis stems from classical parabiosis experiments in which cell fate of embryonic quail mesodermal tissue was tracked once grafted into the head region of stage 9-10 chick embryos (Feinberg and Noden, 1991;Pudliszewski and Pardanaud, 2005). Intussusceptive angiogenesis refers to a process whereby vessels are capable of dramatically increasing the overall vascular bed volume of existing vascular networks. The basic driving event in intussusceptive vessel growth involves the formation of transvascular tissue pillars. Transvascular tissue pillars are composed of adjacent endothelial cells that invade the luminal space. The outcome of pillar development has differing consequences which are based upon their location, timing, and frequency (Burri et al., 2004). While formation of vascular pillars in small capillaries and venuoles contributes to increases in the overall size and complexity of the vascular tree, pillars that occur in small arteries or veins may contribute to changes in branching geometry or pruning (Burri *et al.*, 2004). Organs in which intussusceptive angiogenesis plays a defining role include the lungs, eyes,

intestinal mucosa, kidneys, ovaries and uterus (Burri *et al.*, 2004). As is the case with sprouting angiogenesis, intussusceptive angiogenesis occurs primarily during embryonic development and the early postnatal period.

RTK-Dependent Angiogenic Signaling:

Angiogenesis involves a tightly orchestrated activation/inactivation of pro and antiangiogenic cell signaling pathways. Many of these signaling pathways originate at the plasma membrane and emanate from the activation of prototypical endothelial cell RTKs such as vascular endothelial growth factor receptors 1, 2 and 3 (VEGFR-1,2&3), basic fibroblast growth factor receptor 1 (bFGFR-1), Tie1 and Tie2/Tek receptors.

VEGFRs

The most extensively examined family of vascular RTKs is the VEGFRs. The VEGFR family consists of three primary receptors, VEGF 1-3, and two co-receptors, neuropillin 1 and 2 (NRP 1&2). VEFGR-2, also known as Flk-1/KDR, is a 200-230 kDa high affinity receptor for vascular endothelial growth factors (VEGF) A, E and snake venom $_{sv}$F as well as the processed forms of C and D (Junqueira, I et al., 2001;Takahashi et al., 2004;Takahashi and Shibuya, 2005). High levels of VEGFR-2 expression have been reported in blood and lymphatic endothelial cells as well as macrophages and hematopoietic stem cells (Katoh et al., 1995;Takahashi *et al.*, 2005). VEGFR-2 is a key mediator of blood and lymphatic endothelial cell migration, mitogenesis, differentiation, morphogenesis, survival and permeability. Diverse physiological effects elicited by VEGFR-2 activation can be attributed to phosphorylation of several tyrosine residues

located in the carboxy-terminus of the receptor. For instance, VEGF A-induced phosphorylation of VEGFR-2 tyrosine 951 has been shown to recruit VEGFR associated protein (VRAP) (Wu et al., 2000), while phosphorylation of tyrosine 1175 serves as a multi-docking site for Sck (Warner et al., 2000), Shb (Holmqvist et al., 2004) and phospholipase C γ (PLC γ) (Takahashi et al., 2001). Weak activation of phosphatidylinositol-3 kinase (PI3 kinase)/Akt pathway following VEGFR-2 activation is thought to synergize through cross talk with several different integrins to promote a VEGF mediated pro-survival effect. VEGF, originally identified as vascular permeability factor, is well known for its ability to potently induce vascular leakage. The exact mechanisms that underlie this are not completely understood but mouse genetic studies demonstrate that this effect primarily mediated VEGFR-2 (Brekken et al., 2000). Activation of downstream pathways including Cdc42, Src and PI3 kinase have been shown to be critical to VEGF-induced permeability (Takahashi *et al.*, 2005).

Although most of the proangiogenic effects of the VEGFR family are ascribed to VEGFR-2, several more recent findings highlight an important role for VEGFR-1 in various pathological conditions such as cancer angiogenesis, inflammation and ischemia (Autiero et al., 2003). VEGFR-1/Flt-1 is a 180 kDa endothelial enriched RTK, that when compared to VEGFR-2, has very little kinase activity (Shibuya and Claesson-Welsh, 2006). VEGFR-1 has affinity for VEGF A, B and placental growth factor (PlGF). Initially, during embryonic development, VEGFR-1 acts as an inhibitor of angiogenesis. One prevailing explanation for this fact is borne from the observation that VEGFR-1 may act like a VEGF sink due to its much higher affinity for VEGF A than VEGFR-2 (Shibuya *et al.*, 2006). Because VEGFR-1 and VEGFR-2 possess similar and overlapping

ligand binding characteristics, it has been difficult to ascribe particular cellular events to either one or the other receptor. Antibodies that specifically block the ability of VEGFR-1 to bind VEGF A inhibit endothelial migration but not proliferation indicating a role for VEGFR-1 in cell migration (Kanno et al., 2000;Takahashi *et al.*, 2005). Unlike VEGFR-2 driven migration, which preferentially phosphorylates focal adhesion kinase (Fak) and Paxillin to rearrange the actin cytoskeleton, VEGFR-1 drives cytoskeletal rearrangement through activation of p38 mitogen activated protein kinase (MAPK), suggesting that VEGFR-1 and 2 act collaboratively to affect migration processes (Kanno *et al.*, 2000;Rousseau et al., 2000).

VEGFR-3/Flt-4 is a 195 kDa RTK which possesses specificity for VEGF C and D. Expression of VEGFR-3 is primarily restricted to lymphatic endothelial cells and has been shown to play a critical role in lymphangiogenesis, including the initiation of prosurvival signals through activation of PI3 kinase and mitogenic signals by way of protein kinase C (PKC) activation (Veikkola et al., 2001). The actual synthesis of VEGFR-3 differs significantly from that of VEGFR-1&2. VEGFR-3 is proteolytically cleaved into two pieces during synthesis and reassembled via the introduction of a disulphide bridge (Lohela et al., 2003).

Tie Receptors and the Angiogenic Response:

Concurrent with the identification and characterization of the VEGFR family a distinct family of RTKs was cloned. These receptors, named Tie 1 and Tie 2/Tek are primarily expressed on endothelial cells and their progenitors and have since been shown in mouse studies to be indispensable to normal embryonic vessel development (Dumont et al.,

1993;Puri et al., 1999). Despite the high degree of structural homology to Tie 2, until recently, Tie 1 has remained an orphan receptor. Because of this fact, much of what is know about Tie 1 function revolves around many different genetic studies (Puri et al., 1995;Sato et al., 1995b;Patan, 1998), while mechanistically, the role of Tie1 has remained enigmatic. Mice engineered to lack Tie 1 die *in utero* from approximately E13.5 until birth of hemorrhage and edema suggesting primary defects in vessel integrity (Puri *et al.*, 1995;Sato *et al.*, 1995b). Early reports suggested that Tie 1 was incapable of binding, and responding to, native angiopoietins (Angs)-the cognate ligands of Tie 2 (Davis et al., 1996;Maisonpierre et al., 1997;Valenzuela et al., 1999). More recently Saharinen P., and Alitalo K, et al. present compelling evidence to suggest that Tie 1 can be activated by several different native and recombinant chimeric forms of angiopoietin (Saharinen et al., 2005). In addition to binding and activating the Tie 1 receptor, these ligands are thought to facilitate heterotypic clustering of Tie 1 and Tie 2; an event predicted to be critical in cross talk and collaborative modulation of Tie 1/Tie 2 downstream signaling pathways (Marron et al., 2000a;Tsiamis et al., 2002;Saharinen *et al.*, 2005). As is the case with VEGFR-1 and 2, Tie 1 can inducibly undergo proteolytic cleavage of the extracellular ligand binding portion, leaving behind a transmembrane bound endodomain. Release of the Tie 1 ectodomain occurs in response to stimulation with VEGF, phorbol myristate acetate, PKC and shear stress (Yabkowitz et al., 1997;McCarthy et al., 1999;Yabkowitz et al., 1999;Chen-Konak et al., 2003). Although unable to bind ligand, the endodomain has been found to associate with SHP2, suggesting that Tie 1 may also be able to function in a ligand-independent manner (Marron et al., 2000b). Tie 2, like Tie 1 was initially an orphan receptor and as such many of the same

difficulties existed in determining its functional role in angiogenesis. Genetic ablation of the Tie 2 receptor results in gross defects in sprouting angiogenesis, cardiac morphogenesis, widespread hemorrhage, loss of endothelial cells, poor recruitment of perivascular cells and overall decreases in vascular complexity which ultimately lead to embryonic lethality by day E9.5-12.5 (Dumont *et al.*, 1993;Sato *et al.*, 1995b;Suri et al., 1996;Patan, 1998). To avoid repetition, Tie 2 dependent signaling will be discussed below in the context of the Angs, with distinction made to highlight non-Tie 2 mediated Ang signaling events.

Angiopoietins 1-4

Subsequent to the cloning of Tie 2 a family of highly conserved Tie 2 ligands was identified. Angiopoietins (Ang) 1-4 have all been shown to bind to and activate Tie 2 receptor tyrosine kinase activity to differing extents (Davis *et al.*, 1996;Maisonpierre *et al.*, 1997;Valenzuela *et al.*, 1999). Ang 1 and 4 appear to be the primary agonists while Ang 2 and 3 act in a context dependent agonist/antagonist manner (Davis *et al.*, 1996;Maisonpierre *et al.*, 1997;Valenzuela *et al.*, 1999). Ang 3 and 4 are mouse and human orthologues which are more divergent structurally than Ang 1 and 2 (Valenzuela *et al.*, 1999). All the Angs are characterized structurally by an N-terminal super clustering domain (SCD) followed by a coiled-coil domain (CCD) and a C-terminal fibrinogen-like domain (FLD). Functional studies have highlighted a role for the SCD and CCDs in forming high order Ang homotypic multimers. The specific nature of these multimers is variable and seems to be unique to each Ang family member. Binding specificity of the Angs for the Tie 2 receptor has been ascribed to the FLD. Taken together, unique

structural attributes of each Ang family member promotes binding and differential clustering of Tie 2. The pleiotropic physiological effects of Ang 1-4 are thought to at least in part be mediated by appropriate and specific clustering of the receptor. For instance mice engineered to overexpress the CCD of Ang 1, capable of heterodimerizing with endogenous Angl produced in the same cell, caused improper patterning of the coronary vessels. Furthermore, chimeric forms of Ang 1 engineered to contain the C-terminal FLD and one of several different CCDs differed in their ability to activate the Tie 2 receptor.

Genetic knock out or transgenic overexpression of the Angs in mice has revealed a central role for these growth factors in angiogenesis, heart development and primitive hematopoiesis. Genetic loss of Ang 1 closely phenocopies the Tie 2 knock out mice. These embryos die by E12.5 due to endocardial and myocardial defects, decreased vascular complexity and an inability of the endothelial cells to form necessary interactions with periendothelial cells (Suri *et al.*, 1996). Ang 2 knock out mice have been examined in the context of two different genetic backgrounds, 129SV and C57BL/6. Knock out mice generated on the 129SV background are born live but succumb to defects associated with lymphatic drainage (Gale et al., 2002), while loss of Ang 2 in the C57BL/6 background results in non-lethal defects associated with lymphatic hypoplasia and aberrant recruitment of periendothelial cells to lymphatic vessels (Shimoda et al., 2007). Forced overexpression of Ang 1 and 2 has been reported in the literature with some conflicting observations most likely related to differing experimental approaches. Overexpression of Ang 1 under control of the keratin 14 (k14) promoter gives rise to viable mice with larger, more branched vessels of the skin that are resistant to VEGF

induced permeability, while Ang 1 overexpression under the control of heart specific promoter MHCA, results in the death of 90% of the mice at E12.5-15.5 due to dilated atria, significant thinning of the myocardial wall and outflow tract disturbances (Ward et al., 2004b). Mice engineered to overexpress Ang 2 under control of the Tie 2 promoter are non-viable by day E10.5 and succumb to defects in endocardial-myocardial separation and vascular defects described as "moth eaten" in appearance (Maisonpierre *et al.*, 1997). In a different approach, Bureau and Dumont *et. al.*, examined the effects of systemically elevated Ang 2 concentration produced transgenically by way of the liver-specific Lap tTa promoter (Bureau et al., 2006). These animals survive gestation but exhibit enlarged myocardial and pulmonary vasculature, decreases in endocardial vasculature and vascular leakage which could not be attributed to concomitant increases in VEGF secretion (Bureau *et al.*, 2006). The studies mentioned above serve to highlight the degree to which not only Ang 1 and 2 concentrations must be regulated, but also the critical necessity for exquisite temporal and spatial control of expression.

Angiopoietin-Dependent Cell Signaling

Although genetic studies have provided a gross understanding of the indispensable role that Tie 2 and the Angs play in the growth and maintenance of the embryonic and adult vasculature, much has been learned from examinations of Ang-dependent cell signaling events. While they elicit very different physiological effects in the context of the animal, Ang 1 and 2 seem to activate many of the same signaling pathways *in vitro*, the best known of which are MAPK and Akt. Interestingly, activation of Tie 2-dependent MAPK does not result in a pro-proliferative response, but rather is thought to contribute to the

morphogenic effects seen upon receptor activation (Suri *et al.*, 1996;Koblizek et al., 1998;Witzenbichler et al., 1998;Kwak et al., 1999). Low levels of constitutive Tie 2 phosphorylation have been reported in quiescent endothelium and it is believed that this event provides pro-survival, maturation and stabilization signals to the endothelium via activation of Akt (Kwak *et al.*, 1999). Furthermore, Tie 2-dependent Akt activation has been shown to directly phosphorylate eNos, an enzyme known to be a key mediator of endothelial cell growth, survival, angiogenesis and vascular tone (Babaei et al., 2003;Chen et al., 2004). Ang 1 and 2 have also been identified as key regulators of inflammatory response and seem to play opposing roles. Using experimental models of peritonitis Fiedler U., and Augustin HG., *et. al.*, described a mechanism in which autocrine secretion of Ang 2 was shown to antagonize Ang 1-dependent inhibition of NF-κB, while the pro-inflammatory effects of Ang 2 include NF κB-dependent increases in ICAM-1 and VCAM-1, molecules which play a critical role in leukocyte-endothelial cell adhesion and extravasation (Fiedler et al., 2006). Activation of the Tie 2 receptor has also been shown to facilitate release of MMPs 2 and 9 and promote migration through recruitment of a signaling complex which includes Dok-R, Nck and Pak. Tie 2-independent signaling has recently been described for both Ang 1 and 2. Dallabrida S., *et. al.*, reported a dose-dependent cell adhesion effect for Ang 1 and 2 in non-Tie 2 containing cells such as skeletal and cardiac myocytes (Dallabrida et al., 2005). The increased adhesion noted could be blocked by the addition of cationic chelators or RGD-based peptides, suggesting a role for integrins in mediating this effect (Dallabrida *et al.*, 2005). In support of this theory, several independent reports have demonstrated Ang 1 can directly bind several different integrins including $\alpha 2\beta 1$, $\alpha 5\beta 1$, $\alpha v\beta 3$ and $\alpha v\beta 5$

(Carlson et al., 2001;Weber et al., 2005;Cascone et al., 2005;Dallabrida *et al.*, 2005). Consistent with these findings, Ang 1 has been shown to confer potent anti apoptotic effects to myocytes that have been either serum starved or treated with taxol. These effects are significantly blocked upon pre-incubation with anti-β3 antibodies or through chemical inhibition of Akt or MAPK (Dallabrida *et al.*, 2005).

Therapeutic Antiangiogenesis

Post embryonic angiogenesis and vasculogenesis occur rarely in a normal physiological setting. Wound repair and female reproductive health are two exceptions which necessitate a functional and intact angiogenic response. The overwhelming majority of post embryonic vessel formation or pruning involves some form of pathology. As such, the ability to modulate the angiogenic response for therapeutic gain has been profusely examined. Two primary forms of pathological angiogenesis exist. They can be crudely characterized by a relative absence or reduction in vascular density as seen in ischemic limb disease or following myocardial infarct or an overgrowth of dysfunctional poorly ordered and leaky vessels as seen in cancer, rheumatoid arthritis, psoriasis or diabetic retinopathy.

Inhibition of angiogenesis as an adjunct to conventional cancer therapy has recently moved into practice. Drugs like Avastin, which was specifically engineered to target vascular endothelial growth factor (VEGF), are showing high clinical efficacy by cutting off critical blood flow to tumor cells. At least 70 antiangiogenic compounds are in the development pipeline of various pharmaceutical companies. Although much of the focus

in reducing pathological angiogenesis has been firmly aimed at improving cancer treatment outcomes, a more recent concept which examines angiogenesis and its influence in modulating inflammation, has lead to great interest in the potential of treating chronic inflammatory disease states with endothelium-specific compounds. For instance, Regeneron Pharmaceuticals is currently exploring the utility of treating age related macular degeneration and diabetic retinopathy, two disease states characterized by overgrowth of fragile, leaky, inflamed vessels in the eye, with the VEGF TRAP, a novel VEGF inhibitor (Saishin et al., 2003;Nguyen et al., 2006). Inflammatory diseases of the bowel, such as Crohn's and ulcerative colitis, are now thought in part to be regulated by a chronically inflamed endothelium which promotes leukocyte extravasation and resulting tissue remodeling (Hatoum et al., 2006). It however remains unknown whether endothelial inflammation in the bowel is a consequence or a contributing factor to the underlying etiology of the disease. Therapeutic approaches aimed at treating microvascular disturbances in diseases of the bowel are currently undergoing investigation and may be useful in resolving this issue. Perhaps the best evidence in support of the treatment of a chronic inflammatory state with an angiomodulatory approach derives from studies of rheumatoid arthritis (Veale and Fearon, 2006). Angiogenesis is thought to play a central role in the development of pannus formation in the inflamed synovium of affected joints and inhibition of this process with the antiangiogenic compound endostatin has been shown to offer benefit in an experimental mouse model of rheumatoid arthritis (Stupack et al., 1999;Matsuno et al., 2002).

Therapeutic Angiogenesis

Although surgical approaches to correct and restore collateral blood flow to ischemic tissues have steadily improved many pathological states exist due to defects in small vessels. These vessels are not amenable to correction with surgical approaches, and even if they were, the underlying defects that lead to the dysfunctional vasculature are likely to remain. Because of this, therapeutic modulation of angiogenesis with delivery of interventional compounds continues to be an intense field of research. Clinical trials have explored the possibility of promoting angiogenesis through the application of proangiogenic growth factors. Thus far, clinical studies aimed at promoting angiogenesis have focused primarily on the addition of a single growth factor-most often VEGF. VEGF is highly angiogenic but produces vessels that are poorly ordered and highly leaky. Many other studies, designed to specifically assess the benefit of proangiogenic growth factors including PDGF, FGF2, EGF, and TGFβ in the treatment of poorly vascularized foot and leg ulcers have been reported in the literature (Robson et al., 1992;Falanga et al., 1992;Richard et al., 1995a;Harding et al., 2002). However, thus far, only Becaplermin (Regranex ®, Johnson & Johnson), a topical preparation of PDGF BB, has received regulatory approval for the treatment of diabetic foot ulcers, and its utility in increasing angiogenesis in these wounds remains unclear. The lack of success noted in these trials is perhaps due to the fact that while in theory, it is possible to inhibit angiogenesis by targeting a single determinant event, promoting angiogenesis generally requires the coordinated action of several different growth factors.

Therapeutic modulation of the Tie 2 receptor

Therapeutic modulation of the Tie 2 receptor may present benefits that exist beyond that which most other single growth factors demonstrate due to its highly pleiotropic nature (Peters et al., 2004;Li et al., 2005;Brindle et al., 2006). Data in support of this hypothesis are many fold. For instance, beyond the well known roles of Ang 1 in promoting endothelial cell migration, reducing apoptosis and providing signals necessary to recruit supporting periendothelial cells, Ang 1 has more recently been studied for the profound anti inflammatory properties it demonstrates on the endothelium (Thurston et al., 1999). In addition to counteracting VEGF-induced vascular permeability and restoring normal vessel growth patterning (Thurston *et al.*, 1999), Ang 1 has been shown to reduce Endothelin-1 release, a potent proinflammatory mediator known to be important in many diseases such as atherosclerosis and acute respiratory disease syndrome (ARDS) (McCarter et al., 2006). Autocrine regulation of Tie 2 by Ang 2 has been shown to increase the expression of ICAM-1 and VCAM, sensitize endothelial cells to the effects of TNF α, and thus promote extravasation of leukocytes (Fiedler *et al.*, 2006). Fiedler U., *et al.*, propose a mechanism whereby in vascular endothelial cells Ang 1 acts as a counterbalance to the proinflammatory effects of Ang 2, and that the anti-inflammatory effects of Ang 1 are mediated through inhibition of NF-κB (Fiedler *et al.*, 2006). The work of Fiedler U., *et al.*, provide the specific framework for explaining the pro and anti-inflammatory effects of Ang 2 and 1 respectively, while also offering a more general understanding of the importance of vascular inflammation and its role in the genesis or progression of many different inflammatory disease states.

Targeted Use of Ang 1 in Wound Care

Traditional wound care treatments such as sutures and staples are rapidly being replaced by advanced therapeutic modalities such as moist type dressings and advanced products which contain natural growth stimulating factors. Clinical indications for wound therapy and management include trauma and surgery, diabetes, and age related skin or pressure ulcers. Global estimates report that 12.5 million patients worldwide suffer from chronic wounds and of the estimated 4 million in the U.S., approximately 3 million suffer from pressure ulcers and diabetic foot ulcers. The morbidity associated with these ulcers includes pain, odor, infection, sepsis, and amputation. Wound healing involves a well choreographed series of molecular activities that ultimately lead to wound closure. These events are driven by three processes: inflammation, cellular proliferation and angiogenesis. The inflammatory response, thought to occur just after wounding, involves the transmigration of macrophages through blood vessels into the wound site. The macrophages secrete a complex mixture of growth factors and cytokines which promote cellular proliferation and angiogenesis. Defects in any single key process will severely hamper or prevent wound closure from occurring. Chronic wounds, such as neuropathic or neuroischemic foot ulcers, are often seen in diabetic patients. These wounds may arise from the patient's inability to sense injury (neuropathic) or due to microvascular defects (neuroischemic). It is well known that many of the perturbations noted in diabetics are linked to micro and macroangiopathies (Parving et al., 1976;Williamson and Kilo, 1977;La Fontaine et al., 2006). The principle functions of the small vessels in the foot are to supply nutrients and remove metabolites. This exchange occurs at the capillary layer, but in the case of advanced diabetes, thickening of the basement membrane surrounding

the capillaries, impairs normal hemodynamics (Parving et al., 1983). Parving H., *et al.*, were the first to describe the hemodynamic defects associated with the diabetic foot (Parving *et al.*, 1983). In so doing, they described a situation in which hyperglycemic-dependent increases in microvascular blood pressure contribute to basement membrane thickening, vascular permeability, and endothelial wall sclerosis (Parving *et al.*, 1983). These changes contribute to an overall inability of the vasculature to autoregulate and eventually will lead to endothelial cell death. Animal studies have highlighted a potential role for the Angs in the genesis and progression of diabetic angiopathies. Specifically, Kampfer H., *et al.*, examined the steady-state and post wound expression profiles of Ang 1, 2 and VEGF in normal and the genetically diabetic mouse line (db/db) (Kampfer et al., 2001). They find that whereas Ang 2 is increased in both the normal and db/db mouse upon wounding, only in the case of the db/db mouse does this induction persist beyond seven days (Kampfer *et al.*, 2001). And while this increase in Ang 2 was marked by a concomitant and transient increase in VEGF expression and resultant angiogenesis in the early phase of diabetic wound healing, the protracted increase in Ang 2 levels lead to marked vascular death (Kampfer *et al.*, 2001). Moreover, the authors report a general reduction in Tie 2 levels in the skin of db/db mice and an almost complete loss of Tie 2 expression in wounded tissue (Kampfer *et al.*, 2001). These data provide strong support for the notion that the Tie 2 signaling axis may be perturbed during human diabetic wound healing and provide impetus for experimental approaches which are designed to reestablish appropriate Tie 2 functioning. Subsequently, Cho C., *et al.*, were able to demonstrate the therapeutic benefit of treating excisional full thickness, db/db mouse skin wounds with a topically administered Ang 1 mimetic, COMP-Ang 1 (Cho et al., 2006).

They show that improved wound closure times correlate with increases in angiogenesis, blood flow, lymphangiogenesis and dermal and epidermal reconstruction (Cho *et al.*, 2006). Described herein (Chapter 2) we examine the use of our own novel Ang 1 mimetic, Vasculotide, in the treatment of excisional full thickness wounds performed in the context of the db/db mouse strain. We detail the construction of this novel proangiogenic compound, its target specificity for the Tie 2 receptor, its use in several *in vitro* and *in vivo* angiogenesis assays and propose future experiments to address potential therapeutic efficacy in experimental models of diabetic retinopathy, ARDS and hypertension (Chapter 2 and 5).

Chapter 2

Construction and Characterization of Vasculotide: A Novel Angiopoietin Peptide-Mimetic

A version of this chapter is submitted to Diabetes and is currently under review:

Van Slyke, P., Alami, J., Martin, D., Kuliszewski, M., Leong-Poi, H., Sefton, M., and Dumont, D. (2006) **"Acceleration of Diabetic Wound Healing by an Angiopoietin Peptide-Mimetic".**

Abstract:

Global estimates report that 12.5 million patients worldwide suffer from chronic wounds and a significant number of these individuals suffer from decubitus ulcers and diabetic foot ulcers. Wound healing involves a well choreographed series of molecular activities that ultimately lead to wound closure. These events are driven by three interrelated processes: inflammation, cellular proliferation and angiogenesis. Impaired angiogenesis is one of several primary defects reported in diabetic patients. These patients often suffer from impaired wound healing, and as such suffer significant morbidity associated with vascular compromise (Dinh and Veves, 2005). Many of the signalling pathways that drive the angiogenic response originate at the plasma membrane and emanate from the activation of endothelial cell receptor tyrosine kinases, including Tie2/Tek (Jones et al., 2001a;Olsson et al., 2006). Angiopoietin (Ang) members 1-4 constitute a family of protein growth factors, all of which have been shown to activate Tie 2 receptor activity to differing extents. Primary defects in growth factor secretion and/or proteolytic cleavage of growth factors in diabetic wounds has been reported suggesting therapeutic application of these factors may be beneficial (Wieman et al., 1998;Tsang et al., 2003). Here we show that a short angiopoietin peptide-mimetic can increase the production of vessels in a diabetic wound leading to accelerated wound closure. We found that when a short peptide known to bind to Tie2 was tetramerized it led to the activation of Tie2 and its associated signalling pathways. This activation resulted in the production of both *in vitro* and *in vivo* angiogenic responses. Our results demonstrate that activation of the pleiotropic Tie2 signalling axis by a single compound can produce blood vessels that are highly organized and well supported by myogenic support cells. Although the potential of

this novel proangiogenic compound in regenerative medicine is not strictly limited to topical administration, we provide evidence as a proof of principle in support of its efficacious use in wound healing.

Rationale: The impetus for the work described in this chapter is several fold. Initially, the desire to construct a novel Ang mimetic stemmed from the fact that purification of native family members 1-4 has proven very difficult and only recently have these ligands become a commercially available reagent. Once purified in their commercial form, stability, solubility, cost and batch variation of the Ang's has severely hampered basic research on the native ligands and their receptors Tie 1 and 2. Early work aimed at elucidating the developmental/physiological/pathophysiological role of Tie2 and the Ang signaling axis relied heavily upon gene ablation and overexpression studies. *In vitro* studies conducted by the Dumont lab and others have utilized Ang 1 and 2 conditioned media and Tie2 transfection studies. Although these approaches were state of the art at the time and yielded much of what is currently known about Tie2 and the Ang's they were difficult to manipulate and to interpret. In addition to the obvious benefits derived from having a high quality source of native ligand for research purposes, there has been growing interest in the potential therapeutic application of these ligands. This interest, discussed in several critical reviews in the field, highlights the beneficial, or potential benefit of using the Ang's to modulate activity and/or function of the Tie 2 receptor. Several groups have attempted to circumvent the inherent difficulties associated with working with the native Ang's by constructing novel chimeric proteins. These variant forms include COMP-Ang 1, Ang 1* and Bow-Ang 1. This chapter describes the novel

invention and early characterization of Vasculotide, a novel, semi-synthetic Ang peptide mimetic.

Introduction:

All the Ang's are characterized structurally by an N-terminal super clustering domain (SCD) followed by a coiled-coil domain (CCD) and a C-terminal fibrinogen-like domain (FLD) (Ward and Dumont, 2002;Tsigkos et al., 2003). Functional studies have highlighted a role for the SCD and CCD's in forming high order homotypic Ang multimers (Procopio et al., 1999). The specific nature of these multimers is variable and seems to be unique to each Ang family member. Binding specificity of the Ang's for the Tie 2 receptor has been ascribed to the FLD. Taken together, unique structural attributes of each Ang family member promotes binding and differential clustering of Tie 2. The pleiotropic physiological effects of Ang 1-4 are thought to at least in part be mediated by appropriate and specific clustering of the receptor (Procopio *et al.*, 1999;Davis et al., 2003;Cho et al., 2004;Ward et al., 2004a). Gene ablation and transgenic approaches in mice have highlighted an indispensable role for Ang 1 and 2 in the development and maintenance of the blood and lymphatic vascular systems as well the hematopoietic system. Non-genetic studies of the Ang's have been hampered by the inherent difficulty associated with their purification, stability and solubility.

Materials and Methods:

Cell Culture and Western Blotting: Human umbilical endothelial cells (HUVEC) (Cambrex, New Jersey) were grown on 6-well or 10 cm plates (Nunc) coated with gelatin

(Sigma). Huvec's were cultured in F12 growth medium containing 10% fetal bovine serum (FBS), 0.1 mg/ml heparin sulphate, 1x penicillin, 1x streptomycin, 1x glutamine, VEGF 10ng/ml, EGF 10ng/ml, bFGF 5ng/ml. All Huvec's were used between passage 3-9. Cos 1 and C166 cells (ATCC) were maintained on 10-cm-diameter plates (Nunc) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (FBS), 1x penicillin, 1x streptomycin, and 200 mM L-glutamine (all Gibco BRL) in a 5% CO2 incubator at 37°C. Cell lysate preparation and Western blotting was performed as previously described (Jones and Dumont, 1998b).

Antibodies For Western Blotting, Immunofluorescence and IHC: The following commercially available antibodies were used: monoclonal anti Tie 2 (Pharminogen), polyclonal anti pY992 Tie 2, (Cell Signaling Technology), polyclonal anti Mapk (Cell Signaling Technology), monoclonal anti phospho Mapk (Cell Signaling Technology), polyclonal ant Akt (Cell Signaling Technology), polyclonal anti pS473 Akt (Cell Signaling Technology), monoclonal anti pS1177 eNos (BD Biosciences), polyclonal ant pY397 Fak (Biosource), polyclonal anti Fak (Santa Cruz), polyclonal anti smooth muscle actin-Cy3 direct conjugate (Dako), polyclonal anti PECAM (Pharminogen), polyclonal anti smooth muscle actin 1 (Sigma).

Peptide Synthesis and Biotinylation: Solid Phase Peptide Synthesis (Fmoc-SPPS) was utilized to manufacture Vasculotide peptide. Briefly, peptides were synthesized using Applied Biosystems' ABI433A Peptide Synthesizer (Foster City, CA, USA) using manufacturer's instructions with slight modification. Modifications include the following: coupling time was extended to 17.5 min. 2) HBTU was substituted by HATU. The resin used was Wang resin. T7 amino acid sequence (N-terminus to C terminus): Cys-His-His-

His-Arg-His-Ser-Phe. The protection groups for the side chain residue were: Cys-(tBu), His-(Trt), Arg-(Pbf), Ser-(tBu). Amino acid derivatives used here were the L-form to mimic the natural folding conformation in mammalian cells. The cleavage cocktail used to cleave T7 peptide off the solid phase resin support consist of: 90%Trifluoro acetic acid (TFA), 8% TIPS, 2% EDT. The cleavage was performed under nitrogen gas for 3 hours. The cleavage cocktail and the resin mixture were separated by means of Kimax Fritted funnel. T7 peptide was then precipitated with cold ether and centrifuged at 2700x g for 5 min at 4 ^{0}C. The pellet was washed for a total of four times with cold ether. Each wash was carried out by 30 seconds vortexing, and 5 min centrifugation at 2700x g at 4 ^{0}C. Finally, T7 peptide pellet was dried under nitrogen gas. Biotin was conjugated to the above T7 peptide by using EZ-link-PEO-maliemide-biotin (Pierce) according to the manufacturer's instruction.

Stimulations: Cells were stimulated in full serum containing growth media for indicated times with angiopoetin 1(R&D Systems), or VEGF (R&D Systems). Biotinylated Vasculotide peptide was either left unclustered or preclustered with a 4:1 molar ration of peptide:avidin in PBS for 2 hours at 4^{o}C. Stimulations with Vasculotide were performed in full serum containing growth media for 15 minutes unless otherwise indicated.

Boyden Chamber Migration Assay: HUVEC cells were seeded at a density of 8.4 x10^4 cells in 500 ml of F12 media plus 0.1% FBS in the upper chamber of an 8um-pore modified Boyden chamber (Falcon). 500 ul of F12 media plus 0.1% FBS plus various growth factors or controls (as indicated) were placed in the bottom chamber. Cells were allowed to migrate for 4 h in a 37 °C, 5% CO2 incubator. Nonmigrating cells were scraped off, and filters were fixed in 100% methanol for 5 min, stained with

Harris'Hematoxylin (BDH) for 10 min, and washed twice with tap water for 3 min each. Filters were then mounted using Aquapolymount mounting medium. Cells that had migrated more than 50% of the way through the pore were scored as positive for migration.. Student's *t* test was used to test the statistical significance with a 95% confidence interval. All experiments were performed with three replicates each and 5 random counts were taken per replicate.

Zymography: Huvec's cultured in F12 media plus 0.1% FBS were stimulated for 16 h with various factors as indicated. Conditioned media was centrifuged to remove cellular debris and prepared for gel electrophoresis using non-reducing conditions. Gels were washed twice for 30 minutes in 2.5% Triton X-100 to remove sodium dodecyl sulfate and were then incubated in substrate buffer (50 mmol/L Tris-HCl, pH 8.8, 5 mmol/L CaCl2) for 16 hours at 37°C. Gels were then stained with 0.5% Coomassie blue in 30% methanol/10% acetic acid for 2 hours at room temperature and destained in 50% methanol/10% acetic acid. The presence of metalloproteinases was indicated by unstained proteolytic zones in the gel.

Matrigel Plug Assay: Growth factor reduced matrigel (BD Biosciences), impregnated with various factors, was injected subcutaneously into the flank region of 3 month old CD1 mice. 14 days post implantation and immediately prior to sacrifice FITC lectin (100 μg) was injected IV and allowed to circulate for 10 minutes. Plugs were surgically resected and fixed in 4% PFA for 16 h. Images of vascular topology were taken using a Zeiss dissecting microscope at 4x magnification. Upon fixing, all plugs were whole mount stained with anti-Sma1-Cy3. Plugs were analyzed on a Zeiss Axiovert 100 M confocal microscope (Carl Zeiss) for three dimensional image reconstruction and photos

were processed using LSM Image Browser and Adobe Photoshop 7.0. Morphometric quantification of vessel characteristics was performed by skeletonizing confocal image projections with Image Processing Tool Kit 5.0 (IPTK 5.0). Student's *t* test was used to test the statistical significance with a 95% confidence interval. All experiments were performed with three replicates each, with at least 6 representative image stacks.

Wound Healing Assay: Pathogen free, nine week old B6.Cg-m(+/+)Lepr(db)/J (db/db) mice were purchased from Jackson Laboratories. Circular, full excisional, 6mm diameter wounds were performed according to animal committee approval guidelines. Indicated factors were suspended in sterile Intrasite Topical Gel (Smith and Nephew) at noted concentrations. Treatments were as follows: Vehicle n=8, bFGF (10μg/ml) n=8, clustered Vasculotide (20nM) n=4, clustered Vasculotide (5nM) n=4. Preparations were applied at day 0 (time of wounding), day 2, 4 and 6. Standardized images were taken on days 0, 2, 4, 6 and 7 using a Canon EOS digital Rebel camera. Image J (NIH) was used to manually outline wound perimeter from which total pixel counts (wound area) were determined. Mice were sacrificed on day 7 and full wound beds including margin were excised and fixed in 4% PFA. Wounds were bisected and each half was prepared for either paraffin or frozen sections. Wax sections were processed according to standard protocols for H&E and Masson's Trichrome stain. Images were captured using a Leica DMLS compound light microscope and Pixel Link camera at 5X and 20X. Student's *t* test was used to test the statistical significance.

Histological Score: Histologic sections were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned and stained with hematoxylin and eosin and Masson's trichrome for histologic evaluation.

Sections were evaluated by a pathologist for re-epithelialization, granulation tissue formation, and inflammation.

Criteria used for scoring skin:

Re-epithelialization:

0 - none

1 – minimal (0-20% regrowth from wound margins)

2 – mild (25% to 50% regrowth)

3 – moderate (>50% regrowth, up to 100% but not > 2 cells thick along length)

4 – complete regrowth (epithelium > 1 cell thick along length, keratinized)

Granulation tissue (GT) and neovascularization:

0 - none

1 – minimal (1-3 small, isolated islands of GT at margins of defect)

2 – mild (multifocal, patchy islands of GT underlying tissue defect, <10 new blood vessels)

3 – moderate (locally extensive bands of GT underlying length of defect)

4 – marked (dense bands of GT at margins and underlying length of defect with collagen fibrils and numerous blood vessels)

Inflammation (within defect):

0 - none

1 – minimal (a few scattered neutrophils at margins of defect)

2 – mild (multifocal aggregates of up to 5 neutrophils underlying defect)

3 – moderate (multifocal aggregates of up to 10 neutrophils and occasional macrophages, minimal to mild edema)

4 – marked (locally extensive, dense neutrophilic infiltrates with lesser numbers of macrophages, mild-moderate edema)

Adenoviral Infections: Cos1 or C166 cells were infected (MOI 30) overnight with adenoviruses encoding one of either EGFP or Tie 2. Infection efficiency was confirmed by epifluorescence (EGFP), or by immunoblot (Tie 2).

Cell Death ELISA and MTS Assay. Huvec's were maintained in F12 media plus 0.1% FBS for 16 h in the presence of various concentrations of unclustered or clustered Vasculotide. Analysis of apoptosis was performed using Cell Death ELISA Plus (Roche) according to manufacturers' specifications. C166 cells infected with either EGFP or Tie2 were maintained in DMEM plus either 10% FBS or 0% FBS. Cells were treated with avidin, unclustered Vasculotide or clustered Vasculotide for 16 h after which MTS reagent was applied to all samples for 4 h. Asbsorbance at 492 was measured using a multiwell plate reader (Power Wave X340, Biotek Instruments Inc).

Pull-Down Assay: EaHy926 endothelial cell lysate prepared in Phospho Lipase Cγ lysis buffer (50mM Hepes buffer pH 7.5, 150mM NaCl, 10% glycerol, 1% Triton X-100, 1.5mM MgCl2, 1.0mM EGTA, 10mM NaPPi, 100nM NaF, 2mM Na3VO4, 1x aprotinin, 1x leupeptin and 1x PMSF) was mixed with either biotinylated Vasculotide or biotinylated irrelevant peptide. Biotinylated peptides were isolated with avidin agarose and subsequently tested for their ability to precipitate Tie 2 via immunoblot analysis.

Results:

Recently Tournaire and Pouyssegur *et al.*, using a phage display screening approach, identified several short Tie 2 binding peptides (Tournaire et al., 2004). Here we use the

core sequence of one of those peptides and knowledge of optimal Tie 2 receptor clustering (Procopio *et al.*, 1999;Davis *et al.*, 2003) to specifically engineer a novel receptor agonist. T7 peptide (-His-His-His-Arg-His-Ser-Phe-) (Tournaire *et al.*, 2004) was modified by addition of an amino terminal cysteine to facilitate sulfhydryl-mediated addition of a 29 angstrom biotinylated polyethylene oxide (PEO)-maleimide moiety (Fig 2.1a). Post synthesis clustering of this biotinylated peptide-PEO, referred to hence forth as Vasculotide, with avidin gave rise to an obligate tetrameric compound. The ability of Vasculotide to bind the Tie 2 receptor, despite the engineered modifications, was tested using an *in vitro* pull down assay. Non clustered Vasculotide was mixed with whole cell lysate isolated from EaHy926 endothelial cells (EC's). Vasculotide, but not irrelevant biotinylated peptide, was able to precipitate Tie 2 (fig 2.1b). Addition of a ten fold excess of Vasculotide preclustered with soluble avidin was able to compete for available Tie 2 and was able to abolish this interaction (final lane).

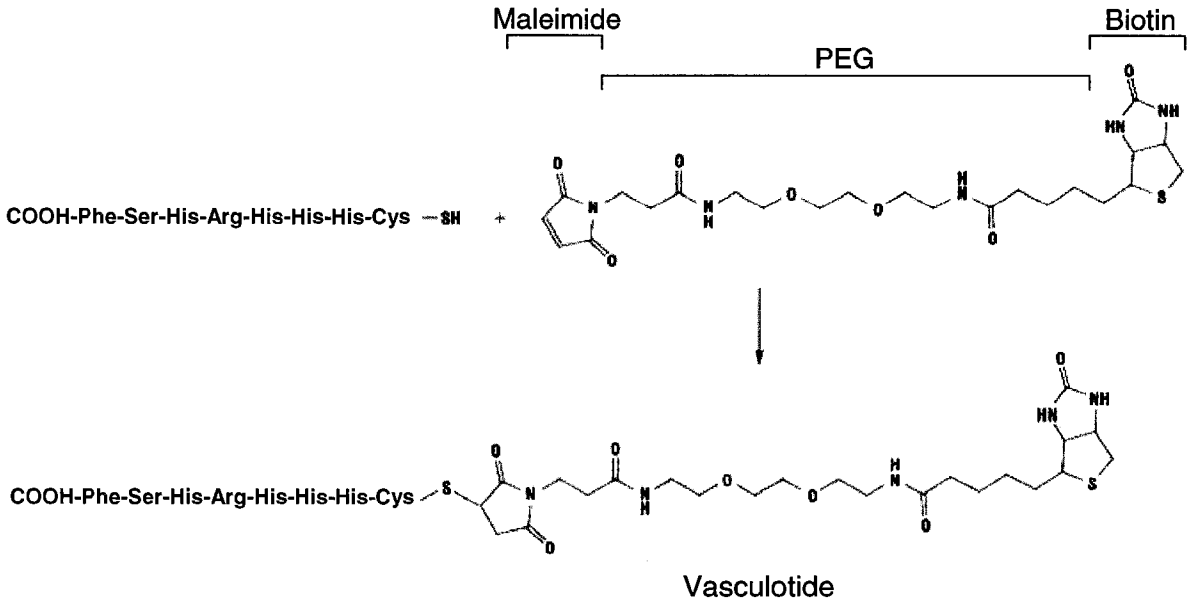


Figure 2.1a: Schematic of unclustered Vasculotide. An eight amino acid, Tie 2 binding peptide (Cys-His-His-His-Arg-His-Ser-Phe-)was covalently linked to a 29 angstrom maleimide-PEO2-biotin spacer. The resulting construct, termed Vasculotide, was subsequently clustered by way of avidin.

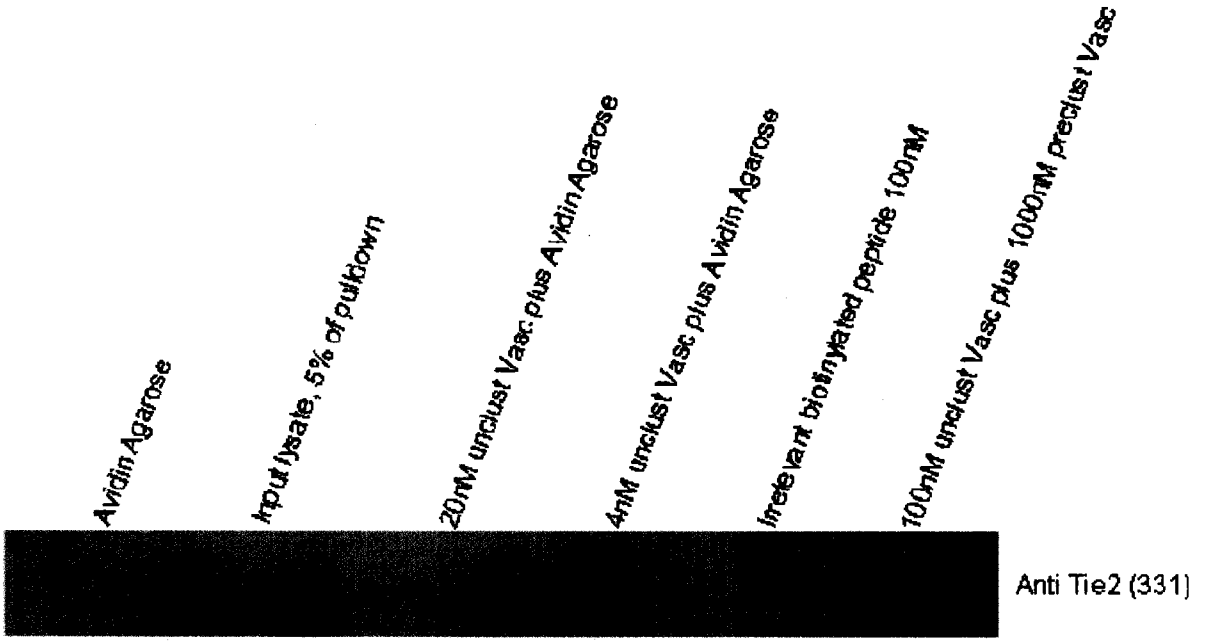


Figure 2.1b: Modified T7 (Vasculotide) Co-precipitates Tie2. Unclustered Vasculotide, but not irrelevant biotinylated peptide, specifically binds Tie2 in an in vitro pull-down assay.

Having established that Vasculotide was able to bind Tie 2 we tested its ability, when clustered with avidin in a 4:1 ratio (Vasculotide:avidin), to activate the Tie 2 receptor and several well established downstream signalling pathways (Fujikawa et al., 1999;Kim et al., 2000;Babaei *et al.*, 2003). Human umbilical endothelial cells (HUVEC) were stimulated with each one of the following: Ang 1, Vasculotide, avidin or clustered Vasculotide at various doses. Immunoblot analysis of whole cell lysates revealed that clustered Vasculotide activates Tie 2 (pY992 Tie 2) at concentrations ranging from 20nM to 750pM, with 5-10nM appearing optimal (fig 2.1c). Significantly, only when preclustered with avidin was Vasculotide able to stimulate Tie 2 phosphorylation (see avidin alone and unclustered Vasculotide). Coincident with receptor activation we show phosphorylation of several known Tie 2-responsive proteins including Mapk, Akt and eNos. Paradoxically, high concentrations of clustered Vasculotide are not capable of activating Tie 2 receptor activity in HUVEC's (see clustered Vasculotide 50nM). We reasoned that this was due to the fact that high ratios of clustered Vasculotide:Tie 2 would decrease receptor clustering. To test this hypothesis we utilized Eahy926 EC's which express much higher levels of Tie 2. Using this cell line we were able to demonstrate specific activation of the Tie 2 receptor at concentrations ranging from 200μM to 750pM (fig 2.1d), suggesting that the ratio of clustered Vasculotide:Tie 2 is critical for activation.

To examine Tie 2 activation kinetics, we stimulated HUVEC's with native Ang 1 or clustered Vasculotide for various times. Overall, activation of the receptor by either Ang 1 (400ng/ml) or clustered Vasculotide (2nM) followed a very similar time course, with phosphorylation returning to almost basal levels sometime after 2 hours (fig 2.1e).

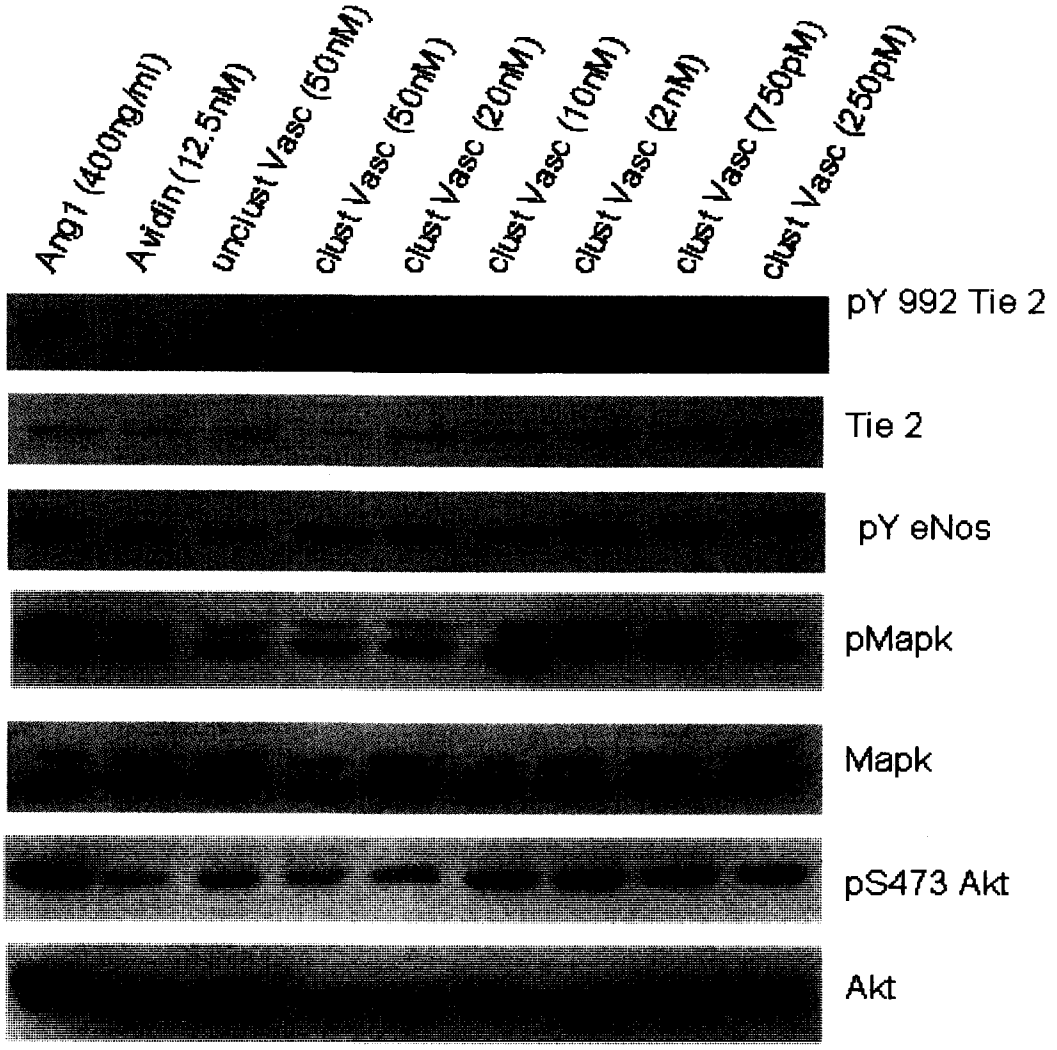


Figure 2.1c: Activation of downstream Tie2-specific signaling pathways. Treatment of HUVEC's with clustered Vasculotide results in the activation of Tie 2 and downstream signaling proteins eNos, MAPK, and Akt.

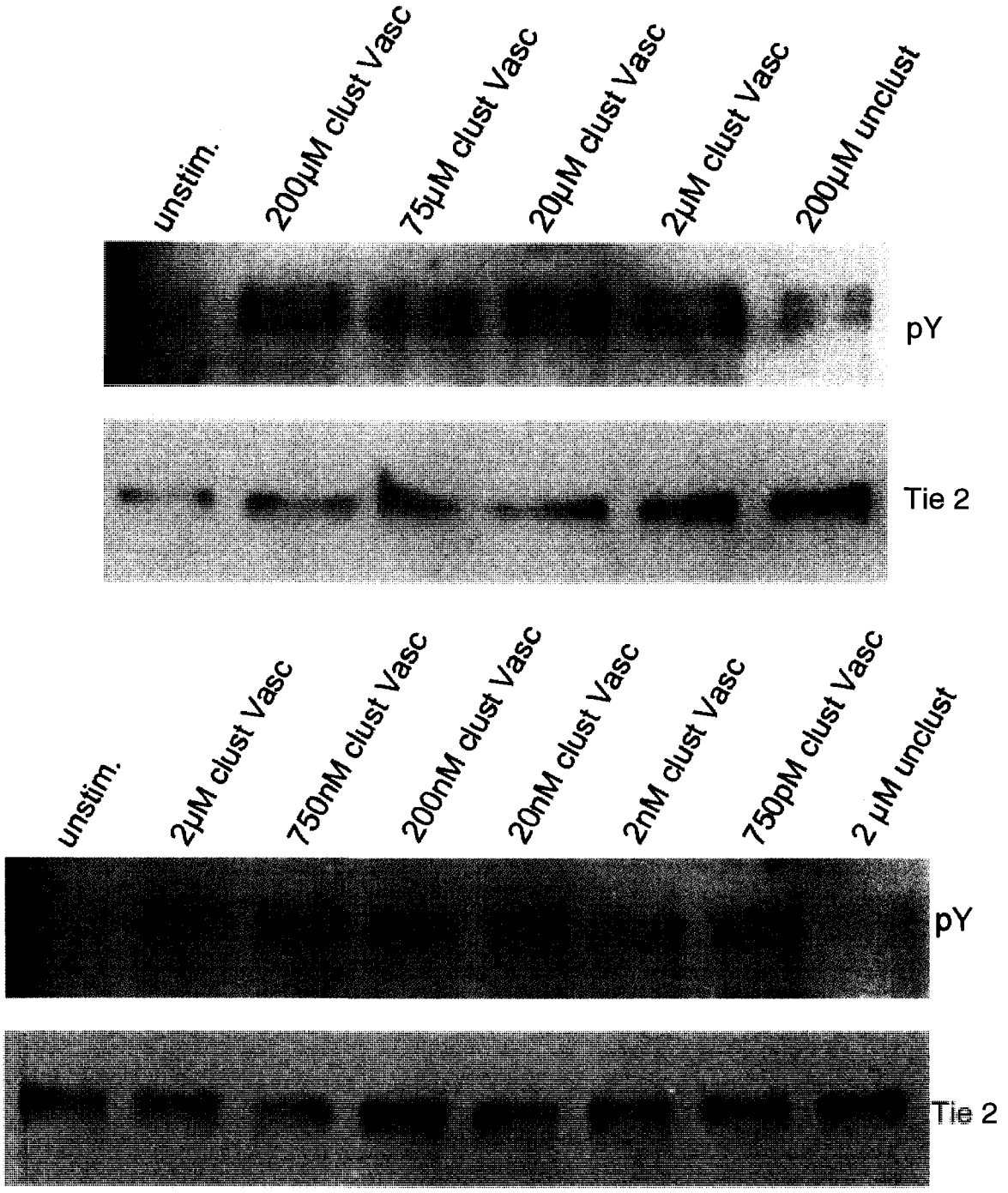


Figure 2.1d: Vasculotide-dependent activation of EaHy946 Tie 2 activity. Immunoprecipitation of Tie 2 followed by anti-phosphotyrosine (4G10) IB demonstrates Vasculotide activates Tie 2 receptor phosphorylation at concentrations ranging from 200μM to 750pM.

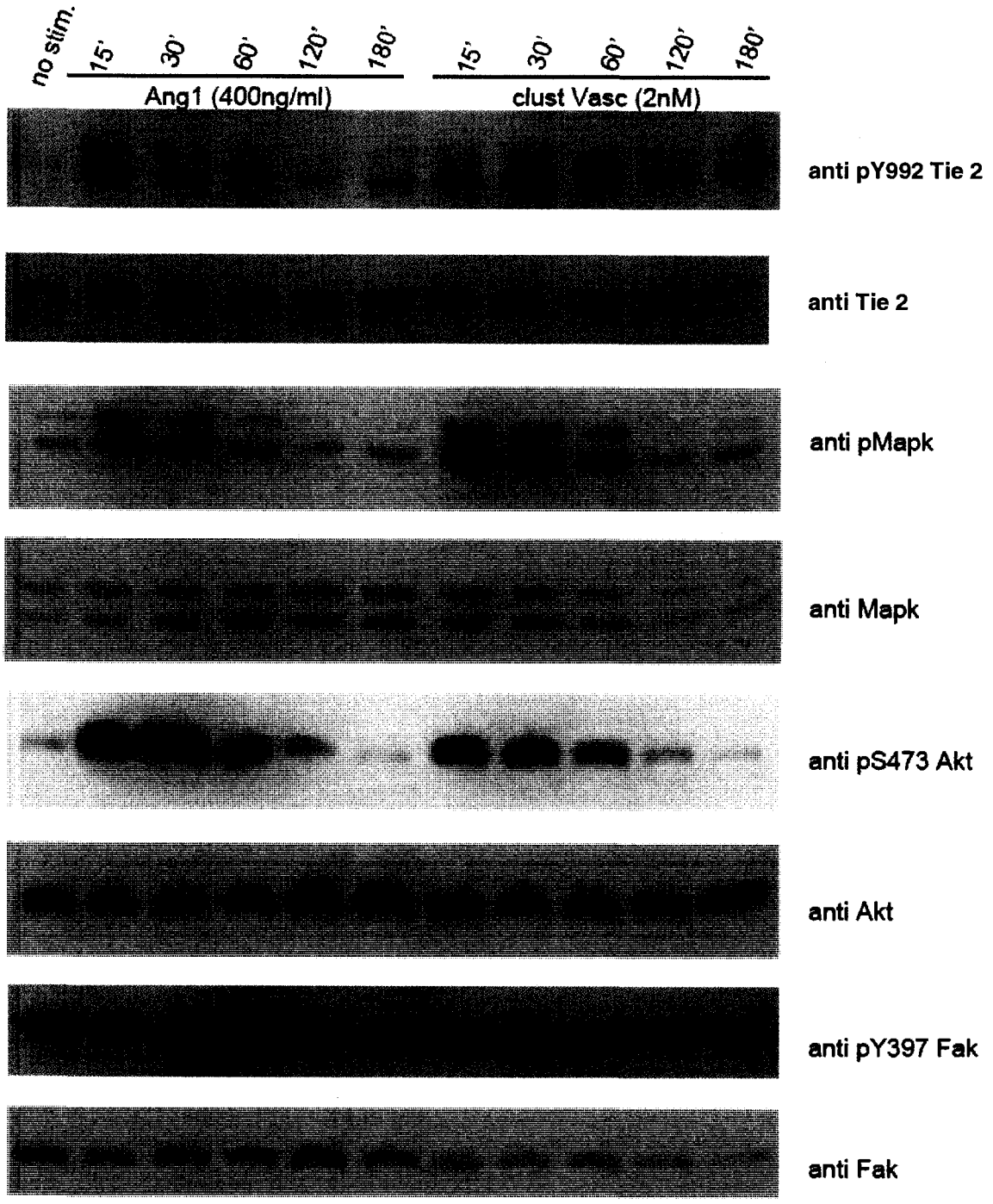


Figure 2.1e: Vasculotide-dependent activation kinetics. Clustered Vasculotide activates Tie 2, Akt and Mapk but not Fak in HUVEC's. Activation of these events closely mimics the kinetics of activation with recombinant Ang1.

Again we noted marked increases in pMAPK and pAkt. Previously Ang 1 and 2 have been shown to activate focal adhesion kinase (Fak), in a Tie 2 independendent manner through direct engagement of αVβ1 integrin (Carlson *et al.*, 2001;Hu et al., 2006). To test whether clustered Vasculotide was capable of activating this arm of the Ang 1 signalling cascade we examined the activation of Fak. As previously shown Ang 1 time dependently activated Fak whereas clustered Vasculotide had no significant effect. These results are suggestive of a more direct role for Vasculotide in eliciting only Tie 2-specific signalling.

To more formally address the specificity of clustered Vasculotide for the Tie 2 receptor we performed reconstitution experiments in Cos 1 fibroblast cells and C166 EC's, both of which are phenotypically null for Tie 2. Cells infected with recombinant adenovirus encoding EGFP were either left unstimulated or stimulated with Vasculotide or clustered Vasculotide. To broadly examine the potential that clustered Vasculotide was activating Tie 2-independent downstream signalling pathways we examined activation of MAPK and Akt in the EGFP infected cells. Neither Vasculotide nor clustered Vasculotide significantly induced phosphorylation of Mapk or Akt above that noted in the non-stimulated samples (fig 2.1f). As expected, overexpression of Tie 2 in either of the cell types resulted in its constitutive activation (see pY992 Tie 2) as well as activation of Mapk and Akt. This fact precluded us from stimulating these cells any further with Vasculotide or clustered Vasculotide (data not shown). Since Mapk and Akt are highly activated downstream of a host of cell surface receptors we reason that these results are highly suggestive of a Tie 2 specific and dependent role for clustered Vasculotide in these events.

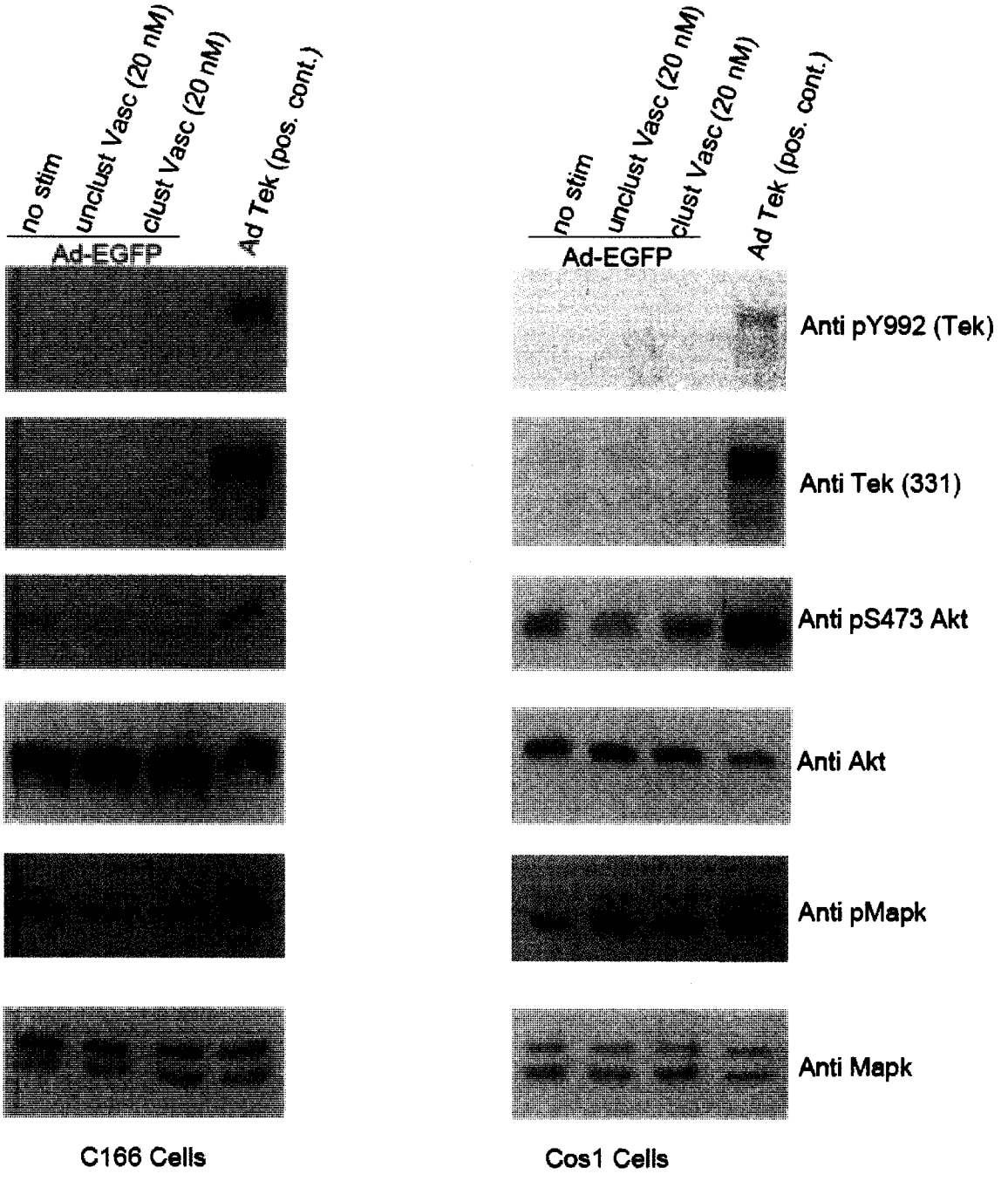


Figure 2.1f: Vasculotide does not activate Akt or Mapk in Tie2-null cells. Clustered Vasculotide was tested in cells phenotypically null for Tie 2. Neither clustered nor unclustered Vasculotide was able to stimulate phophorylation of Akt or MAPK above basal unstimulated levels.

Ang 1, and to a lesser degree Ang 2 have been shown to protect EC's from various different apoptosis-inducing conditions including serum withdrawal (Kwak *et al.*, 1999;Harfouche and Hussain, 2006). Based on the fact that clustered Vasculotide strongly activates the anti apoptotic protein Akt, we wanted to determine if it could protect EC's from serum withdrawal-induced death. We treated HUVEC's maintained in 0.1% FBS for 16 hours with various concentrations of unclustered or clustered Vasculotide. Analysis of cell apoptosis via cell death ELISA revealed a statistically significant decrease in cell death when treated with clustered Vasculotide at 500pM ($p=0.002$) compared to non-treated cells (fig 2.2a). Non clustered Vasculotide and avidin alone also had no effect on survival, further illustrating that Vasculotide is only active when preclustered with avidin.

EC migration is an event deemed critical to revascularization upon injury. Migration must take place in a coordinated fashion with the EC's responding to chemotactic signals from the stroma and in turn secreting proteases necessary to clear a path for their directional migration. Previous studies underline a role for Ang 1 in promoting EC migration and release of the matrix degrading enzyme MMP 2 (Witzenbichler *et al.*, 1998;Kim *et al.*, 2000). To test if Vasculotide, like Ang 1, was capable of promoting these processes we initially tested its influence on migration. Using a modified Boyden chamber assay we subjected Huvec's to various chemotactic agents including Vasculotide alone, or in combination with VEGF. Clustered Vasculotide applied at concentrations of 2nM and 10nM promoted significant increases in chemotactic cell

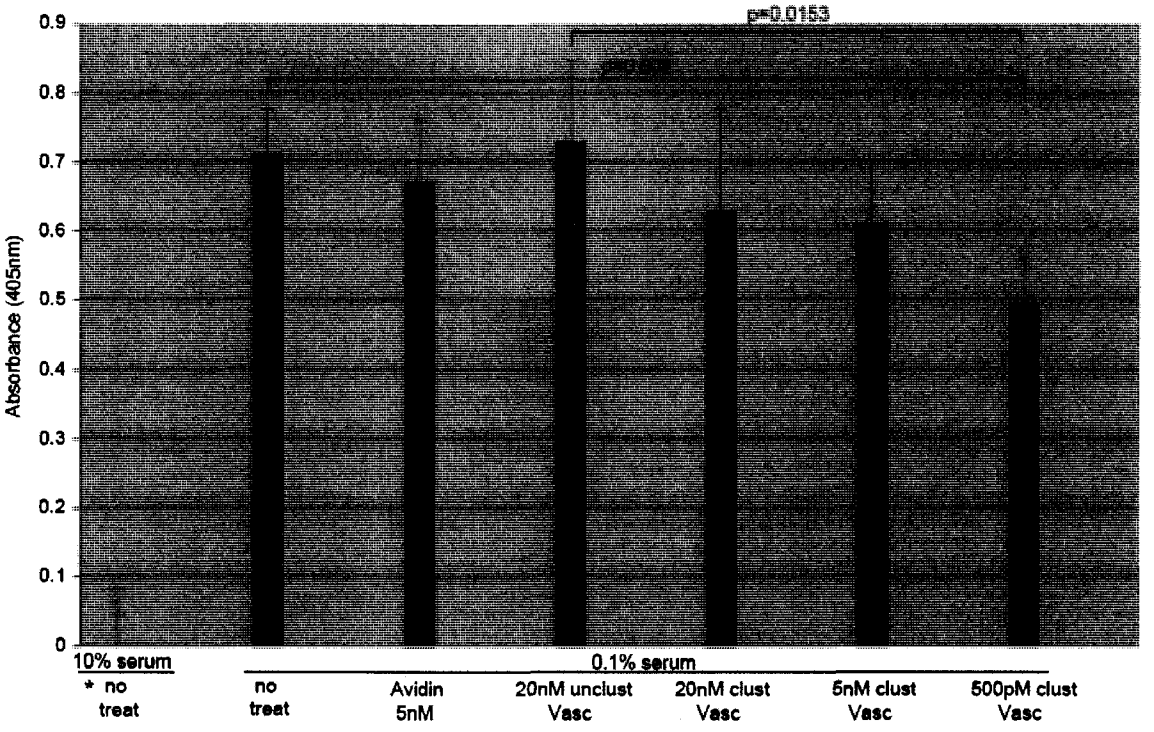


Figure 2.2a: Vasculotide protects against serum withdraw-induced cell death. Clustered Vasculotide reduces serum withdrawal-induced cell death of HUVEC cells. Values were normalized to basal levels of cell death for HUVEC's maintained in full 10% FBS, growth factor supplemented F12 media see (* no treat). Results shown represent the mean of three replicates plus or minus 1 SD. Student's T-test was used for statistical analysis with P value indicated.

migration when compared to no treatment (fig 2.2b). Noted migration was not significantly different than that seen in samples treated with VEGF or Ang 1. Non clustered Vascluotide did not promote migration above that seen in the untreated sample. *In vivo*, EC's can not migrate until they secrete enzymes necessary to break down components of the extracellular matrix. Here we employed gelatin zymography to determine if clustered Vasculotide promoted secretion of MMP2. Analysis of conditioned media revealed that cells treated with clustered Vasculotide secreted a protein that dose-dependently degraded gelatin at a molecular weight that was consistent with pro and active forms of MMP2 (fig 2.2c). Taken together with the results of the migration analysis it appears that application of clustered Vasculotide might offer distinct advantages *in vivo* to migrating endothelial cells.

To further examine this *in vivo* angiogenic potential growth factor reduced Matrigel was impregnated with either clustered Vasculotide or one of several different control factors. These Matrigel samples were injected subcutaneously into adult CD1 mice. After 14 days, and prior to sacrifice, all mice were injected with FITC-lectin to facilitate vessel identification and quantification. Upon surgical removal of the plugs, a distinct and robust angiogenic response was seen in the membranous capsule that surrounded the plugs impregnated with clustered Vasculotide, VEGF or clustered Vasculotide plus VEGF (fig 2.3a). In the case of the clustered Vasculotide samples, clear, large, well arborized vessels were present on the surface. These vessels were well branched and contained a continuum of small and large arterioles and venules. Consistent with literature accounts (Connolly et al., 1989), vessel growth promoted by the addition of

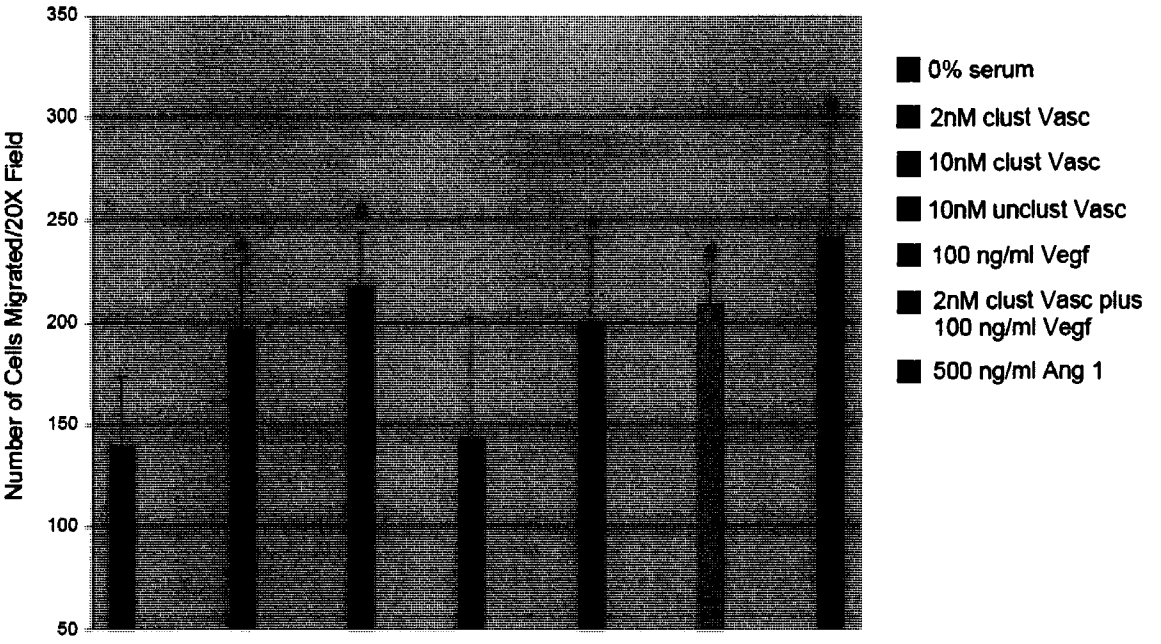


Figure 2.2b: Clustered Vasculotide promotes chemotactic cell migration in a modified Boyden chamber assay that is statistically indistinguishable from VEGF (100ng/ml) and Ang 1 (500ng/ml). Combination of VEGF and clustered Vasculotide does not offer any additional effect. Statistical analysis was performed by way of unpaired student's T test. Results are graphically represented. Stimulations were performed in triplicate and migrating cells in 6 microscopic fields per replicate were counted. Error bars shown represent mean +/- SD. * ($P<0.05$), # ($P<0.01$).

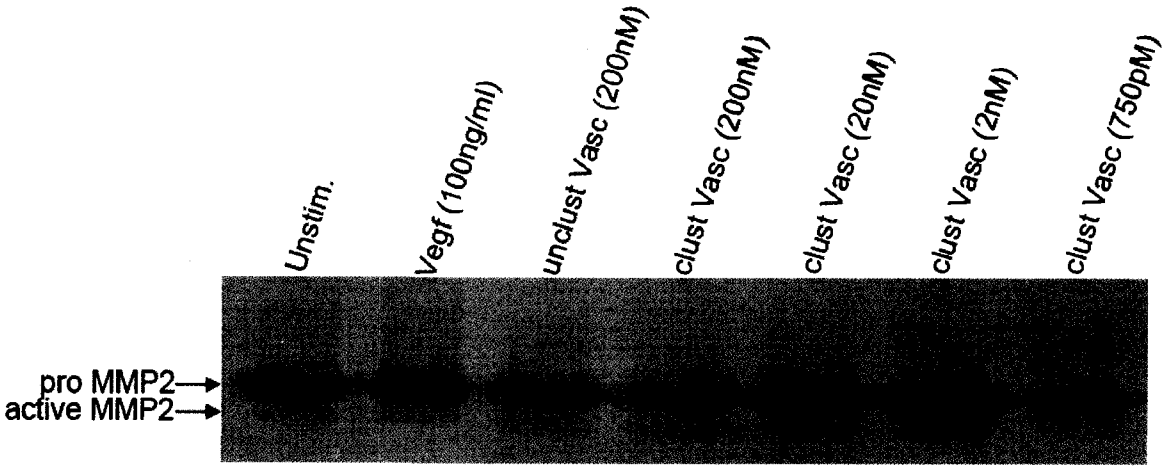


Figure 2.2c: Gelatin zymographic analysis of conditioned HUVEC media supports a role for clustered Vasculotide in promoting the release of MMP2.

VEGF alone was apparent although the nature of these vessels was highly tortuous. Vessels located in the proximal tissue surrounding the VEGF-containing plugs also looked enlarged and inflamed upon examination (data not shown). Application of clustered Vasculotide in combination with VEGF did not seem to increase the overall microvascular density but rather contributed to decreases in the level of the tortuosity seen with VEGF alone. To more fully address and assign quantitative vessel parameters to the different treatment groups the plugs were fixed and interrogated with laser confocal microscopy analysis combined with post processing image analysis (Image Processing Tool Kit 5.0, Reindeer Graphics, NC). No significant vessel growth was noted in plugs containing Matrigel or Matrigel impregnated with non clustered Vasculotide (fig 2.3a,b,c,d,e,f). Plugs containing VEGF, clustered Vasculotide or clustered Vasculotide plus VEGF displayed robust induction of angiogenesis. Statistically, VEGF, Vasculotide or combinations thereof did not differ in total vessel number (fig 2.3c), length (fig 2.3d) or branch points (fig 2.3e). As was noted in the membranous capsule surrounding the plugs, samples that contained Vasculotide displayed more organized branching patterns and less tortuosity than the plugs containing VEGF alone. Ang 1 has been reported to potentiate the recruitment of myogenic support cells (Suri *et al.*, 1996) and inhibit VEGF induced vascular permeability through mechanisms that are not yet well understood (Thurston et al., 2000). To determine if Vasculotide, like Ang 1, was capable of initiating pericyte recruitment we performed whole mount immunofluorescence against smooth muscle actin 1 (Sma1). Matrigel plug analysis of VEGF driven angiogensis revealed a relative absence of Sma1 positive staining cells (fig 2.3f). Samples containing clustered Vasculotide alone or in combination with VEGF displayed a significant increase in

overall Sma1 staining. Sma1 staining was tightly associated with the exterior of the vessels and was enriched on larger vessels. Taken together these results demonstrate potent *in vivo* angiogenic effects of clustered Vasculotide and provide the impetus for follow up experiments to more fully examine the potential that when combined with VEGF, clustered Vasculotide is capable of counteracting VEGF induced permeability and aberrant vessel formation. Chronic wounds, such as neuropathic or neuroischemic foot ulcers, are prevalent in diabetic patients. These wounds may arise from the patient's inability to sense injury (neuropathic) or due to microvascular defects (neuroischemic). Animal models of type II diabetes exist and closely recapitulate wound healing defects seen in human diabetics. Here we used B6.Cg-m(+/+)Lepr(db)/J (db/db), a strain of diabetic mouse that presents with impaired wound healing, to test if clustered Vasculotide could improve wound closure times. Circular (6mm diameter), full excisional wounds were made equal distance apart on the dorsal side of the mice. Intrasite gel (Smith and Nephew), impregnated with PBS, basic fibroblast growth factor (bFGF) or clustered Vasculotide were applied on the day of wounding (day 0), day 2, 4 and 6. Images of the wounds were taken on the same days and on the final day of the assay, day 7 (fig 2.4a). Significant decreases in wound margin in the clustered Vasculotide samples (20nM, $p=0.05$, $n=4$) were noted as early as day 2 (fig 2.4a and b). Coincident with improved wound closure was a change in the appearance of the wounds treated with clustered Vasculotide. These wounds presented with a lighter more mucoid looking scab (fig 2.4c). Margin closure in the clustered Vasculotide treated wounds (20nM and 5nM) continued at an accelerated pace for the duration of the assay. As was the case *in vitro*, clustered

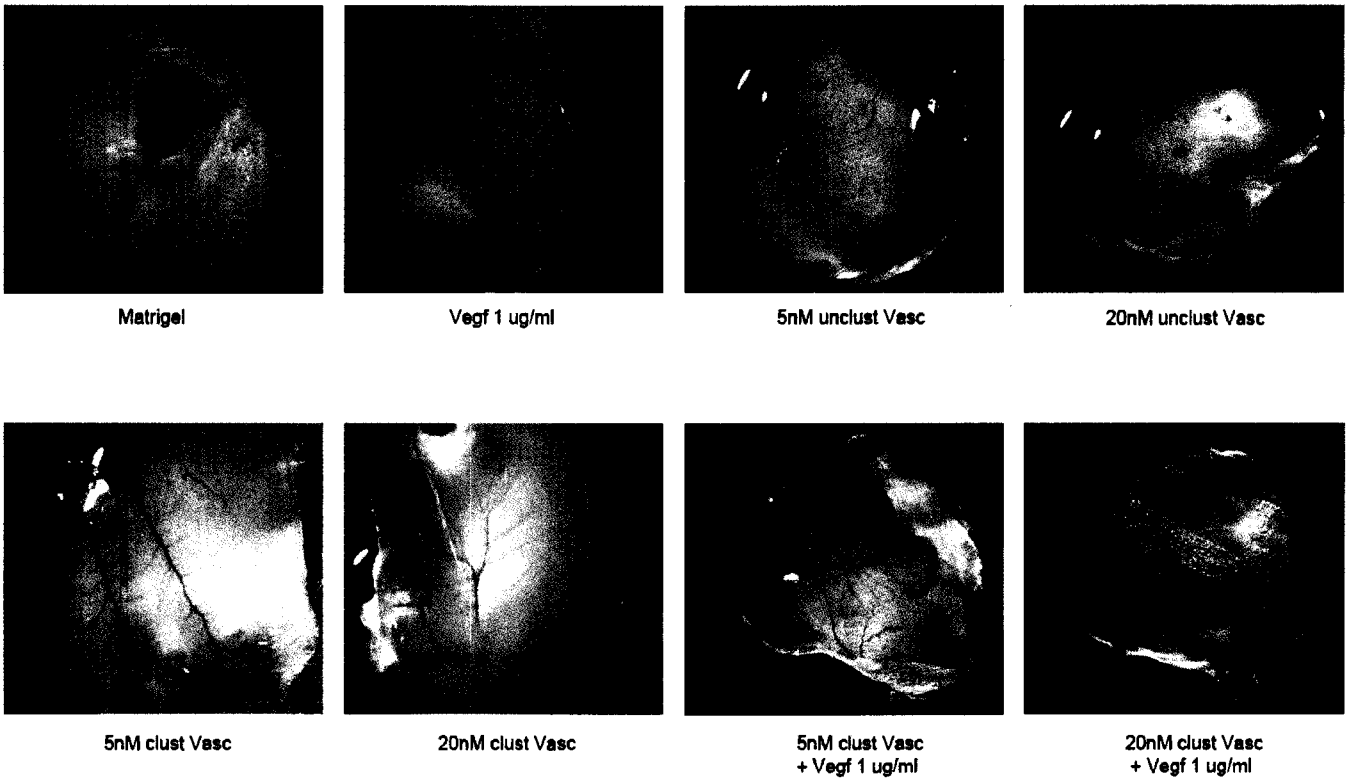


Figure 2.3a: Clustered Vasculotide promotes a robust *in vivo* angiogenic response. Topological features of Matrigel plugs impregnated with clustered and unclustered Vasculotide. Proangiogenic effects of clustered Vasculotide can clearly be seen in the membranous capsule that surrounds resected plugs-note the well branched architecture of vessels present on the 5nM and 20nM clustered Vasculotide plugs.

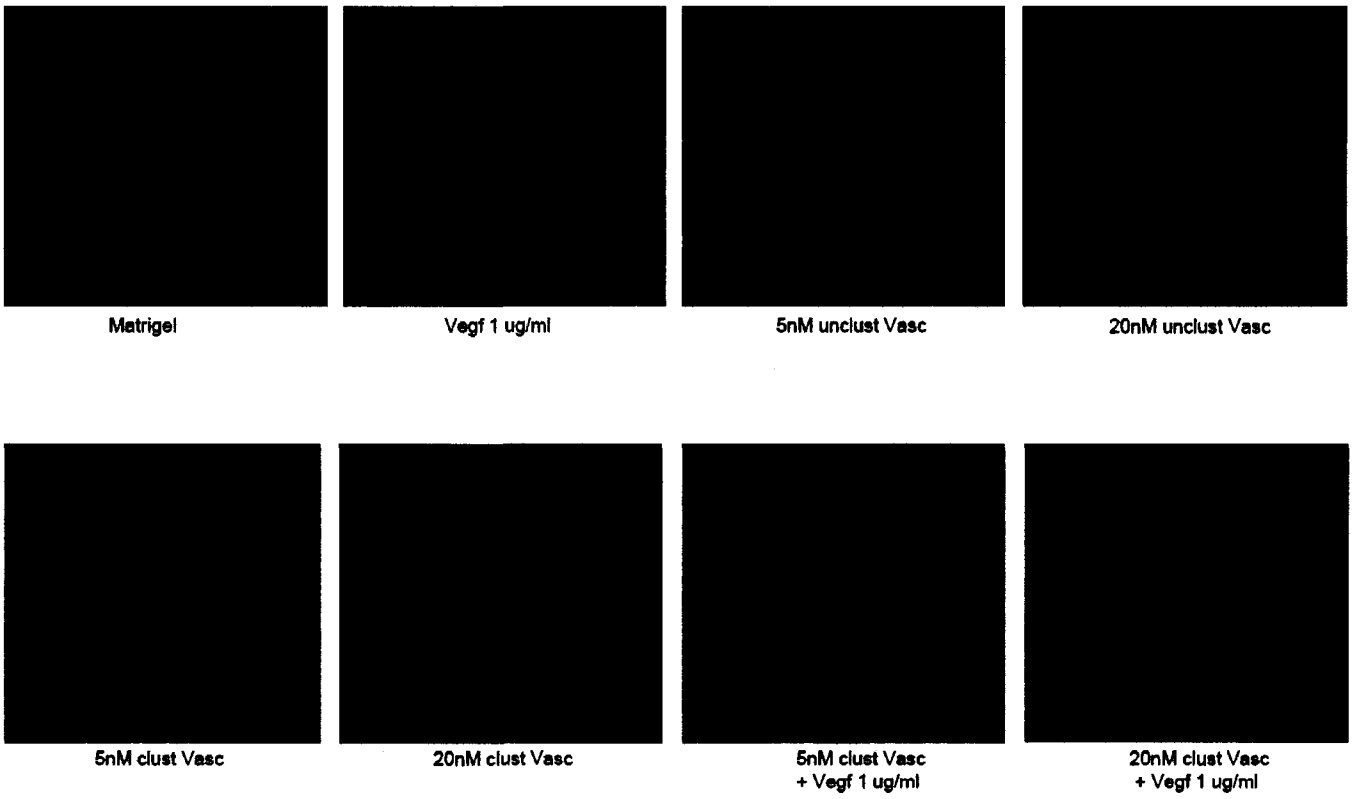


Figure 2.3b: Confocal analysis of resected Matrigel plugs was performed. Prior to sacrifice, mice were injected with FITC-lectin to facilitate analysis of the vasculature. Distinct differences in vessel morphology are clearly apparent in the clustered Vasculotide samples alone when compared to VEGF. Note: poor perfusion of FITC lectin in all 20nM clustered Vasculotide plus Vegf samples impaired confocal visualization.

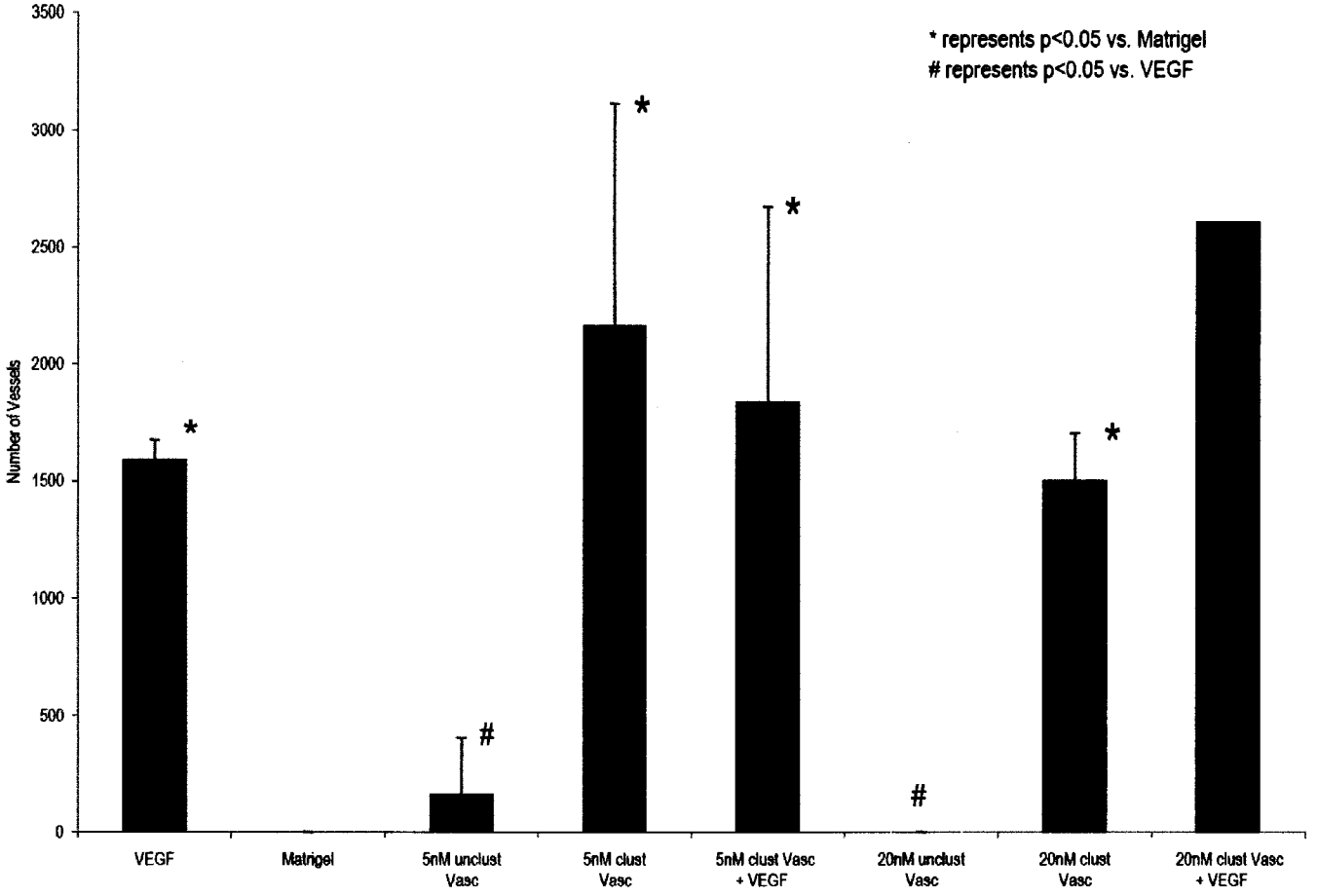


Figure 2.3 c: Statistically, Vasculotide, VEGF or combinations thereof did not differ in total vessel number. Vessel number, was quantified on at least six representative fields (Image Processing Tool Kit 5.0, Reindeer Graphics, NC). Statistical analysis was performed by way of unpaired student's T test. Results are graphically represented. Poor perfusion of FITC-lectin into mice harbouring 20nM clust Vasculotide plus VEGF plugs prevented statistical analysis, therefore bars are the mean of 2 separate fields. All other bars shown represent mean +/- SD. * ($P<0.05$ vs. Matrigel), # ($P<0.05$ vs. VEGF).

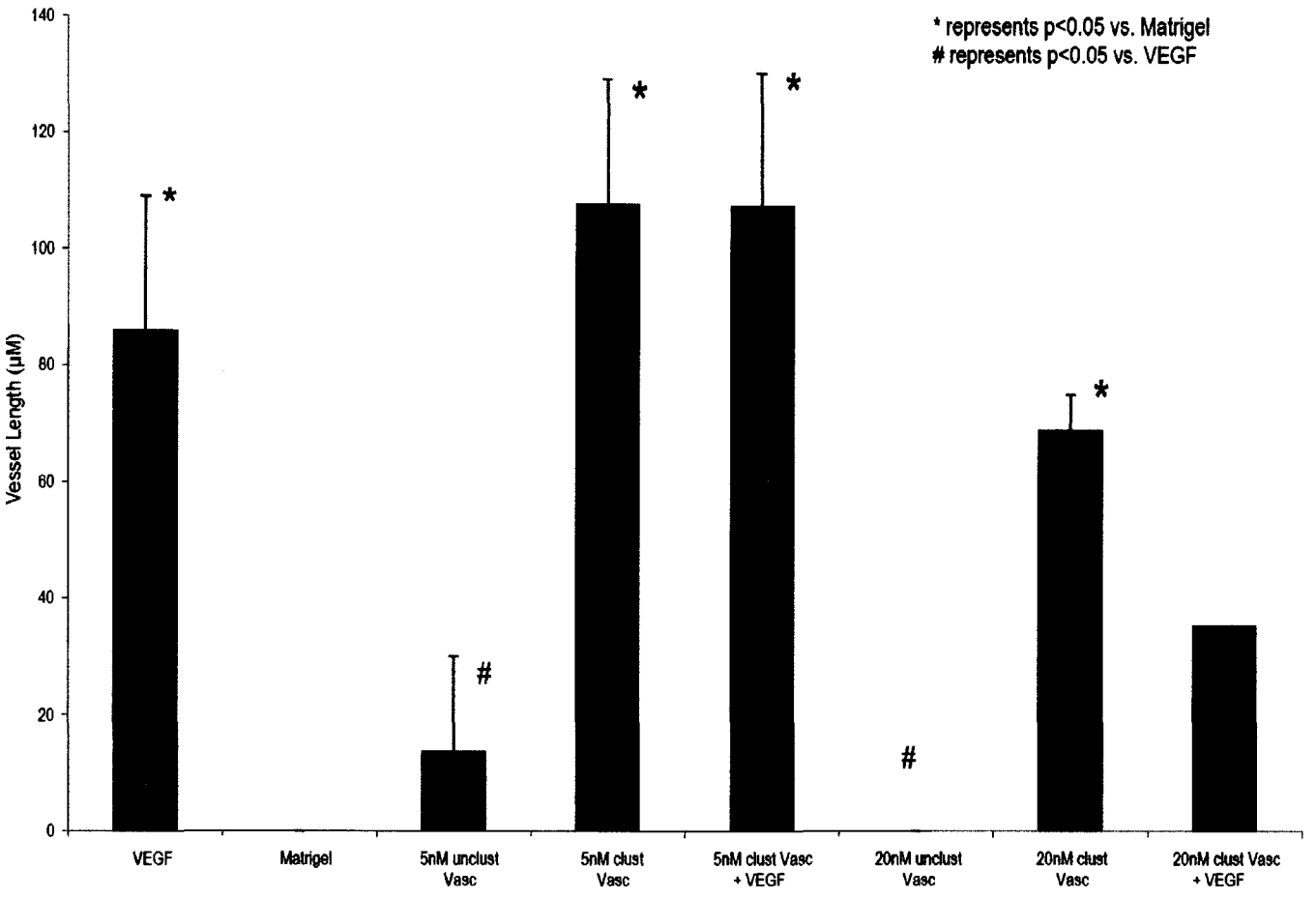


Figure 2.3 d: Statistically, Vasculotide, VEGF or combinations thereof did not differ in total vessel length. Vessel length, was quantified on at least six representative fields (Image Processing Tool Kit 5.0, Reindeer Graphics, NC). Statistical analysis was performed by way of unpaired student's T test. Results are graphically represented. Poor perfusion of FITC-lectin into mice harbouring 20nM clust Vasculotide plus VEGF plugs prevented statistical analysis, therefore bars are the mean of 2 separate fields. All other bars shown represent mean +/- SD. * ($P<0.05$ vs. Matrigel), # ($P<0.05$ vs. VEGF).

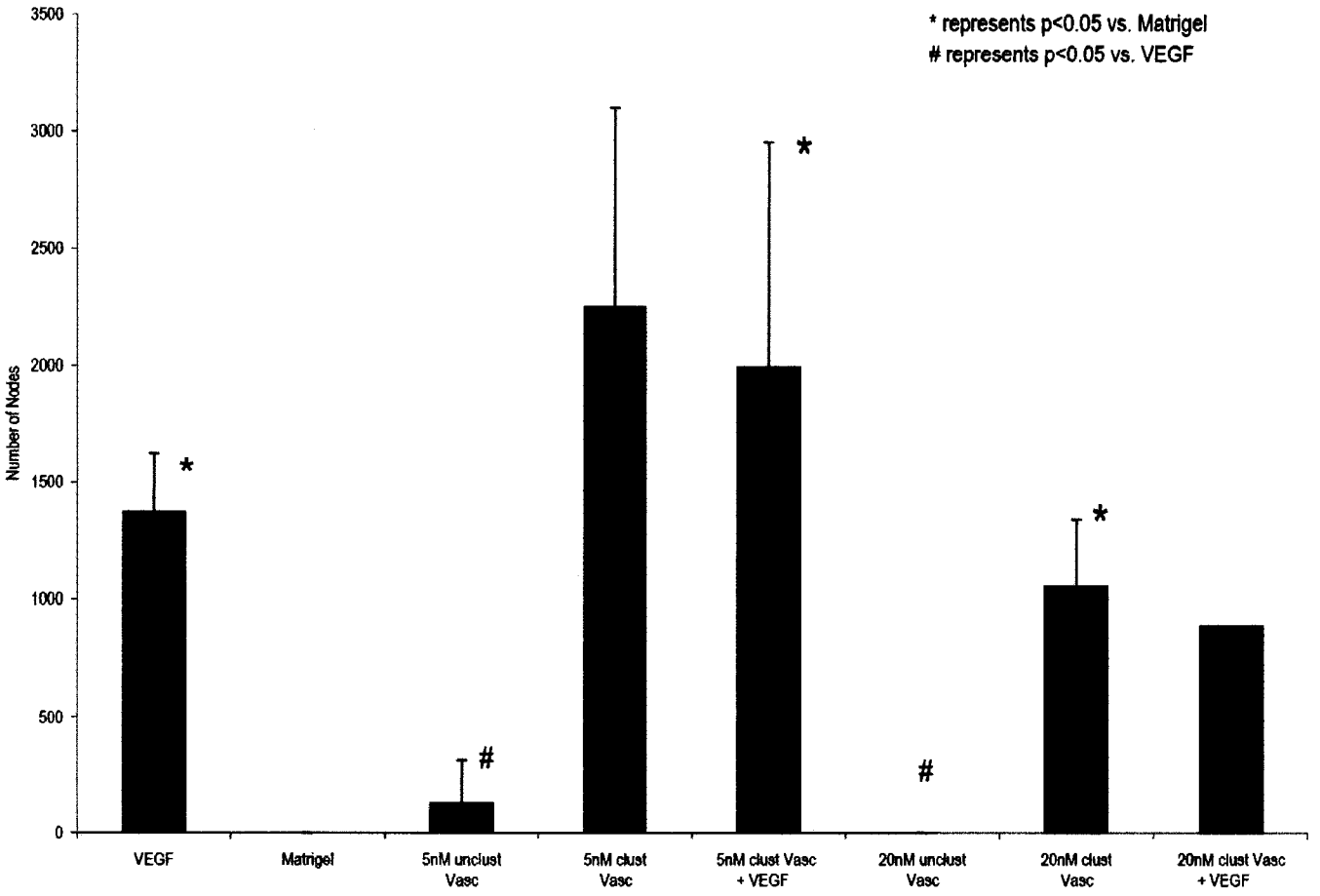


Figure 2.3 e: Statistically, Vasculotide, VEGF or combinations thereof did not differ in total vessel nodes. Vessel branch points, were quantified on at least six representative fields (Image Processing Tool Kit 5.0, Reindeer Graphics, NC). Statistical analysis was performed by way of unpaired student's T test. Results are graphically represented. Poor perfusion of FITC-lectin into mice harbouring 20nM clust Vasculotide plus VEGF plugs prevented statistical analysis, therefore bars are the mean of 2 separate fields. All other bars shown represent mean +/- SD. * ($P<0.05$ vs. Matrigel), # ($P<0.05$ vs. VEGF).

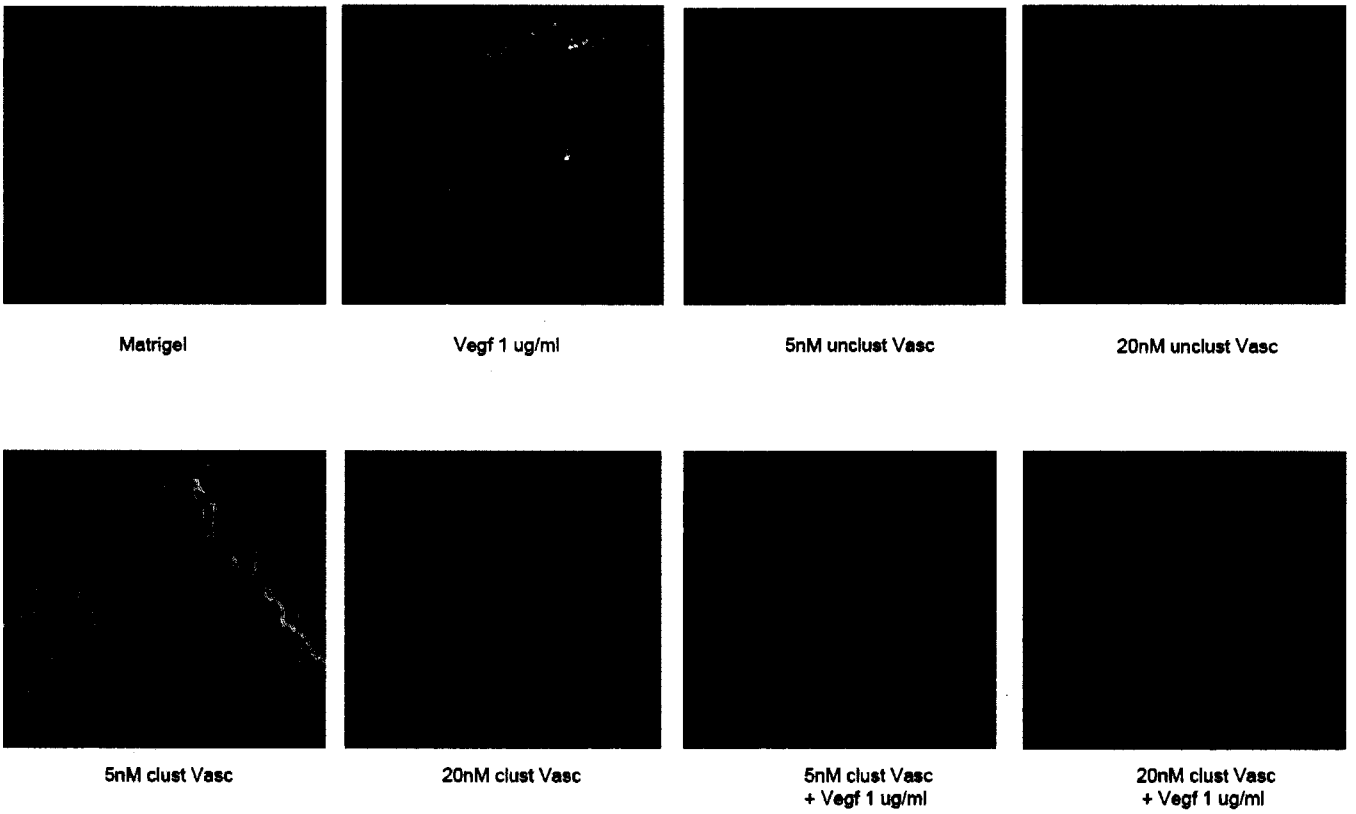


Figure 2.3f: Matrigel plugs were whole mount stained with anti Sma1-Cy3 and interrogated with confocal microscopy. Matrigel plugs that contained VEGF alone displayed fewer Sma1 positive staining myogenic support cells then those which contained clustered Vasculotide alone or in combination with VEGF. Note: poor perfusion of FITC lectin in all 20nM clustered Vasculotide plus Vegf samples impaired confocal visualization.

Vasculotide applied at lower concentration seemed to offer more benefit (see day 4&6, 20nM vs. 5nM p=0.05 compared to p=0.01 respectively) (fig 2.4a and b). Although this may seem unusual, similar results were also noted with topical application of becaplermin (Regranex), a topical preparation of modified PDGF (Mustoe et al., 1994). Moreover, these results further highlight the need to optimize the Vasculotide:Tie 2 ratio to facilitate appropriate clustering of the receptor. Although Vasculotide provided the fastest healing at early time points by day 7 of treatment wound closure promoted by bFGF at 10μg/ml was statistically indistinguishable from clustered Vasculotide at 20nM and 5nM. Paraffin embedded cross sections of all wounds stained with H&E and Masson's trichrome, a chemical stain used to highlight collagen fibers, (fig 2.4d) were given to a pathologist for independent blind analysis. Scores ranging from 0-4 were assigned for each of the following: re-epithelialization, granulation tissue and neovascularisation and inflammation (see methods for in-depth scoring criteria). Differences between vehicle and bFGF although apparent in wound closure times, existed only at the level of inflammation (p=0.0486)(Fig 2.4e). Application of clustered Vaculotide resulted in delayed re-epithelialization (20nM, p=0.0004 and 5nM, p=0.029) but promoted profound increases in regranulation and neovascularization (20nM and 5nM p=0.0006). Inflammation noted in the clustered Vasculotide samples was only slightly increased at 20nM (p=0.034) and was not significantly different from vehicle at the 5nM dose (p=0.0979). Immunohistochemical analysis of the wounds with antibodies directed against Pan EC, PECAM1, ICAM 1, podoplannin and smooth muscle actin demonstrated

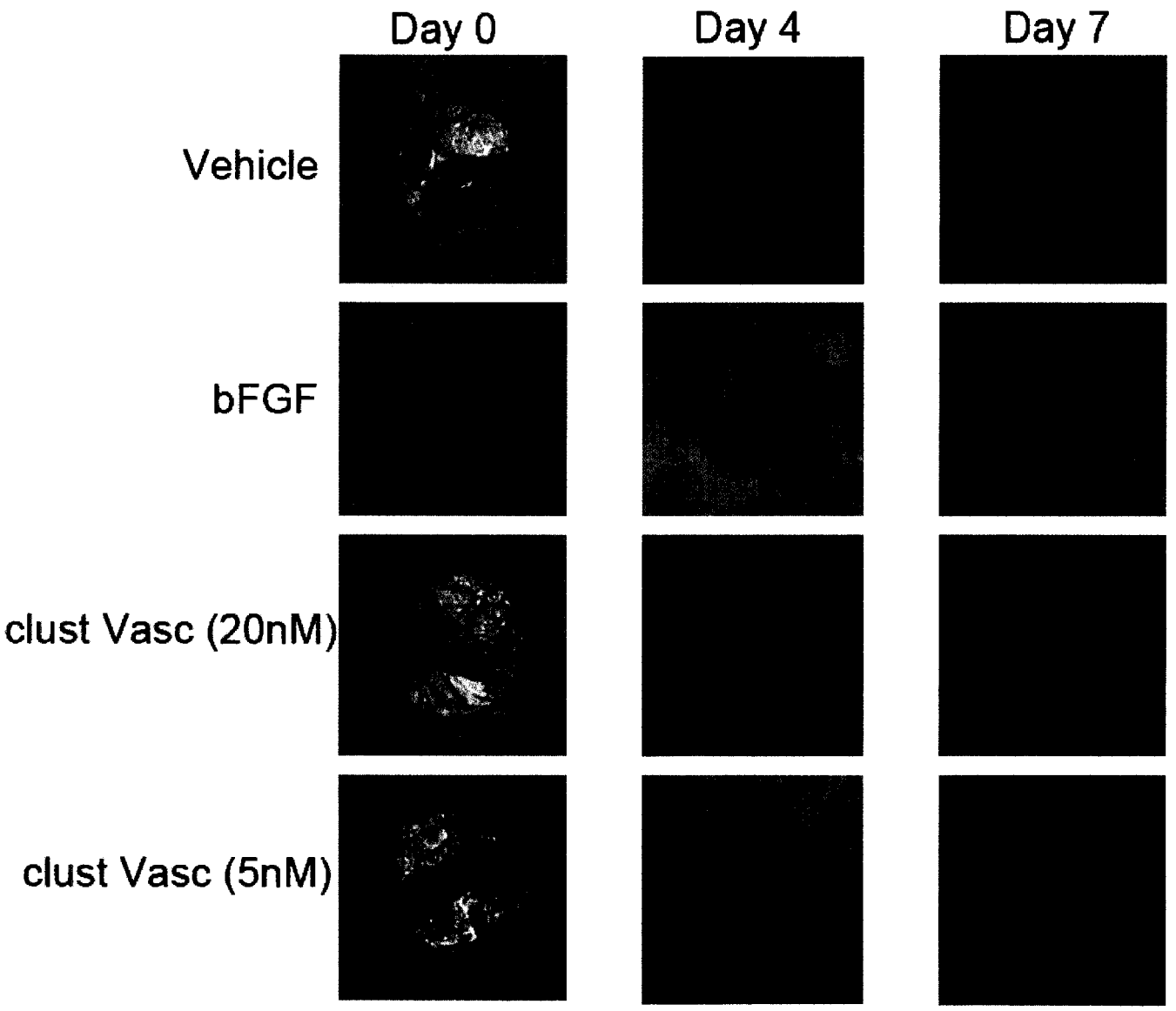


Figure 2.4a: Clustered Vasculotide promotes accelerated wound closure in genetically diabetic mice. 6mm excissional wounds were made on day 0 and digitally documented in a standardized manner. Sterile Intrasite Topical Gel (Smith and Nephew) impregnated with PBS, bFGF (10ug/ml) or clustered Vasculotide (5nM or 20nM) was applied on days 0, 2, 4 and 6. Images of representative wounds are shown for days 0, 4 and 7.

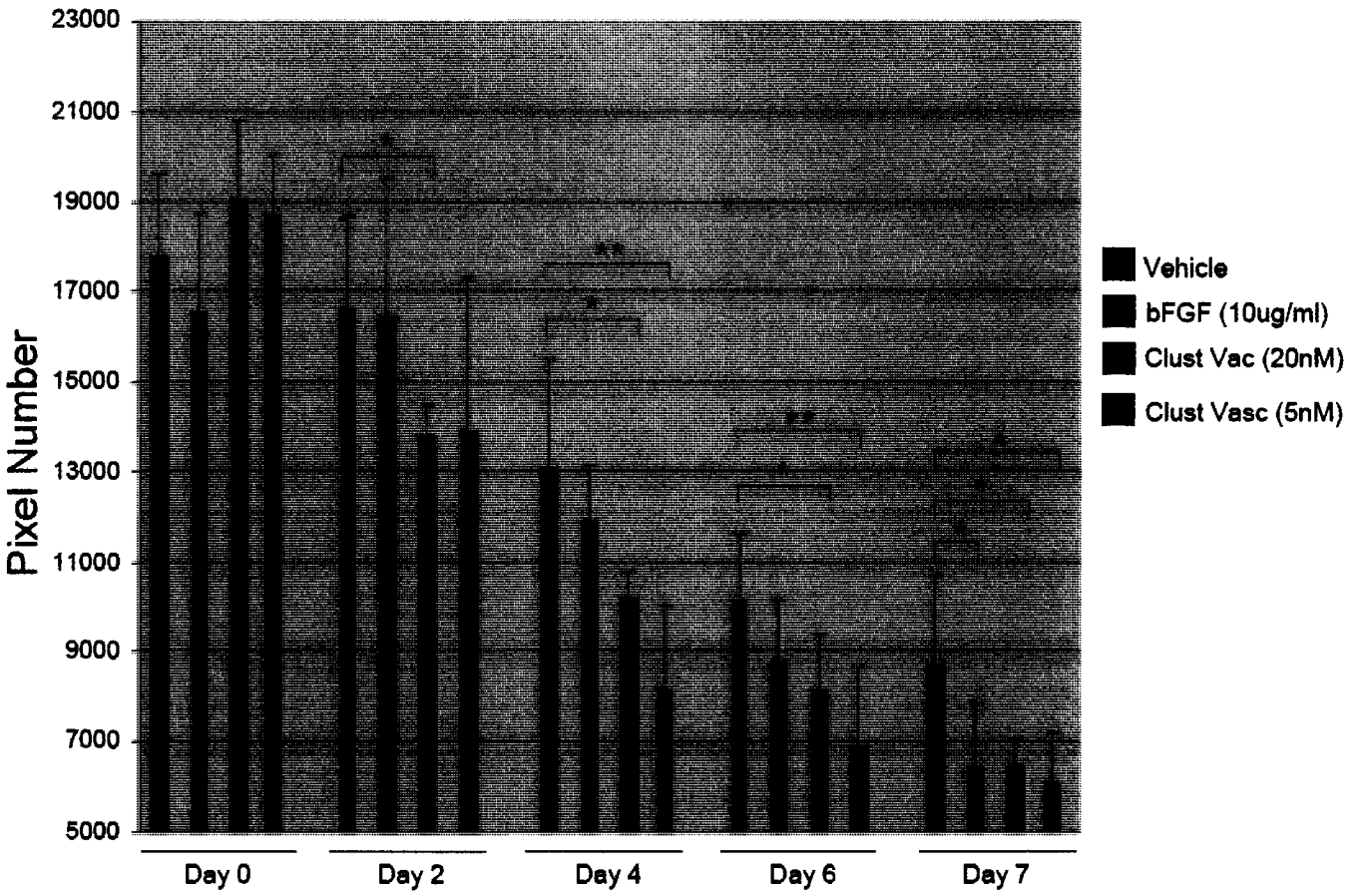


Figure 2.4b: Quantification of Vasculotide treated wound healing. Standardized wound images were recorded on days 0, 2, 4, 6, and 7. Digital tracing of the wound margins were performed and total pixel counts were recorded using Image J software (NIH). Results are graphically shown. Statistical analysis was performed by way of unpaired student's T test. Clustered Vasculotide 5nM and 20nM n=4, vehicle and bFGF n=8. All bars shown represent mean +/- SD. * ($P<0.05$), **($P<0.01$).

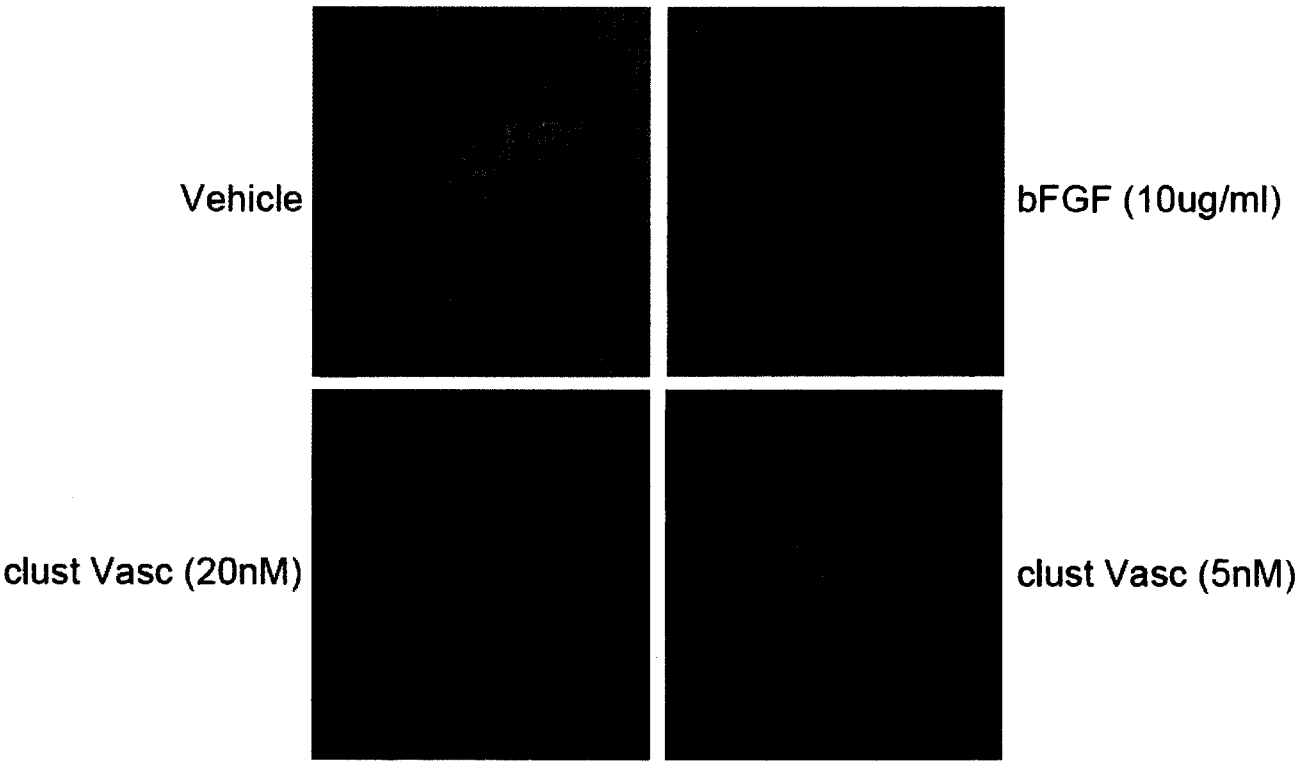


Figure 2.4c: Clustered Vasculotide alters appearance of fibrin clot. Close-up of wounds 48 hours post injury. Note the lighter, more mucoid appearance of the fibrin clot.

clear differences between clustered Vasculotide-treated and untreated samples (Fig 2.4f I,II,III,IV,Vrespectively). of the new vessels in the granulation tissue were accompanied by SMA-positive support cells. Furthermore, these vessels were also positive for ICAM 1, a marker of activated endothelial cells (Sluiter et al., 1993), demonstrating an active wound healing process was occuring. The increase in vessel density was surprisingly not accompanied by an increase in lymphangiogenesis as detailed by similar numbers podoplannin-positive vessels (data not shown). Podoplannin is a mucin-type glycoprotein specifically expressed on lymphatic ECs and not blood Ecs. Overall collagen deposition, as assessed by Masson's Trichrome staining, was dramatically increased at both of the clustered Vasculotide concentrations compared to the vehicle and bFGF samples (data not shown for clustered Vasculotide 20nM). Collagen deposition, primarily mediated by activated fibroblasts, plays a necessary role in contracting the wound margins, offers tensile strength, provides a scaffold for neovascularisation and facilitates cellular signalling to migrating cells via integrin engagement. Based on pathological and immunohistochemical analysis we conclude that the improved wound closure times noted in the clustered Vasculotide samples are a likely consequence of strong contraction effects brought about by increases in collagen deposition and neovascularisation.

Discussion

Herein we describe the rationale design of a novel Ang mimetic. *In vitro* analysis of its properties profile effects that very closely resemble other native Ang's. Specifically, we show activation of Tie 2 and signalling pathways that have been classically described downstream of Tie 2 including MAPK, AKT, and eNOS. However, unlike the effects of

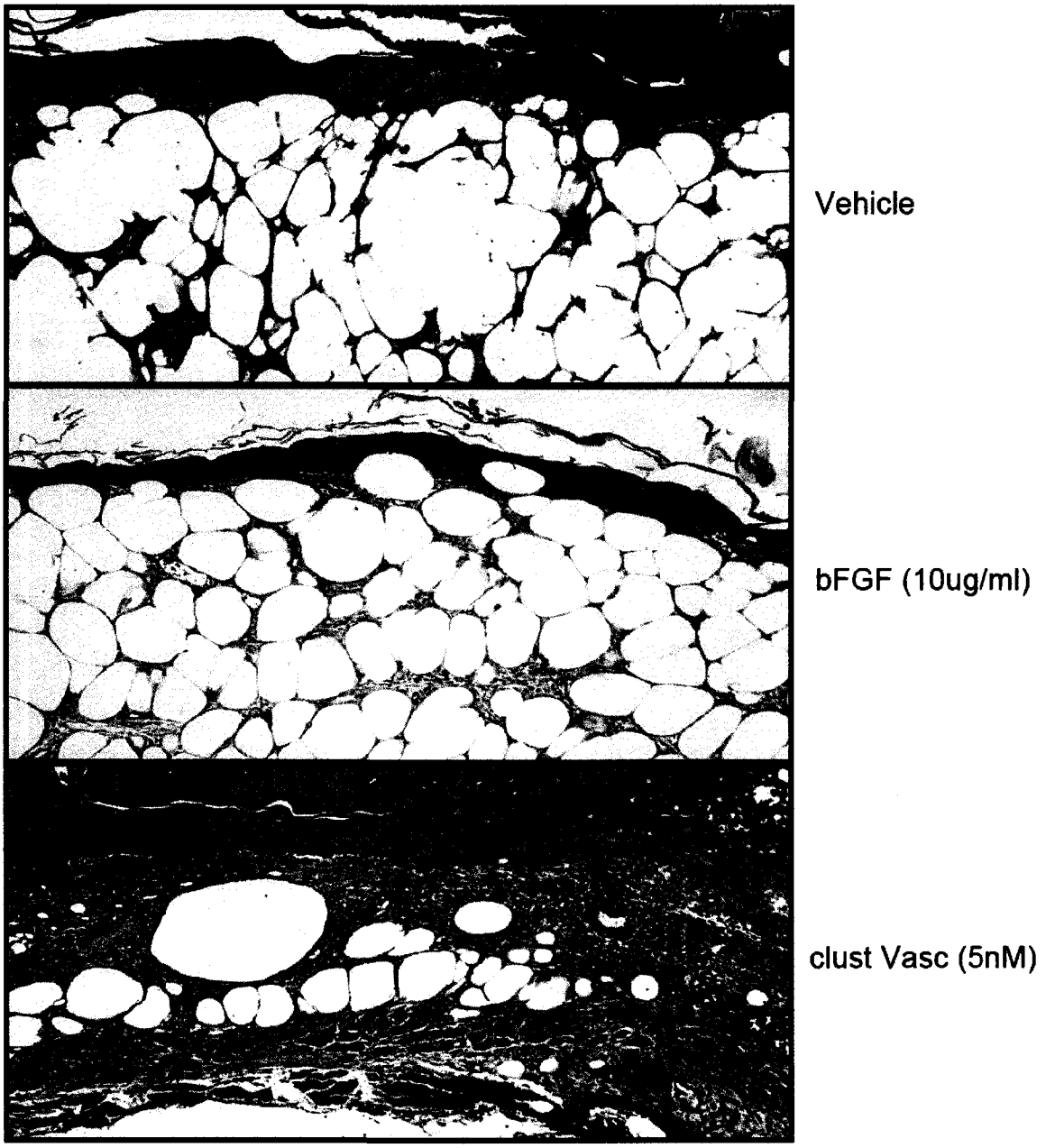


Figure 2.4d: Clustered Vasculotide promotes dramatic increases in granulation tissue, Representative photomicrographs of H&E stained skin sections from wounds treated with PBS, bFGF (10ug/ml) or clustered Vasculotide (5nM).

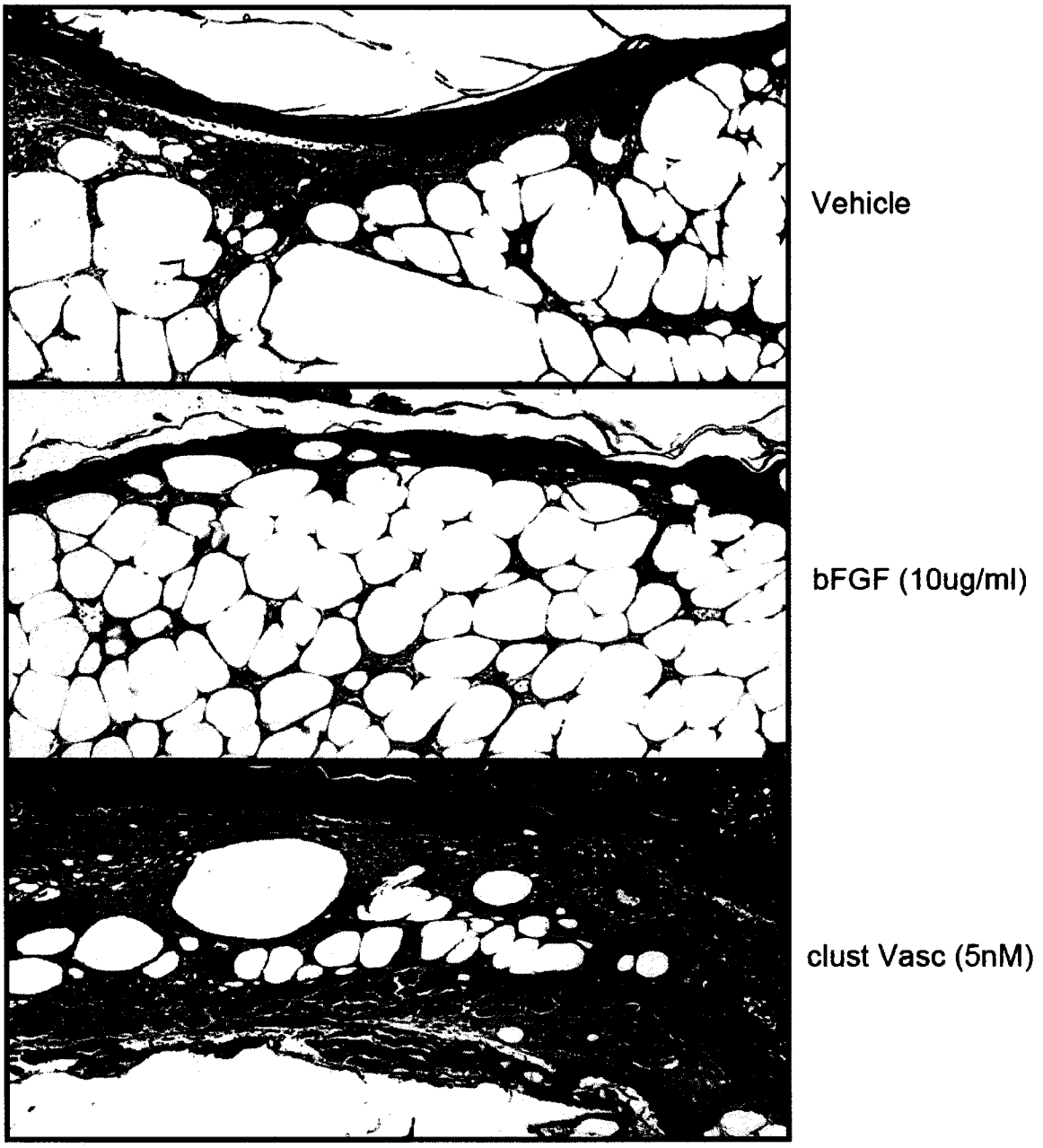


Figure 2.4d: Clustered Vasculotide promotes dramatic increases in Collagen Deposition. Representative photomicrographs of Masson's Trichrome stained skin sections from wounds treated with PBS, bFGF (10ug/ml) or clustered Vasculotide (5nM).

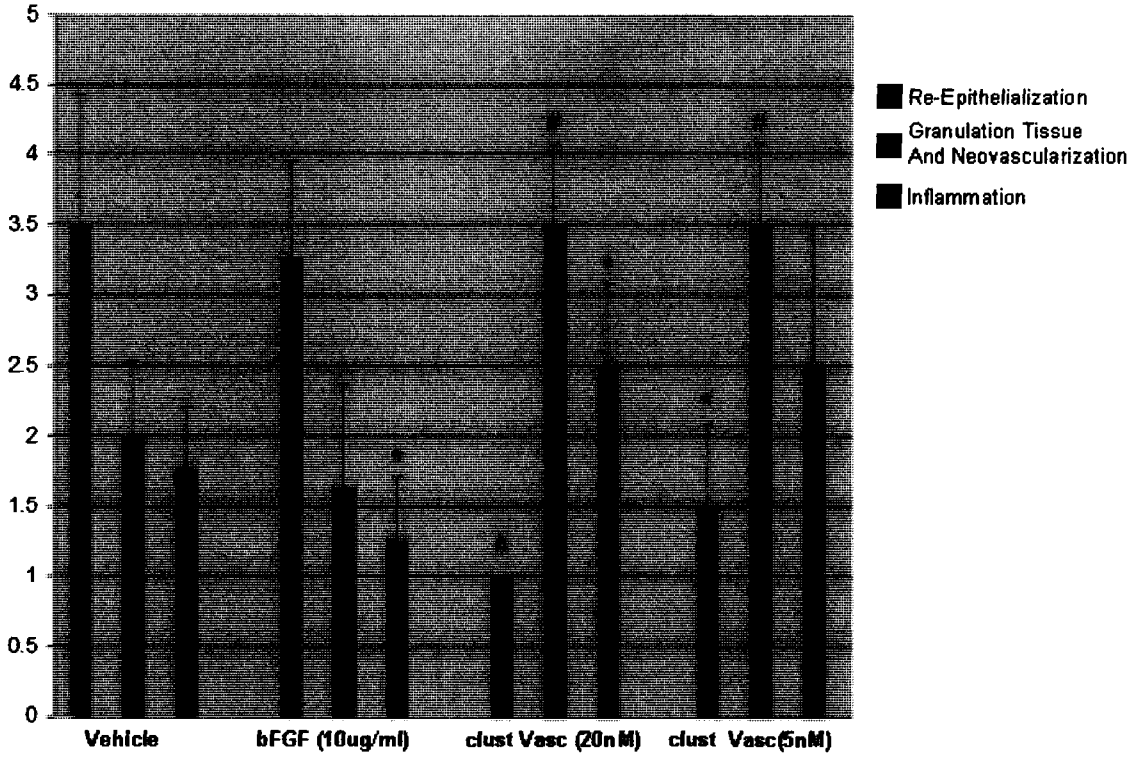


Figure 2.4e: Blind histolopathological wound scoring was performed by an independent pathologist. Scores raging from 0-4 were assigned for re-epithelialization, granulation and neovascularisation and inflammation. Statistical analysis was performed by way of unpaired student's T test. Results are graphically represented, clustered Vasculotide 5nM and 20nM n=4, vehicle and bFGF n=8. All bars shown represent mean +/- SD. * ($P<0.05$), # ($P<0.001$).

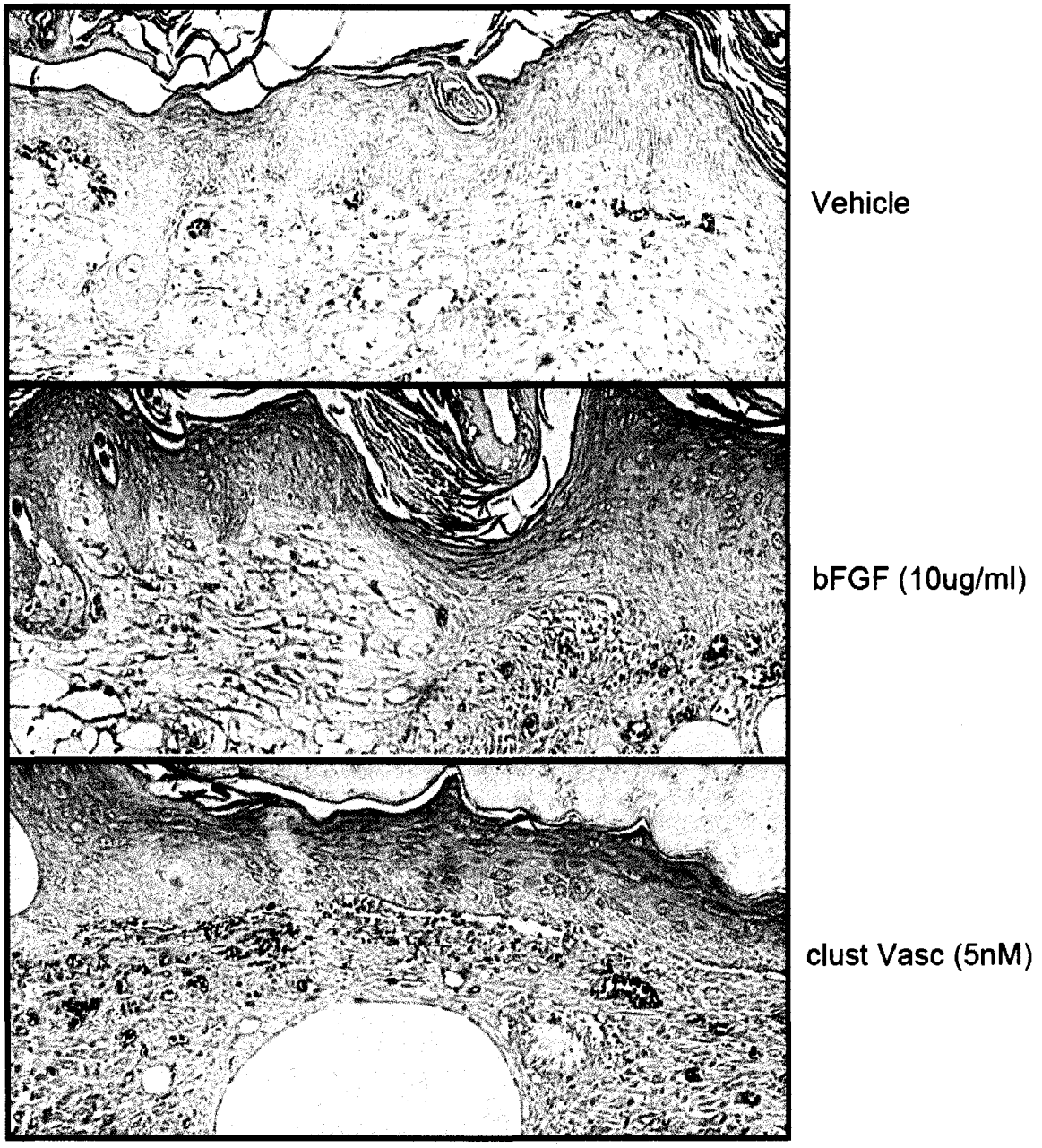


Figure 2.4f I: Representive fields of wound area sections treated with vehicle, bFGF (10ug/ml) clustered Vasculotide (5nM), are peroxidise stained with antibody against PanEC. Note, histopathological comparison of 5nM and 20nM clustered Vasculotide treatments revealed no statistically significant difference (data not shown). For presentation purposes only the 5nM clustered Vasculotide samples are shown.

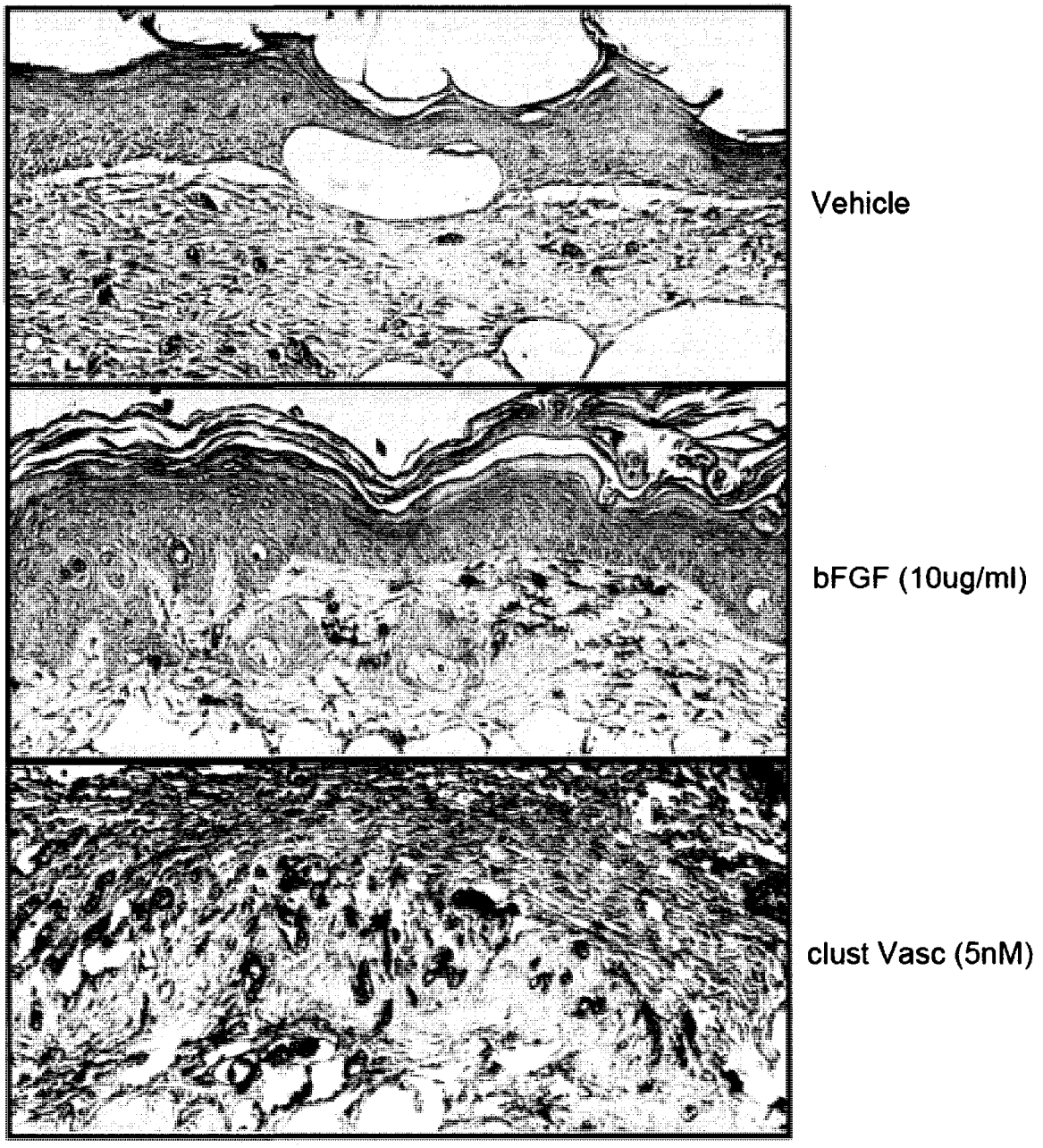


Figure 2.4f II: Representive fields of wound area sections treated with vehicle, bFGF (10ug/ml) clustered Vasculotide (5nM), are peroxidise stained with antibody against PECAM. Note, histopathological comparison of 5nM and 20nM clustered Vasculotide treatments revealed no statistically significant difference (data not shown). For presentation purposes only the 5nM clustered Vasculotide samples are shown.

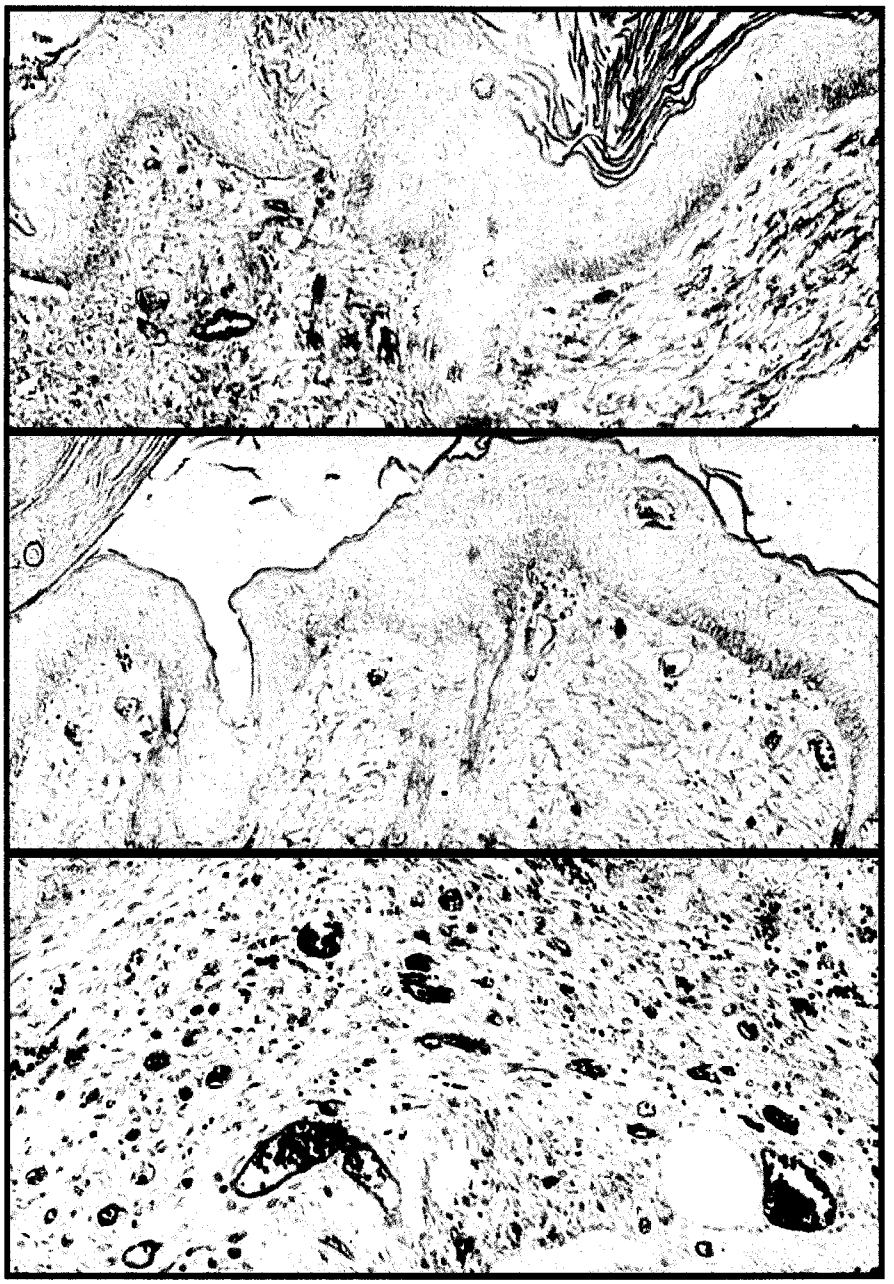


Figure 2.4f III: Representive fields of wound area sections treated with vehicle, bFGF (10ug/ml) clustered Vasculotide (5nM), are peroxidise stained with antibody against ICAM. Note, histopathological comparison of 5nM and 20nM clustered Vasculotide treatments revealed no statistically significant difference (data not shown). For presentation purposes only the 5nM clustered Vasculotide samples are shown.

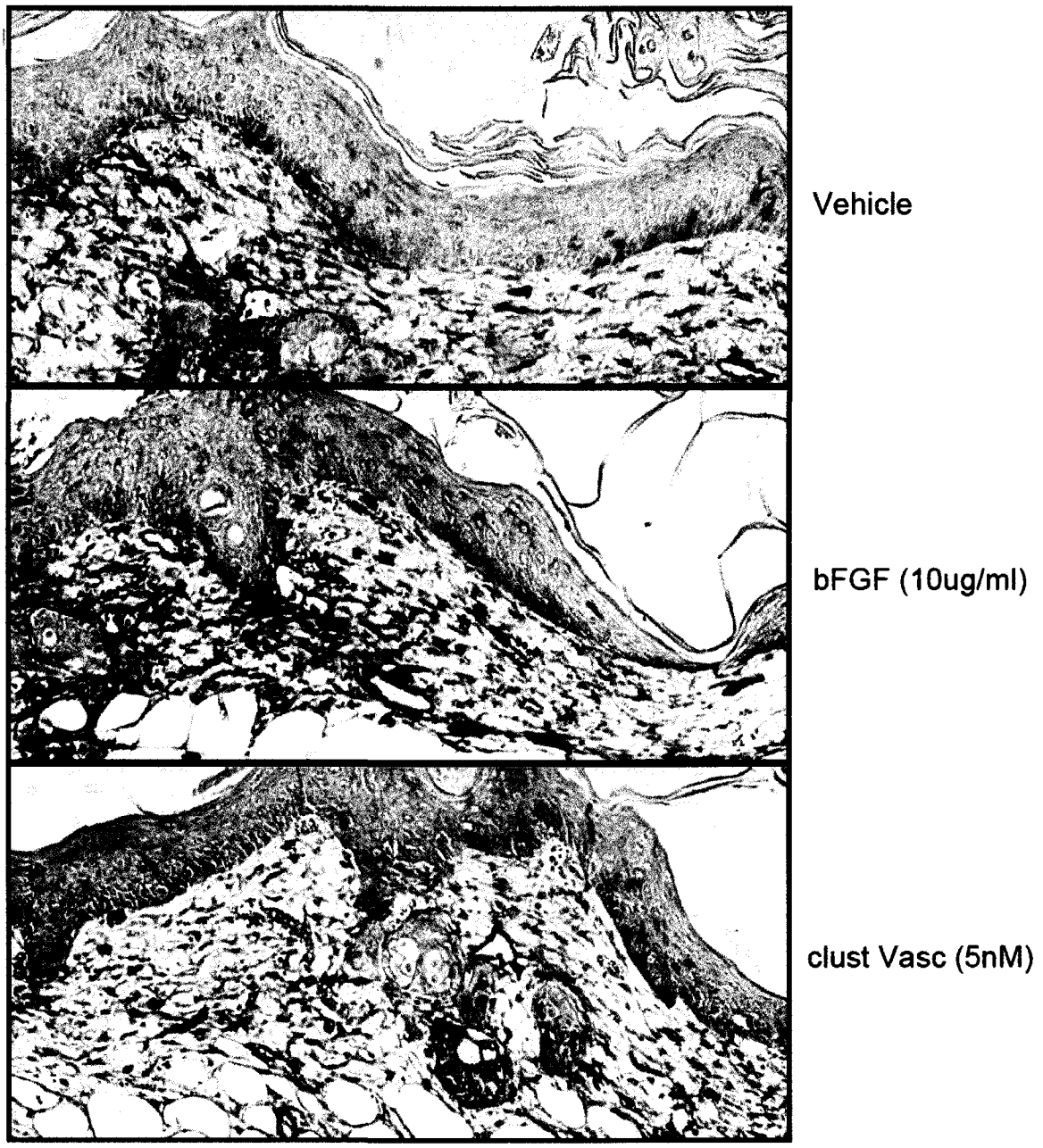


Figure 2.4f IV: Representive fields of wound area sections treated with vehicle, bFGF (10ug/ml) clustered Vasculotide (5nM), are peroxidise stained with antibody against Podoplanin. Note, histopathological comparison of 5nM and 20nM clustered Vasculotide treatments revealed no statistically significant difference (data not shown). For presentation purposes only the 5nM clustered Vasculotide samples are shown.

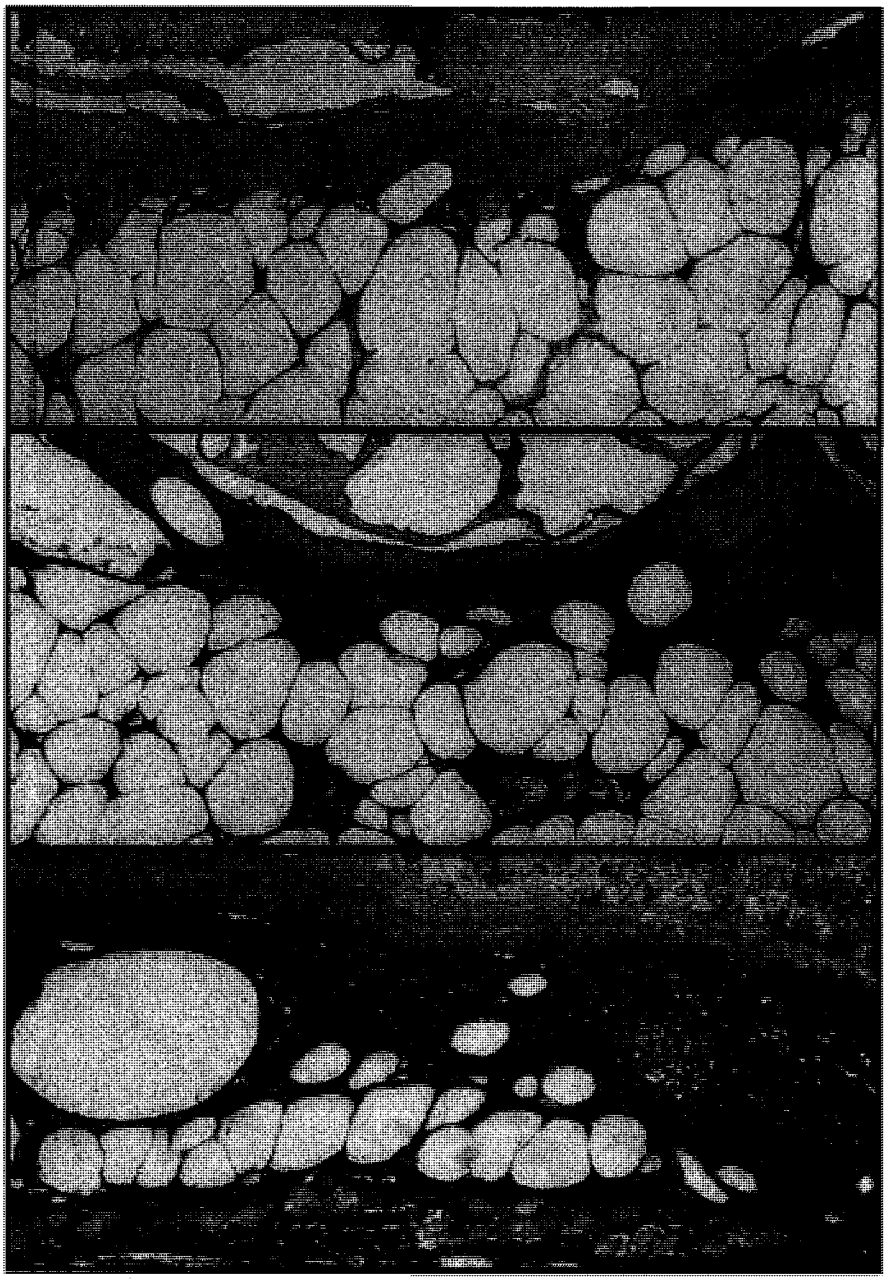


Figure 2.4f V: Representive fields of wound area sections treated with vehicle, bFGF (10ug/ml) clustered Vasculotide (5nM), are peroxidise stained with antibody against Sma1. Note, histopathological comparison of 5nM and 20nM clustered Vasculotide treatments revealed no statistically significant difference (data not shown). For presentation purposes only the 5nM clustered Vasculotide samples are shown.

Ang 1, we illustrate with the use of reconstitution experiments, that Vasculotide uncouples Tie 2 signalling from β1 integrin. We report that treatment of primary endothelial cells with clustered Vasculotide results in induction of cell migration, MMP2 release and protection from serum withdrawal-induced apoptosis. *In vivo* we show, when impregnated in Matrigel, clustered Vasculotide promotes a robust proangiogenic response that is marked by the production of well arborized vessels that stain positively for the myogenic support cell marker, Smal. Significantly, we show that clustered Vasculotide, when combined with VEGF, does not increase vessel number, vessel length or number of branch points, but rather mitigates the tortuosity seen with the addition of VEGF alone. We have examined the utility of clustered Vasculotide in the treatment of experimentally induced wounds using a widely accepted animal model of diabetes mellitus type II. We show that clustered Vasculotide offers accelerated wound closure when compare to vehicle or bFGF controls. Using this model of impaired wound healing we elucidate that the improved wound closure times noted for clustered Vasculotide derive from increased granulation tissue and neovascularization of the wound. Currently there are 16 million people with diabetes in the U.S. with 798,000 new cases reported annually and a prevalence of approximately 6% of the population. Estimates report that 10–15% of diabetics will go on to develop foot ulcers of which 14-20% will require amputation. Foot ulceration is the precursor to approximately 85% of lower extremity amputations. This work provides the impetus to further explore the therapeutic potential of clustered Vasculotide in the treatment of complex wounds and its potential in promoting angiogenesis in a number of other regenerative medicine endeavors.

Chapter 3

Processive recruitment of c-Src and Csk to Dok-R

A version of this chapter is published in: Van Slyke, P., Loza Coll, M., Master, Z., Kim, H., Filmus, J., and Dumont, D. (2005) **"Dok-R mediates attenuation of EGF-dependent MAPK and Akt activation through processive recruitment of c-Src and Csk"**. MCB. (25): 3831–3841.

Abstract

The Dok family of proteins, which includes Dok, Dok-R, DokL, Dok-4, Dok-5 and possibly IRS1-3, are docking proteins that are recruited to a wide array of activated receptor tyrosine kinases. Dok-R has previously been shown to associate with the Epidermal Growth Factor Receptor (EGFR) and become tyrosine phosphorylated in response to EGF stimulation. The recruitment of Dok-R to the EGFR, which is mediated through its phosphotyrosine binding (PTB) domain, results in attenuation of mitogen activated protein kinase (MAPK) activation. Dok-R's ability to attenuate EGF-driven MAPK activation is independent of its ability to recruit rasGAP, a known attenuator of MAPK activity, suggesting an alternate Dok-R-mediated pathway. c-Src family kinases (SFK) play multiple roles in transducing signals from a diverse family of receptors, including the EGFR. Herein, we have determined the structural determinants within Dok-R that are required for its ability to attenuate EGF-signaling, to associate with c-Src and with the SFK-inhibitory kinase, Csk. We demonstrate that Dok-R associates constitutively with c-Src through an SH3-dependent interaction and that this association is essential to Dok-R's ability to attenuate c-Src activity and diminish MAPK and Akt/PKB activity. We further illustrate that EGF-dependent phosphorylation of Dok-R requires SFK activity and, more specifically, that SFK-dependent phosphorylation of tyrosine 402 on Dok-R facilitates the inducible recruitment of Csk. We propose that recruitment of Csk to Dok-R serves to bring Csk to c-Src and down-regulate its activity resulting in a concomitant attenuation of MAPK and Akt/PKB activity. Furthermore, we demonstrate that Dok-R can abrogate c-Src's ability to protect the breast cancer cell line, SKBR3 from anoikis and that an association with c-Src and Csk is required for this

activity. Importantly, we demonstrate that this multiprotein complex can be coimmunoprecipitated from early embryos illustrating that the complex exists *in vivo.* Collectively we demonstrate that Dok-R acts as an EGFR-recruited scaffolding molecule that processively assembles c-Src and Csk to attenuate signaling from the EGFR.

Introduction

The precise spatial and temporal control of signals emanating from an activated receptor tyrosine kinase (RTK) depends in part on the diverse repertoire of recruited proximal signaling proteins. These recruited proteins can serve to both augment the signal from the receptor or to attenuate the signal, the balance of which is crucial to normal cell physiology (Fiorini et al., 2001). Docking proteins have been shown to play a pivotal role in transducing signals from activated RTK's. In addition to being constitutively bound to signaling molecules these specialized types of polypeptides also become tyrosine phosphorylated upon recruitment to RTKs. These tyrosine phosphorylation events establish high-affinity phoshotyrosine-based binding sites for the recruitment of additional signaling molecules (Pawson and Scott, 1997;Hlavacek et al., 2003). As such, docking proteins function much like a scaffold protein, locally enriching the quantity and diversity of signaling proteins necessary to elicit a defined response to RTK activation.

One family of docking proteins that appears to have a negative role in RTK or cytokine signaling is the Dok family of proteins. Based on amino acid sequence homology the Dok family of proteins consists of five members, including Dok, Dok-R, DokL, Dok4, and Dok5 (Yamanashi *et al.*, 1997;Carpino *et al.*, 1997;Di Cristofano et al., 1998;Jones *et*

al., 1998b;Cong et al., 1999;Lemay *et al.*, 2000;Grimm *et al.*, 2001). Structural characteristics of this family make them most similar to the insulin receptor substrate family of proteins (Cong *et al.*, 1999). The Dok family of proteins contain three distinct protein domains or regions, which include an amino terminal pleckstrin homology (PH) domain, a central phosphotyrosine binding (PTB) domain and a carboxy terminal proline rich region (PRR). Dok4 and Dok5 have been shown to potentiate signals emanating from the c-Ret receptor (Grimm *et al.*, 2001) while Dok, Dok-R and DokL have all been shown to primarily mitigate signals downstream of a wide array of receptor and non-receptor tyrosine kinases (Nelms *et al.*, 1998;Jones and Dumont, 1999;Cong *et al.*, 1999;Yamanashi *et al.*, 2000;Lemay *et al.*, 2000;Zhao *et al.*, 2001). It has been proposed that family members Dok, Dok-R and DokL are phylogenetically distinct from Dok4 and Dok5 and that they therefore be considered a separate subgroup of the family based upon functional differences and differing patterns of expression (Grimm *et al.*, 2001).

Based upon structure-function analysis it seems apparent that Dok, Dok-R and DokL mediate negative signaling events by recruiting, and locally enriching negative signaling proteins in the proximal region of transduction cascades. For example Dok, Dok-R, and DokL have been shown to inducibly interact with the lipid phosphatase SHIP1 (Lemay *et al.*, 2000;Latour et al., 2001;Ott et al., 2002) while Dok and DokL also interact with Csk, a potent negative regulator of c-Src family kinase members (Shah and Shokat, 2002).

Both Dok and Dok-R have been shown to inducibly interact with the GTPase activating protein p120 RasGAP suggesting a negative regulatory role in Ras/Raf/Mek/MAPK

signaling pathway (Yamanashi *et al.*, 1997;Carpino *et al.*, 1997;Di Cristofano *et al.*, 1998;Jones *et al.*, 1998b). Supporting the significance of the Dok/RasGAP association, Songyang *et al.* demonstrated in v-src transformed cells that overexpression of Dok leads to a dramatic reduction in Ras GTP loading and a concomitant decrease in cellular transformation (Songyang *et al.*, 2001). Furthermore, genetic ablation of the Dok locus initiates leukemogenesis, an event which correlates with increased and prolonged MAPK activation (Di Cristofano et al., 2001). In addition, Gugasyan *et al.* demonstrate that bone marrow cells retrovirally infected with Dok-R are deficient in their ability to reseed lymphoid organs and that this defect is dependent on intact RasGAP binding sites (Gugasyan et al., 2002). Notwithstanding, a clear consensus in the literature with regards to the functional importance of this association has not been developed. Kashige *et al.* demonstrated that Bcr-Abl-dependent recruitment of RasGAP to p62 Dok results in dramatically decreased RasGAP activity, suggesting the existence of alternate mechanisms, other than the Dok/Dok-R-RasGAP association in attenuating tyrosine kinase driven activation of MAPK (Kashige et al., 2001). Consistent with this notion, Zhao *et al.* found that Dok–dependent attenuation of MAPK activity following PDGF stimulation was not dependent on its ability to recruit RasGAP or Nck, but rather was a function of some unknown mechanism involving phosphatidylinositol 3 (PI3) kinase activity (Zhao *et al.*, 2001). Studies by our group on EGFR signaling (Jones *et al.*, 1999) have further indicated that RasGAP recruitment by Dok-R is not essential for inhibition of MAPK. And finally, further contributing to the unknown role of RasGAP in modulating the down-regulation of signaling from the Dok–family of proteins is data

demonstrating that DokL, which intrinsically lacks RasGAP binding sites, retains the ability to attenuate MAPK activity in Bcr-Abl transformed cells (Cong *et al.*, 1999).

Herein we attempt to elucidate in further detail what mechanisms other than RasGAP may mediate Dok-R-dependent attenuation of MAPK. Optimal recruitment of Dok-R to the activated EGFR and its subsequent phosphorylation requires both a functional PH and PTB domain, which is required for Dok-R's ability to inhibit downstream activation of MAPK (Jones *et al.*, 1999). Importantly, these effects were shown not to be a consequence of Dok-R displacing the Shc/Grb2/Sos complex from the receptor (Jones *et al.*, 1999) suggesting a more specific role for Dok-R in down-regulating MAPK activity than mere competition for binding sites on the EGFR. Moreover, expression of a form of Dok-R no longer able to interact with RasGAP illustrates that recruitment of RasGAP to a phosphorylated Dok-R is not required to mitigate MAPK activation downstream of EGFR (Jones *et al.*, 1999). Collectively these results support a role for attenuation of MAPK through a rasGAP-independent mechanism that requires recruitment of Dok-R to the EGFR. Here we describe a novel mechanism whereby Dok-R facilitates the formation and recruitment of a multi-protein complex to the activated EGFR resulting in the attenuation of signals from this receptor. We present data to support a model whereby Dok-R is constitutively associated with c-Src and upon recruitment to the EGFR Dok-R becomes phosphorylated on tyrosine residue 402, which serves to recruit the negative regulator kinase, Csk. We show that this processive recruitment of a Dok-R/c-Src/Csk complex to the EGFR serves to provide the scaffolding that ultimately leads to

the attenuation of c-Src kinase activity as well as diminished MAPK and Akt/PKB activity.

Results

The carboxyl portion of the proline rich region of Dok-R is required for EGF-dependent Erk-2 attenuation.

Previously we demonstrated that Dok-R is able to attenuate EGF-induced Erk-2 activity in EGF-stimulated Cos1 cells in a RasGAP-independent manner (Jones *et al.*, 1999). In this study we set out to define the alternative pathway(s) utilized by Dok-R to attenuate signaling from the EGFR. Towards this goal, a truncation mutant was engineered for these studies, which completely deletes the PRR (Dok-R ΔPRR). The effect of an empty vector, Dok-R and Dok-R ΔPRR on Erk-2 activation was monitored by co-transfection of Cos1 cells with these constructs and Gst-Erk-2 as previously described (Jones *et al.*, 1999). Serum-starved EGF-stimulated cells transfected with vector cDNA produced the predicted time-dependent increase in Erk-2 activation as assessed by immunoblotting for phopho-specific p42,44 MAPK (Figure 3.1). As previously reported, expression of Dok-R in EGF-stimulated cells resulted in a dramatic decrease in the induction of Erk-2 activation as well as a delay in the activation kinetics (Figure 3.1), while Dok-R ΔPRR completely lost this Erk-2 attenuating capacity which demonstrates that the key residues for mediating this attenuation are found within the PRR.

C-Src Kinase Activity is Necessary for Full EGF-Dependent Activation of MAPK

Since our previous studies had demonstrated that Dok-R mediated attenuation of Erk2 activity is RasGAP independent we sought to analyze other cognate MAPK activators

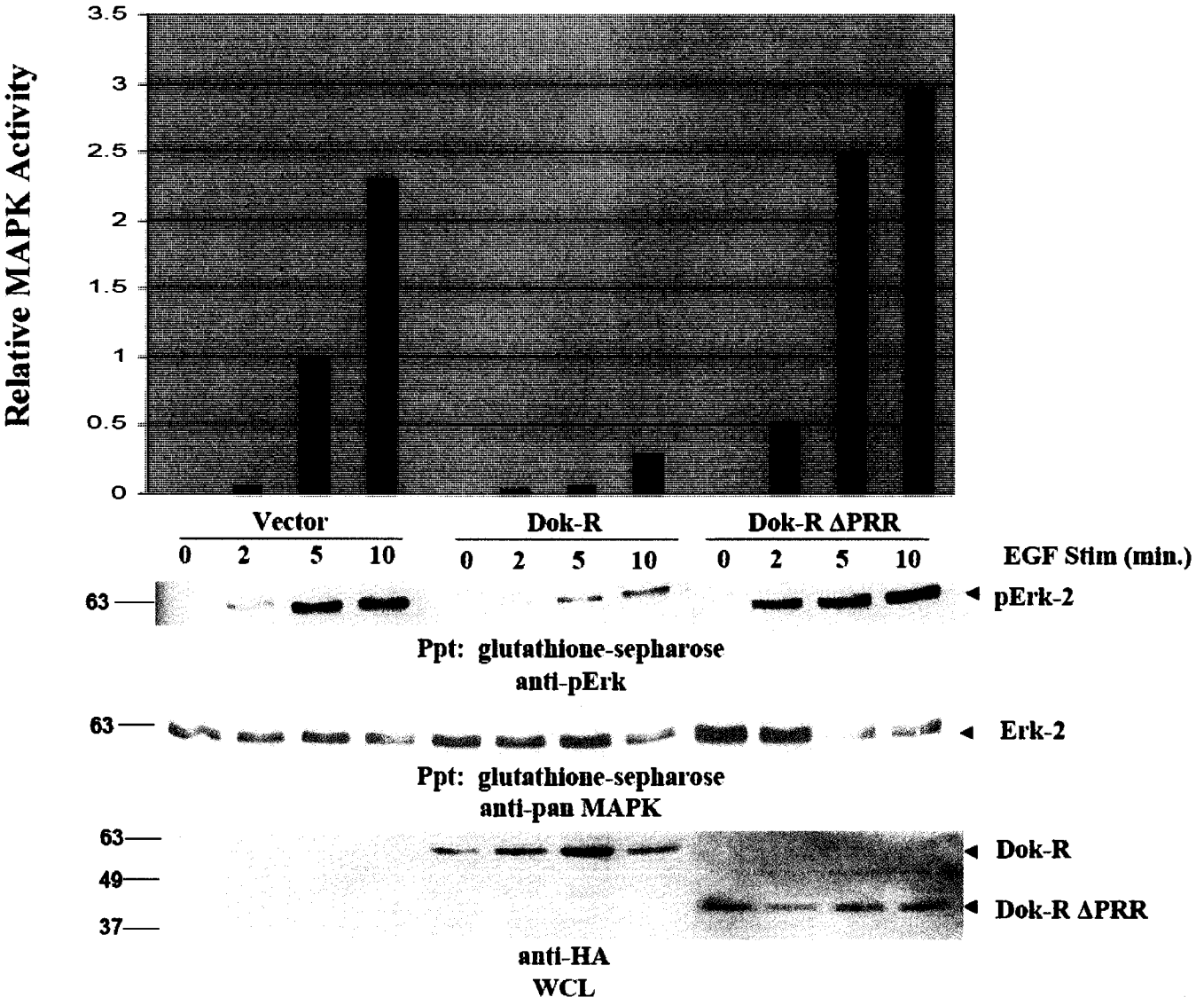


Figure 3.1. The carboxy terminal region of Dok-R is required for suppression of EGF-dependent MAPK activation. Cos 1 cells were transiently transfected with Gst-Erk 2 and one of either vector, Dok-R, or Dok-R ΔPRR. Serum starved Cos 1 cells were left unstimulated (0), or stimulated with 100 ng/ml of EGF for indicated times (2, 5, 10) minutes. Cleared lysates were prepared and glutathione sepharose was used to specifically purify the exogenous Gst-Erk 2. Immunoblot analysis with phospho-specific MAPK, pan MAPK (Gst-pull-downs) as well as anti HA (WCL) was performed. The graph represents relative MAPK activation determined via anti phosho-MAPK immunoblot analysis when normalized to pan Erk 2 levels using densitometry (Molecular Dynamics and ImageQuant 5.0). Results are representative of four independent experiments and serve to demonstrate that the PRR of Dok-R is necessary to attenuate the EGF-dependent MAPK activation.

downstream of the EGFR. EGF-dependent recruitment and activation of c-Src has been shown to contribute to the potentiation of several distinct signaling pathways which culminate in Erk2 activation (Belsches et al., 1997). Thus, we were interested in examining whether Dok-R may be playing a role in attenuating signals from the activated EGFR through modulation of c-Src-dependent kinase activity. In order to determine whether Src-family kinases (SFK) are involved in Erk-2 activation in our cell system Cos 1 cells transfected with the Erk-2-Gst plasmid were serum starved and stimulated with EGF in the presence or absence of the SFK inhibitor, PP1. Basal Erk-2 phosphorylation was apparent in the untreated cells prior to EGF stimulation and there was a time dependent increase in Erk-2 activation up to and including 10 minutes of stimulation (Figure 3.2). Cells that had been pre-treated with PP1 demonstrated a potent loss of Erk-2 phosphorylation in both the unstimulated and stimulated samples. Longer exposure of the immunoblots illustrated that although there is a detectable pErk-2 signal in the PP1 treated lanes, the activation is severely impaired (data not shown). Because high levels of PP1 are known to inhibit EGFR activation in certain systems (Hanke et al., 1996) we probed our samples for phosphorylated EGFR and demonstrated that the levels of PP1 used in these experiments did not dramatically affect the activation of the EGFR, suggesting that these effects can be attributed to inhibition of SFKs.

Dok-R Potently Attenuates c-Src Kinase Activity

Our results demonstrate that EGF-mediated Erk-2 activation requires the activity of one or more SFK's. We next wanted to examine if Dok-R mediated attenuation of EGF-dependent Erk2 activation was facilitated through modulation of c-Src kinase activity.

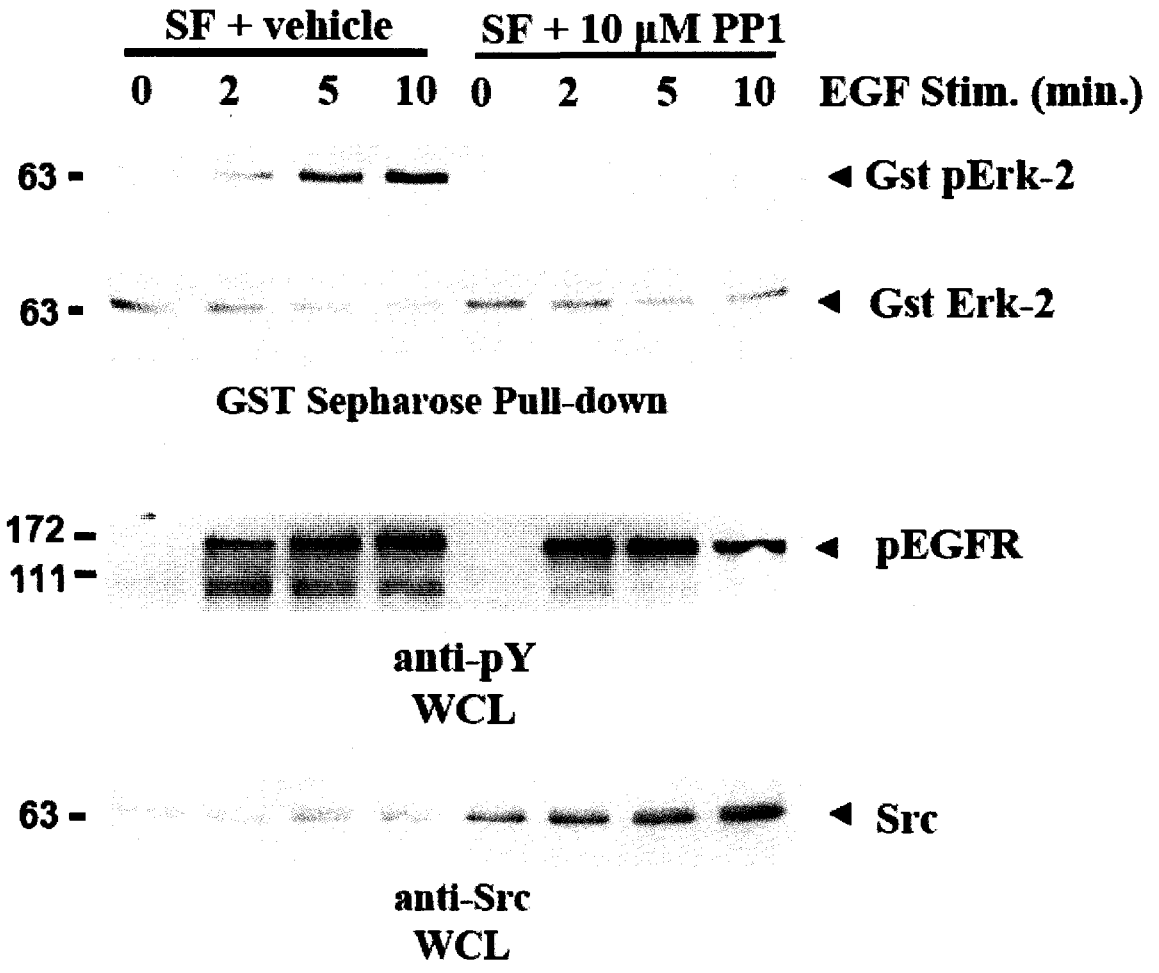


Figure 3.2. Src-family kinase (SFK) activity is required for full activation of EGF-dependent MAPK activity. Gst-Erk 2 transfected Cos1 cells were serum starved for 16 hours prior to being treated with SFK inhibitor PP1 (10 μM) or vehicle (DMSO) for 2 hours. Cells were either left unstimulated (0) or stimulated with EGF 100 ng/ml for indicated times (2, 5, 10) minutes. Gst-Erk 2 was purified from cleared lysates with glutathione sepharose and this exogenous Erk 2 was assayed for activation via immunoblotting with phosho-specific MAPK antibody. Inhibition of SFK's (PP1) but not vehicle dramatically decreases EGF-dependent MAPK activation in Cos1 cells. Differences in MAPK activation could not be attributed to total Erk 2 levels (anti pan MAPK). Treatment with PP1 at 10 μM does not influence activation of the EGFR (anti pY EGFR).

To aid in delivering our constructs of interest into cells we developed recombinant adenoviruses expressing Dok-R and Dok-R ΔPRR. Cos 1 cells were infected with one of either ad-LacZ (vector), ad-Dok-R or ad-Dok-R ΔPRR and incubated for indicated times in the presence or absence of EGF. c-Src was immunoprecipitated from equal amounts of protein lysate and *in vitro* kinase assays were performed using enolase as an exogenous substrate (Cooper and Hunter, 1983). Several independent *in vitro* c-Src kinase assays demonstrated that Dok-R potently inhibits EGF-induced c-Src kinase activity whereas this inhibition is completely abrogated in Dok-R ΔPRR infected cells, as they responded similarly to vector infected cells (Figure 3.3). Interestingly the levels of c-Src kinase activity in unstimulated Cos 1 cells appeared similar in ad-vector, ad-Dok-R, and ad-Dok-R ΔPRR samples indicating that Dok-R does not appear to influence basal c-Src kinase activity, but rather, Dok-R functions to specifically inhibit EGF-induced c-Src kinase activity. Control immunoblots indicate that differences in Src kinase activity could not be accounted for by differing levels of total Src protein, or activation of the EGFR (see WCL anti src IB, anti 4G10 IB).

Dok-R Strongly Induces EGF-Dependent Phosphorylation of c-Src in the Negative Regulatory Tyrosine 527

Src kinase activity is tightly and dynamically regulated by the opposing actions of several different phosphatases (Cooper and King, 1986;den Hertog et al., 1993;Fang et al., 1994;Peng and Cartwright, 1995;Zheng et al., 2000) and almost exclusively, the carboxy-terminal Src kinase, Csk (Okada et al., 1991). Csk down-regulates c-Src kinase activity by catalyzing the addition of a phosphate group to the negative regulatory tyrosine

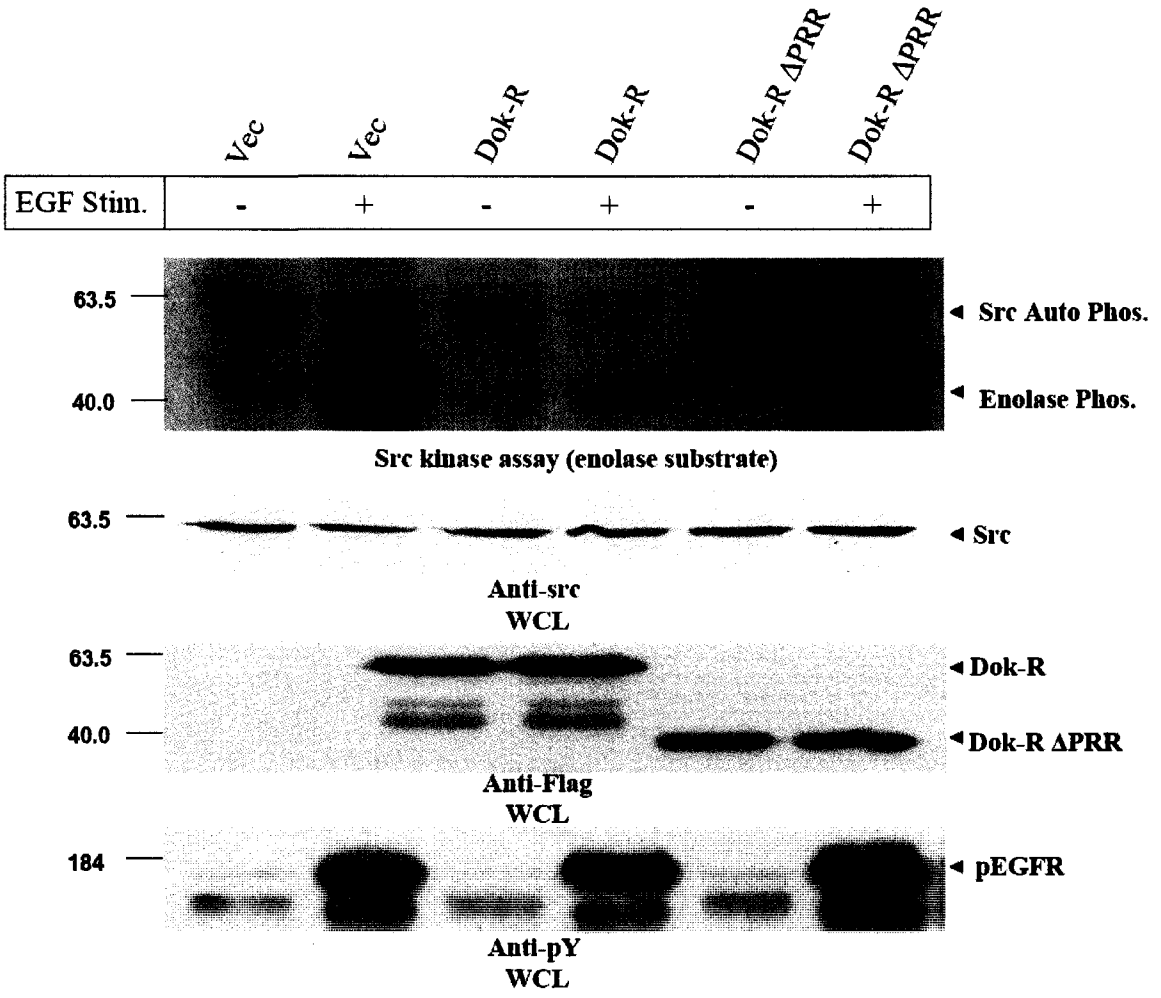


Figure 3.3. Dok-R, but not vector or Dok-R ΔPRR inhibits EGF-dependent Src kinase activity. Cos1 cells were infected with adeno viruses engineered to express one of either LacZ (vector), Dok-R, or Dok-R ΔPRR. Serum starved cells were either left unstimulated (-) or stimulated (+) for 5 minutes with EGF (100 ng/ml). Src immunoprecipitates from infected cells were subjected to an *in vitro* kinase assay using rabbit enolase as a substrate. Src-mediated enolase phosphorylation in the vector, Dok-R, and Dok-R ΔPRR remained at a basal level in the unstimulated cells. Upon EGF stimulation cells infected with vector or Dok-R ΔPRR demonstrated a dramatic increase in enolase phosphorylation while overexpression of Dok-R completely abolished this effect. Immunoblot analysis of Src levels (anti Src IB) reveals that these effects are not due to differing levels of total Src protein nor are can they be accounted for by differential EGFR activation (anti pY EGFR).

(Y527) located in the carboxy terminal region of c-Src. To examine the possibility that Dok-R was mediating its attenuation of c-Src through modulation of the intracellular localization and/or activity of Csk we set out to specifically examine the phosphorylation of c-Src tyrosine residue 527. Cos1 cells were transfected with one of either vector, Dok-R, or Dok-R ΔPRR and allowed to grow for 24 hours under normal growth conditions. Cells were then serum starved and stimulated with EGF as in our previous experiments. Immunoblot analysis with an anti-phospho-tyrosine-527 (pY527) Src-specific antibody reveals that Y527 is hyperphosphorylated in the presence of over-expressed Dok-R (Figure 3.4a). Importantly, these results are in accordance with our previous *in vitro* kinase assay results (Figure 3.3) and they suggest that the attenuation of c-Src activity is dependent upon recruitment of Dok-R to the activated EGFR. Upon longer exposure of the cells to EGF stimulation we see a time dependent loss of c-Src pY527 signal in the vector and Dok-R ΔPRR sample lanes indicative of c-Src activation that is not noted in the Dok-R transfected lanes. To determine how universal this effect was, we performed the same assay in NIH 3T3 cells and observed a similar, albeit less dramatic hyperphosphorylation of c-Src tyrosine 527, which most probably reflects the higher expression levels of Dok-R achieved in Cos1 cells (data not shown). Collectively, these results suggest that the PRR of Dok-R is required in both Cos1 and NIH 3T3 cells to promote EGF-dependent hyperphosphorylation of c-Src Y527, and it is this hyperphosphorylation event that results in mitigated c-Src kinase activity.

To examine if this effect was specific to EGFR mediated signaling events we co-transfected Cos1 cells with either vector or Dok-R and Gst-Erk2. Serum starved cells were either left unstimulated or stimulated with PMA for indicated times. Transfection

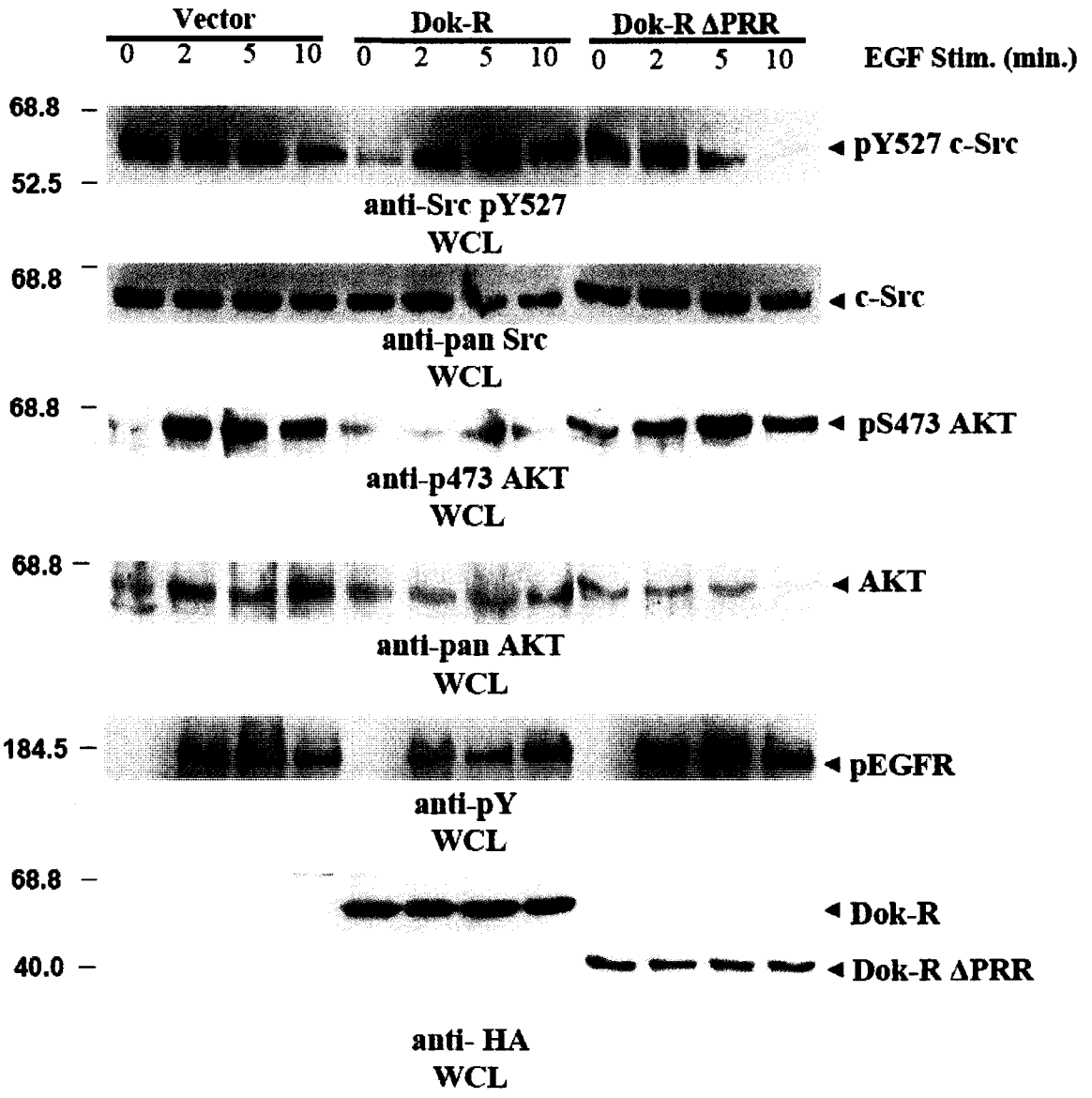


Figure 3.4. Overexpression of Dok-R facilitates EGF-dependent hyperphosphorylation of autoinhibitory Src Y527. (**a**) Serum starved Cos1 cells transfected with one of either vector Dok-R or Dok-R ΔPRR were either left unstimulated (0) or stimulated with EGF 100 ng/ml (2, 5, 10 minutes) for indicated times. Cleared lysates were prepared and subjected to SDS PAGE. Immunoblot analysis of WCL demonstrates that overexpression of Dok-R but not vector or Dok-R ΔPRR results in an EGF-dependent hyperphosphorylation of Src on tyrosine 527. These results cannot be accounted for by overall Src levels (anti Src IB) or differences in EGFR activation (anti pY EGFR). Coincident with the Dok-R-dependent hyperphosphorylation of Src Y527 is a dramatic decrease in Akt activation (anti Akt pS 473) which can not be accounted for by overall Akt protein levels (anti pan Akt).

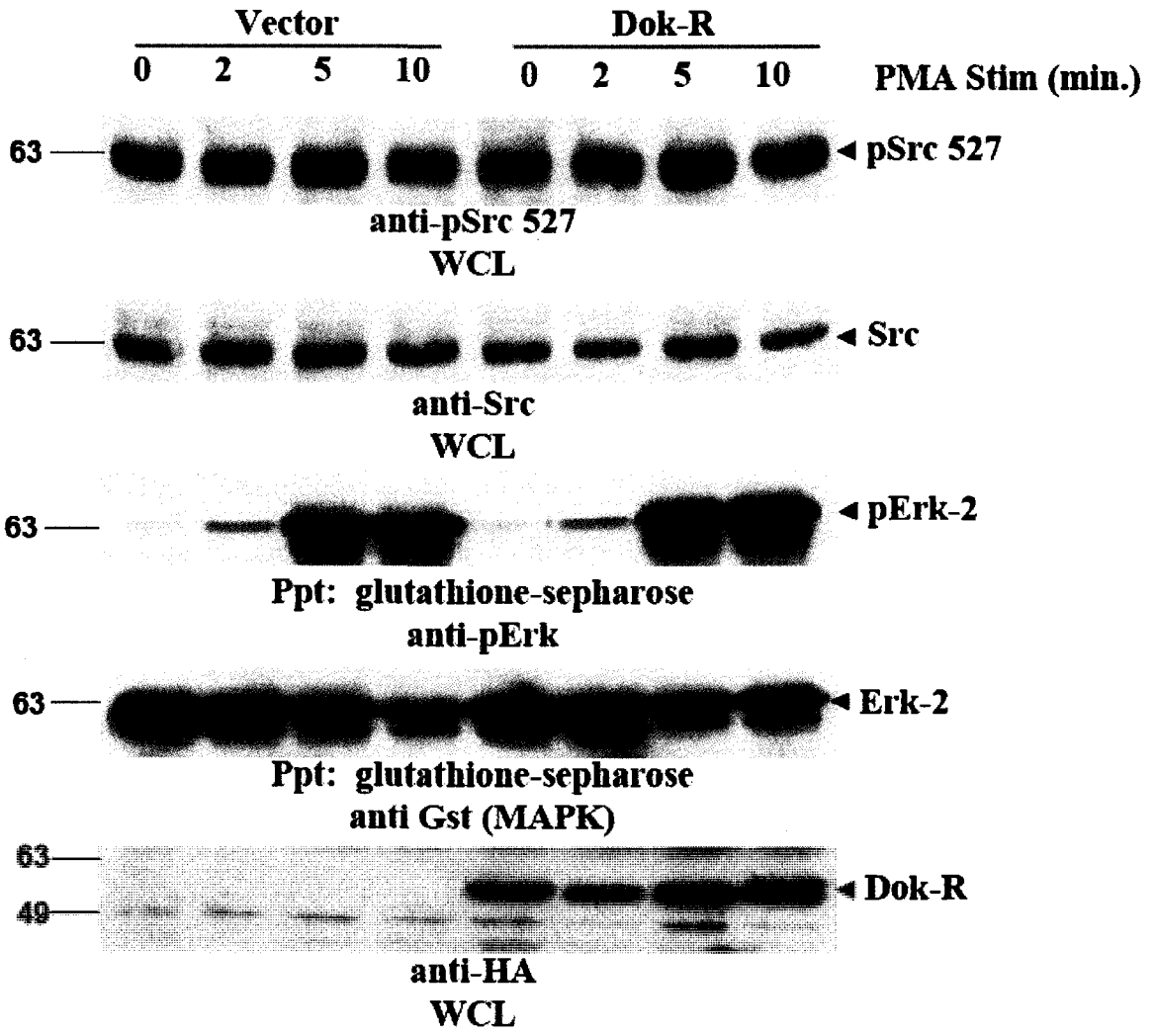


Figure 3.4. Dok-R does not inhibit Src kinase or MAPK activation in response to PMA stimulation. **(b)** Cos1 cells co-transfected with one of either vector or Dok-R and Gst-Erk2 were left unstimulated (0) or stimulated for indicated times (2, 5, 10 minutes) with 200 nM PMA. Cleared lysates were prepared and subjected to SDS PAGE. Immunoblot analysis demonstrates that overexpression of Dok-R does not result in PMA-dependent inhibition of Src kinase activity (anti Src pY527) or MAPK activation (Gst Ppt/anti pErk) when compared to vector transfected cells.

of Dok-R did not alter the activation of Erk2 (anti pErk) or the phosphorylation of c-Src pY527 (anti Src pY527) in PMA stimulated cells (Figure 3.4b), when compared to vector transfected controls. These results suggest that c-Src independent pathways leading to MAPK activation are not perturbed by overexpression of Dok-R protein but rather Dok-R functions to specifically inhibit EGF-induced c-Src activation (Figure 3.3 and 3.4a).

Dok-R is a Substrate of c-Src Family Kinases

The Dok-family of proteins is known to be tyrosine phosphorylated by several distinct protein tyrosine kinases, including Tek/Tie2, Hck, Src, and Abl (Jones *et al.*, 1998b;Lock et al., 1999;Master *et al.*, 2003). In previous studies we have demonstrated that Dok-R becomes tyrosine phosphorylated upon EGF stimulation in Cos1 cells (Jones *et al.*, 1999). In these studies the kinase that phosphorylated Dok-R was not determined experimentally thus we set out to further define the kinase(s) responsible for EGF-dependent Dok-R phosphorylation. Our initial experiments using the SFK inhibitor PP1 (Figure 3.2) suggested that in Cos1 cells SFK activity is absolutely required for full activation of Erk2, thus we focused our studies on SFK's. Cos1 cells infected with either ad-Dok-R or ad-Dok-R ΔPRR were treated with PP1 or vehicle (DMSO) prior to stimulation with EGF. Precleared whole cell lyates were analyzed for evidence of stimulation dependent Dok-R tyrosine phosphorylation via immunoblot analysis with an anti phosphotyrosine antibody (Figure 3.5). Inhibition of SFK activity with PP1 resulted in a dramatic reduction of Dok-R phosphorylation. Interestingly the inability of c-Src to physically associate with Dok-R ΔPRR (see also Figure 3.7a) does not fully abrogate its SFK-dependent phosphorylation as indicated by the fact that Dok-R ΔPRR to a small

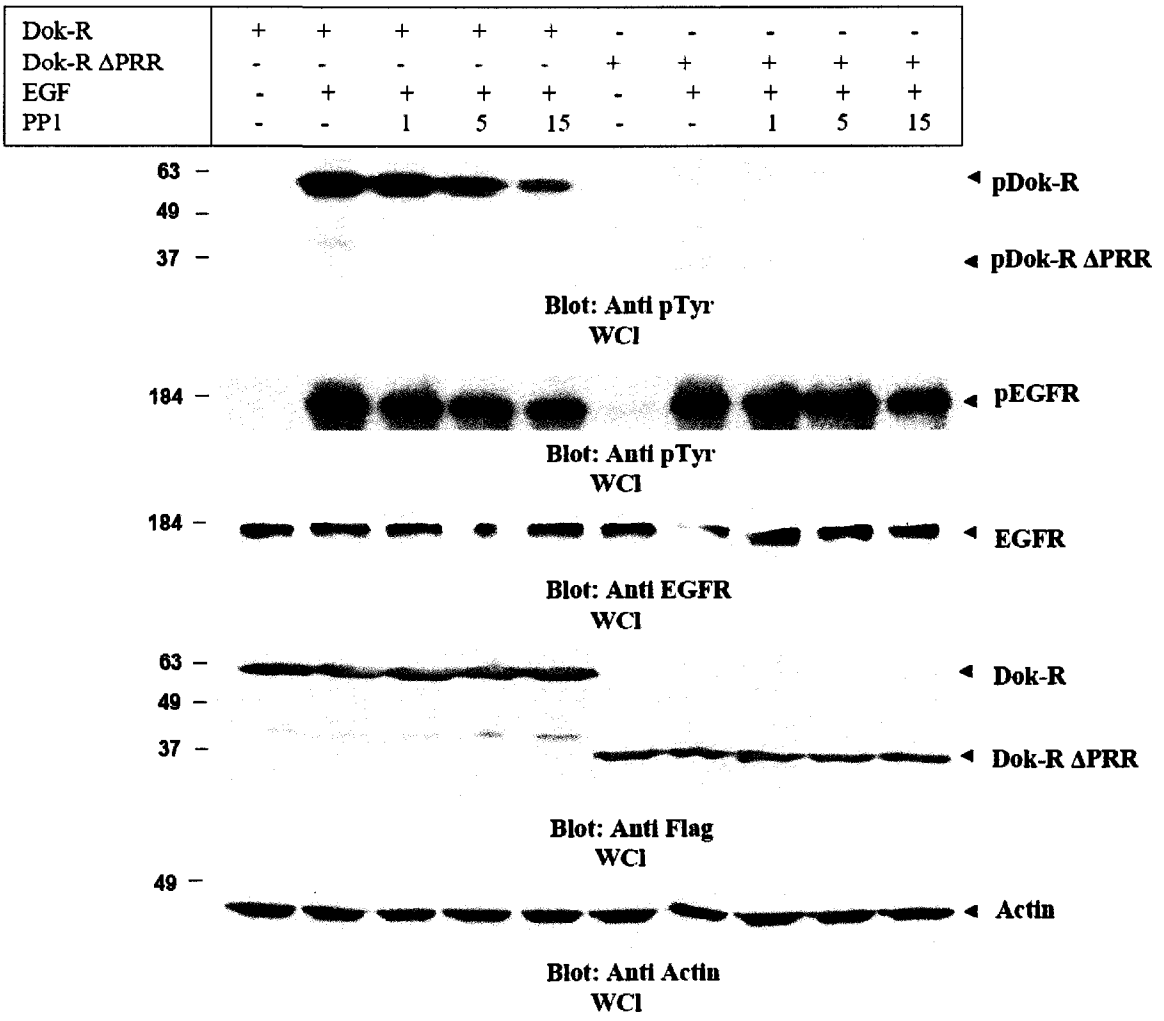


Figure 3.5. Dok-R is a substrate of SFK's. Cos1 cells transfected with either Dok-R or Dok-R ΔPRR were serum starved for 16 hours prior to a two hour pretreatment with PP1 (1, 5, 15 μM) or vehicle (-). Following the pretreatment cells were either left unstimulated (-) or stimulated (+) for 5 minutes with 100 ng/ml EGF. Cleared lysates were resolved via SDS PAGE. Immunoblot analysis of lysates with an anti pY antibody demonstrates that Dok-R and to a much lesser degree Dok-R ΔPRR become tyrosine phosphorylated in response to EGF stimulation. Increasing concentrations of PP1 are able to completely abolish phosphorylation of Dok-R ΔPRR while phosphorylation of Dok-R is severely impaired in a dose-dependent manner by PP1 treatment. Changes in Dok-R phosphorylation are not a reflection of changes in total Dok-R or Dok-R ΔPRR protein levels (anti HA, WCL), nor are they due to PP1-dependent changes in EGFR activation (anti pY EGFR) or EGFR levels (anti EGFR).

degree, is tyrosine phosphorylated, and this phosphorylation is lost upon treatment with as little as 1 μM PP1. PP1 sensitive phosphorylation of Dok-R ΔPRR suggests that at least one tyrosine residue upstream of the PRR is phosphorylated upon EGF stimulation and that this specific phosphorylation event is mediated by a SFK member. These results may be consistent with those of Songyang *et al.* as it has recently become apparent that Dok-R, like Dok becomes phosphorylated on tyrosine residue 142 (unpublished data, Biosource USA), the analogous residue to Dok tyrosine 146 (Songyang *et al.*, 2001). Our results demonstrate that upon recruitment of Dok-R to the activated EGFR it becomes tyrosine phosphorylated and the phosphorylation of these tyrosine residues is primarily mediated by a SFK.

Endogenous Co-immunoprecipitation of Dok-R/EGFR/c-Src/Csk

The preceding structure-function studies required transfection and viral experimental approaches, thus to further validate that these interactions occurs *in vivo* we tested for the presence of this multiprotein complex in an instance where the EGFR was presumed to be activated *in vivo*. Embryonic day 12.5 CD1 mouse embryos were harvested on ice and then disaggregated in EDTA/EGTA. In an attempt to further insure maximal activation of the EGFR half of the cell suspension was stimulated with EGF while the other half was left unstimulated. Cell lysates were prepared and a single immunoprecipitation was conducted for each of the stimulated and unstimulated samples using equal quatities of either covalently cross linked anti Dok-R sepharose or irrelevant control rabbit IgG sepharose (non cross linked). Each immunoprecipitation was split into four equal portions and individual Western blots for c-Src, EGFR, Csk and Dok-R were performed (Figure

3.6). The pre-treatment of disaggregated embryonic cells with EGF did not increase the relative phosphorylation levels of EGFR, suggesting that the EGFR is already fully activated in early embryos (Figure 3.6). Immunoprecipitations utilizing the anti Dok-R antibody specifically associated with large amounts of c-Src and Csk, while EGFR was co-purified with Dok-R, albeit at lower amounts. There was a complete absence of c-Src, Csk, EGFR and Dok-R in the immunoprecipitations with control rabbit IgG. Attempts to resolve Dok-R from the IgG heavy chain have proved futile (see bottom panel Figure 3.6). As such, covalent cross linking of the anti Dok-R antibody facilitates relative determination of the quantity of Dok-R immunoprecipitated in this experiment. The total quantity of IgG heavy chain noted in the Csk Western blot is approximately equal to that in the Dok-R Western blot. The increase in the signal pertaining to the Dok-R immunoprecipitation/ Western blot when compared to the Dok-R immunoprecipitation/Csk Western blot can be attributed solely to the quantity of Dok-R that was removed from the embryonic lysates. The large amount of IgG heavy chain noted in the rabbit IgG immunoprecipitation/Dok-R Western blot is a reflection of the fact that this IgG was not covalently crosslinked to the beads. The high activation state of the EGFR in lysates produced from early embryos precluded us from detailing the inducibility of these interactions.

Constitutive interaction between Dok-R and c-Src

Towards further defining the type of interactions occurring between Dok-R/Src/Csk/EGFR, we performed detailed structure-function studies utilizing the Cos-transfection system described earlier. SFK's have been shown to interact in both

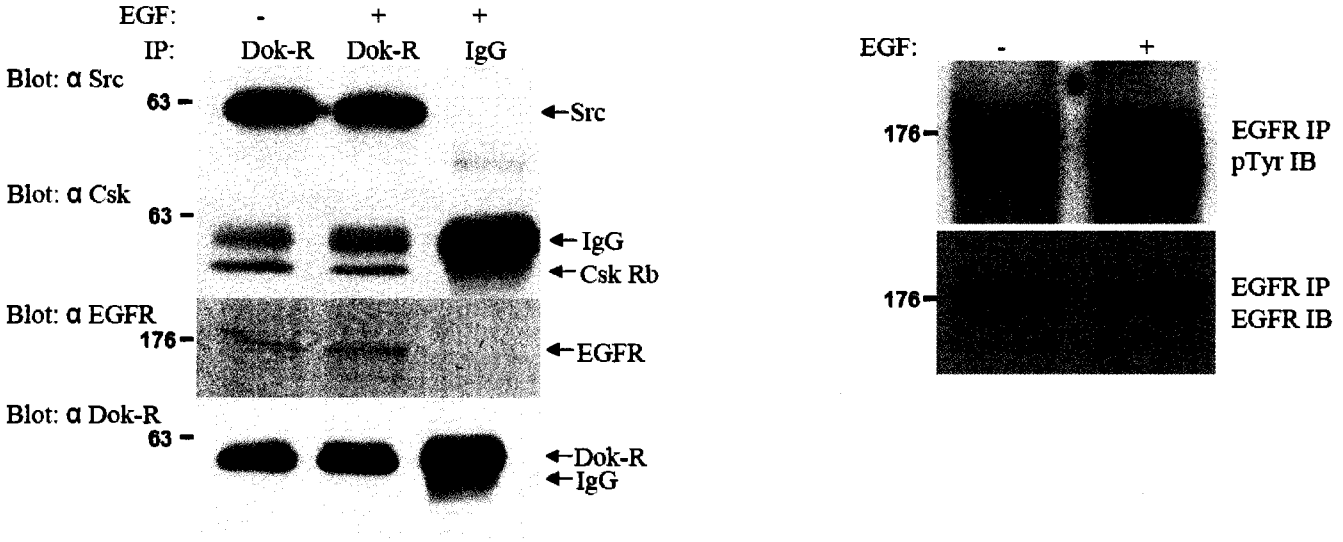


Figure 3.6. Dok-R/Src/Csk/EGFR can be coimmunoprecipitated *in vivo*. Disaggregated embryonic day 12.5 mouse embryos were either left unstimulated or stimulated with EGF for 10 minutes. Lysates prepared from these samples were subjected to immunoprecipitation with either anti sera to Dok-R (covalently cross linked to sepharose beads) or control rabbit IgG (non-cross linked). Immunoprecipitations containing the Dok-R anti sera co-purified c-Src, Csk, and EGFR while these proteins were not detected in the rabbit IgG control lanes. Western blots were performed to assess the relative activation of EGFR in response to EGF stimulation. Based on this analysis it appears that the EGFR is already maximally phosphorylated in the context of the embryonic tissue used for this experiment (see EGFR IP/pTyr IB and EGFR IP/EGFR IB).

inducible and constitutive manners with numerous signaling molecules (Thomas and Brugge, 1997), thus, we sought to determine if Dok-R was able to associate with c-Src. Cos1 cells were co-transfected with plasmids encoding c-Src and either vector, Dok-R, a truncation mutant engineered to delete the final 76 amino acids of the PRR (Dok-R ΔC PRR), or Dok-R ΔPRR. Transfected serum starved cells were left either unstimulated or stimulated for 5 minutes with EGF. Immunoprecipitations for either c-Src or Dok-R (Figure 3.7a) demonstrated that these two proteins were found associated with each other in a non-EGF-stimulation dependent manner. Furthermore, neither of the truncation mutants, Dok-R ΔC PRR or Dok-R ΔPRR were able to associate with c-Src demonstrating the region on Dok-R required for interaction with c-Src resides in the last 76 amino acids of Dok-R. To delineate the region on c-Src responsible for the constitutive interaction with Dok-R purified Gst-SH3 fusion proteins were tested for their ability to bind Dok-R in *in vitro* pull-down assays. Pull-down experiments with several different SH3-Gst domains demonstrated that Dok-R was unable to associate with purified SH3 domains of Vav, Spectrin, p85 and Crk, whereas it is able to associate with the SH3 domain of c-Src, Lck, Fyn, and Abl (Figure 3.7b). These results illustrate that c-Src is bound to Dok-R in a constitutive fashion and that this association requires the SH3 domain of c-Src and the last 76 amino acids of Dok-R.

Recruitment of Csk to Dok-R is mediated by phosphorylation of tyrosine 402

Since previous reports have demonstrated that Dok and DokL both inducibly interact with Csk (Lemay *et al.*, 2000;Shah *et al.*, 2002) we decided to explore the possibility that Dok-R serves as a scaffolding protein that recruits Csk into close proximity of c-Src. As

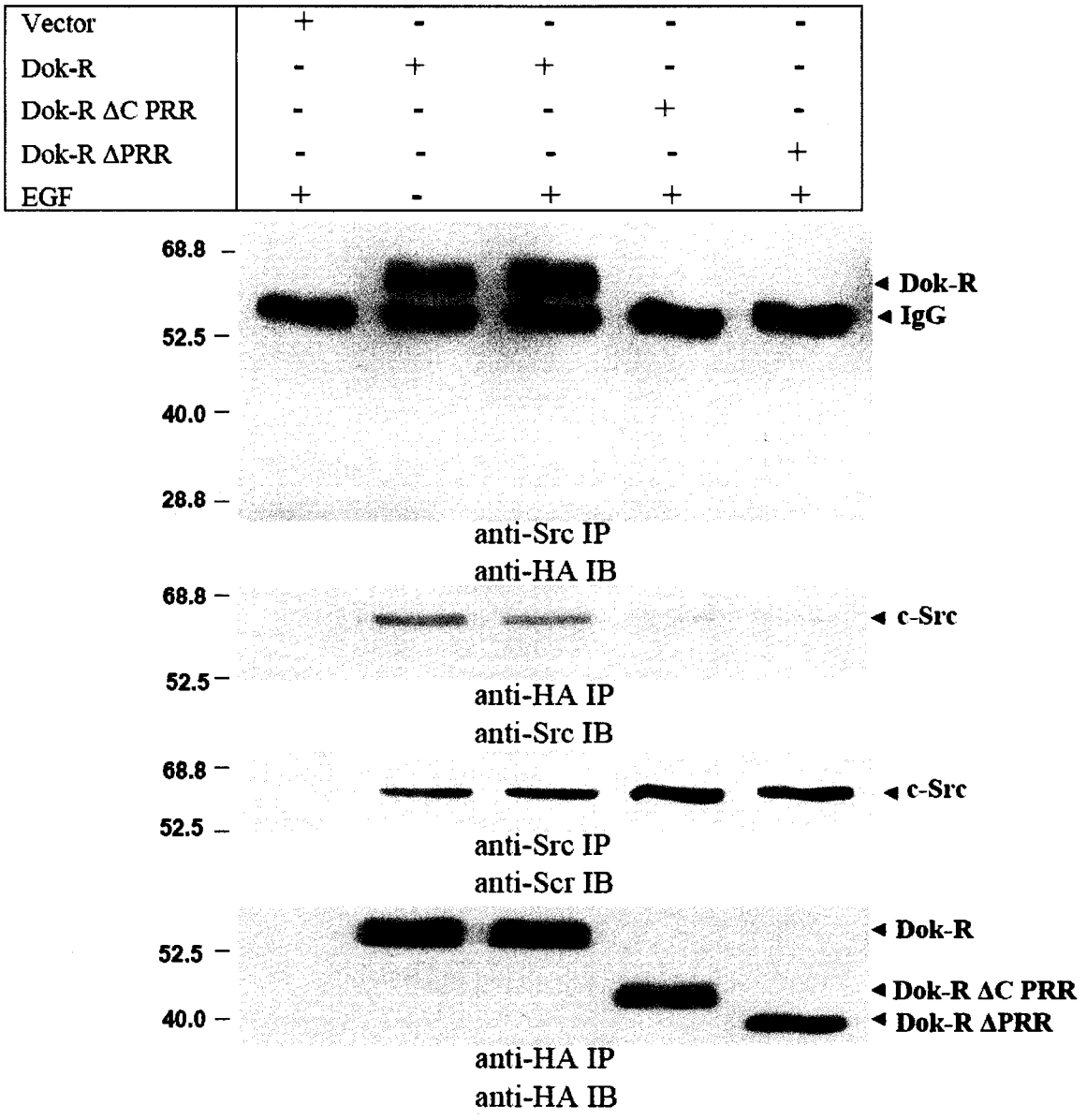


Figure 3.7. Dok-R and Src constitutively co-immunoprecipitate from Cos1 cells and this association is mediated through Dok-R's PRR. (**a**) Serum starved Cos1 cells co-transfected with Src and one of either vector, Dok-R, Dok-R ΔC PRR, or Dok-R ΔPRR were either left unstimulated (-) or stimulated (+) with EGF 100 ng/ml for 5 minutes. Cleared lysates were prepared and immunoprecipitations were performed for either Src or HA (Dok-R constructs). Reciprocal experiments were performed in which Src immunoprecipitations were immunoblotted for HA or HA immunoprecipitations were immunoblotted for Src. In both cases co-immunoprecipitation of Dok-R and Src was noted and was not dependent on EGF stimulation (compare lane 2 and 3). Membranes were stripped and reprobed (Src IP/Src IB and HA IP/HA IB) and serve to demonstrate that the inability of Dok-R ΔC PRR, and Dok-R ΔPRR to co- immunoprecipitate with Src was not due to a relative lack of these proteins in the immunoprecipitation.

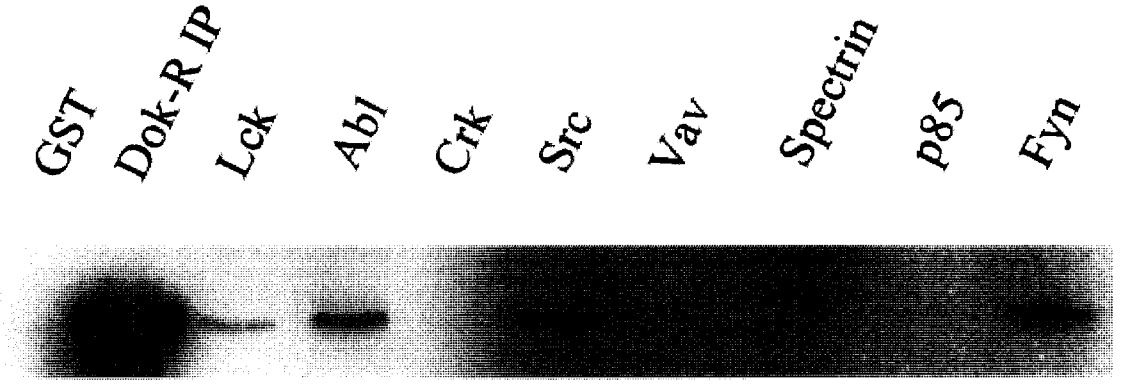


Figure 3.7. (b) The SH3 domain of Src is capable of binding Dok-R *in vitro*. COS1 cell lysates transfected with HA Dok-R were mixed with Gst or Gst-SH3 domain fusions of Lck, Abl, Crk, Src, Vav, Spectrin, p85 or Fyn and separated by SDS PAGE. Anti HA immunoblots revealed that specifically the SH3 domains of Lck, Abl, Src and Fyn precipitated Dok-R and not Gst or the other Gst-SH3 fusion proteins.

noted earlier the relatively high degree of EGFR activation found in mouse embryonic lysates (Figure 3.6) precluded us from defining the nature of the Dok-R/Csk interaction. As such, we set out to specifically determine whether the SH2 domain of Csk could associate with Dok-R or Dok-R ΔPRR isolated from EGF-stimulated Cos 1 cells. To this end, *E. coli* purified Csk SH2 was tested for its ability to associate with Dok-R in an *in vitro* pull-down assay. We demonstrate that the SH2 of Csk is able to interact with Dok-R in an EGF inducible manner and that this interaction is dependent on the PRR (Figure 3.8a). Furthermore, this interaction is partially impaired upon pretreatment of the Cos1 cells with PP1, suggesting the tyrosine on Dok-R that is responsible for this interaction is phosphorylated by a SFK member. Lending credence to this result is the fact that the most carboxy terminally located tyrosine in Dok-R Y402 is nested in a consensus sequence (YXXV) that has been previously identified to be both phosphorylated by SFK members and bound by Csk. To test the hypothesis that Dok-R Y402 is the tyrosine that mediates the interaction with Csk peptide competition assays were performed. Phosphorylated and non phosphorylated peptides spanning the region surrounding Dok-R Y402 were manufactured and tested for their ability to compete for Csk SH2 binding in the presence of Dok-R. Results clearly demonstrate that the phosphorylated peptide, but not the non phosphorylated peptide compete for Csk SH2 binding and that this competition is concentration specific (Figure 3.8b). Note, we were not able to fully compete off all of the Dok-R bound Csk SH2 even at high molar equivalents however, we believe that this is due to a minor non-specific association that occurs between Dok-R and the Gst moiety (see figure 3.8a lanes 3 and 4).

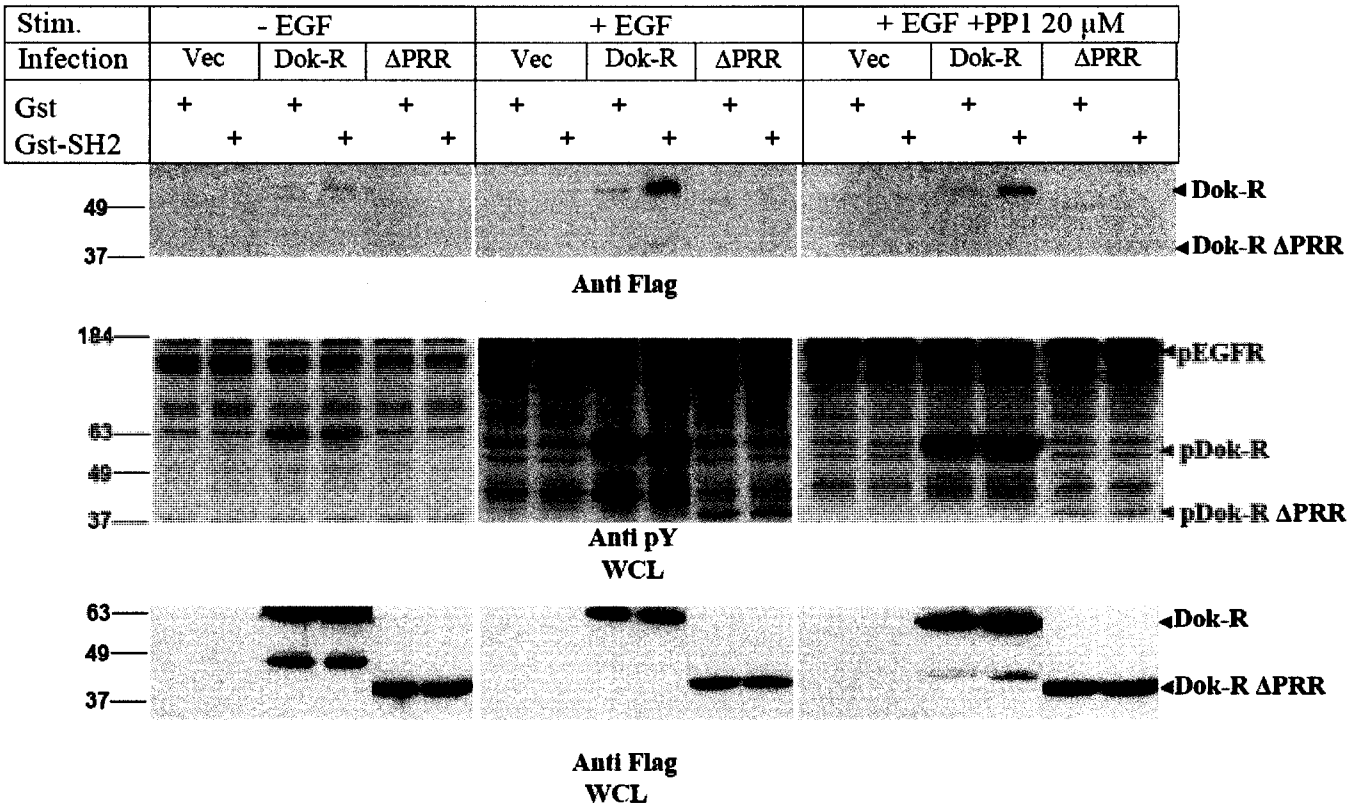


Figure 3.8. **(a)** Dok-R, but not vector or Dok-R ΔPRR inducibly interact with the SH2 domain of Csk. Cos1 cells were infected with adeno viruses engineered to express either vector, Dok-R or Dok-R ΔPRR. The cells were either left unstimulated, stimulated with EGF or stimulated with EGF plus PP1. Cleared lysates of these cells were subjected to Gst sepharose alone or Gst-Csk SH2 sepharose in an *in vitro* pull-down assays.

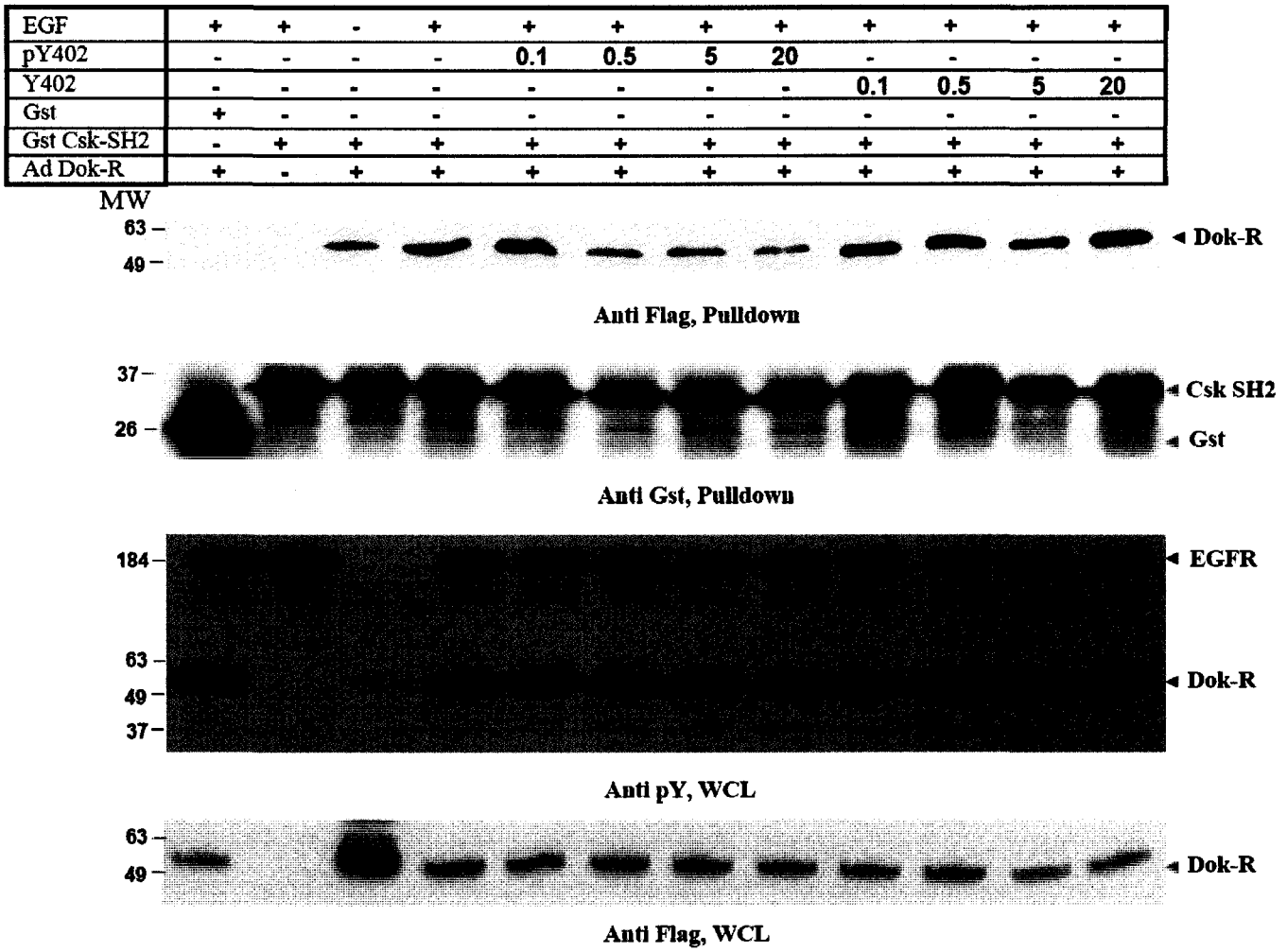


Figure 3.8. **(b)** Src-dependent phosphorylation of Dok-R Y402 mediates Csk SH2 binding. Cos1 cells infected with Dok-R or vector adeno viruses were left either unstimulated or stimulated with EGF. Lysates from these cells were collected and phosphorylated or nonphosphorylated peptides corresponding to the region spanning Dok-R Y402 were tested for there ability to compete for Gst-Csk SH2 binding. Peptide concentration was calculated as molar equivalents when compared to total Gst-Csk SH2, i.e. moles of peptide/moles of Csk SH2.

Dok-R attenuates EGF-Dependent activation of Akt

Several biologically important kinases are found in signaling pathways that are down-stream of other kinases. These kinase-kinase cascades serve to amplify and diversify signals. One such kinase is the serine/threonine kinase, Akt/PKB. In a recent report Kong *et al.* demonstrated that full EGF-dependent activation of Akt requires Src family kinase activity in rat hepatocytes (Kong *et al.*, 2003). Thus, we set out to determine whether Dok-R's ability to attenuate c-Src and ultimately Erk2 activation could also result in the attenuation of other kinases such as Akt. Cos1 cells expressing Dok-R or Dok-R ΔPRR were either left unstimulated or stimulated with EGF for the indicated times and lysates were probed with an activation-specific antibody directed to phosphorylation of Akt serine residue 473 (James et al., 1996). Cells transfected with either empty vector or Dok-R ΔPRR produced very similar levels of activated Akt, such that phospho-Akt levels increased rapidly upon stimulation, persisted for at least 5 minutes, and then began to decline considerably by 10 minutes post stimulation (Figure 3.4a). In dramatic contrast to this, cells expressing Dok-R demonstrated severely impaired levels of Akt activation. Control immunoblots of lysates from these samples suggests that the stark differences in Akt activation between these experimental groups of samples can not be accounted for by differing levels of EGFR activation (anti pY blot), total Akt levels (pan Akt), or overall protein levels (anti β-actin). The central role that c-Src plays in normal cellular physiology suggests that Dok-R's ability to attenuate c-Src activity may extend to numerous pathways that are down-stream of c-Src, including Erk2 and Akt, the latter playing a key role in mediating cell survival.

Potentiation of anoikis by Dok-R

Expression of c-Src or v-Src has been shown to play a pivotal role in counteracting cell detachment-induced apoptosis (anoikis) (Coll et al., 2002;Windham et al., 2002). The breast cancer cell line SKBR3, is known to express high levels of EGFR and c-Src and display increased resistance to anoikis (Belsches-Jablonski et al., 2001;Wang et al., 2001). Thus, we utilized this cell line to examine if expression of Dok-R within these cells could sensitize them to anoikis. The colorectal carcinoma cell line, HT29, also known to express very high levels of c-Src (Windham *et al.*, 2002) was included to examine the relative importance of EGFR signaling. Both cell lines were cultured in monolayer and then infected with one of either ad-LacZ (vec), ad-Dok-R or ad-Dok-R ΔPRR. After 24 hours the cells were trypsinized, counted and equal numbers of cells were seeded into 6-well dishes that were either coated with agarose (suspension) or not (monolayer). Immunoblot analysis of protein lysates from infected cells demonstrate that both Dok-R cDNAs were expressed to similar levels (data not shown). HT29 cells were unaffected by either of the Dok-R expressing viruses. Paradoxically SKBR3 cells expressing Dok-R ΔPRR were somewhat sensitized to anoikis when compared to parental (ad-LacZ) SKBR23 cells while cells expressing Dok-R were highly sensitized (Figure 3.9).

Discussion

Since their cloning it has been known that Dok and DokL bind Csk although the functional significance of this interaction has remained elusive. Our data now clearly demonstrate that Dok-R also binds Csk, further lending support to the notion that Dok, DokL, and Dok-R belong to a subfamily of the larger Dok family. These data provided

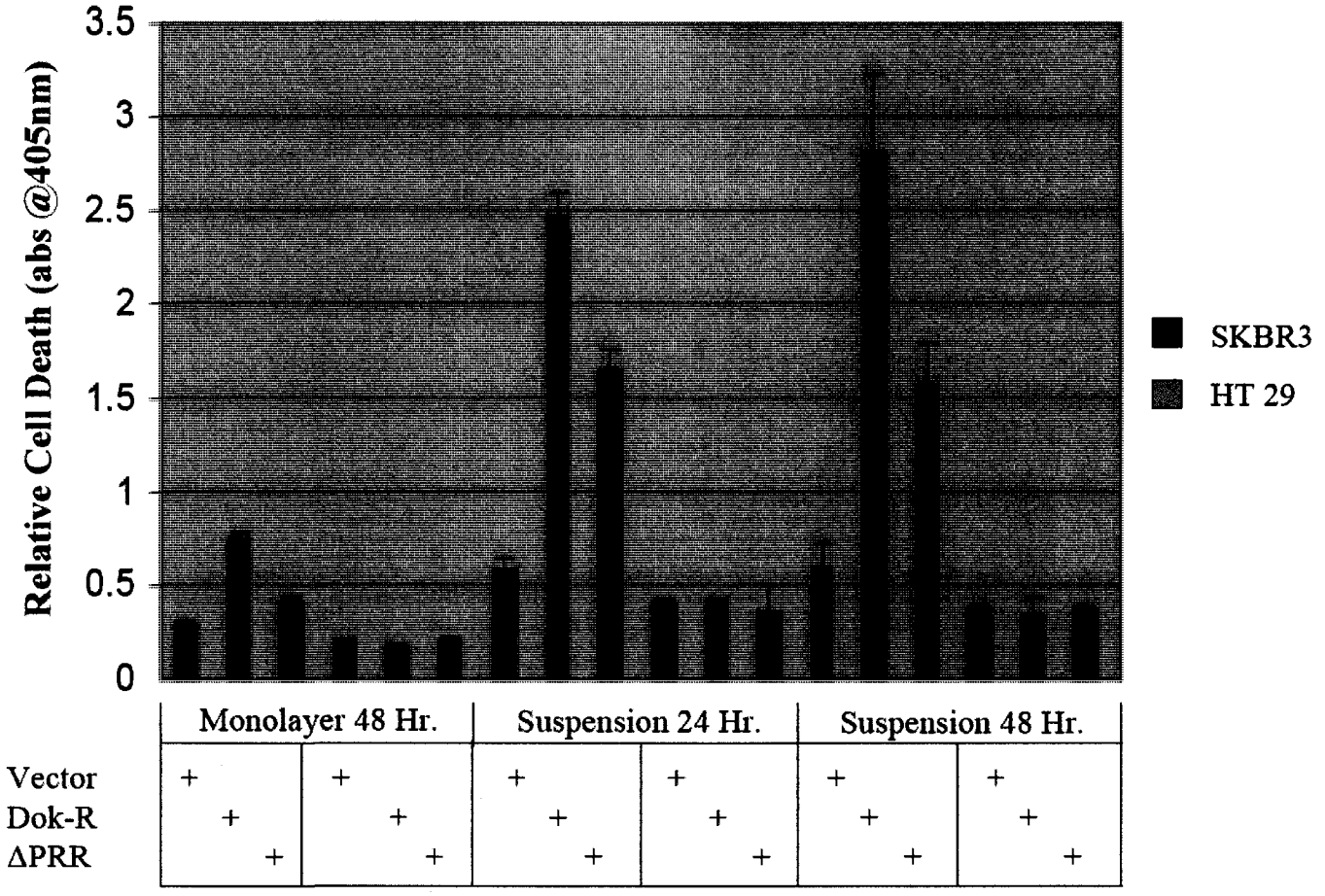


Figure 3.9. Overexpression of Dok-R sensitizes SKBR3, but not HT 29 cells to cell detachment induced cell death. SKBR3 and HT 29 cells were infected with adeno viruses engineered to express one of either vector, Dok-R, or Dok-R ΔPRR. 24 hours post-infection cells were placed in monolayer or suspension culture for indicated times. Triplicate samples were analyzed for cell death with Cell Death ELISA Plus kit (Roche). Results are representative of three separate experiments performed in triplicate.

the impetus for us to test the hypothesis that Dok-R attenuates MAPK activation by specifically modulating EGF-dependent c-Src kinase activity, and begs the question that in general this subfamily of proteins may function universally to modulate c-Src kinase activity. As such, one would imagine, based upon shared sequence and structural characteristics that this subfamily shares similar and, or overlapping functions. These three family members are generally known as context specific attenuators of receptor and non-receptor tyrosine kinase signaling. More specifically, they have been shown in many different settings to attenuate downstream activation of MAPK and thus, counteract mitogenic cell signaling. The mechanism by which this takes place is still not clear and herein we present an alternate novel mechanism using the EGFR and Dok-R as prototype members to gain valuable insight into the role of Csk and c-Src in these events. We show processive assembly of an EGF-inducible quaternary complex consisting of the EGFR, Dok-R, c-Src and Csk. Previous work in our laboratory has shown that Dok-R is recruited to an activated EGFR via association of its PTB domain with phosphorylated tyrosine residues 1086 and 1148 of the EGFR (Jones *et al.*, 1999). Here we demonstrate that Dok-R is able to shuttle constitutively associated c-Src to the EGFR, whereupon there is a transient activation of c-Src that results in tyrosine phosphorylation of the PRR of Dok-R. Phosphorylation of these sites is SFK dependent and serves to recruit downstream signaling proteins to Dok-R. One such protein is the negative regulator of c-Src, Csk. We show that one of the sites that c-Src specifically phosphorylates on Dok-R, Y402, is nested within an ideal consensus sequence (YXXV) for SH2-dependent binding of Csk. The inducible recruitment of Csk to this complex positions c-Src and Csk in such a way that Csk is able to hyperphosphorylate the negative regulatory tyrosine of c-Src, Y527

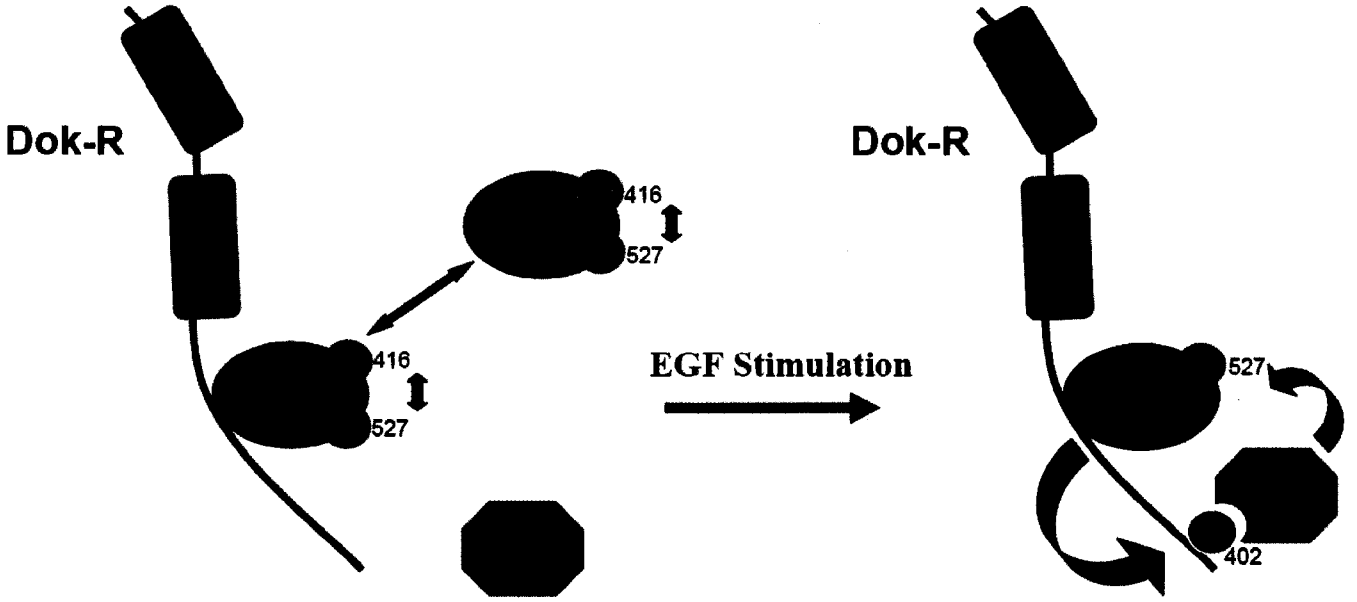


Figure 3.10. Dok-R mediates attenuation of EGF-dependent MAPK and Akt activation through processive recruitment of c-Src and Csk. c-Src is tightly and dynamically maintained between active (pY416) and inactive (pY527) conformations. In the absence of EGF stimulation a portion of c-Src constitutively interacts with the PRR of Dok-R. Upon EGF stimulation Dok-R is recruited to the EGFR where a transient activation of c-Src occurs. c-Src-dependent phosphorylation of Dok-R tyrosine 402 facilitates the recruitment of Csk and subsequent inhibition of Src kinase activity.

(Figure 3.10, schematic). Furthermore, the noted hyperphosphorylation of c-Src Y527 correlates with a dramatic loss of EGF-dependent activation of c-Src kinase, MAPK and Akt that occurs as soon as 2 minutes post stimulation and persists for at least 10 minutes.

EGF driven cell proliferation is a highly conserved cellular event that requires exquisite regulation. Tyrosine residues 845 and 1101 of the EGFR have been shown to be phosphorylated *in vivo* in response to recruitment and activation of c-Src (Poppleton et al., 1999;Tice et al., 1999), while *in vitro* tyrosine residues 891 and 920 are c-Src specific substrates (Stover et al., 1995). Although mutation of tyrosine 845 to phenylalanine does not effect the overall kinase activity of the EGFR, c-Src-dependent phosphorylation of this residue has been shown to be a critical event for EGF-driven mitogenesis (Maa *et al.*, 1995;Tice *et al.*, 1999). Two primary mitogenic pathways that are activated by the EGFR are the Ras and the PLC-gamma pathway. Full activation of either of these pathways requires EGFR-c-Src complex formation (Maa *et al.*, 1995;Sato et al., 1995a;Biscardi *et al.*, 2000). Dok and Dok-R's ability to inducibly associate with the inhibitor of Ras signaling, RasGAP, suggests that the Dok's may impinge on the MAPK pathway by promoting the turnover of GTP bound Ras to GDP bound Ras, thus inactivating the Ras-dependent arm of MAPK activation. Some evidence has been presented to support this idea (Yamanashi *et al.*, 1997;Carpino *et al.*, 1997;Yamanashi *et al.*, 2000;Songyang *et al.*, 2001;Shah *et al.*, 2002). However, we demonstrate here and in previous work (Jones *et al.*, 1999) that Dok-R is capable of strongly inhibiting EGF-dependent c-Src kinase activity and that the noted attenuation of MAPK associated with enforced Dok-R expression is more likely a consequence of c-Src inhibition than a function of its ability to recruit RasGAP and shut down Ras signaling. Importantly, these two phenomena are

difficult to differentiate and ultimately may lead to similar outcomes. For instance, if EGF-dependent c-Src activity is diminished, there will be a concomitant decrease in phosphorylation of EGFR substrates Shc and PLC-gamma and thus, the Ras signaling pathway. Alternatively, if Dok-R was, through recruitment of RasGAP, attenuating Ras activation one would observe the same effects, starting from Ras GTP loading down to MAPK activation. Our previous work (Jones *et al.*, 1999) however, demonstrates that site directed ablation of the RasGAP binding sites on Dok-R does not mitigate its ability to attenuate MAPK activation nor does Dok-R displace Shc from being recruited to the EGFR, further lending support to the idea that Dok-R inhibits MAPK activation by modulating c-Src kinase activity.

Negative feedback loops in signal transduction serve to limit the intensity and duration of a particular signal, and are therefore necessary to maintain cellular homeostasis (Fiorini *et al.*, 2001). For instance, the activation of cell signaling events in T cells are tightly regulated and are dependent upon antigen receptor ligation, and subsequent activation/inactivation of Lck, Fyn (Mustelin and Tasken, 2003). Specifically in the case of TCR activation Lck and Fyn phosphorylate key tyrosine residues that reside within receptor associated chains- ITAM's as well as within PAG/Cbp (Lindquist et al., 2003). Phosphorylation of PAG/Cbp on tyrosine 317 facilitates the recruitment of Csk. Although little is currently known about Csk activation, it has been shown that binding of Csk to PAG/Cbp and its juxtaposition to Lck and Fyn is sufficient for Csk to phosphorylate the carboxy terminal inhibitory tyrosine of these SFK members (Takeuchi et al., 2000). Furthermore, it is the intensity of the original signal that dictates the degree to which the signal will be allowed to propagate implying a high degree of regulation at

the level of both activation and inactivation. Dok-R, like PAG/Cbp is an adapter/scaffolding protein. Unlike PAG/Cbp which is constitutively associated with gycosphingolipid-enriched microdomains, Dok-R is inducibly recruited to phosphoinositol phosphates by virtue of its PH domain. The significance of this difference is not currently known but it would suggest an even greater level of regulation that is facilitated through EGF-dependent changes in phosphorylation-dephosphorylation status of proximal inositol lipids. Like TCR activation, signaling events initiated by the EGFR involve activation of the SFK members. Here we show that activation of the EGFR results in SFK-mediated tyrosine phosphorylation of Dok-R, recruitment of Csk, and a subsequent attenuation of c-Src kinase activity, thereby specifically inhibiting c-Src-dependent aspects of EGFR signaling.

Aside from mitogenesis, c-Src is involved in many other cellular functions including regulation of the cytoskeleton, vascular permeability and apoptosis. In the case of apoptosis Windham *et al.* and Coll *et al.* examined the role of c-Src in counteracting cell detachment-induced cell death-anoikis (Coll *et al.*, 2002;Windham *et al.*, 2002). Coll *et al.* found that v-Src induced activation of Mek/MAPK leads to increased expression of the anti apoptotic protein Bcl-xL, while Windham *et al.* demonstrate that increased levels of c-Src expression and or activity in colon epithelial cells results in a survival advantage conferred by Akt/PKB activation. Stover *et al.* have investigated the collaborative role EGFR and c-Src play in recruiting the p85 subunit of the anti apoptotic protein PI3 kinase to the EGFR. Their work demonstrates that c-Src-dependent phosphorylation of EGFR tyrosine residue 920 facilitates recruitment of PI3 kinase in cancer cell lines DLD-1 and MCF 7 (Stover *et al.*, 1995). Although c-Src-dependent recruitment of p85 to an activated

EGFR is interesting, it does not, in and of it self address whether this interaction is sufficient to activate PI3 kinase and its downstream substarte Akt/PKB. Kessenbrock *et al.* have since demonstrated that PP1 treatment of T47D cells blocks EGF-induced activation of the anti apoptotic protein kinase AKT/PKB suggesting that this event requires c-Src kinase activity (Kassenbrock et al., 2002). Herein we describe a Dok-R-dependent decrease in EGF-driven c-Src kinase activity that correlates with a decrease in both MAPK and consistent with the above studies, a concomitant decrease in Akt activation. Our current studies demonstrate that Dok-R is able to sensitize SKBR3 cells, one of the two cell lines tested, while the other line HT29, was completely unaffected. We believe that one possible explanation for this is the EGFR status of each of the cells lines. While, both cell lines express high levels of c-Src, SKBR3 cells have much higher levels of EGFR (Belsches-Jablonski *et al.*, 2001) (and data not shown). As detailed in our previous studies (Jones *et al.*, 1999) Dok-R must be recruited to the EGFR to efficiently attenuate MAPK activity. Here, we show that this attenuation is dependent on Dok-R's ability to modulate c-Src kinase activity, suggesting that cell lines possessing intrinsically low levels of EGFR, such as HT29, would not be sensitive to Dok-R mediated effects. Quite unexpectedly we find that Dok-R ΔPRR was able to partially sensitize SKBR3 cells to cell detachment–induced cell death. Although the origin of this result is not currently known we are examining the possibility that there are functional Dok-R domains outside of the PRR that participate in apoptosis. One possibility is phosphorylation of Dok-R tyrosine 142. Songyang *et al.* demonstrated that the equivalent tyrosine in Dok, tyrosine 146, becomes phosphorylated in a v-Src specific manner and that this phosphorylation event facilitates clustering of additional Dok proteins via

homotypic PTB-based interactions (Songyang *et al.*, 2001). If this proves to be the case for Dok-R, the Dok-R ΔPRR may be recruiting wild type endogenous Dok-R to activated EGF receptors in the SKBR3 cells.

Perhaps the most intriguing concept to arise from this work is the notion that Dok, Dok-R and DokL are global inhibitors of SFK members. This is not a novel idea though; Lemay *et al.* coined this idea upon cloning of Dok 3 (DokL), suggesting that Dok and DokL may inhibit SFK's by virtue of their ability to recruit Csk (Lemay *et al.*, 2000). Until now there has been no evidence to substantiate this hypothesis. Dok-R, unlike Dok associates with c-Src via a constitutive SH3 mediated interaction not an inducible SH2 based interaction. Gst pull-down assays conducted in our laboratory (Figure 3.3) serve to demonstrate that the SH3 domains of SFK member c-Src, Lck, Fyn as well as Abl are able to directly interact with Dok-R. Although we did not examine the binding of additional SFK members, due to the highly conserved nature of their SH3 domains, it seems possible that Dok-R may bind several as yet undetermined SFK members. Taken together with the fact that Dok-R associates with Csk in an inducible fashion and that Dok-R binds many receptor and nonreceptor tyrosine kinases it is intriguing to consider the possibility that the phenomenon that we see with the EGFR may be a highly conserved manner of attenuating RTK-induced SFK signaling.

Experimental Procedures

Plasmids

The plasmids encoding HA-Dok-R, HA-Dok-R ΔPRR and HA-Dok-R ΔC-PRR have previously been described elsewhere (Master *et al.*, 2003). HA-Dok-R ΔPRR, HA-Dok-

RΔC-PRR were engineered to contain premature TAG stop codons situated at nucleotides 815 and 1010 respectively. HA-Dok-R Y402F, built from the wild type HA-Dok-R plasmid, was engineered to express a phenylalanine residue in place of tyrosine 402. This construct and all other Dok-R constructs were confirmed for sequence integrity. Strep tagged Csk SH2 and SH3 were a generous gift of Dr. Kari Alitalo. Bacterial Gst fusion proteins including Lck, Abl, Vav, Crk, Src, Spectrin, p85 and Fyn were a kind gift of Dr. Jane McGlade. The plasmid encoding Gst-tagged Erk2 has previously been described (Jones *et al.*, 1999). Adenoviruses expressing Flag-Dok-R, Flag-Dok-R ΔPRR were constructed and purified by Dr. Andrea Gambotto (Pittsburgh viral vector core facility).

Cell Lines and Cell Culture

Cos-1 monkey fibroblasts (ATCC), SKBR3 breast carcinoma cell lines (gift of Dr. Joyce Slingerland), and HT-29 were all maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1X penicillin and 1X streptomycin (Life Technologies). All transient cell transfections were performed with 5 ug or 10 ug of plasmid DNA utilizing Lipofectamine (Gibco) according to the manufacturer's instructions. Adenoviral infections were performed in normal 10% FBS, DMEM at a final MOI of 25.

EGF Cell Stimulation, PMA and PP1 Treatment

Cos-1 cells were serum starved for a period of at least 18 hours prior to EGF stimulation for indicated times. Purified recombinant EGF (R&D) diluted to 100 ng/mL in serum free DMEM was applied to indicated samples and allowed to incubate at 37°C, 5% CO_2. Immediately after timed EGF stimulation cells were rinsed in ice cold PBS and lysed on

ice. PP1 and PMA were suspended in reagent grade DMSO (Sigma). Cells were treated with indicated concentrations of either PP1, PMA or vehicle.

Antibodies Used for Immunoprecipitation and Western Blotting

Commercially available antibodies were used as follows: monoclonal anti-Src GD-11, monoclonal anti-phospho tyrosine 4G10 and polyclonal Dok-R (Upstate Biotech); monoclonal anti-phospho specific p42, 44, monoclonal anti-phospho serine 473 Akt/PKB and monoclonal anti-Akt (Cell Signaling Technology); monoclonal anti-HA HRP conjugated 12CA5 and monoclonal anti-HA 12CA5 (Roche); monoclonal anti-Actin AC-40 and monoclonal anti-Flag M2 (Sigma); polyclonal anti-phospho Y527 Src (BioSource); polyclonal anti-Gst Z-5 and polyclonal anti-EGFR 1005 (Santa Cruz); monoclonal anti-Csk (Transduction Laboratories). Co-immunoprecipitation and Western blotting procedures have previously been described (Jones *et al.*, 1998b;Jones *et al.*, 1999).

***In vitro* Src kinase Assay**

c-Src activity was measured using an *in vitro* kinase assay as described previously (Cooper *et al.*, 1983). Briefly, equal quantities of cell lysate were immunoprecipitated with 1 μg of monoclonal anti-Src antibody and 30 μl of 50% agarose-bound protein A. Immunoprecipitates were washed three times with 500 μl of lysis buffer and three times with 100 μl of 10 mM HEPES (pH 8.0). Beads were then resuspended in 35 μl of reaction mixture (45 mM HEPES (pH 8.0); 150 mM NaCl; 50 mM MgCl2; 10 μM Na3VO4; 2 μM ATP; and 10 μCi of [γ-32P]ATP) containing 0.04 μg/μl acid-treated enolase (see below) and incubated at 25 °C for 15 min. Reactions were stopped by addition of 6 μl of 5X SDS-polyacrylamide gel electrophoresis loading buffer and boiling

for 5 min. Samples were run through a 7.5% SDS-polyacrylamide gel, and the dried gels were analyzed by autoradiography. The protocol for acid treatment of enolase was adapted from Shen et al. (Shen et al., 1999). Briefly, 0.6 µl of enolase suspension (Sigma) was mixed with 0.6 µl of 60 mM HEPES (pH 8.0), 2.4 mM dithiothreitol, and 60% glycerol and added to 1.2 µl of 500 mM acetic acid. After incubation at 37 °C for 15 min, the reaction was stopped with 2.4 µl of 100 mM Tris-HCl (pH 8.0) and 20 mM MgCl2.

Cell Death ELISA

SKBR3 and HT29 cells were infected with one of either adLacZ (vector control), adDok-R or adDok-R ΔPRR. 24 hours post infection cells were trypsinized and counted in trypan blue. 50,000 viable cells were added to each well of a six well plate either coated with 1% agarose (suspension) or uncoated (monolayer) and were then cultured for an additional 24 hours in 10% DMEM, 37°C, 5% CO_2. Samples were analyzed according to manufacturers specifications (Roche, Cell Death ELISA Plus).

***In Vitro* Pull-Down Assay**

Recombinant Gst fusion proteins were raised as previously described (Master *et al.*, 2003). Purity of each Gst construct and quantification was accomplished via PAGE analysis. Each pull-down experiment utilized 2 µg of purified recombinant Gst fusion protein. Cos1 cells were lysed and pull downs were executed as previously described (Master *et al.*, 2003). Peptide sequences were as follows: Dok-R Y402, Biotin-PQATEYDNVILKK, Dok-R pY402, Biotin- PQATEpYDNVILKK.

Chapter 4

Characterization of Dok-R ΔPRR: In Vivo and In Vitro Approaches

Rationale: Two widely accepted methods of investigating the developmental role of a particular mouse gene involve genetic ablation or transgenic overexpression. Although these traditional approaches have yielded many key scientific discoveries, used in their simplest form, they suffer from deficits in regulation. That is, researchers are unable to temporally or spatially control the expression of a particular gene; consequently the information that could be gleaned from these manipulations was often severely limited. Here we use the binary pTetOS/tTA system to inducibly overexpress Dok-R ΔPRR under the control of tissue specific promoters. Conception of this project occurred late in 2000, at which point three Dok family proteins had been identified, p62 Dok 1, Dok-R/Dok 2/FRIP, and Dok 3/DokL. Each of the proteins had been shown to contain highly conserved amino terminal PH and PTB domains, while within the more divergent carboxy terminal regions several motifs known to engage SH3, SH2 and PTB domains where present. Subsequent to the cloning of Dok 1-3 it became apparent that all three family members interact with distinct and overlapping repertoire of receptor and non-receptor protein tyrosine kinases via the PTB domain, and while doing so, attenuate downstream signals including MAPK and Akt. A model was described wherein Dok 1 and Dok-R bind to a specific kinases, become phosphorylated, and recruit the negative regulatory protein, RasGAP (Park *et al.*, 1993;Carpino *et al.*, 1997;Nelms *et al.*, 1998;Jones *et al.*, 1998b). Interestingly, Dok 3, which lacks the motifs in the carboxy terminus necessary to recruit RasGAP was also shown to attenuate MAPK signaling, a fact which is still lacking full explanation (Cong *et al.*, 1999;Lemay *et al.*, 2000;Honma et al., 2006). Tissue expression profiles for the Dok family members have been described in the literature and although enriched in hematopoietic lineages there is a wide range of

overlapping tissues that co-express two or more Dok's. Our mouse genetic approach was aimed at combating some of the inherent risks associated basic genetic ablation or overexpression of Dok-R. We designed and built a model wherein we overexpress a dominant interfering form of Dok-R consisting of the PH and PTB domains. The resulting construct, which we call Dok-R ΔPRR, was cloned into the pTetOS expression vector such that when co-expressed with promoter-specific tTA mouse driver lines the effects of tissue specific expression patterns could be examined. The decision to overexpress a dominant interfering form of Dok-R derives from the concern that the Dok family of proteins may serve compensatory roles within the cell and to delete and single family member might not produce a discernable phenotype. Precedence for this phenomenon includes early attempts to elucidate the developmental role of Src in mouse knock out studies which were hampered by compensatory signaling of other family members(Bock and Herz, 2003;Kanda et al., 2003;Kotani et al., 2007). Yamanashi and Baltimore *et al.*, were the first to describe a Dok family knock out with Dok 1(Yamanashi *et al.*, 2000). The report described a minor hyperproliferative B-cell phenotype, in which the authors highlight the possibility of compensatory mechanisms which may be masking the effects of the Dok 1 deletion (Yamanashi *et al.*, 2000). The approach described herein was aimed at overexpressing a dominant interfering protein, Dok-R ΔPRR, that would bind to and prevent or displace binding of endogenous Dok 1, Dok-R and Dok 3. With this approach we endeavored to mitigate potential compensation and examine on a more global level, what the developmental roles of Dok family members are. It was reasoned, that based on pre-existing structure function studies, the Dok PH and PTB domains were necessary to localize and engage specific target proteins, but without the carboxy

terminus the noted attenuation of downstream signaling would not occur. Based on this understanding our hypothesis was that the mouse line engineered to overexpress Dok-R ΔPRR would have severe defects related to unmitigated PTK or RTK-dependent activation of MAPK.

Introduction: Dok-R, also known as Dok2 and FRIP, was identified by three separate groups by virtue of its ability to associate with, and become phosphorylated by receptor and non-receptor tyrosine kinases IL-4, Tek/Tie 2, and p210bcr-abl (Di Cristofano *et al.*, 1998;Nelms *et al.*, 1998;Jones *et al.*, 1998b). Dok-R is a 56 kDa tyrosine phosphorylated protein, with an N-terminal pleckstrin homology domain, a central phosphotyrosine binding domain as well as several other features of a signaling molecule, including 13 potential tyrosine phosphorylation sites and 6 PXXP motifs (Di Cristofano *et al.*, 1998). Dok-R was the second in a family of 7 docking molecules to be identified and most closely resembles family member Dok-1 and insulin receptor substrates 1 and 3 (IRS 1&3) (Jones *et al.*, 1998b). Dok-R becomes tyrosine phosphorylated in response to stimulation of several other RTK's including insulin receptor, epidermal growth factor receptor, cytokine receptors including IL-2, IL-3, IL4, erythropoietin receptor, hematopoietic growth factors such as CSF, M-CSF and GM-CSF as well as non-receptor protein tyrosine kinases including Lyn, Hck, Src, Lck, Abl, p210bcr-abl and Tec. Transcriptional regulation of Dok-R has been shown to exist in response to stimulation with M-CSF, GM-CSF, and IL-3. Cytogenetic studies have revealed that Dok-R maps to the hairless mouse locus on chromosome 14D2-D3 (Nelms *et al.*, 1998;Jones and Dumont, 1998a). Mutations within the hairless loci result in a dramatic decrease in Dok-R expression which coincides with occurrence of lymphadenopathy, and increased T cell

proliferation (Nelms *et al.*, 1998). Much of what is known about Dok-R function is ascribed to its ability to recruit signaling proteins such as Nck, p120 RasGAP, Src and Csk (Di Cristofano *et al.*, 1998;Jones *et al.*, 1998b;Van Slyke *et al.*, 2005). For instance, retroviral-mediated overexpression of Dok-R in bone marrow cells was shown to dramatically inhibit their capacity to form colonies in vitro as well as impair the ability of these cells to repopulate lethally irradiated mice. These effects were shown to be dependent on Dok-R's ability to associate with Nck and p120RasGAP (Gugasyan *et al.*, 2002). Further, Angiopoietin1-mediated migration of endothelial cells has been shown to be dependent on Dok-R's ability to associate with Tek/Tie2 and recruit Nck (Master *et al.*, 2001). A central and prevailing theme of Dok signaling has been their ability to attenuate mitogen activated protein kinase (MAPK) signaling events downstream of receptor and non-receptor kinases (Nelms *et al.*, 1998;Jones *et al.*, 1999;Suzu et al., 2000;Grimm *et al.*, 2001). Although RasGAP recruitment has been implicated in these events there are several lines of evidence to suggest that Dok-R mediates MAPK attenuation in conjunction with, or independent of RasGAP association (Jones *et al.*, 1999;Kashige *et al.*, 2001). One such mechanism, described by Van Slyke and Dumont *et al.*, demonstrates that Dok-R acts as an EGFR-recruited scaffolding molecule that processively assembles c-Src and Csk to attenuate MAPK signaling (Van Slyke *et al.*, 2005).

Although collectively, Dok 1, Dok-R, and Dok 3 have been primarily studied in the context of negative regulatory cell signaling within the cytoplasm, recent reports examining the subcellular localization of Dok 1 describe a significant nuclear distribution (Smith *et al.*, 2004;Niu et al., 2006). Two separate groups in 2004 described a nuclear

enrichment of Dok 1 protein, the functional significance of which remains unknown (Smith *et al.*, 2004;Niu *et al.*, 2006). Evidence in support Dok 1 nuclear localization also derives from the fact that Niu Y and Sylla B, *et al.*, characterized a functional CRM1-dependent nuclear export sequence (NES) located within the PRR (Niu *et al.*, 2006). The exact mechanism by which Dok 1 gains entry to the nucleus has not been determined, but data presented suggests that Src family-dependent kinase activity dramatically limits Dok 1 nuclear entry, suggesting a physiologically inducible shuttling of Dok 1 between the cytoplasm and the nucleus. In the case of Dok-R and Dok 3, no literature reports have examined their presence or function within the nucleus. Several different advanced protein localization software packages including WoLF PSORT (Horton et al.), pTARGET (Doyle et al., University of Albany), and LOCtree (Nair R, and Rost B.,) (Nair and Rost, 2005) predict, with a high degree of certainty, Dok-R and Dok 3 to localize to the nucleus and to a lesser degree the cytoplasm. While in the nucleus, LOCtree (Nair R, and Rost B.,) (Nair *et al.*, 2005) does not predict a viable interaction with DNA for either Dok-R or Dok 3. As more data accumulates to demonstrate a conserved nuclear/cytoplasmic shuttling mechanism for Dok 1, Dok-R and Dok 3 researchers are no closer to assigning a function, if any, to these negative regulatory molecules within the nucleus.

Herein we describe the construction and characterization of a line of mice genetically engineered to over express a dominant interfering form of Dok-R under the control of the endothelial specific drivers Tie1, enhanced Tek/Tie2 (enhTek) and Vascular Endothelial Cadherin (VE Cad). We find that these mice are born in normal Mendelian ratios, absent of any overt phenotype. These mice overexpress the transgene as expected and survive as

long as wild type or single transgenic littermates. Close histological examination of double transgenic mice reveals lymphocyte infiltration surrounding vessels of the liver and minor increases in the Bowman's space in the kidney. Consistent with the anatomical defects noted in the kidney, these mice present with proteinuria. A comprehensive hematological assessment (full CBC) of Dok-R ΔPRR mice reveals a significant four fold increase in blood monocytes with non-significant, but consistent increases in all other WBC populations examined, suggesting that Dok-R plays a pivotal role in negatively regulating hematopoiesis. In vitro studies of Dok-R ΔPRR revealed overall increases in translocation to the nucleus, while its nuclear export was dependent on CRM1 activity. The origin of this result, consequences to the phenotype of the Dok-R ΔPRR mice, and the overall cell physiological effect of increased Dok-R ΔPRR nuclear localization remain unclear.

Methods and Materials:

Generation, Genotyping, and Treatment of Transgenic Mice

Construction of the responder transgene, pTetOS-Dok-R ΔPRR, was as follows: Dok- R ΔPRR shuttled from pcDNA3.1HA2 vector via double restriction digest with EcoR1 and Xba1. The gel purified fragment containing Dok- R ΔPRR was cloned into the EcoR1/Xba1 sites of the pFlagCMV2 vector. Dok- R ΔPRR, including the 5' Flag tag (from the pFlagCMV2 vector) were excised from this vector with Nde1 and BamHI. The resulting gel purified DNA fragment was blunt-ended with klenow and cloned into pTetOS. Driver and responder transgenes, p*Tie1-tTA*, p*enhTek-tTA*, pV*e Cad-tTA* and pTetOS-*nlsLacZ*, have been described previously (Sarao and Dumont, 1998;Gory et al., 1999;Voskas et al., 2005). All transgenic driver lines were maintained and genotyped as

previously described (Sarao *et al.*, 1998;Jones et al., 2001b). The pTetOSDok-R ΔPRR responder transgene was amplified using forward 5' CGA TCC TGA GAA CTT CAG G 3' and reverse 5'GCG ATG ACT AAT ACG TAG A 3' and primers. Males were housed with a single female for 14 to 20 days and then separated into individual cages. CD1 mice were maintained in a barrier or barrier-free facility. All adult mice used in these studies were 4 to 6 months old and unless otherwise specified, mice from each age group (neonates or adults) were generated, treated, and biochemically or immunohistochemically examined at least in triplicate (n =3).

Lac Z Expression Analysis

Adult mouse tissue was fixed in 4% paraformaldehyde (pH 7.4) (Fisher Scientific, Pittsburgh, PA) for 4 hours at room temperature, cryoembedded in optimal cutting temperature (OCT) compound (VWR International, Mississauga, Ontario, Canada), sectioned (8 μm) on SuperFrost glass slides (Fisher Scientific), and processed and stained for nlsLacZ expression as previously described in Dumont and colleagues(Voskas *et al.*, 2005). Tissues were subsequently counter stained with eosin and mounted with permount (Fisher Scientific, Pittsburgh PA). Embryos were fixed in 4% paraformaldehyde (pH 7.4) (Fisher Scientific, Pittsburgh, PA) overnight at 4^0C and processed for nlsLacZ staining as above. Whole mount embryos were examined on a Leica dissection microscope (Leica Microsystems, Germany) and photographed with a CoolSnap camera (Roper Scientific Inc, Germany).

Tissue Processing and Histological Staining

Mouse tissues were fixed in 4% paraformaldehyde (pH 7.4) solution overnight at 4^0C, embedded in paraffin, sectioned (6 μm) on SuperFrost glass slides, and processed and

stained for hematoxylin and eosin (H&E) using standard techniques. Slides were analyzed on a Leica compound light microscope (Leica Microsystems, Germany) and photographed with a CoolSnap camera (Roper Scientific Inc, Germany). Photos were processed using Adobe Photoshop v7.0 (Adobe Systems, San Jose, CA).

Complete Blood Counts and Blood Smear Analysis

Whole blood was collected from anaesthetized (3% isoflourane in oxygen) mice via the left heart ventricle. Blood samples were immediately transferred to heparin-containing microtubes. Complete blood counts and Wright's stained blood smears were prepared by Laboratory Services located at Sunnybrook and Women's College Health Science Centre, Toronto, Ontario, Canada.

Miles Assay for Vascular Permeability

Six month old anesthetized (3% isoflourane in oxygen) ST/WT or DT (pTie1-tTA X pTetOS-Dok-R ΔPRR) mice received tail vein injections (25 gauge needle) of Evan's blue dye (EVD)(100μl, 1.25% in PBS) or EVD (as above) containing 1μg, VEGF 121 (R&D Systems, Minneapolis, MN). Mice were maintained under anesthesia for 5 minutes post injection while the dye was allowed to circulate. Mice were then perfused with 60 ml of PBS via the left ventricle to remove all intravenous EVD. Extravassated EVD was extracted by placing heart and lung samples in equal volumes of formamide and maintaining at 55^0C for 3 days. Post EVD extraction, samples were centrifuged at 14,000g for 15 minutes and a supernatant sample was removed for spectrophotometric analysis (Bio-rad, Hercules, CA, USA) at 650nm.

Urine Collection and Protein Identification (MS/MS)

Urine was collected directly into an eppendorf tube from ST/WT or DT (Ve Cad X Dok-R ΔPRR) mice each morning. Centrifuge cleared urine supernatants (14,000g for 15 minutes) were used to determine overall protein content spectrophotometrically (Bio-rad, Hercules, CA, USA) by way of Bradford Assay (Bio Rad, Hercules, CA, USA) at 592nm. Cleared urine samples were also examined by polyacrylamide gel electrophoresis. Urinary proteins contained in the polyacrylamide gels were visualized with Colloidal Coomassie brilliant blue R (Bio Rad, Hercules, CA, USA) according to manufacturer's specifications. In gel tryptic digestion was performed according to Shevchenkko A, and Mann M, *et al.,* (Shevchenko et al., 1996). *De novo* sequencing was performed by the Sunnybrook Hospital proteomics core facility using an ABI/MDS Sciex QSTAR-XL.

Cell Culture and Adenoviral Infections

Cos-1 monkey fibroblasts, purchased from (ATCC) were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1X penicillin and 1X streptomycin (Life Technologies). Adenoviral infections were performed in normal 10% DMEM at a final MOI of 25.

EGF Cell Stimulation and PP1 Treatment

Cos-1 cells, cultured on glass cover slips, were serum starved for a period of at least 18 hours prior to EGF stimulation for indicated times. Purified recombinant EGF (R&D Systems, Minneapolis, MN) diluted to 100 ng/mL in serum free DMEM was applied to indicated samples and allowed to incubate at 37°C, 5% CO_2 for 10 minutes. PP1 was suspended in reagent grade DMSO (Sigma) and used at a working concentration of 20μM

for 1 hour treatment times. Lyophilized Leptomycin B, from Streptomyces, (Sigma) was resuspended in 100% ethanol and used at a working concentration of 20ng/ml for a treatment time of 2 hours. Immediately following timed treatments cells were rinsed in ice cold PBS and fixed in 4% paraformaldehyde (pH 7.4) solution for 15 minutes.

Immunofluorescent Staining

Cells were fixed in 4% paraformaldehyde for 15 min and permeabilized in phosphate-buffered saline containing 1% Nonident P40 (Sigma) for 10 min. Samples were blocked in 5% goat serum (Jackson ImmunoResearch Laboratories Inc.) and then incubated with an antibodies to Flag (1:1000) for 2 h at room temperature. Samples were washed and incubated with secondary antibody conjugated to fluorescein isothiocyanate (FITC) (1:200) for 1 h at room temperature. Slides were mounted using Aquapolymount (Polysciences Inc.) and visualized using ZeissAxiovert 100 M laser scanning microscope (LSM) confocal microscopy. Analyses were performed using LSM510 (version 2.3) scanning software (Zeiss), and images were captured using 10X objective.

Results and Discussion:

To examine the effects of Dok-R ΔPRR expression we have used the previously described (Voskas *et al.*, 2005) pTetOS/tTA binary transgenic system. Briefly, the tetracycline transactivator driver gene (tTA) was expressed under control of one of several different endothelial-specific promoters including Tie1, enhTek, and VE CAD. When these mice were crossed to mice carrying the pTetOS-Dok-R ΔPRR responder transgene only those offspring which contained the driver gene and the responder gene were capable of expressing the Dok-R ΔPRR protein (Fig 4.1a). Tissue specificity of the

Tie1 endothelial driver line was tested by mating mice which were positive for one of either the pTetOS NLS LacZ gene or Tie1 tTa. Betagalactosidase staining of these double transgenic (DT) offspring (pTetOS NLS LacZ/Tie1 tTa) reveals a largely vascular pattern (Fig 4.1b whole mount and Fig 4.1c cross sections). Pronuclear injection of the pTetOS-Dok-R ΔPRR construct into 15 fertilized oocytes yielded stable chromosomal integration in only one founder (Fig 4.2-top panel). To expand the Dok-R ΔPRR mouse line this male founder was mated to wild type CD1 female mice. All mice were genotyped from collected tail DNA samples as described in materials and methods. Offspring of these matings that tested positive for the responder transgene (Fig 4.2-bottom panel) were then crossed with the Tie1 tTA driver line to examine the effects of endothelial-specific expression of Dok-R ΔPRR. Double transgenic offspring from these matings where born in expected Mendelian ratios suggesting that overexpression of the Dok-R ΔPRR transgene in the endothelial cell compartment did not have lethal effects on embryonic development. The DT mice were indistinguishable from their single transgenic (ST) or wild type (WT) littermates at birth. DT mice, developed normally without overt defects, survived as long as WT or ST littermates and were fertile (data not shown).

Six month old DT, ST, and WT littermates were sacrificed to examine potential morphological or histological differences. Necroscopies of these mice did not reveal any gross morphological differences. Organs including lung, heart, liver, kidney, spleen and small intestine (Fig 4.3a, b, c, d and e) were examined histologically by hematoxylin and eosin (H&E) staining. Although not dramatic we consistently found differences in the liver and the kidneys of DT mice (pTetOS-Dok-R ΔPRR /Tie1 tTa) when compared to

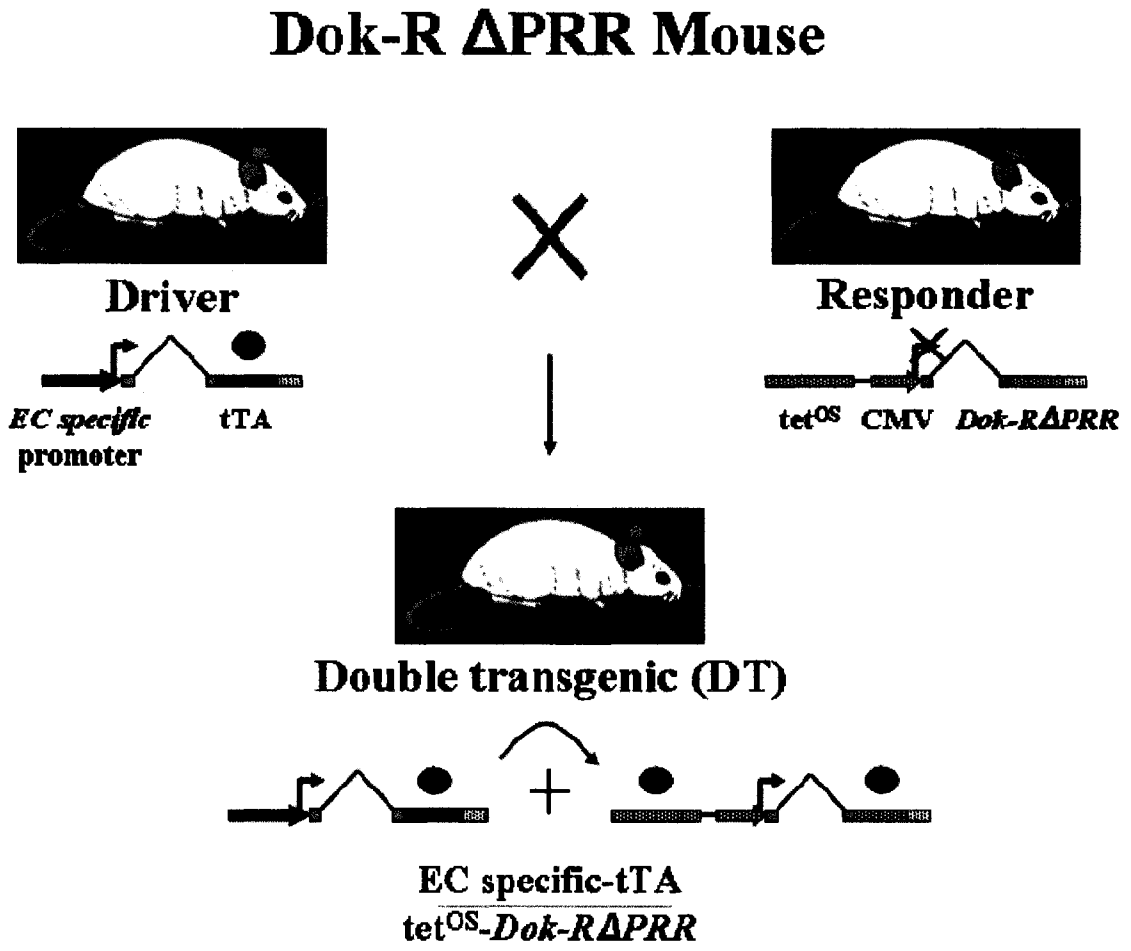


Figure 4.1a: Binary transgenic overexpression of Dok-R ΔPRR. A binary transgenic mouse system was used to induce the conditional expression of Dok-R ΔPRR in vivo. The tetracycline-responsive transactivator (tTA) expressed from the EC-specific promoters (driver transgenic line) binds to the tTA-binding site (tetOS) upstream of the mouse Dok-R ΔPRR cDNA (responder transgenic line).

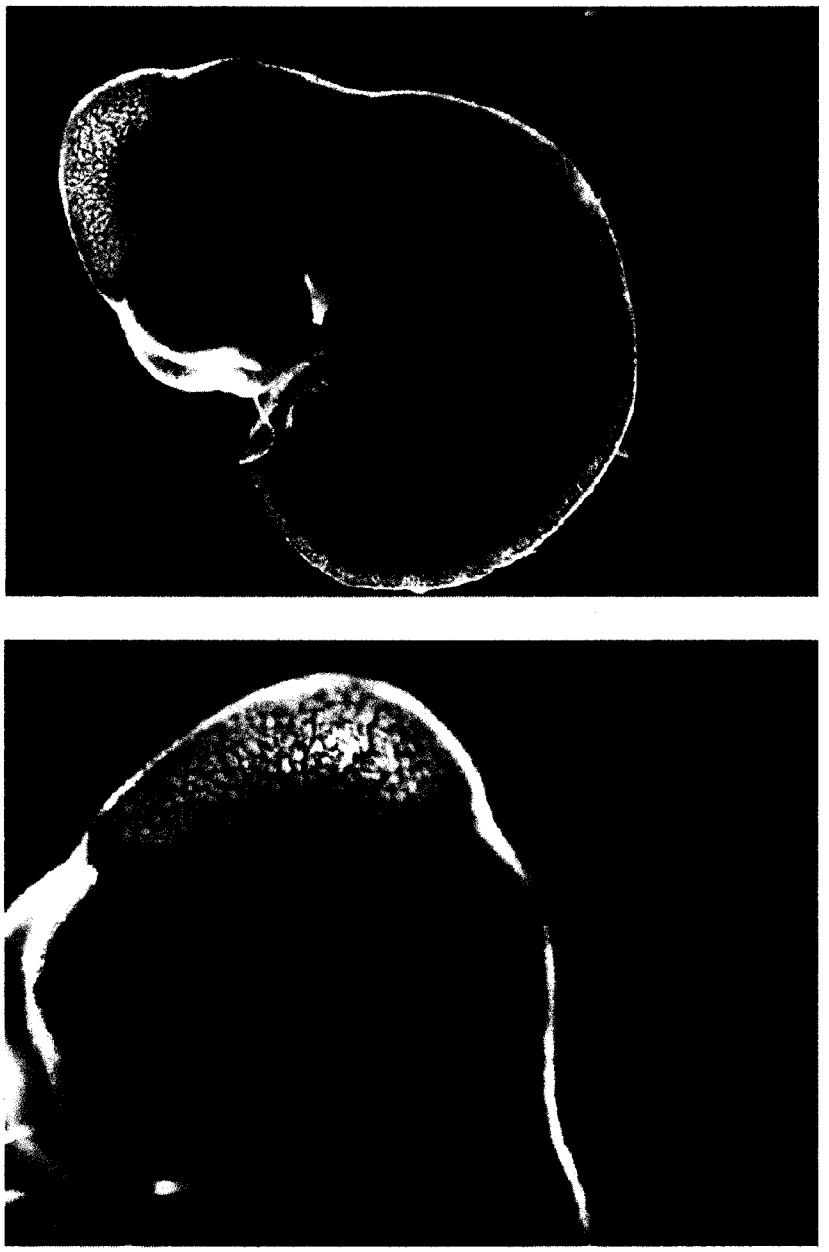


Figure 4.1b: Whole mount analysis of embryonic Tie1 promoter activity. Tie1 tTA mice crossed to a pTetOS NLS LacZ line were analyzed by whole mount beta galactosidase staining. Only mice that were PCR positive for both the driver and responder transgene displayed marked color development. Staining was primarily limited to developing vessels. Mice single transgenic for either transgene did not display a positive color reaction (not shown).

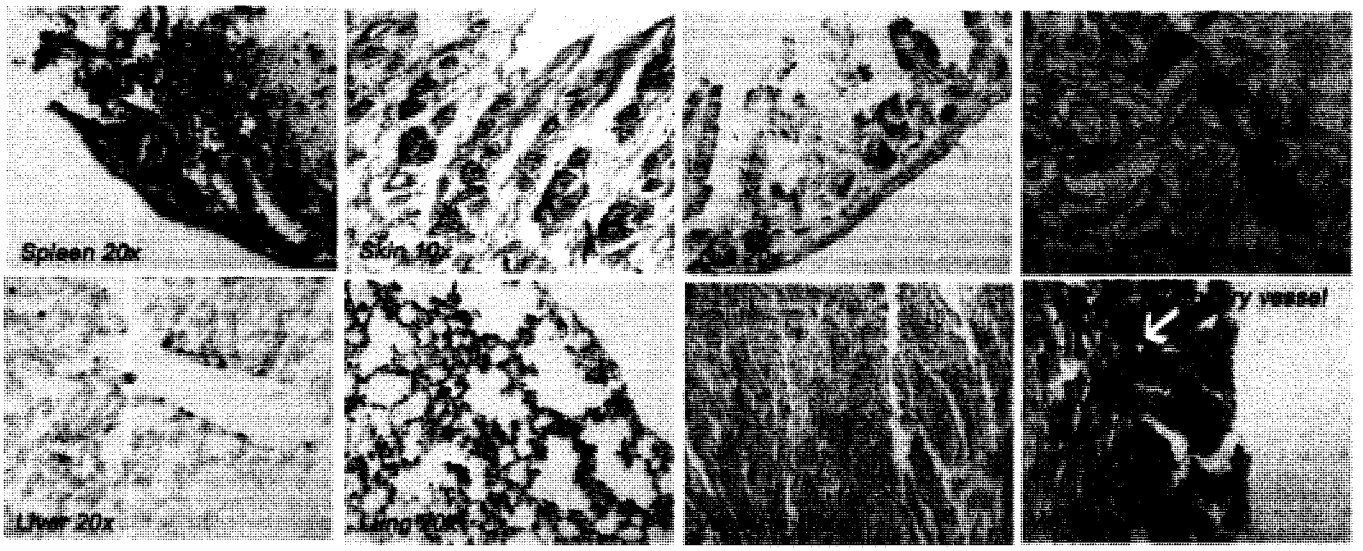


Figure 4.1c: Analysis of Tie1 promoter activity in adult tissues. Various tissues derived from double transgenic mice (Tie1 tTa and pTetOS NLS LacZ) were sectioned and examined by beta galactosidase staining. Positive staining was limited to the vascular compartment. Magnification is indicated for each image.

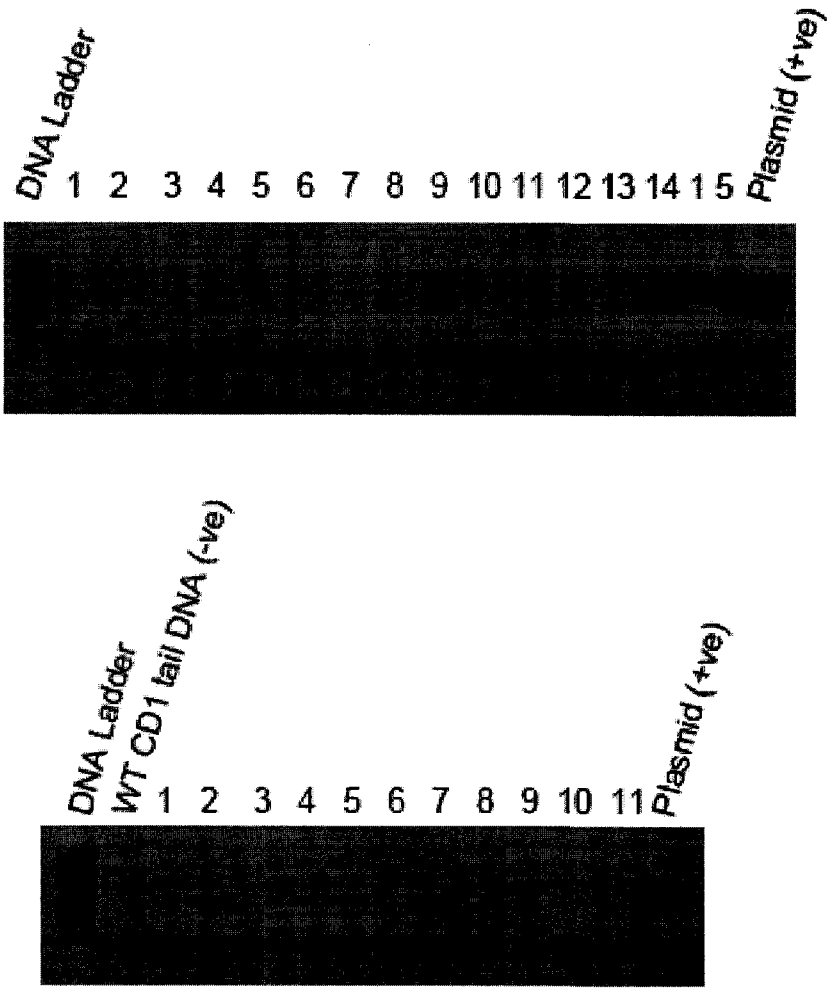


Figure 4.2: Genotype analysis of Dok-R ΔPRR mice. Top panel- Tail DNA from pronuclear injected offspring was examined for the presence of the Dok-R ΔPRR transgene. Of the 15 offspring tested only a single transgene positive mouse was identified. Bottom panel- Mouse number 9 (shown in top panel) was bred to a female WT CD1 mouse. PCR results examining the presence of the Dok-R ΔPRR transgene are shown.

ST or WT littermates. Within the kidneys of DT mice there was a marked dilation of the Bowman's space (region between the glomerulus and Bowman's capsule)(Fig 4.3c). The Bowman's space, also called the urinary space, is the space within the Bowman's capsule surrounding the lobules of the glomerulus. This is the space into which the plasma filtrate collects as it leaves the capillaries through the filtration membrane. Increases in Bowman's space may occur for various different reasons including vasoconstriction of the vessels of the glomerulus, decrease in size of the glomerulus or disturbances in tubular absorption that increase hydrostatic pressure in this region (Zhong et al., 1998). We examined the overall size of the glomeruli in the DT, ST and WT mice and found that increased Bowman's space in the DT mice was not likely a consequence of atrophic glomerular tufts as they were indistinguishable from their WT and ST counterparts (Fig 4.3c). Currently, the origin of increased Bowman's space in the DT mice remains unknown. Independent examination of blinded liver histology sections by a pathologist confirmed a marked lymphocyte infiltration surrounding a high proportion of the vessels within the liver of DT mice but not ST or WT littermates (Fig 4.3d). Transendothelial cell migration of lymphocytes into surrounding tissue often occurs in response to localized inflammation. Mechanisms which facilitate extravasation of leukocytes are well described in the literature and involve adhesion and rolling of these cells along endothelial cell surface proteins such as ICAM 1, VCAM, P Selectin, and PECAM (Ala et al., 2003). Deregulation of proteins critical to extravasation, either on the endothelial cells, or leukocytes, may lead to defects in this process. For instance, Graesser D, and Madri J, describe results in which mice genetically deficient for PECAM exhibit robust

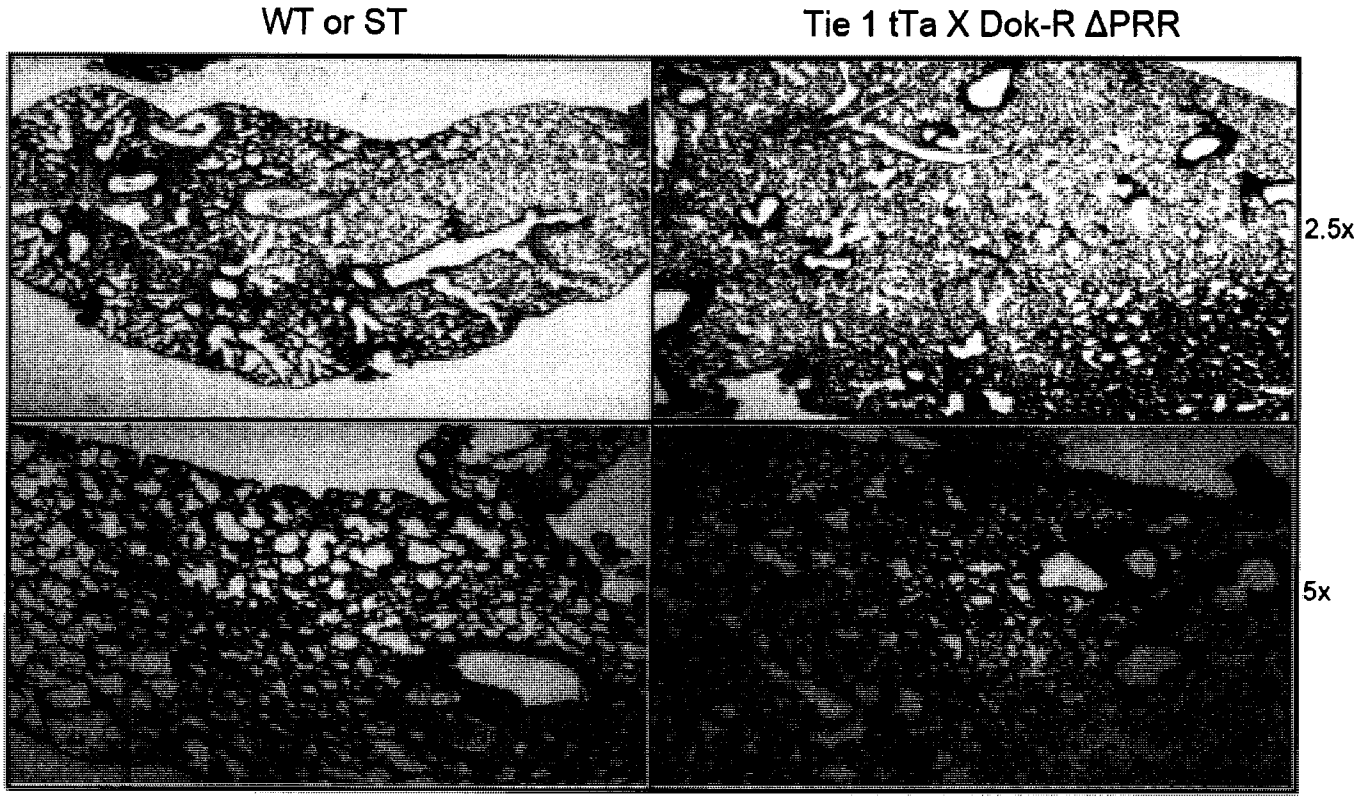


Figure 4.3a: Histological examination of Dok-R ΔPRR transgenic mice. Tissues from 6 month old double transgenic mice (pTetOS Dok-R ΔPRR/tTa Tie1) were sectioned and examined by H&E staining. No morphological differences were noted in lung sections.

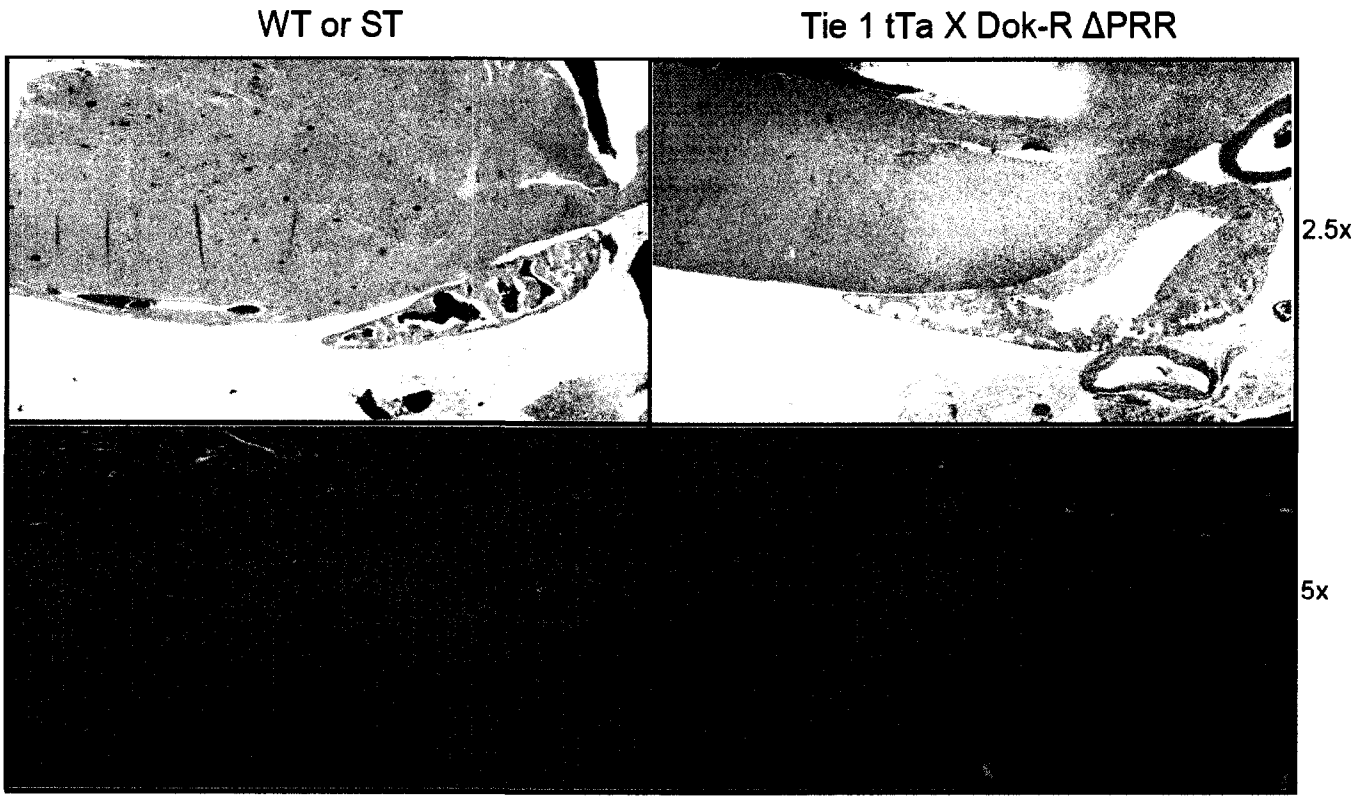


Figure 4.3b: Histological examination of Dok-R ΔPRR transgenic mice. Tissues from 6 month old double transgenic mice (pTetOS Dok-R ΔPRR/tTa Tie1) were sectioned and examined by H&E staining. No morphological differences were noted in heart sections

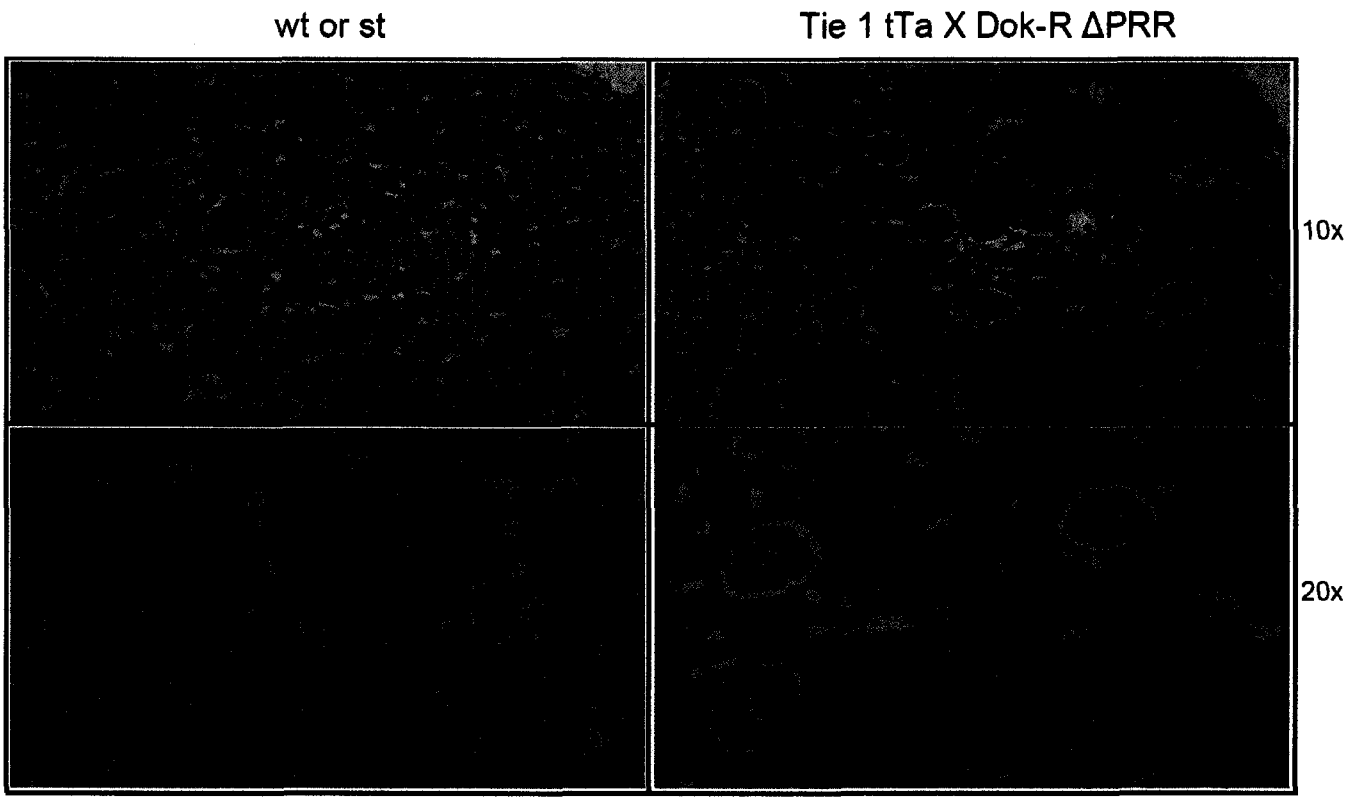


Figure 4.3c: Histological examination of Dok-R ΔPRR transgenic mice. Tissues from 6 month old double transgenic mice (pTetOS Dok-R ΔPRR/tTa Tie1) were sectioned and examined by H&E staining. Consistent morphological differences corresponding to increased glomerular space were noted in the kidneys of DT mice.

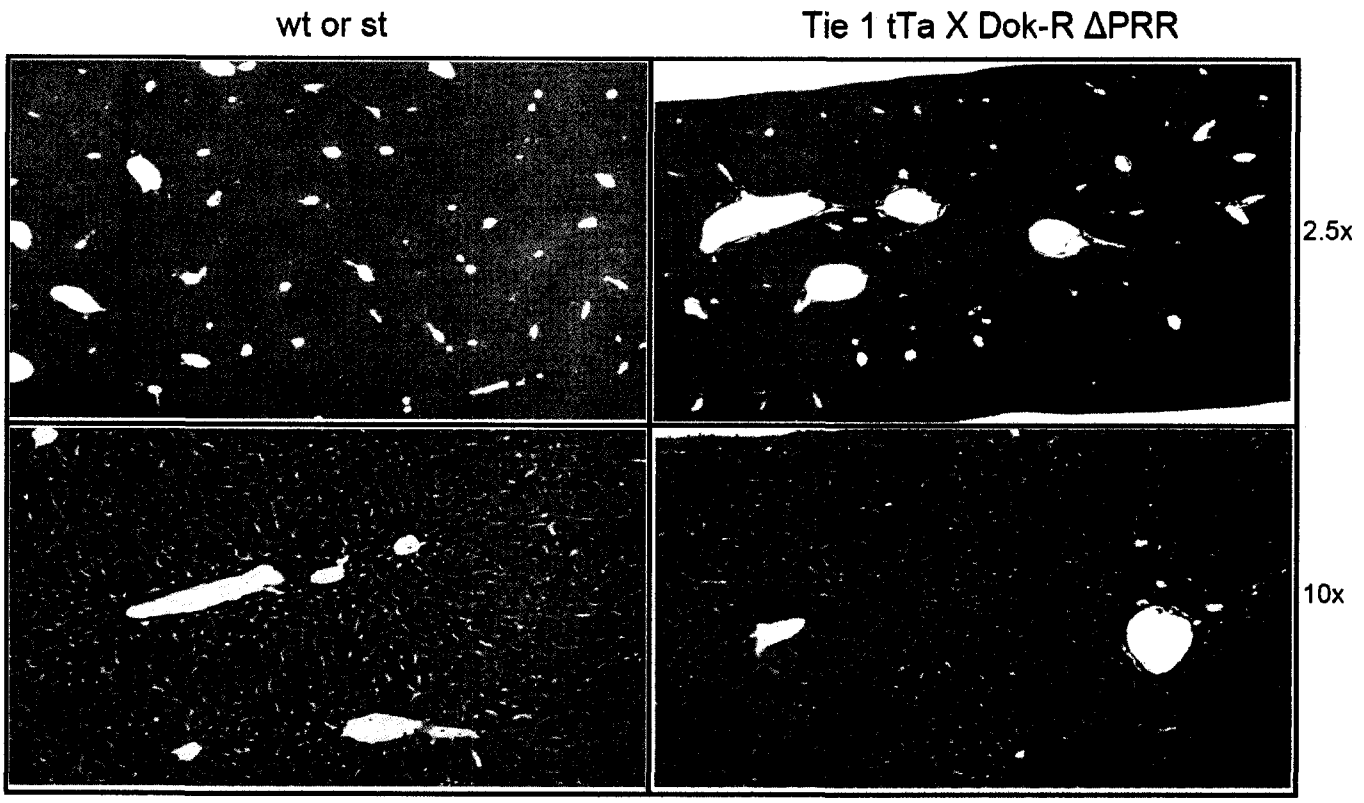


Figure 4.3d: Histological examination of Dok-R ΔPRR transgenic mice. Tissues from 6 month old double transgenic mice (pTetOS Dok-R ΔPRR/tTa Tie1) were sectioned and examined by H&E staining. Mononuclear infiltrates were noted surrounding a high proportion of hepatic vessels in the DT mice.

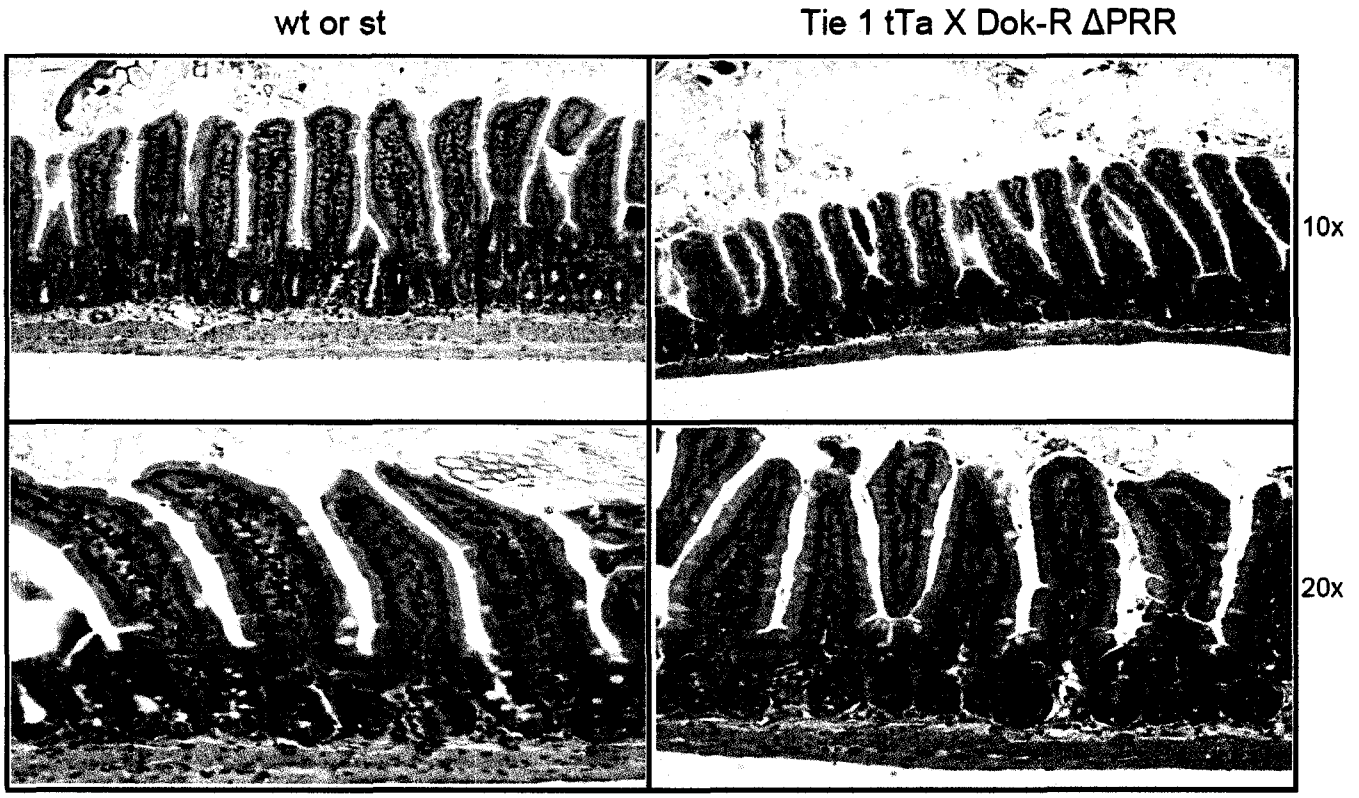


Figure 4.3d: Histological examination of Dok-R ΔPRR transgenic mice. Tissues from 6 month old double transgenic mice (pTetOS Dok-R ΔPRR/tTa Tie1) were sectioned and examined by H&E staining. No morphological differences were noted in small intestines of DT mice.

infiltration of leukocytes surrounding the vessels in the cerebellum (Fig 4.4) (Graesser et al., 2002). The perivascular mononuclear cell infiltrate observed in this mouse model of autoimmune encephalomyelitis very closely resembles that which is seen in the liver of the DT mice overexpressing Dok-R ΔPRR (Graesser *et al.*, 2002). Leukocyte extravasation may also be mediated by alterations in the barrier properties of the endothelium. For instance, leukocyte transendothelial migration has been shown to be increased by vasoactive substances that cause a breakdown of endothelial cell junctions (Singhal et al., 2000). Loss of productive endothelial:endothelial cell adhesion also results in increased vascular permeability. To examine what was promoting the noted perivascular extravasation of lymphocytes we investigated the possibility that there was an endothelial cell barrier disruption in the DT mice. To do so we employed the Mile's assay. The Mile's assay is a specific assay to quantify basal or induced vascular permeability by measuring the ability of the albumin-binding dye, Evan's Blue, to extravasate to surrounding tissues. We examined the basal and VEGF-induced vascular permeability of DT, ST, and WT mice. We find that in the heart and lungs the DT mice display small increases in basal and VEGF induced vascular permeability (Fig 4.5). Vascular permeability of the liver proved difficult to assess because of the fenestrated nature of the endothelium. This discontinuous endothelium found in the liver allowed the Evan's Blue dye to passively diffuse into the liver sinusoids or the "space of Disse" and differences between DT, ST, and WT mice were not noted (data not shown). Consequently, we were not able to determine whether the noted lymphocyte extravasation in the livers of the DT mice was due to an endothelial cell:cell adhesion defect or an inflammatory defect.

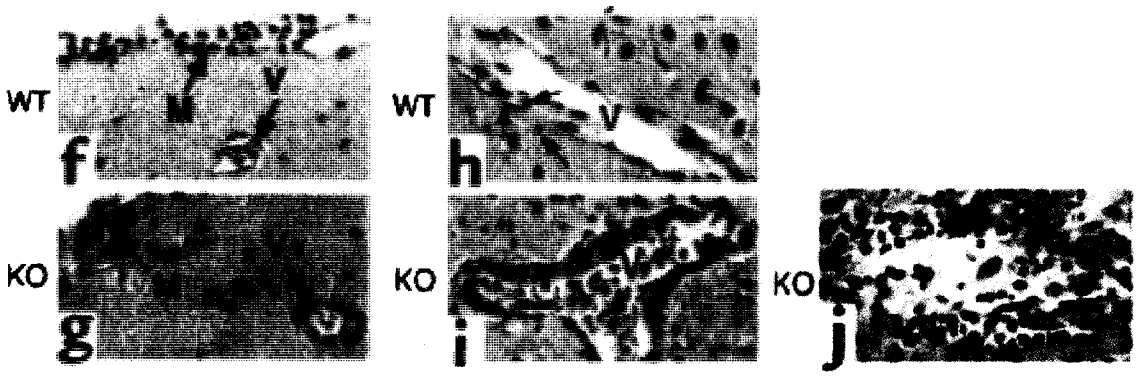


Figure 4.4: PECAM KO Mouse. 20 days post initiation of autoimmune encephalitis PECAM KO mice show (**g,i,j**) show increased perivascular and parenchymal infiltrates of the cerebellum compared to WT littermates (**f and h**). Arrows indicate perivascular mononuclear cells. V, vessels; M, meninges. Adapted from Grasser D, *et al.*, ***JCI***, (2001).

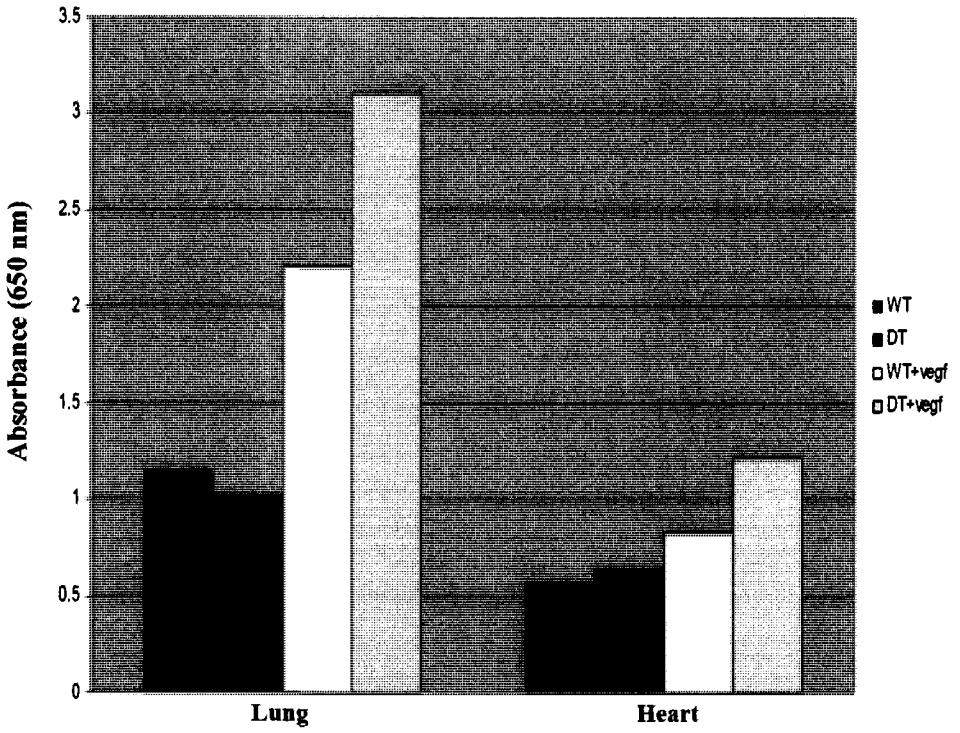
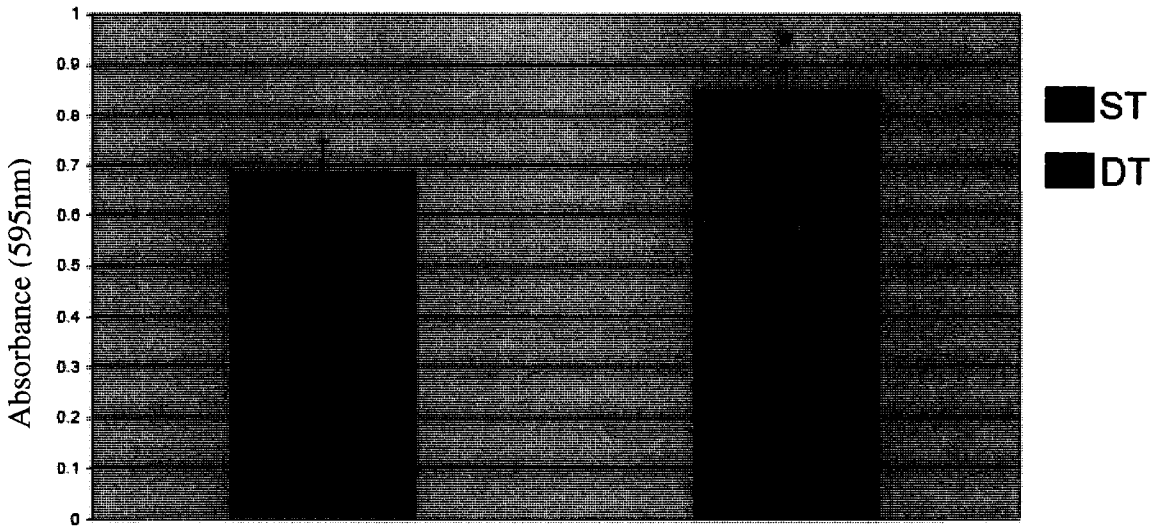
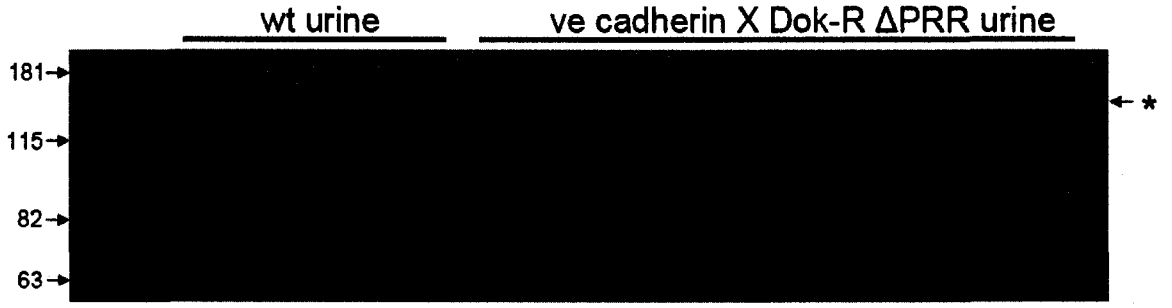


Figure 4.5: Miles assay for vascular permeability. DT mice (pTetOS Dok-R ΔPRR/Tie1 tTA) displayed modest increases in basal and VEGF-induced vascular permeability in the lungs and heart when compared to WT and ST littermates. Tissues were weighed and values were calculated as absorbance 650nm/mg tissue weight.

To investigate if there were functional implications due to the noted kidney defect in the DT mice we collected and examined urine samples for protein concentration and protein identity. Statistically significant increases in total urine protein content were noted in DT mice when compared to ST or WT mice ($p<0.05$) (Fig 4.6a). Analysis of urine samples by SDS PAGE revealed a consistent increase in high molecular weight proteins of the DT mice (Fig 4.6b). One protein, of approximately 160 kDa, which was particularly enriched in the urine of the DT mice, was excised from the gels and subjected to MS/MS-dependent protein identification. A high probability identification of prepro-epidermal growth factor (EGF) was made from three separate DT urine samples. Prepro-EGF, also known as Urogastrone is a kidney enriched, unprocessed form of EGF that is normally excreted in urine (Parries et al., 1995). The precursor form of EGF is present on the apical plasma membrane of EGF producing cells in the kidney which are localized to the ascending limb and distal convoluted tubules (Nouwen et al., 1994). In its unprocessed form, prepro-EGF acts as a renal cell mitogen and differentiation factor (Nouwen *et al.*, 1994;Parries *et al.*, 1995). Although we were attempting to isolate urinary proteins from the DT mice that may correlate or be predictive of kidney defects it is well documented that expression of prepro-EGF in the kidney and its liberation into the urine are quickly abolished during several types of drug or ischemia-induced acute renal failure and also in ureteral obstruction. Moreover, its expression is restored only after morphological and functional recovery of the kidney (Nouwen *et al.*, 1994). As such, the increased prepro-EGF noted in the urine of the DT mice appears paradoxical and remains to be explained



a



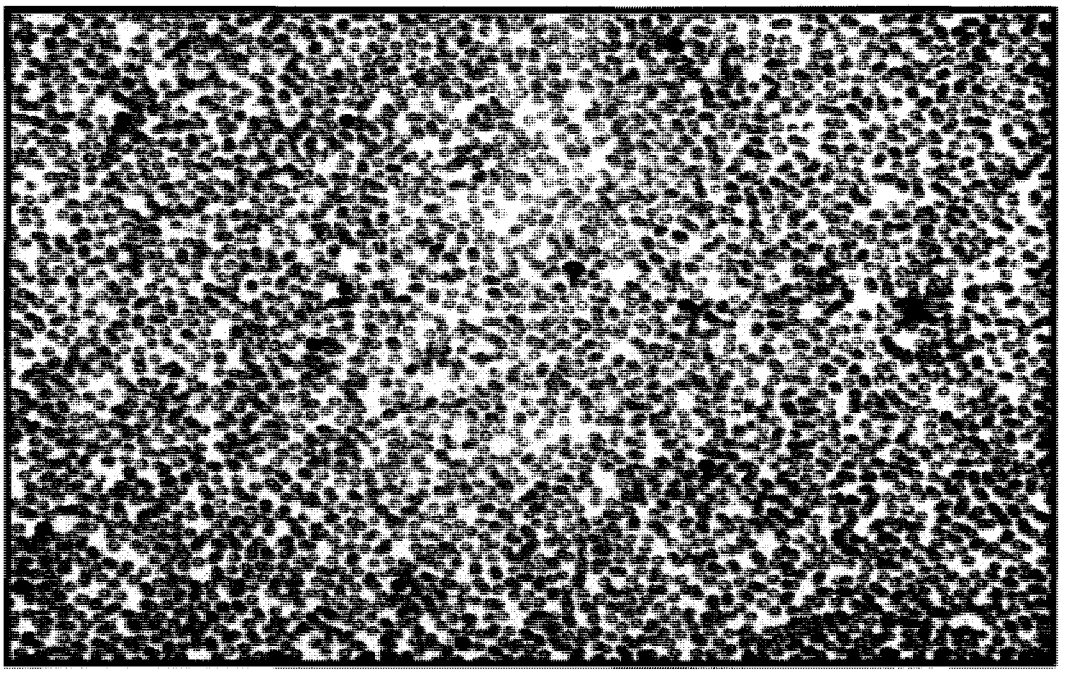
b

Figure 4.6: Urinalysis of Dok-R ΔPRR Transgenic Mice: a) The urine of DT mice (pTetOS Dok-R ΔPRR/VE Cad tTA) displayed increased protein concentration when compared to ST littermates (student t-test, $p<0.05$, $n=4$). **b)** The urine of DT mice contained increased prepro-EGF (*) when compared to WT urine.

but does not seem to correlate with known form of kidney disfunction. One possible explanation to account for the increase in not only prepro-EGF, but rather the total amount of protein measured in the urine of DT mice would include defects in barrier or filtration function of the nephron. Glomerular filtration is best known to be regulated by visceral epithelial cells (podocytes) and a complex glomerular basement membrane. Recently it has become apparent that the degree to which the fenestrated glomerular endothelial cells are permeable plays a vital role in modulating the passage of solutes into the collected urine (Eremina and Quaggin, 2004). Disturbance of this function may cause a reduction in glomerular filtration rate or proteinuria. Permeability of endothelial cells within the glomerulus is in part regulated by podocyte-dependent secretion of VEGF. Ang 1, also expressed by the podocytes, is unique in its ability to reduce endothelial permeability and antagonize the effects of VEGF in its permeability and angiogenesis-inducing actions (Satchell and Mathieson, 2003). Understanding of the regulation of the filtration barrier is incomplete but the expression of Ang's and their cognate receptor Tie 2 expressed on the endothelial cell of the glomerulus suggests a mechanism for maintenance of the glomerular endothelium and modulation of the actions of glomerular VEGF. Dok-R, one of the first described Tie 2 binding partners has been shown to play a critical role in Ang 1-driven migration and cytoskeletal reorganization of endothelial cells (Master *et al.*, 2001). Because of these facts, we hypothesize that overexpression of Dok-R ΔPRR in DT mice may contribute to barrier dysfunction of the endothelial cells and consequently proteinuria by perturbing the Ang 1/Tie 2 signaling axis.

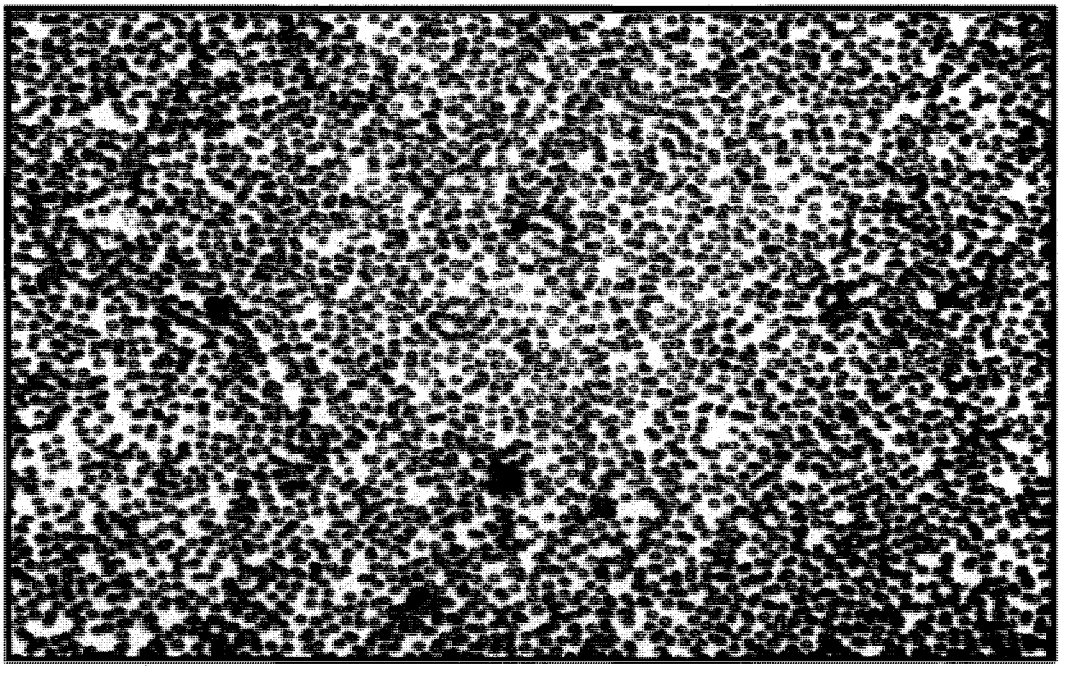
To investigate the possibility that the leukocyte infiltrate in the liver was a function of chronic inflammatory state, or leukocytosis, we performed blood smears and complete

blood counts (CBC's) on 6 month old DT (pTetOS-Dok-R ΔPRR/enh Tek tTa), ST, and WT littermates (Fig 4.7a and b). CBC's revealed a statistically significant (p=0.0139), four-fold increase in the number of circulating monocytes in DT mice compared to littermate ST and WT controls (Fig 4.4b). Overall, DT mice showed increases in all white blood cell populations measured, narrowly missing statistical significance in the lymphocyte and eosinophil populations (n=3). Erythrocyte and platelet numbers counts were normal, as were other descriptive measures of erythrocytes including MCV, MCH, MCHC, hematocrit and hemoglobin (Fig 4.7b). Subsequent to these findings Niki M, and Pandolfi P (2004), found that mice concomitantly engineered with inactivated Dok 1 and Dok-R presented with aberrant hemopoiesis and Ras/MAP kinase activation. Strikingly, all Dok-1/Dok-R double KO mutants spontaneously developed transplantable CML-like myeloproliferative disease due to increased cellular proliferation and reduced apoptosis. Most dramatic increases existed in the neutrophil and monocyte populations. Only double knock out mice displayed this phenotype while single KO mice possessed normal steady-state hematopoiesis (Niki *et al.*, 2004). The findings by Niki M, and Pandolfi P support a role for the Dok's in negatively regulating hematopoiesis and reinforce the potential that the DT mice (pTetOS-Dok-R ΔPRR/enh Tek tTa) may suffer from a primary hematopoiesis defect. Support for such a hypothesis is borne from the fact that the enhTek tTa drives expression of the transgene in primative hematopoietic lineages, suggesting a cell autonomous effect for Dok-R ΔPRR in these findings and that the Dok-R ΔPRR construct was engineered to serve as a dominant negative for Dok 1, Dok-R and Dok 3. Additionally, instead of performing more longitudinal CBC studies we examined what we believed would be steady state hematopoiesis at six months of age. Niki M, and



20x

St Dok-R ΔPRR



20x

Dt enh Tek tTa X Dok-R ΔPRR

a

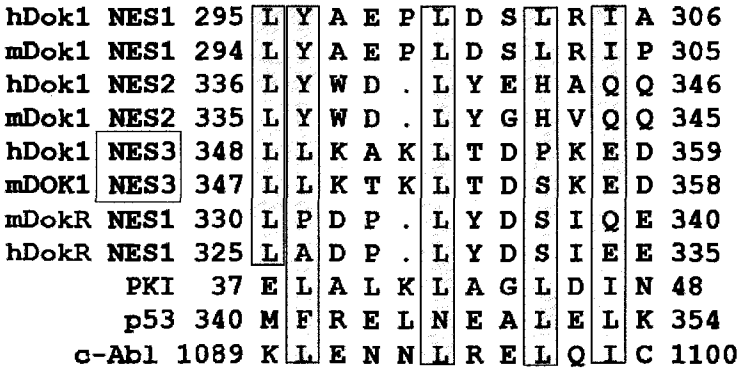
	Reference Values (Mouse)	St, Ave.	St, Std. Dev	Dt, Ave.	Dt Std. Dev.	p-value	
WBC, 10e9/L	5.0-13.7	4.143333	1.571507	7.4975	3.018823	0.072242	
Neu, 10e9/L	0.4-2.7	0.578	0.121618	0.8345	0.250459	0.084242	
Lym, 10e9/L	7.1-9.5	3.326667	1.340982	6.33	2.751569	0.073576	
Mono, 10e9/L	NA	0.052667	0.059936	0.19675	0.062447	0.013896	*
Eos, 10e9/L	NA	0.051667	0.045742	0.1125	0.036756	0.053486	
Baso, 10e9/L	NA	0.011333	0.019630	0.0275	0.02716	0.212915	
%Neu	NA	15.70667	7.124334	11.46	1.746539	0.145198	
%Lym	NA	81.93333	5.500303	83.65	3.175426	0.310183	
%Mono	NA	1.088	1.055371	2.8075	0.99151	0.038927	*
%Eos	NA	1.03	0.930537	1.65	0.804487	0.193545	
%Bas	NA	0.234333	0.405877	0.44825	0.455747	0.274668	
RBC, 10e12/L	7.9-10.1	9.953333	0.652865	9.93	0.633614	0.481927	
HGB, g/L	110-145	149.3333	6.110101	147.5	5.972158	0.353449	
HCT, L/L	0.37-0.46	0.501667	0.032021	0.50225	0.014268	0.487436	
MCV, fL	NA	50.4	1.479865	50.7	3.163332	0.443407	
MCH, pg	NA	15	0.458258	14.875	0.492443	0.373258	
MCHC, g/L	NA	298	7.937254	293.5	10.11599	0.277235	
Plat, 10e9/L	600-1200	885.6667	323.3146	885.75	43.36954	0.4998	

b

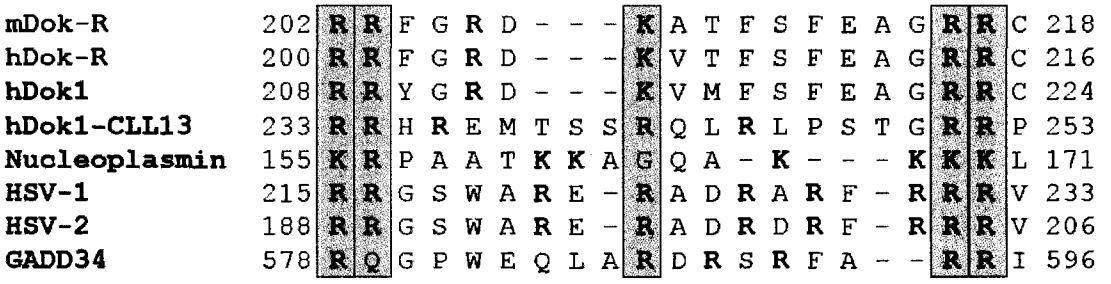
Figure 4.7: Complete blood count analysis of Dok-R ΔPRR transgenic mice. a) Representative DT (pTetOS Dok-R ΔPRR/enh Tek tTA) and ST blood smears are shown. No morphological difference between DT and ST, RBC or WBC populations was noted. **b)** Quantitative complete blood counts of DT and ST blood revealed a statistically significant increase in monocyte numbers (*, t-test, p=0.0139, n=3) and non significant increases in all other WBC populations of DT mice when compared to ST littermates. Erythrocyte and platelet numbers as well as other descriptive measures of erythrocytes including MCV, MCH, MCHC, hematocrit and hemoglobin counts were normal and did not differ between ST and DT animals.

Pandolfi P found that double knock out mice for Dok 1 and Dok-R did not start to display myeloproliferative disease until approximately ten months of age, suggesting that we may have missed further increases in white blood cell numbers as the DT mice aged.

Recently Niu Y, *et al.*, described a Crm1-dependent nuclear export sequence located in the carboxy terminus of the Dok 1 protein (Niu *et al.*, 2006). Because of the high degree of sequence homology between Dok 1 and Dok-R we sought to determine if Dok-R contained a similar NES. This information was deemed important for two reasons-the first of which was to gain a better mechanistic understanding of how Dok-R functions; the second reason involved the possibility that we may have inadvertently truncated a portion of Dok-R critical to its proper subcellular localization when we constructed the Dok-R ΔPRR mouse. Sequence alignment analysis of Dok 1 and Dok-R revealed a putative NES located in the carboxy terminus of Dok-R that corresponded very well to that which was identified in mouse and human Dok 1 (Fig 4.8a). The putative NES identified in Dok-R was carboxy terminal to the deletion that was performed when constructing the Dok-R ΔPRR mouse suggesting that if functional, the mutant Dok-R protein may be mislocalized. Further sequence interrogation also revealed a putative bipartite NLS located within the PTB of Dok-R. This sequence closely resembles previously identified bipartite NLS's of GADD 34, HSV-1 and 2, Nucleoplasmin and is also closely represented in the human Dok 1 protein amino acid sequence (Fig 4.8b). Taken together, these observations suggest that Dok-R, like Dok1, may be capable of inducibly transiting the cell nucleus. To formally investigate the subcellular localization of Dok-R, Cos1 cells were adenovirally infected with constructs corresponding to WT Dok-R or


hDok1 NES1 295 L Y A E P L D S L R I A 306
mDok1 NES1 294 L Y A E P L D S L R I P 305
hDok1 NES2 336 L Y W D . L Y E H A Q Q 346
mDok1 NES2 335 L Y W D . L Y G H V Q Q 345
hDok1 NES3 348 L L K A K L T D P K E D 359
mDOK1 NES3 347 L L K T K L T D S K E D 358
mDokR NES1 330 L P D P . L Y D S I Q E 340
hDokR NES1 325 L A D P . L Y D S I E E 335
PKI 37 E L A L K L A G L D I N 48
p53 340 M F R E L N E A L E L K 354
c-Abl 1089 K L E N N L R E L Q I C 1100

a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hDok1 208 R R Y G R D - - - K V M F S F E A G R R C 224
hDok1-CLL13 233 R R H R E M T S S R Q L R L P S T G R R P 253
Nucleoplasmin 155 K R P A A T K K A G Q A - K - - - K K K L 171
HSV-1 215 R R G S W A R E - R A D R A R F - R R R V 233
HSV-2 188 R R G S W A R E - R A D R D R F - R R R V 206
GADD34 578 R Q G P W E Q L A R D R S R F A - - R R I 596

b

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301 egeyavpfdt vahslrksfr glltgpppll pdplydsiqe dpgaplpdhi ydepegvaal
361 slydrtqrps getwreqata dggpsslqqd ssvpdwpqat eydnvilkkg pk
```

|= end of Dok-R ΔPRR
|= end of Dok-R ΔC-PRR

= Putative NLS
= Putative NES

c

Figure 4.8: Identification of Putative Dok-R NES and NLS. a) Amino acid sequence alignments of human and mouse Dok-R with Dok 1 reveal a putative CRM1-specific NES located in the PRR. Three putative CRM1-dependent Dok 1 NES's were previously identified and functionally tested. A NES located in the PRR of Dok 1 corresponding to NES3 (red box) was shown to mediate Dok 1 nuclear export (Niu *et al.*, 2006). Green shading indicates residues thought to be important in mediating CRM1-specific export. **b)** The PTB domain of Dok-R contains a stretch of basic amino acids that share homology to other proteins with identified NLS's. Basic amino acid residues thought to be critical to NLS function are boxed in red, while other basic amino acids are shown in bold text. **c)** Shows the localization within Dok-R of putative NES (turquoise box) and NLS (red box) and their relative position to truncation mutants Dok-R ΔPRR and Dok-R ΔC-PRR.

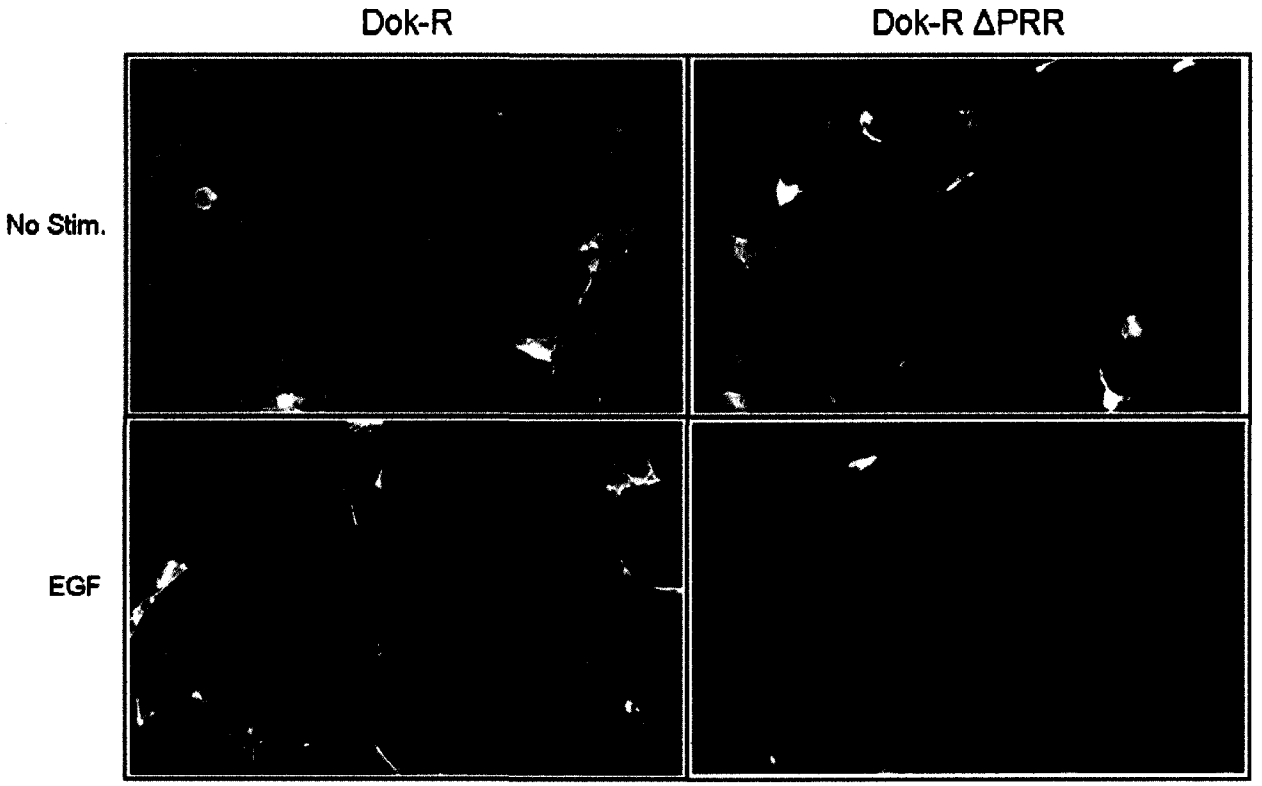
Dok-R ΔPRR. Immunofluorescent staining for WT Dok-R revealed diffuse cytoplasmic and nuclear localization that did not change significantly upon acute EGF stimulation (10 minutes) (Fig 4.9a). Dok-R ΔPRR protein primarily localized to the nucleus with faint and diffuse staining in the cytoplasm. Stimulation with EGF did not significantly change the localization of WT Dok-R or Dok-R ΔPRR. These results suggest that while WT Dok-R and Dok-R ΔPRR are able to enter the nucleus, the carboxy PRR plays a critical role in nuclear export. Entry to the nucleus, although mapping to a region upstream of the PRR, may be intrinsic to Dok-R or facilitated extrinsically by association to some other protein that shuttles to the nucleus. Alternatively, Dok 1 and Dok-R have been shown to inducibly homodimerize and heterodimerize (Songyang *et al.*, 2001;Boulay *et al.*, 2005). As such it is intriguing to consider a Dok nuclear localization mechanism similar to that of Stats (Lim and Cao, 2006) in which it is thought that a discontinuous NLS is formed upon dimerization. The possibility of Dok-R entering the nucleus by this mechanism has not been tested.

Crm1-dependent export of Dok-R from the nucleus was specifically evaluated by treating cells with Leptomycin B (LMB). Cells treated for 1 hour with LMB showed very significant accumulation of WT Dok-R protein in the nucleus suggesting that nuclear export of Dok-R is Crm1 mediated (Fig 4.9b). Further nuclear accumulation of Dok-R ΔPRR was not noted following LMB treatment (Fig 4.9b).

Although treatment of cells with LMB increased the overall proportion of WT Dok-R that partitioned to the nucleus a small cytoplasmic fraction still persisted. These results suggested that Dok-R, like Dok 1, might preferentially localize to the nucleus only when not phosphorylated on tyrosines by SFK's. To test this possibility Cos 1 cells infected

with WT Dok-R or Dok-R ΔPRR were pretreated with the SFK-inhibitor PP1 alone or in conjunction with LMB prior to immunofluorescent staining. Interestingly, Dok-R but not Dok-R ΔPRR showed a very pronounced perinuclear staining pattern when treated with PP1 (Fig 4.9b). It is currently unknown if the noted ring-like staining appearance of WT Dok-R occurs on the outer or inner surface of the nuclear membrane. Cells simultaneously treated with PP1 and LMB showed increased Dok-R nuclear localization compared to LMB alone suggesting that SFK-dependent phosphorylation of Dok-R is inhibitory to nuclear entry (Fig 4.9c).

Once in the nucleus no role has ever been ascribed to any of the Dok's. Software such as LOCtree (Nair *et al.*, 2005) does not predict a viable interaction with DNA for Dok 1, Dok-R or Dok 3. However, interestingly, the nuclear localization of Dok-R appears restricted to regions referred to as speckles which are involved in mRNA processing, while Dok-R ΔPRR displays a more diffuse and uniform nuclear staining pattern with a loss of speckling (Fig 4.9d). Similar observations were made for the Dok-R homologue IRS 3, in which the authors describe independent nuclear targeting functions to the PH and PTB domains (Maffucci *et al.*, 2003). In addition, Maffucci T, *et al.*, also found that once in the nucleus, the truncation of the carboxy terminus portion of IRS3 destroys the proteins ability to localize to speckles. Finally, Kabuta T, et al., find that while localized to the nucleus IRS 3 possesses transcriptional activation properties that map to the carboxy terminus of the protein(Kabuta et al., 2002). While the role of Dok-R in the nucleus remains unknown it is possible that, like IRS 3, Dok-R and perhaps other Dok's function in a multifactoral manner, both at the plasma membrane to inhibit signal transduction and within the nucleus to regulate transcription.



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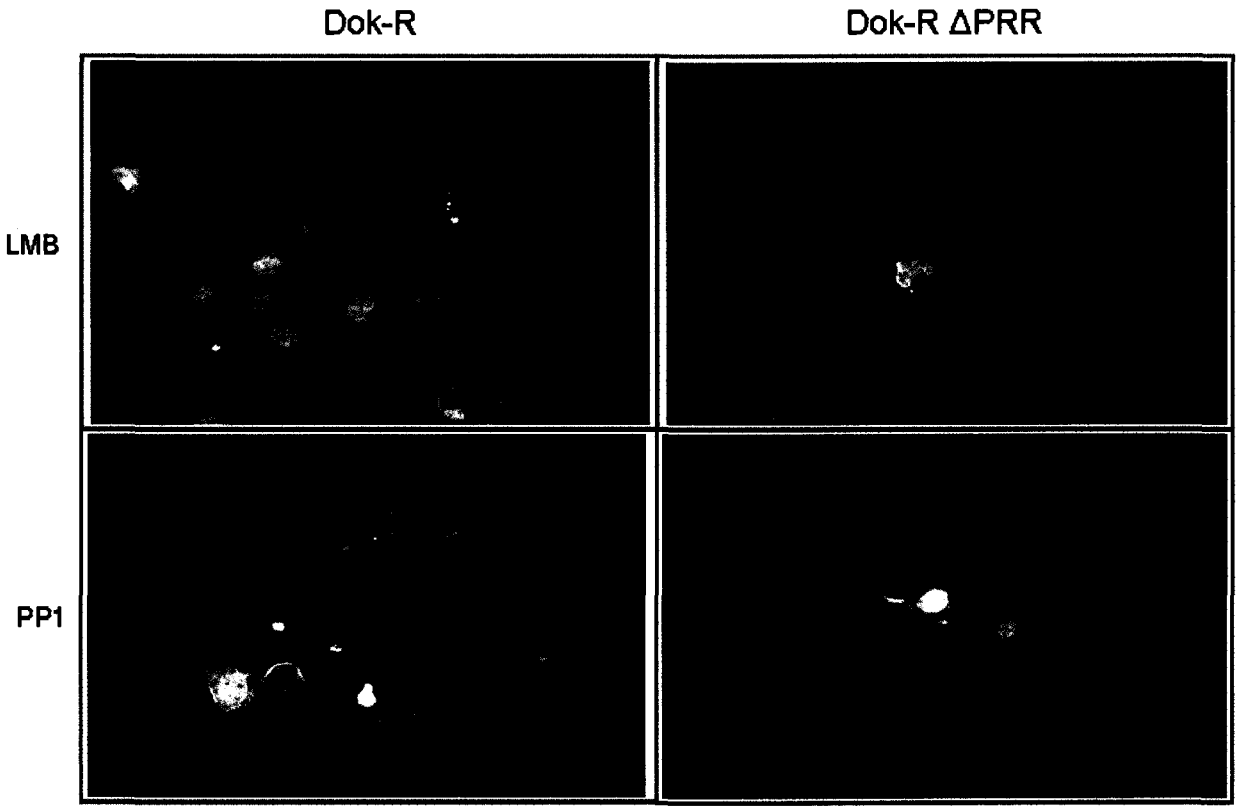

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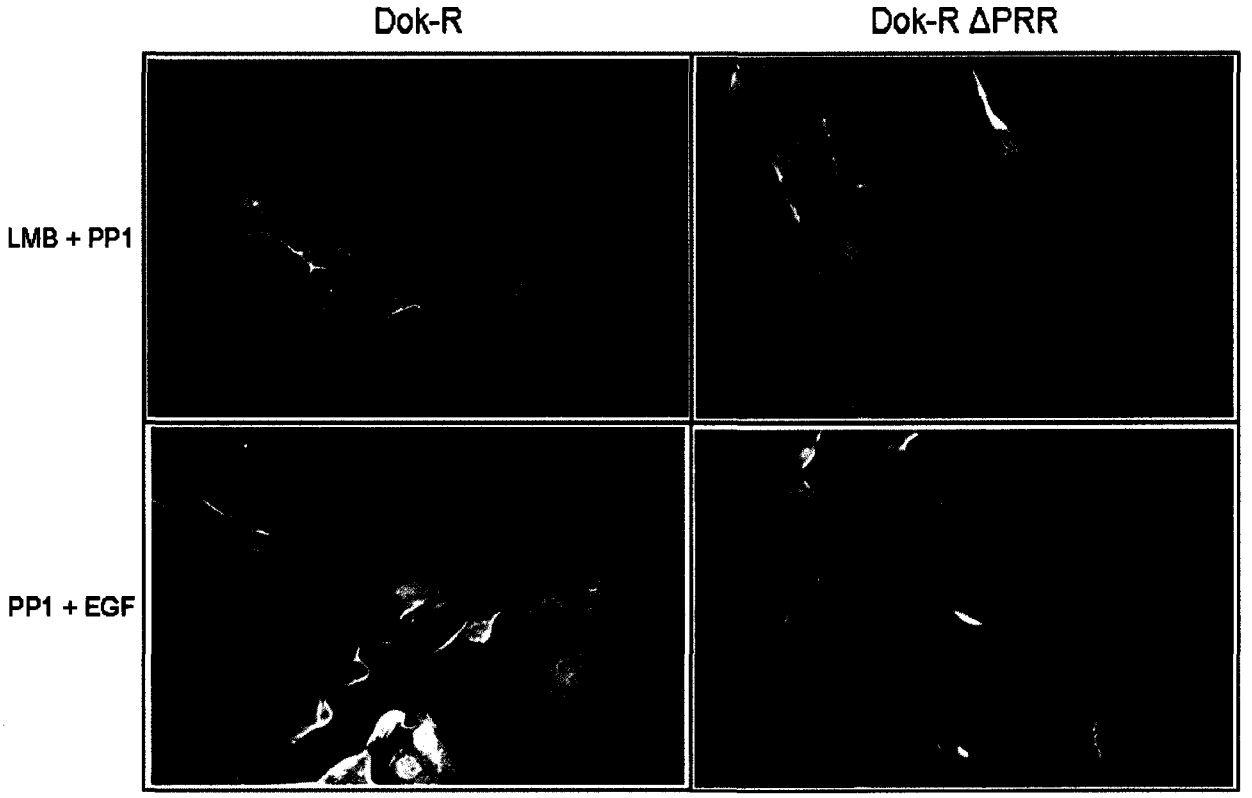

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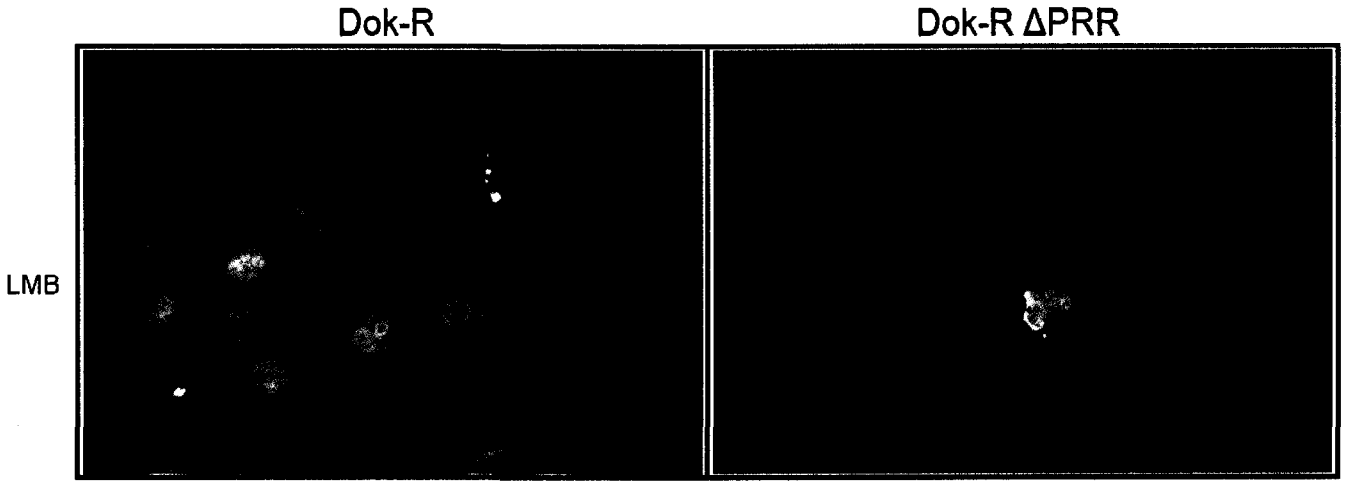

d

Figure 4.9: Subcellular localization of Dok-R and Dok-R ΔPRR. Cos 1 cells, grown on chamber slides, were infected with adeno viruses encoding either WT Dok-R or Dok-R ΔPRR. Immunofluorescenct staining (monoclonal anti-Flag M2) was performed on fixed cells and images were recorded using a Zeiss Axiovert 100 M laser scanning microscope (LSM). Cells were left **a**) unstimulated or stimulated with EGF (100 ng/ml 10min), **b**) treated with LMB (20ng/ml, 1 hour) or PP1 (20 μM, 1 hour), **c**) LMB (20ng/ml, 1 hour) plus PP1 (20 μM, 1 hour) or PP1 (20 μM, 1 hour) plus EGF (100 ng/ml 10min). Panel **d**, an increased magnification image of the LMB-treated cells shown in **b**, is included to highlight differences in nuclear compartmentalization of the WT Dok-R and Dok-R ΔPRR protein.

Discussion:

Herein we describe the preliminary characterization of a genetically modified mouse line engineered to overexpress Dok-R ΔPRR in the vascular and hematopoietic cell lineages. We find that under these conditions loss of the carboxy terminal portion of Dok-R results in minor defects in the kidney and liver including increased Bowman's space and perivascular extravasation of lymphocytes respectively. Kidney defects associated with Dok-R ΔPRR overexpression also correlate with increases in to protein content within the urine. Defects in hempatopoiesis remain to be more fully examined, however early analysis suggests that the Dok-R ΔPRR mice may present with a myloproliferative disease latter in life similar to that which was noted by Niki M, *et al.,* in the Dok 1 and Dok 2 compound knock out mice (Niki *et al.*, 2004). In follow up analysis of the Dok-R ΔPRR construct we find that this protein unexpectedly does mislocalize predominantly to the nucleus; a likely result of truncating a putative CRM 1 NES located in this region. Substantiating evidence for this derives from LMB-dependent accumulation of Dok-R and Dok-R ΔPRR in the nucleus of Cos 1 cells. We further show that pretreatment of cells with PP1 increases the proportion of Dok-R that can enter the nucleus when concomitantly exposed to LMB suggesting that SFK activity is critical to Dok-R's cytoplasmic/plasma membrane localization. Finally, we present novel data to show that Dok-R, once in the nucleus, localizes to nuclear speckles, and that localization to these microdomains requires the amino terminal portion for nuclear entry and the carboxy terminal PRR for speckle location. These data suggest that while much of the Dok-R ΔPRR was mislocalized in follow up *in vitro* studies a significant portion of this protein does remain in the cytoplasm. We believe that this portion was sufficient in displacing or

perturbing the endogenous functions of Dok-R and perhaps Dok 1 and Dok 3. As such, the results presented in this chapter, and pertaining to the characterization of the Dok-R ΔPRR mouse, are believed to be in many ways be representative of an overall reduction in endogenous Dok-R signaling.

Chapter 5

Discussion and Future Directions

Research Impetus and Literary Context

Part 1

The primary objective of the research presented herein was to elucidate the physiological functions of Dok-R. Two separate approaches were employed to this end- *in vitro* experiments examined the role of Dok-R in attenuating EGF-dependent MAPK activation while an *in vivo* mouse molecular genetic approach was aimed at more globally assessing the developmental consequences of inhibiting endogenous Dok-R within the vascular compartment.

While early studies of Dok-R function highlighted its role in attenuating RTK and PTK-induced MAPK activation, the specific mechanism by which this occurred remained controversial. Initial observations by Songyang *et al.*, demonstrate that family member prototype Dok 1 serves as a scaffold protein to recruit RasGAP, leading to the early conclusion that MAPK attenuation occurs by virtue of decreases in overall Ras GTP loading (Park *et al.*, 1993;Carpino *et al.*, 1997;Nelms *et al.*, 1998;Jones *et al.*, 1998b). Support for the functional significance of the Dok-R/RasGAP interaction derives from the observation that bone marrow cells retrovirally infected with Dok-R are deficient in their ability to reseed lymphoid organs and that this defect is dependent on intact RasGAP binding sites (Gugasyan *et al.*, 2002). It was not until Jones *et al.*, made the novel observation that a Dok-R mutant, engineered to be deficient in RasGAP binding sites, was still able to attenuate EGF-driven MAPK activation that the current Dok signaling paradigm was questioned (Jones *et al.*, 1999). Additional evidence in support of the Jones *et al.*, finding was described in several subsequent reports that included those of Kashige et al., who demonstrated that once bound to Dok 1, the activity of RasGAP was

almost completely abolished (Kashige *et al.*, 2001) and Cong et al., who found that Dok L could attenuate Bcr-Abl driven MAPK activity despite the absence of RasGAP binding motifs (Cong *et al.*, 1999). Not long after the initial cloning of Dok 1 Songyang *et al.*, examined its potential involvement in mitigating signals emanating from constitutively active Src Y527F. Because Csk mediates phosphorylation of inhibitory Y527, and Dok 1 had been shown to recruit Csk, the authors were able to specifically address Csk-independent cell signaling events with this approach. Data clearly demonstrated that enforced expression of Dok 1 was sufficient to counteract Src Y527F-induced cell transformation (Songyang *et al.*, 2001). The authors mapped the region of Dok 1 that mediates this effect to a stretch of 27 amino acids (336-363) located in the PRR. Loss of this region severely limited the ability of Dok 1 to bind RasGAP and counteracted Src Y527F-dependent transformation suggesting that RasGAP may play an important role in these events. Interestingly, Songyang *et al.*, also examined the ability of Dok 1 to counteract c-Src-induced cell transformation. Enforced expression of full length Dok 1, but not Dok 1 deficient in the PRR, resulted in a nine fold reduction in Src-induced cell foci formation. Although these results would seem to correlate with those that were noted in the Src Y527F experiments the possibility that Dok 1 attenuated c-Src-induced cell transformation through a Csk-dependent mechanism can not be excluded. In fact, the Dok 1 mutants that could address this lingering question were not examined in conjunction with c-Src, but were instead only used in the Src Y527F experiments. Alternatively, Wick J, *et al.*, report that targeted disruption of Dok 1 at Y362 and Y398 diminished the interaction between Dok 1 and RasGAP and decreased the inhibitory effect of Dok 1 on the insulin-stimulated activation of Ras and Akt, but not mitogen-activated protein kinase

(Wick et al., 2001). The findings of Wick J, et al., suggest two alternate explanations to those put forth by Songyang. First, Dok 1, deficient in its ability to bind RasGAP is capable of attenuating MAPK activation. Second, the novel finding that Dok 1 strongly attenuates Akt through some undefined mechanism supports the possibility that loss of Akt regulation and not necessarily Ras activity was the contravening force behind Dok 1-dependent inhibition of Src-mediated transformation. Finally, given that high expression or hyperactivity of Src has been shown to correlate with poor cancer prognosis (Russello and Shore, 2004;Wilson et al., 2006), the very important question of examining the ability of Dok 1 to attenuate signals from wild type Src, and whether these events are RasGAP or Csk-dependent, was left unanswered.

Dok-R Dependent Cell Signaling

To investigate RasGAP independent mechanisms we utilized the EGFR as a prototypical RTK that had previously been identified to associate with Dok-R (Jones *et al.*, 1999). It was reasoned that Dok-R-dependent mechanisms that participate in attenuating MAPK downstream of EGFR activation would likely be similar to those downstream of a broad range of other RTK's known to bind Dok proteins. Early in our experiments we were able to map the region of Dok-R responsible for attenuating EGF-driven MAPK activation to the PRR. Mapping this region was critical to defining any similarities that exist between Dok 1, Dok-R and Dok 3. At the outset of this investigation it was known that Dok 1 and Dok-R were able to bind RasGAP, and NCK, while Dok 1 and Dok 3 had both been shown to bind Csk and various SFKs in their PRR's (Park *et al.*, 1993;Neet and Hunter, 1995;Carpino *et al.*, 1997;Nelms *et al.*, 1998;Jones *et al.*, 1998b;Lemay *et al.*,

2000;Shah *et al.*, 2002). Much of the research at the time had focused on the role of Dok 1-recruited RasGAP and Nck in MAPK attenuation and cell migration respectively. The functional significance of the Src/Csk interactions remained elusive until data presented in chapter three of this thesis was published (Van Slyke *et al.*, 2005).

Using a candidate molecule approach, we focused our attention on the possibility that Dok-R, like Dok 1 and Dok 3 could bind Src and Csk. We demonstrated *in vitro,* and *in vivo,* the existence of an EGF-inducible quaternary complex consisting of the Dok-R, EGFR, Src and Csk. We showed that Dok-R was able to shuttle constitutively associated Src to the EGFR, whereupon there was a transient activation of Src that resulted in tyrosine phosphorylation of the PRR of Dok-R. Phosphorylation of these sites proved to be SFK dependent and served to recruit downstream signaling proteins to Dok-R. One such protein was Csk. We showed that one of the sites that Src specifically phosphorylates on Dok-R, Y402, is nested within an ideal consensus sequence (pYXXV) for SH2-dependent binding of Csk (Songyang et al., 1994). The inducible recruitment of Csk to this complex positioned Src and Csk in such a way that Csk was able to hyperphosphorylate the negative regulatory tyrosine of Src, Y527. Furthermore, the noted hyperphosphorylation of Src Y527 correlated with a dramatic loss of EGF-dependent activation of Src kinase and MAPK. These key findings were the first to describe a mechanism in which the significance of Dok-recruited Src and Csk was addressed.

Subsequently Zhao M, *et al.*, described a mechanism in which PDGF-elicited cell signaling was attenuated by the juxtaposition of Csk and active Src within the Dok 1 PRR. However, inconsistent with our findings (Chapter 3), Zhao M, *et al.*, report that the

consequence of Dok 1-mediated decreases in Src kinase activity translated into reduced c-myc induction, while MAPK activation was unaffected (Zhao *et al.*, 2006). Instead, they report that Dok 1 negatively regulates PDGF-induced MAPK activation by acting on RasGAP and at least one other undefined protein (Zhao *et al.*, 2006). While the interpretations of their findings differ from ours, they do so when examined with different proteins in a different cellular context, the impact of which is not known. It may be that the mechanisms that control the downstream functions of different Dok family members are overlapping and distinct or it is possible that the interpretation of results by independent researchers differs. For instance, what remains unclear about the Zhao M, *et al.*, findings is how they were able to dissect Src and MAPK dependent and independent events when investigating levels of c-myc mRNA. It is well known that growth factor mediated activation of MAPK increases the expression of early response genes c-fos and c-myc (Dunn *et al.*, 2005). As such, Dok 1 mediated attenuation of MAPK would likely decrease PDGF-induced expression of c-myc, an event that Zhao M, *et al.*, deemed MAPK independent and thus by default, SFK specific. Our results, in which we chemically inhibit SFK's prior to EGF stimulation, clearly support a central role for SFK's in activating MAPK and as such likely c-myc expression as well.

Crystallographic analysis of Src in its autoinhibited state has revealed a functional model in which a single SH2 and SH3 domain form intramolecular associations necessary to lock the kinase lobes into an inactive conformation (Boggon and Eck, 2004).

Phosphorylation of the C-terminal tail on Y527 promotes assembly of the SH2, SH3, and kinase domains into an autoinhibited conformation maintained by intimate interactions among these domains (Sicheri et al., 1997; Williams et al., 1997;Xu et al., 1997)

(Sicheri et al., 1997;Williams et al., 1997;Xu et al., 1997). Perturbations of intramolecular SH2 and/or SH3 domain interactions which result from binding of cognate ligands or dephosphorylation of Y527 have been shown to activate Src kinase activity (Brown and Cooper, 1996;Moarefi et al., 1997). As such, there remains some question as to, why, when bound to Dok-R, Src is not in a constitutively active state. Our data clearly demonstrates that Src constitutively interacts with Dok-R via Src's SH3 domain and a proline rich sequence located in the carboxy terminus of Dok-R. In addition, we can not rule out the possibility that Src also associates with Dok-R via an SH2-mediated interaction, as was reported for Src and Dok 1(Richard et al., 1995b), but we find this unlikely because EGF-induced phosphorylation of Dok-R did not increase Dok-R's ability to recruit Src. We find that cells overexpressing full length Dok-R exhibit global increases in phosphorylation of Src Y527, implicating the actions of Csk in these events. In addition, we demonstrate that Dok-R-dependent increases in Src Y527 phosphorylation correspondingly correlate with decreased Src kinase activity. Zhao M, *et al.*, report similar findings for Dok 1 following PDGF stimulation. Taken together, the consistency of these findings suggests at least two different explanations. It is possible that Dok-R and/or Dok 1 may possess functions analogous to PAG/Cbp. PAG/Cbp, a transmembrane scaffold protein that localizes to glycosphingolipid microdomains, is perhaps best known for the role it plays in modulating T cell receptor signaling. SFK member Fyn has been shown to constitutively interact with PAG/Cbp via its SH3 domain

(Brdicka et al., 2000). Once bound, Fyn and Lck-dependent phosphorylation of PAG/Cbp has been shown to be a critical event in recruiting Csk and attenuating localized SFK activity (Lindquist *et al.*, 2003). In addition to phosphorylating the negative regulatory tyrosine of SFK members, Csk has also been shown to interact with the protein tyrosine phosphatase PEP (Cloutier and Veillette, 1996). PEP in turn has been shown to dephosphorylate the activating tyrosine of Src kinases and the Csk-PEP complex has been shown to be more effective at attenuating Src kinase activity than either Csk or PEP alone (Cloutier and Veillette, 1999;Gjorloff-Wingren et al., 1999). Such a model, if shared by Dok 1 and Dok-R would explain the strong inhibition of Src kinase activity and would suggest that a very transient or subtle activation of Src, sufficient to recruit Csk and perhaps PEP, could strongly limit overall Src kinase activity. Such a mechanism is consistent with the high degree of Csk-dependent Src Y527 phosphorylation noted in our experiments. Additionally, we know from PP1 inhibition studies that a SFK member phosphorylates tyrosine residues in the PRR of Dok-R critical for Csk SH2 recruitment. While these data are consistent with mechanisms explaining PAG/Cbp-dependent regulation of SFK's an alternate explanation is possible. Recent reports examining the function of DOC-2/DAB2, a potent tumor suppressor, detail a mechanism in which the SH3 domain of Src engages a DOC-2/DAB2 proline rich sequence in such a manner as to maintain Src in an inactive state (Zhou et al., 2003). Consequences of this interaction include decreases in EGF-dependent phosphorylation of Src Y416, Erk 1/2 activation (Zhou *et al.*, 2003) and Akt activity (Zhoul et al., 2005), while phosphorylation of Src Y527 remained unaffected (Zhou *et al.*, 2003). Interestingly, by overexpressing DOC-2/DAB2, Zhou J, *et al.*, were able to almost exactly recapitulate Dok-R-dependent effects

downstream of EGFR activation. One inconsistency that favors Dok-R more closely behaving like PAG/Cbp is the fact that Src Y527 phosphorylation did not increase in the DOC-2/DAB2 experiments, suggesting Dok-R functions in a Csk-dependent manner (Zhou *et al.*, 2003).

While it is probably an oversimplification to state that one pathway over another is the most important in Dok-mediated attenuation of MAPK several key findings have come to light due to this controversy. Undisputed is the fact that Dok-R, ablated in its ability to bind RasGAP, is still able to attenuate MAPK (Jones *et al.*, 1999). This finding in conjunction with the fact that Dok 3, intrinsically deficient in RasGAP binding, is capable of attenuating BCR-Abl mediated MAPK activation supports a RasGAP independent role for the Dok family in regulation of MAPK. Our group and Zhao M, *et al.*, independently find that Dok-R and Dok 1 serve as scaffold proteins for Src and Csk, and in so doing, decrease Src activation in response to EGF and PDGF stimulation respectively. Although not formally investigated, based on structural similarities and the ability to bind Src and Csk, it is possible that Dok 3 may also participate in such a signaling paradigm. Controversy in the literature surrounding RasGAP dependent and independent functions for Dok 1 and Dok-R has brought forth a more full understanding of Dok functioning, one which would be best described as a collaborative influence on MAPK activity based on the sum total of recruited proteins, including RasGAP, Src and Csk. As this controversy continues it is likely that differences in data will arise that are representative of cell context, the RTK or PTK examined, or subtle differences in Dok family members.

Currently the full utility of the Dok 1/Dok-R single or compound knock mouse lines have not been fully realized. Although the compound Dok 1/Dok-R mice develop a spontaneous myeloproliferative disease resembling human CML, the origin of this disease and investigation into the potential of more cryptic phenotypes has not been reported.

To diminish potential biases associated with selecting a particular RTK or cell type to examine, and to avoid potential issues associated with *in vitro* analysis, we constructed a line of transgenic mice engineered to overexpress a dominant negative form of Dok-R. Overexpression of Dok-R ΔPRR within the vascular compartment proved to be non lethal to developing mice, resulting in only minor defects within the liver, kidney and hematopoietic compartment. Follow up *in vitro* analysis of the overexpressed transgene revealed alterations in the subcellular localization of Dok-R ΔPRR. Specifically, Dok-R ΔPRR localized primarily to the nucleus. As a consequence, the degree to which the mouse data presented in chapter four represents a dominant negative role for Dok-R ΔPRR at the plasma membrane or within the cytoplasm remains unknown. Consistent with the findings of Niu Y, *et al.*, pertaining to Dok 1 our data suggests that SFK-dependent phosphorylation inhibits nuclear entry of Dok-R ΔPRR (Niu *et al.*, 2006). As such, within cells actively engaged in SFK-mediated signaling events, exclusion of Dok-R ΔPRR from the nucleus may be sufficient to elicit a dominant negative effect to the signaling events of endogenous Doks. Alternatively, phenotypic changes in the Dok-R ΔPRR mice may have occurred due to specific and unforeseen effects within the nucleus. For instance, it has yet to be determined what, if any, the role of the Doks play within the

nucleus. If the PRR is required for some nuclear function, and not just nuclear export, the phenotypic changes noted in the Dok-R ΔPRR mice may be representative of disturbances in Dok-R nuclear function. The fact that Dok 1 and Dok-R have evolved such elaborate and inducible nuclear shuttling mechanisms suggests that the Doks may have heretofore undetermined nuclear functions. As such, it would seem unlikely, and rather inefficient, that these localization mechanisms have evolved to simply sequester Doks away from active signaling complexes at the plasma membrane. In addition, many potentially useful analogies can be drawn between Dok 1, Dok-R and the STAT family of proteins. In response to growth factor or cytokine stimulation STATs, like Dok 1 and Dok-R become phosphorylated on tyrosine residues and homo or heterodimerize via reciprocal binding of SH2 or PTB domains respectively. STAT dimers are released from activated receptors, translocate to the nucleus via association with importins, bind to specific DNA-binding elements, and activate cytokine responsive genes (Lim *et al.*, 2006). Currently the mechanism by which the Doks enter the nucleus is not well understood. Deletional mapping of Dok 1 highlights a short stretch of basic amino acids that reside within the PTB domain (Lee et al., 2004). Unfortunately, deletion of these key arginine residues may have also critically damaged the functionality of the PTB as it is thought that two of these arginines are invariants to phosphotyrosine binding. If such is the case, it may be possible that deletion of these key arginine residues crippled nuclear transport not by disrupting an NLS, but rather by destroying the ability of Dok 1 to form homodimers as is the case with STATs. To examine this possibility further deletions or point mutations within this region, that do not destroy PTB domain function, need to be performed. Evidence that argues against dimer-dependent nuclear import of Dok 1 or

Dok-R is the fact that SFK dependent phosphorylation inhibits nuclear entry but is necessary for dimerization (Songyang *et al.*, 2001). Once in the nucleus it is predicted, based on structural characteristics, that Dok 1 or Dok-R, unlike the STATs, would be incapable of interacting with DNA (LOCtree). However, these predictions have not been experimentally examined, nor has the potential that Dok 1 or Dok-R act as co-activators or co-repressors of gene transcription. Export of Dok 1, Dok-R and the STATs is facilitated by the actions of CRM 1. Although more experimental detail is required, the structural and regulatory characteristics of Dok 1 and Dok-R implicate these proteins in both tyrosine kinase mediated cell signaling and potentially gene transcription regulation. If future experiments successfully identify a role for Dok-R and/or Dok 1 in the nucleus the Dok-R ΔPRR mouse line may become a valuable *in vivo* tool to separate nuclear dependent and independent effects of the Doks.

Vasculotide-A Novel Angiopoietin Mimetic

Part 2

The rational design of a drug therapeutic involves first identifying and validating a key target or pathway to be modified, followed by construction of an agent that is exquisitely specific for this target. Drugs that aim to modify RTK signaling by increasing or decreasing kinase activity have suffered from difficulties broadly associated with specificity. In the case of small molecule kinase inhibitors, the highly conserved nature of RTK kinase domains has posed considerable challenges tied to inhibition of related non-target receptors (Verkhivker, 2007). Non small molecule biotechnology approaches such as therapeutic monoclonal antibodies, blocking peptides, activating peptides, aptamers,

although often more specific than small molecules, may be limited by the shared goal of small molecules in identifying a target RTK that is uniquely specific to the disease state that one aims to treat. This feat has proven difficult, as most RTK's are widely expressed. In the case of angiogenesis, RTK's have been shown to play a central and defining role in the angiogenic response. Growth factor receptors such as PDGFR, MET and Eph's have all been shown to be absolutely critical to developmental and adult morphogenic vascular processes (Peters *et al.*, 2004). Unfortunately, interest in utilizing these receptors as targets for pro or anti angiogenic modulation has decreased due to their wide tissue distribution outside of the vasculature. Mouse molecular genetic approaches have highlighted the indispensable role of the Vegf receptors and Tie 1&2 receptors in development and maintenance of a functional vasculature. These studies, combined with the fact that VEGFR's and Tie's are predominantly restricted in expression to blood and lymphatic endothelial cells, or their precursors, offers the potential of an increased therapeutic index for agents that target these receptors. In addition to acting directly on the endothelium, adenovirus driven expression of VEGF, or Ang 1 in conjunction with VEGF, has been shown to have coincidental effects on the vasculature by mobilizing circulating endothelial progenitor cells or priming these cells for mobilization respectively (Hattori et al., 2001).

Herein we have described the rationale design of a novel Ang mimetic (chapter 2). *In vitro* analysis of its properties profile effects that very closely resemble other native Ang's. Specifically, we show activation of Tie 2 and signalling pathways that have been classically described downtstream of Tie 2 including MAPK, AKT, and eNOS. However,

unlike the effects of Ang 1, we illustrate with the use of reconstitution experiments, that Vasculotide uncouples Tie 2 signalling from β1 integrin. We report that treatment of primary endothelial cells with clustered Vasculotide results in induction of cell migration, MMP2 release and protection from serum withdrawal-induced apoptosis. *In vivo* we show, that when impregnated in Matrigel, clustered Vasculotide promotes a robust proangiogenic response that is marked by the production of well arborized vessels that stain positively for the myogenic support cell marker, Sma1. Significantly, we show that clustered Vasculotide, when combined with VEGF, does not increase vessel number, vessel length or number of branch points, but rather mitigates the tortuosity seen with the addition of VEGF alone. We have examined the utility of clustered Vasculotide in the treatment of experimentally induced wounds using a widely accepted animal model of diabetes mellitus type II. We show that clustered Vasculotide offers accelerated wound closure when compare to vehicle or bFGF controls. Using this model of impaired wound healing we elucidate that the improved wound closure times noted for clustered Vasculotide derive from increased granulation tissue and neovascularization of the wound.

Diabetic ulceration is a common and complex condition that often arises in the lower extremities of afflicted individuals due to microangiopathy, peripheral neuropathy, heightened immune response, leaky vessels, and poor blood perfusion (1993;Laing, 1998;Reiber et al., 1999;Martin et al., 2003). Although microangiopathies are thought to be a precipitating event which leads to diabetic foot ulceration, conventional medical treatments focus almost exclusively on offloading, microbial management, debridement,

suitable dressing application, cellular or acellular grafts and when necessary, amputation (Keyser, 1993;Steed et al., 1996). Several clinical and pre-clinical therapeutic approaches aimed at specifically treating vascular dysfunction have been evaluated (Miller, 1999;Jacobi et al., 2002;Galiano et al., 2004;Cho *et al.*, 2006). Adoption of these treatment strategies has been slow to gain in popularity due to high cost and generally low efficacy. Exogenous growth factor application in particular has been examined extensively in the literature (Bennett et al., 2003) however, with the exception of topical PDGF application, which demonstrated statistically significant improvements in non-healing, well vascularized diabetic foot ulcers (Smiell et al., 1999), widespread use of growth factors has not occurred. Whether the failure of human growth factor trials to treat diabetic foot ulcers is a function of inefficacy, trial design, single agent design, or selection of initial animal models which do not adequately recapitulate human disease is unknown. Recently Galiano RD, *et al.*, and Cho CH, *et al.*, presented promising work which demonstrates that therapeutic augmentation of vascular-specific growth factors, VEGF and COMP Ang1 respectively, can indeed provide wound healing improvements in a mouse model of diabetic wound healing (Galiano *et al.*, 2004;Cho *et al.*, 2006). Because of the complexity of chronic wounds, and the diversity of underlying etiology, it has been suggested that therapeutic approaches to treatment would benefit from the simultaneous application of multiple different growth factors. For instance, in the Beclapermin (Regranex ®) trials, PDGF-dependent improvements in wound closure were only noted in well vascularized wounds, a state which is often scarcely present in diabetic foot ulcers. Thus, the potential benefit of combining Beclapermin with some agent or growth factor capable of improving vascularization is an approach that may yield added

benefit. Alternatively, this approach may increase the cohort of individuals in which use of Beclapermin is indicated. To this end we would like to, in future experiments, assess the potential benefit of combining Beclapermin with Vasculotide in an experimental mouse model of diabetic wound healing.

While we clearly show Vasculotide-dependent activation of Tie 2, it remains unknown whether the downstream effects mimic those of Ang 1, Ang 2 or some combination thereof. In fact, this question has proven difficult to address, as distinguishing features downstream of Ang 1 or Ang 2 dependent activation of the Tie 2 receptor are not well elucidated. For instance, both Ang 1 and 2 activate a grossly overlapping repertoire of proteins including Akt, eNOS and MAPK which mediate endothelial cell migration, morphogenesis, and inhibit permeability through the Tie 2 receptor (Davis *et al.*, 1996;Koblizek *et al.*, 1998;Witzenbichler *et al.*, 1998;Kwak *et al.*, 1999;Babaei *et al.*, 2003;Chen *et al.*, 2004;Daly et al., 2006). The context dependency of these effects and the use of modified recombinant or chimeric forms of Ang 1 and Ang 2 to examine Tie 2-dependent cell signaling contributes to, but at the same time obscures, our current understanding of the endogenous functions of Ang 1 and 2. The central dogma of Ang signaling was the concept that Ang 2 behaved as a context-dependent antagonist or a competitive antagonist of Ang 1 function (Suri *et al.*, 1996;Maisonpierre *et al.*, 1997). However, more recently we, and others have shown that Ang 2 is more likely a partial agonist of the Tie 2 receptor, and that under conditions of endothelial stress Ang 2

possesses many of the same endogenous functions of Ang 1, including resistance to vascular leak and apoptosis (Bogdanovic et al., 2006;Daly *et al.*, 2006).

Although the exact origin(s) of the differences and similarities that exist between the actions of Ang 1 and 2 are poorly understood, unique structural characteristics differentiate these two ligands. Ang 1 and 2 are structurally characterized by an N-terminal super clustering domain (SCD) followed by a coiled-coil domain (CCD) and a C-terminal fibrinogen-like domain (FLD)(Ward *et al.*, 2002;Tsigkos et al., 2006). Binding specificity of the Ang's for the Tie2 receptor has been ascribed to the FLD, with both ligands interacting with the same receptor pocket, while functional studies have highlighted a role for the SCD and CCD's in forming high order homotypic Ang multimers (Procopio *et al.*, 1999). The specific nature of these multimers is variable and seems to be unique to Ang 1 and 2. Taken together, unique structural attributes of each Ang family member promotes binding and differential clustering of Tie2. The pleiotropic physiological effects of Ang 1 and 2 are thought to, at least in part, be mediated by appropriate and specific clustering of the receptor(Procopio *et al.*, 1999;Davis *et al.*, 2003;Cho *et al.*, 2004;Ward *et al.*, 2004a). Vasculotide was engineered to structurally mimic binding and clustering properties of Ang 1. The strength to which Vasculotide is able to activate the Tie 2 receptor, as tested in endothelial cell culture, suggests that it behaves as intended. Consistent with the actions of native Ang 1, we note Vasculotide-dependent increases in endothelial cell MMP2 secretion, migration, and resistance to serum withdrawal-induced apoptosis. However, *in vivo*, the activities of Vasculotide appear to resemble something of a hybrid between those of Ang 1 and 2. For instance, using Vasculotide in an *in vivo* Matrigel plug assay we show a potent induction of

neovascularization, an action which requires initial vessel destabilization and more closely resembles the reported actions of Ang 2. While in the same assay we note that plugs containing Vasculotide are well supported by periendothelial cells, an event previously ascribed to Ang 1-dependent induction of PDGF (Lindahl et al., 1998). In this sense our results are consistent with those of Koh GY *et al.*, who report that use of their chimeric form of Ang 1, COMP Ang 1, both promotes neovascularization of the cornea and recruits perivascular support cells (Cho *et al.*, 2004). One possible explanation for these results derives back to the unique structural characteristics of native Ang 1 and 2. In the case of Kok GY *et al.*, synthesis and purification of COMP Ang 1 gave rise to a heterogeneous mixture of multimers, with pentamers comprising the most abundant species. The synthesis of Vasculotide results in an obligate tetrameric structure. However, no formal studies have been conducted to assess the stoichiometric binding of Vasculotide to Tie 2. As such it remains unknown whether one molecule of Vasculotide is capable of binding four Tie 2 monomers. Factors that contribute to functional tetramerization of Tie 2 by Vasculotide are theorized to involve the angle of T7 peptide presentation as determined by avidin structure, the flexibility and length of the PEG molecular spacer, and the ratio Vasculotide to free Tie 2 receptor available for binding. Given these considerations, it is possible that Vasculotide may be capable of engaging the Tie 2 receptor monomerically, dimerically, trimerically, or tetramerically. Data presented (chapter 2) suggests that Vasculotide may mimic the effects of Ang 1 and 2, and as such, may predominantly engage the Tie 2 receptor as a dimer and/or a tetramer.

Because one of the major underlying defects noted in diabetic foot ulcers is microangiopathy the potential role that Ang 1 and Ang 2 play in this condition was recently examined (Kampfer *et al.*, 2001). Kampfer H., *et al.*, find that while Ang 2 levels are constitutively elevated in the db/db diabetic mouse, there is further induction of Ang 2 and VEGF expression upon wounding and in the early phases of wound healing. They propose that Ang 2 and VEGF collaborate to promote vessel destabilization and neovessel formation. However, they report that the increase in VEGF is transient and in the absence of elevated VEGF it is thought that persistently high levels of Ang 2 contribute to further vessel destabilization and regression. Consistent with these findings Holash J., *et al.*, and Stratmann A., *et al.*, find that when VEGF is expressed in the presence of Ang-2 in tumor growth and metastasis, vessels respond with angiogenic actions like sprouting and ingrowth, but in the absence of VEGF, Ang 2 destabilizes vessels and is found to induce vessel regression (Stratmann et al., 1998;Holash et al., 1999). Given these findings, therapeutic augmentation of Ang 1 or treatment methodologies which include application of an Ang 1 mimetic in diabetic wound healing may be beneficial in re-establishing a balance in the Tie 2 signaling axis.

A dynamic interplay between inflammation and angiogenesis has been shown to exist, however the precise mechanisms that regulate this relationship are poorly understood. Unabated, angiogenesis and inflammation contribute to the genesis of pathological conditions such as arthritis, atherosclerosis, psoriasis and retinopathy. Mounting evidence suggests that Ang 1 and 2 play key roles in regulating the balance that exists between neovascularization/inflammation and vessel stabilization/anti-inflammation (Thurston *et*

al., 1999;Jones *et al.*, 2001a;Fiedler *et al.*, 2006). Use of Vasculotide in the treatment of full thickness dermal wounds performed in the context of db/db diabetic mice promoted a robust production of granulation tissue which included modest, but statistically significant increases in markers of inflammation such as mutifocal neutrophil aggregates, macrophage infiltration and mild edema. Vasculotide-dependent production of granulation tissue correlated with dramatic increases in neovascularization while significantly impairing re-epithelialization. Interestingly, despite reductions in re-epithelialization, Vasculotide treated wounds displayed overall decreases in exposed wound area, a likely consequence of strong wound contraction. If Vasculotide functions as an Ang 1 mimetic, the results of our db/db wound assays appear somewhat contrary to the well accepted paradigm that Ang 1 inhibits inflammation and promotes vessel stabilization. However, literature reports repeatedly highlight the context dependent effects of both Ang 1 and 2 (Lobov et al., 2002;Daly *et al.*, 2006). Wound healing is a dynamic process which requires a prompt re-establishment of hemostasis, neovascularization and inflammation. These activities differ greatly from the developmental context in which much of the Ang 1 research has been conducted. In the context of wound healing little is known about the specific effects of Ang 1. Cho CH., *et al.*, recently examined the effects of COMP Ang 1 in a full thickness cutaneous model of diabetic wound healing (Cho *et al.*, 2006). Consistent with our findings, the authors note early and dramatic increases in granulation tissue and neovascularization of treated mice by day 14. Subsequently, COMP Angl treated wounds displayed reductions in granulation tissue, thinning of the epidermis and persistence of increased vascular density through days 28-56 (Cho *et al.*, 2006). Our analysis focused on the early events of wound

healing such that we were able to assess vascular specific improvements, not later stage remodeling events. In doing so, our analysis precludes us from making statements about more long term effects of Vasculotide. Unfortunately, Cho CH., *et al.*, do not comment on inflammatory response at all, nor did they measure re-epithelialization until 14 days post wounding. Follow up studies of Vasculotide in db/db diabetic mouse wound healing assay are planned to measure late stage events such as length of time to total wound closure, resolution of inflammation, stabilization of underlying vessels, collagen deposition and scarring. Furthermore, although Vasculotide treatment promoted dramatic effects on the production of granulation tissue and neovascularization further studies aimed at optimizing dose and/or delivery method are likely to provide added benefit.

While establishing optimal dosing and delivery strategies for Vasculotide in the treatment of diabetic wounds is likely to improve its therapeutic index, many questions currently exist regarding the significant delay in epithelialization at 14 days of treatment. Re-epithelialization and neovascularization/granulation tissue formation are both integral to successful wound healing. Re-epithelialization is necessary to produce a physical barrier required to exclude bacteria and yeast, maintain homeostasis, prevent protein and fluid loss, while neovascularization/granulation tissue formation provides growth factors and nutrient exchange necessary for regeneration, tensile strength, and wound contraction. Chronic wounds, particularly of the foot, are subjected to flexional and frictional forces associated with walking and as such, in the absence of the sufficient tensile strength these wounds are prone to re-injury. Wound granulation, so named for the massive angiogenic invasion of capillaries which result in a granular appearance of tissue underlying a skin

wound, provides the overlying epithelial layer with nutrients and oxygen necessary for efficient wound closure. Events taking place within the granulation tissue consist of elaborate and interrelated processes of angiogenesis, inflammation and cell proliferation. Activated endothelium provide necessary cues in the form of increased expression of I-CAM, V-CAM, P-Selectin and E-Selectin, to facilitate extravasation of neutrophils and macrophages (Muller, 2003). These cells, central players in the inflammatory response, mitigate infection, debride the wound and release a complex mixture of growth factors. While recruitment of these cells to actively healing wounds is beneficial, knockdown and knockout studies have revealed that they may also impair certain aspects of wound healing (Simpson and Ross, 1972;Leibovich and Ross, 1975). For instance, overly robust recruitment of neutrophils results in a spilling of free radicals which is damaging to host tissue repair(Martin and Leibovich, 2005). Moreover, as long as wounds are kept sterile, mice that are given anti-neutrophil antibodies show increased rates of wound healing, suggesting that factors released by neutrophils are not necessary for wound healing, but rather, in excess may impair the process (Dovi et al., 2003). Further evidence for an inhibitory role of inflammatory cells in wound healing derives from studies of damaged oral mucosa. Wounding of the oral mucosa generally leads to dramatically reduced influx of neutrophils and macrophages and concomitantly lower levels of proinflammatory cytokines (Szpaderska et al., 2003). As these wounds are rapid to heal and they generally do so with little to no scarring, these studies suggest that some forms of inflammation retard re-epithelialization and promote fibrosis. In the case of Vasculotide, we note statistically significant increases in wound inflammation as characterized by mild edema and an influx of neutrophils, and macrophages. Consistent

with these findings we report increases in endothelial I-CAM and a coincidental increase in leukocytes associated with the luminal surface of capillaries. The significant delay in re-epithelialization within our studies is currently thought to correspond to an overly strong inflammatory response, likely mediated by the Vasculotide-dependent induction of neovascularization. Although Cho CH., *et al.*, do not specifically comment of re-epithelialization or inflammation in their studies examining COMP Ang 1, based on the dramatic increase in granulation tissue noted at 14 days, it is possible that a pronounced inflammatory response retarded epithelialization early in treatment but these effects were resolved by week 8. Follow up experiments to assess the properties of Vasculotide in long term wound healing are planned. If these experiments demonstrate protracted delays in epithelialization approaches that include real or artificial graft materials in conjunction with Vasculotide will be assessed. In addition to determining the most suitable approach to treating diabetic wounds with Vasculotide, incorporating grafting materials into our analysis could potentially provide a novel benefit to the use of grafts in general. For instance, tissue engineering has provided physicians with several novel skin graft materials such as Integra®, however, despite Integra's® widespread use in the treatment of burns, reconstructive surgery, and acute and chronic wounds, there remains several factors limiting its use. Because the graft is acellular and heals by cell infiltration, there is an obligate need for rapid vascularization to meet with continuously high nutrient requirements. Burn wounds may take up to 2-3 weeks to fully vascularize a graft, and as such, the growth of functional vasculature is often a rate limiting step in graft success (Stern et al., 1990;Heimbach et al., 2003). In an attempt to improve grafting success researchers have embedded fibroblasts and/or keratinocytes in the collagen matrix of skin

substitutes such as Integra® (Coulomb et al., 1998). Again, the absence of a vascular plexus was found to preclude the survival of cells contained within the collagen matrix and increase the chance of infection (Auger et al., 2004). Use of Vasculotide in conjunction with grafting materials may provide additional benefit by virtue of rapidly increasing neovascularization within the graft.

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