Modulation of Endothelial Signaling Molecule Expression by TGF-β

By

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A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Biomedical Sciences

Guelph, Ontario, Canada

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ABSTRACT

Modulation of endothelial signaling molecule expression by TGF-β

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Transforming growth factor-beta (TGF-β) is an important modulator of cancer angiogenesis. Several signaling pathways interact in the process of regulating sprouting angiogenesis. It has been established that the Notch-1/Dll4 signaling pathway affect many biological processes including angiogenic tip and stalk cell differentiation. Our laboratory previously observed that TGF-β1 produced by colorectal cancer cells down-regulates VEGFR2 in endothelial cells. Here I expand on those studies and propose that TGF-β, in combination with other factors, may modulate the phenotype of endothelial cells into tip or stalk cells and that endoglin might affect the availability of TGF-β. In bovine aortic endothelial cells (BAEC), a dose response experiments were conduct to evaluate the protein expression of Notch1, Dll4, endoglin and VEGFR2. Results suggests that endoglin modulates TGF-β1 levels and therefore interferes with its influence on the Notch1/Dll4 and VEGF/VEGFR2 signaling pathways, which play an important role in regulating tumor angiogenesis.
ACKNOWLEDGEMENTS

First, I would like to acknowledge my advisor, Dr. Brenda Coomber for her guidance, wisdom and support throughout the course of my Masters degree. Thank you for being an amazing mentor with great deal of patience and thank you also for always having the time to listen and offer me advice when I was in need of it.

I would also like to thank my advisory committee, Dr. Alicia Viloria-Petit and Dr. Tony Mutsaers for their helpful suggestions and assistance throughout this process. Thanks also go to Lizzie Kuczynski for providing the starting point for my project.

Moreover, I would like to acknowledge all of my past and present labmates, thank you for being great colleagues and fantastic friends and for providing a great work environment and also for being there for me when I was homesick. I would like to thank Leanne Delaney, Nathan Farias, Richard Gilbert, Nelson Ho, Jodi Morrison, Amy Richard, Dr. Kaya Skowronsiki, Dr. Jen Thompson, Sean Masson and Sonja Zours.

Finally, I cannot conclude this series of acknowledgements without mentioning my parents and family, I am a better person because of your love, understanding and unconditional emotional and financial support. I honestly could not have done this without you.
DECLARATION OF WORK PERFORMED

I declare that with the exception of the items indicated below, all work reported in this thesis was performed by me.

Elizabeth Anne Kuczynski collected some of the samples used for analysis in this thesis. Samples of normal epithelial cell of the rat ileum (IEC18) used for analysis was collected by Nelson Ho. The Matrigel cord formation image analysis was performed by the commercial company Wimasis Image Analysis (http://www.wimasis.com).
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<tr>
<td>ALK</td>
<td>Activin-like Kinase</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD105</td>
<td>Cluster of differentiation 105 (endoglin)</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal carcinoma</td>
</tr>
<tr>
<td>DAPI</td>
<td>Diamidinophenylindole</td>
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<tr>
<td>Dll4</td>
<td>Delta like ligand 4</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagles Medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TGFβR</td>
<td>Transforming growth factor beta receptor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
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INTRODUCTION

Cancer is the leading cause of death worldwide, accountable for 7.6 million fatalities in 2008 (http://www.who.int). Colorectal cancer is the second leading cause of cancer-related deaths in Canadians, and is the third most common cancer diagnosis overall (www.cancer.ca/statistics). The median overall survival in metastatic CRC is less than two years, even though there are now six drugs that have been approved by the USA Food and Drug Administration for the treatment of metastatic CRC\textsuperscript{1}. In 2000, Hanahan and Weinberg suggested a list of six hallmarks of cancer that virtually all cancers possessed \textsuperscript{2}, and one of these hallmarks is sustained angiogenesis. This in combination with previous studies led to the rapid exploration of tumor angiogenesis as a treatment target, since tumor angiogenesis provides the tumor with the oxygen and nutrients it needs in order to advance and survive.

Tumor angiogenesis consists of the formation of new blood vessels from the pre-existing vasculature surrounding the tumor. In the course of this process, endothelial tip cells lead the newly formed blood vessel towards the tumor in response to signals from the tumor. One of these signaling molecules is vascular endothelial growth factor A (VEGF-A), the signaling of which stimulates delta-like ligand4 (Dll4) expression in endothelial tip cells. Subsequently, Dll4 activates Notch signaling in endothelial stalk cells, which down regulates stalk-cell sensitivity to VEGF stimulation, and reduces VEGFR-2 and VEGFR-3 expression. This consequently suppresses the endothelial tip-cell phenotype\textsuperscript{3,4}. 
Our laboratory has previously investigated transforming growth factor beta (TGF-β) effects on VEGFR-2 heterogeneity in colorectal tumor angiogenesis and found that, in vitro, TGF-β derived from colorectal cancer cells (CRC) down-regulates VEGFR-2 expression in endothelial cells\(^5\). Therefore, my thesis builds on that result and focuses on studying the influence that TGF-β might have, in combination with other factors; on the reprogramming of the phenotype of endothelial cells into tip and stalk cells.
LITERATURE REVIEW

Angiogenesis

Angiogenesis is the neo-formation of blood vessels from pre-existing vessels. This can be attained either through endothelial cell sprouting or intussusceptive micro-vascular growth\textsuperscript{6,7}. Several \textit{in vivo} and \textit{in vitro} models of angiogenesis have been developed to allow the study of selected aspects of the angiogenic process, such as endothelial migration, proliferation and capillary tube formation\textsuperscript{8}.

Folkman and Haudenschild were the first to place human endothelial cells on dishes coated with a thin layer of extracellular matrix protein, which then organized into tube-like structures\textsuperscript{9}. In vitro assays were further developed using a three-dimensional collagen gel, which demonstrated that when a monolayer of endothelial cells is placed on top of a collagen gel and then is covered with a second layer of collagen, the cells regroup into a network of branching and anastomosing tubules\textsuperscript{10}. In 1991 Grant et al. demonstrated that these capillary-like networks had lumens and that the endothelial cells came to possess membrane changes similar to those observed in vessels \textit{in vivo}\textsuperscript{11}. The progress of these and more \textit{in vivo} and \textit{in vitro} models of angiogenesis contributed to the great advancements observed in angiogenesis studies.

Sprouting Angiogenesis

Activation of vascular sprouting entails the specification of endothelial cells into tip and stalk cells. Endothelial tip cells are mainly migratory and polarized with minimal proliferation; in contrast endothelial stalk cells proliferate throughout
sprout establishment and form the nascent vascular lumen cells\textsuperscript{12}. The specification of endothelial cells as tip or stalk cells is very transient and its reversibility is contingent on the balance between pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) and Jagged-1 (JAG-1), and suppressors of endothelial cell proliferation, such as delta-like ligand 4 (Dll4)-Notch1 signaling pathways\textsuperscript{13-15}. Tip cells express high levels of Dll4, platelet derived growth factor-B (PDGF-B), and VEGF receptor-2 (VEGFR-2), and have low levels of Notch signaling activity\textsuperscript{12,16,17}.

\textbf{Sprouting Angiogenesis in Tumors}

Cancerous growths use a complex process that involves interaction between tumor cells, endothelial cells, phagocytes and their secreted factors to either initiate or inhibit angiogenesis\textsuperscript{18}. The first account of sprouting angiogenesis in tumors was portrayed to have four stages\textsuperscript{6}. This first step involved degradation of the basement membrane on the side of the dilated peritumoral post-capillary venule situated closest to the angiogenic stimulus. Following which endothelial cells migrate into the connective tissue as a result of the weakening of the inter-endothelial contacts, leading in turn to the formation of a solid cord of endothelial cells. Finally, proximal to the migrating front the lumen forms, and functional capillary loops form as a result of the anastomoses of adjoining tubular sprouts. Parallel to the formation of the capillary loops, the new basement membrane is synthesized and pericytes are recruited\textsuperscript{6}.
Role of VEGF and VEGFRs in sprouting angiogenesis

The VEGF family contains six known members referred to as: VEGF-A (VEGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E, and placenta growth factor (PIGF), as well as the three receptors VEGFR1, VEGFR2 and VEGFR3\(^{19}\). VEGF-C and VEGF-D play a key role in lymphangiogenesis where they have high binding affinity to VEGFR3 and low affinity to VEGFR2. VEGF-B binds to VEGFR1 while VEGF binds to both VEGFR1 and VEGFR2. The signaling of VEGF through VEGFR2 accounts for the majority of the angiogenic stimulatory signal necessary for the initiation of sprouting angiogenesis\(^{19}\).

In sprouting angiogenesis the migration of tip cells depends on a gradient of VEGF while the concentration of VEGF regulates stalk cell proliferation\(^{12}\). In response to the VEGF gradient the leading tip cell migrates outward from the pre-existing blood vessel; this VEGF gradient also induces the formation of filopodia in addition to the expression of delta like ligand-4 (Dll4) protein in tip cells\(^{20}\). This increased expression of Dll4 in tip cells leads to the binding of Dll4 to its receptor Notch1 on the neighboring stalk cells, which results in the increased expression of Notch1 in stalk cells\(^{20}\). This increased expression of Notch1 results in the down regulation of VEGFR2 and VEGFR3, which helps maintain the stalk cell phenotype. Next, filopodia merge with those of a nearby tip cell, which leads to the formation of a bridge and the subsequent formation of a new blood vessel\(^{20}\). In turn the increased concentration of VEGF leads to the elongation of the vessel and ensuing formation of a lumen as well as the synthesis of a basement membrane increasing the mass and
surface of the newly formed blood vessel \textsuperscript{20}.

**Notch Signaling Pathway**

The Notch receptor family consist of four type I transmembrane receptors (Notch1, Notch2, Notch3, Notch4), and the Notch Ligand family is comprised of five ligands (Jagged1, Jagged2, Delta-Like 1 (Dll1), Dll3 and Dll4). The mature Notch receptor, which is comprised of two subunits, is formed as a result of cleavage by furin-like convertase of the precursor form of Notch. The first subunit consists of a major share of the Notch extracellular domain (NECD); the second subunit is comprised of the reminder of the NECD, the transmembrane domain and intracellular domain (ICD). Notch signaling is mediated by Notch intracellular domain (NICD), which is the active form of the protein\textsuperscript{21}.

Notch signaling is mediated by cell-to-cell contact, and following the binding of a Notch receptor to its ligand on a neighboring cell, the Notch receptor undergoes conformational changes exposing the S2 cleavage site in the NECD. The resulting S2 cleavage, by the metalloproteinase tumor necrosis factor-\(\alpha\)-converting enzyme (TACE), is followed by the S3 cleavage of the NICD under the influence of the presenilin–\(\gamma\)-secretase complex. This S3 cleavage results in the release of the active NICD and its subsequent translocation into the nucleus. Once the active NICD is in the nucleus it initiates a transcriptional cascade that mediates both the activation and the repression of target genes\textsuperscript{21}.

**Role of Notch pathway in tumor angiogenesis**

The importance of the Notch pathway in angiogenesis is emphasized by the
strong expression of Notch receptors and its ligands in the vasculature. During physiological angiogenesis, vascular endothelial growth factor (VEGF) increases the number of Dll4 expressing endothelial tip cells in pre-existing blood vessels. Endothelial tip cells are non proliferative, however the adjacent stalk cells are proliferative and express Notch and their proliferation leads to the formation of the lumen of the recently formed blood vessel. The Dll4-Notch signaling from tip to stalk cell results in the down regulation of VEGFR2 expression and this subsequently leads to decreased VEGF-induced sprouting\textsuperscript{3,17,22}.

Cancer cell secretion of VEGF, in a hypoxic environment, results in the increased expression of Dll4 by endothelial cells in the stroma. Therefore, inhibiting VEGF expression in these tumors would lead to the decreased expression of Dll4 in the surrounding endothelium\textsuperscript{23,24}. This connection between the expression of VEGF and Dll4 in tumor angiogenesis led to a study that assessed the effect of blocking Dll4-Notch signaling on tumor angiogenesis. It was found that inhibiting Dll4 mediated Notch signaling lead to reduction in tumor growth, however this inhibition was also associated with an increase in the number of newly formed vessels\textsuperscript{25}.

An essential step in the formation of new blood vessels is endothelial cell migration; it was demonstrated that the interaction between TGF-β and bone morphogenetic protein (BMP) pathways with the Notch signaling pathway result in modifications in endothelial cell migration\textsuperscript{26}. Furthermore, other studies found that some BMP family members can induce the expression of the hairy-related
transcription factor 1 (HEY1) synergistically with Notch. Subsequently HEY1 negatively regulated the activity of a promoter of endothelial cell migration ID1, which lead in turn to the inhibition of endothelial cell migration, and functions downstream of the Notch and BMP pathways.

**Transforming Growth Factor-beta (TGF-β) Signaling:**

The transforming growth factor-beta (TGF-β) superfamily of secreted polypeptides is involved in many cellular processes such as proliferation, migration, invasion, epithelial–mesenchymal transition (EMT) and growth inhibition. Nevertheless, in pathological conditions, such as cancer, fibrosis and inflammation, the TGF-β family members are found to be over expressed and therefore possibly drive disease progression. There are more than 30 ligands that belong to the TGF-β superfamily including: TGF-βs, bone morphogenic proteins (BMPs), growth and differentiation factors (GDFs), activins, inhibins, leftys, Nodal and Anti-Müllerian hormone (AMH). The TGF-β family of receptors consist of seven members of the type I receptor, five members of the type II receptor and two type III receptors.

Depending on the type of human tissue, the three homologous isoforms of TGF-β (TGF-β1, TGF-β2 and TGF-β3) are expressed in varying levels, however they share a receptor complex and signal in similar ways. In the majority of cell types the three TGF-β isoforms signal through a SMAD-dependent pathway where the type I receptor activin receptor-like kinase 5 (ALK5) becomes activated and forms a complex with TGF-βRII. The formation of this complex leads in turn to the
phosphorylation of receptor specific SMAD (R-SMAD). These phosphorylated R-SMADs then form a heterodimeric complex with the common mediator SMAD4 and together they translocate to the nucleus. Upon translocation to the nucleus this heterodimeric complex interacts with other transcriptional factors, which result in the regulation of transcriptional responses\textsuperscript{29}.

TGF-\(\beta\) has a biphasic role in tumor progression, which depends on the tumor type and the stage of tumor progression. Moreover, up to 85\% of colorectal carcinoma cell lines are resistant to the growth inhibitory effect of TGF-\(\beta\)\textsuperscript{31,32}. Furthermore, TGF-\(\beta\) levels are highly detectable in serum and plasma samples collected from patients with colorectal carcinoma and these high TGF-\(\beta\) levels are associated with poor prognosis\textsuperscript{32}.

TGF-\(\beta\) in Angiogenesis

The formation of angiogenic sprouts is regulated in endothelial cells by their expression and signaling through an ALK1- TGF-\(\beta\)RII complex. The interactions between the ALK5- TGF-\(\beta\)RII and the ALK1- TGF-\(\beta\)RII signaling pathways are thought to regulate angiogenesis\textsuperscript{33,34}. TGF-\(\beta\) exerts a bi-functional influence on endothelial cells where low doses of TGF-\(\beta\) were found to have a stimulating effect on the proliferation and migration of endothelial cells, while high doses have the opposite inhibitory effect on these functions. The regulatory effect of the balance between these two pathways is apparent in the inhibitory effect that the ALK5/TGF-\(\beta\) pathway has on the migration and proliferation of endothelial cells, while the ALK1/TGF-\(\beta\) pathway prompts endothelial cell migration and proliferation\textsuperscript{34}. 
Several studies were performed to examine the effect of both pathways on endothelial cells. The inhibition of the ALK5/TGF-β pathway was examined in human embryonic stem cells (hESC) derived endothelial cells, where an ALK5 kinase inhibitor inhibited the pathway and resulted in sustained proliferation of the cells by sustaining Id1 (DNA-binding protein inhibitor) expression\textsuperscript{35}. A similar result was shown in a study done with mouse embryonic stem cells (mESC), where the addition of an ALK5 kinase inhibitor increased endothelial cell growth and integrity via upregulation of the tight junction component claudin-5\textsuperscript{36}.

**TGF-β co-receptors in angiogenesis:**

The human type III TGF-β co-receptors endoglin and betaglycan are type I integral membrane proteins\textsuperscript{37,38}. Both endoglin and betaglycan are generally expressed as homodimeric glycoproteins, however a minor subset of heterodimeric complexes of the two co-receptors has been detected in endothelial cells\textsuperscript{39}. Tβ-RIII (betaglycan) is universally expressed on nearly all cell types and is the most highly expressed of the TGF-β superfamily receptors\textsuperscript{39}. However, the expression of betaglycan in some cell types, specifically vascular endothelial cells, appears to be weak or absent, and instead they express the related TGF-β co-receptor, endoglin\textsuperscript{39}. Both endoglin and betaglycan are generally expressed on the cell surface as homodimers, with endoglin homodimers being linked by disulfide bridges; however, it was found that endoglin and betaglycan are capable of forming heterodimeric complexes in microvascular endothelial cells\textsuperscript{39}.
In addition to being found in a membrane-bound form, both type III co-receptors can be found as a soluble form. Endoglin has two splice isoforms termed long endoglin (L-End) and short endoglin (S-End) (Figure 1). L-End is the mostly expressed in endothelial cells while S-End is found to be significantly expressed in liver and lung tissue. Betaglycan shedding is mediated in part by matrix metalloproteinase 14 (MMP 14) and plasmin, while soluble endoglin is produced by cleavage of the membrane-bound endoglin at close proximity to the transmembrane domain by only matrix metalloproteinase 14 (MMP 14).

Endoglin expression is potently stimulated by hypoxia, BMP-9, and constitutively active ALK1, while TNFα exerts an inhibitory effect on endoglin expression in endothelial cells. Furthermore, TGF-β exhibits a potent inhibitory effect on cell proliferation, however, endoglin offsets this inhibitory effect in endothelial cells, which is demonstrated by the upregulation of endoglin expression in the proliferating endothelium of tissue undergoing angiogenesis.

Both betaglycan and endoglin cytoplasmic domains can be phosphorylated by serine/theronine kinases. The phosphorylation of endoglin has been shown to influence its subcellular localization, probably by modulating endoglin’s interaction with adhesive proteins, such as zyxin and ZRP-1, and in doing so modifying the adhesive properties of endoglin expressing cells.

It’s unknown how endoglin regulates TGF-β dependent responses, although a possible mechanism would depend on endoglin’s effect on the phosphorylation of the other two TGF-β receptors. TGFβ-R1 is activated and subsequently phosphorylated by the constitutively active TβRII in response to ligand binding to
TβRII and ligand. However, endoglin association with Tβ-RII results in the altering of Tβ-RII phosphorylation status and ensuing loss of ALK5 from the TGF-β receptor complex, which could explain endoglin’s inhibitory effect on ALK5 signaling\textsuperscript{49}. Furthermore, studies conducted on human umbilical vein endothelial cells proposed that ALK1-dependent inhibition of cell adhesion is counteracted by endoglin phosphorylation\textsuperscript{49,50}. These results suggest that endoglin’s interaction with TGF-β signaling receptors through both its extracellular and cytoplasmic domains might affect cell responses to TGF-β.

**Regulation of TGF-β ligand access to co-receptors**

Betaglycan binds multiple members of the TGF-β family, including TGF-β1, TGF-β2, TGF-β3, Activin-A, BMP-2, BMP-4, and BMP-7\textsuperscript{51-53}. Betaglycan also plays a role in presenting the ligand to TβRII, leading to either enhancing or inhibiting signaling while interacting with the TβRII\textsuperscript{53}. Unlike betaglycan, endoglin binds TGF-β1 and TGF-β3 but not TGF-β2\textsuperscript{54}. Other ligands that endoglin can bind include activins and BMPs and endoglin also can interact with activin type II receptors\textsuperscript{55}. Therefore, both functional differences and similarities that can be found between betaglycan and endoglin can be credited to the differences between these two proteins ligand-binding profiles.

In the case of the type III co-receptor betaglycan, TGF-β binds to the N-terminal region of betaglycan and the residual carboxy-terminal half of the protein is necessary for protein anchoring to the cell membrane\textsuperscript{56}. Moreover, betaglycan carries out its function as a co-receptor to specific members of the TGF-
β superfamily through its ectodomain that consist of two validated and independent ligand-binding domains\textsuperscript{56}. In a comparative study between endoglin and betaglycan intracellular responses to TGF-β signaling, it was found that there is a distinctive role to the extracellular domain of the two co-receptors\textsuperscript{57}. In case of exchanging the extracellular domain between the two co-receptors there was no effect on endoglin ligand binding potential, however, unlike with betaglycan, TβRII presence is essential for endoglin binding of TGF-β1, activin-A, BMP-2, or BMP-7\textsuperscript{55,57}. In another study, the inhibitory effect of the soluble form of endoglin was shown in the reduced binding of TGF-β1. By interfering with its contact with TβRII, it was also demonstrated that soluble endoglin has an adverse effect on TGF-β1 signaling in endothelial cells\textsuperscript{58}.

TGF-β can signal in endothelial cells either through ALK1 or ALK5, both of which are type I receptor pathways\textsuperscript{59}. In the case of TGF-β signaling through the TGF-β/ALK1 pathway, this signaling results in the stimulation of endothelial cell proliferation and migration, whereas in the case of TGF-β signaling through the TGF-β/ALK5 pathway it results in inhibiting these responses\textsuperscript{59}. It has also been observed that forced expression of endoglin led to the inhibition of TGF-β/ALK5 signaling and, as a consequence, inhibition of the TGF-β induced growth inhibitory effect on endothelial cells\textsuperscript{57,60,61}.

Endoglin can also function independently from the TGF-β family, as shown in endothelial cells undergoing hypoxic stress, where endoglin signaling in the absence of TGF-β inhibits apoptosis of endothelial cells\textsuperscript{62}. Endoglin also may be able to
control cell migration independent of TGF-β signaling through its interaction with focal adhesion proteins that contain zyxin and ZRP-1 domain. Additionally, in endothelial cells endoglin cytoplasmic tail interaction with β-arrestin2 leads to endoglin inducing an inhibitory effect on TGF-β induced ERK activation and migration in endothelial cells.

During embryogenesis, inflammation, and wound healing, modifications in vascular structure occur and endoglin expression has been shown to be elevated during these modifications. The importance of endoglin function in maintaining normal vascular structure is underlined by the relationship between mutations in the endoglin gene and hereditary hemorrhagic telangiectasia (HHT), which is a disorder characterized by the formation of small dilated blood vessels and arteriovenous malformations (AVMs) in the vasculature of lung, liver, and brain. Studies done to elucidate the role that endoglin plays in the enhancement of the TGF-β/ALK1 signaling pathway suggest that endothelial cell responses to TGF-β are critically dependent on endoglin functional association with ALK1. The results from these studies concur with what is seen in cases of HHT where there is a mutation of either human endoglin (ENG) or ALK1 (ACVRL1) genes.
Figure 1: Different forms of endoglin and the generation of soluble endoglin. **A.** Endoglin is a dimeric protein and a disulphide bridge connects monomers. Some of the sub domains that have been identified so far include: orphan domains, TGF-β/BMP binding domains, and receptor interacting domains. There are two forms of cell surface bound endoglin: long endoglin (L-End) and the short form (S-End) ⁴⁰,⁴¹. **B.** Soluble form of endoglin (Sol-End) can be found in the circulation by shedding of membrane bound endoglin possibly via matrix metalloproteinase 14 (MMP14) activities.
RATIONALE

Tumor angiogenesis is initiated and regulated by several signaling pathways. This formation of new blood vessels from pre-existing mature vasculature is called sprouting angiogenesis. TGF-β plays an important role in regulating tumor-sprouting angiogenesis. TGF-β exerts a biphasic influence on endothelial cells where low doses of TGF-β were found to have a stimulating effect on the proliferation and migration of endothelial cells, while high doses have the opposite inhibitory effect on these functions\textsuperscript{34,70}.

VEGF (or VEGF-A) is one of the molecules that regulates the initiation of angiogenic sprouting by signaling through its receptor VEGFR2 on endothelial cells. It was found previously in our laboratory that TGF-β derived from colorectal carcinoma (CRC) cells down-regulates VEGFR2 expression in endothelial cells in vitro. Also, it was suggested in several studies that soluble molecules present in the circulation, such as soluble endoglin, might affect TGF-β influences on sprouting angiogenesis. Therefore, I hypothesized that TGF-β, in combination with other factors, may influence the phenotype of endothelial cells into tip and stalk cells. Moreover, changes in endoglin levels may affect TGF-β availability and therefore alter its possible effect on endothelial cells (Figure 2). Consequently, in order to investigate these hypotheses three objectives were undertaken:

**Objective 1:**

Evaluate the expression of endothelial signaling molecules associated with tip and stalk cells when they are exposed to TGF-β.

**Objective 2:**
Quantifying the effect of TGF-β on *in vitro* angiogenesis using a Matrigel cord formation assay

**Objective 3:**

Determine the relationship between endoglin levels and the availability of TGF-β and how this may affect its role in altering endothelial signaling molecule expression and cord formation *in vitro*.

By expanding our understanding of the interactions between TGF-β and other endothelial signaling pathways we gain a better insight into tumor angiogenesis. This knowledge would also be vital in varying vascular diseases, such as diabetic retinopathy, where sprouting angiogenesis would lead to deteriorating of the condition.
Figure 2: Signaling pathways in sprouting angiogenesis. VEGF signaling stimulates Dll4 expression in tip cells, and Dll4 activates Notch signaling in stalk cells, which down regulates stalk-cell sensitivity to VEGF stimulation, and reduces VEGFR2/3 expression, consequently suppressing the tip-cell phenotype. We speculate on soluble endoglin's interference with the possible role of TGF-β's effect on endothelial tip and stalk cells.
MATERIALS AND METHODS

A list of suppliers for chemicals and recipes for solutions preparation are found in Appendices I and II, respectively.

**Cell lines and culture conditions:**

Primary bovine aortic endothelial cells (BAEC) were previously isolated in our laboratory from the aorta of cattle. The BAECs used in the present studies have been previously characterized for TβRII expression and dose-dependent response to TGF-β1.5 Several human colorectal cell lines were obtained from the American Type Culture Collection (ATCC) that include: SW480, LS174T, CaCo2, HCT116 and DLD1, all derived from adenocarcinomas of the colon 71-73. Another cell line included in this study was the normal epithelial cell of the rat ileum (IEC18), also from ATCC 74.

All cultures were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1 mM sodium pyruvate (Sigma-Aldrich) and 0.25 mg/ml gentamicin (Invitrogen) at 37°C in 5% CO2 and 95% atmospheric air. Medium was changed every three to four days and cells were passaged when confluency reached 80% or above. Once cells reached confluency, they were washed with sterile PBS (Sigma-Aldrich), detached using 3 ml of trypsin for a tissue culture dish with a diameter of 10 cm, and centrifuged at 350 X g for 4 minutes. The media supernatant was aspirated and cells were re-suspended in fresh 10% FBS DMEM and plated at a split ratio of 1:5. For experiments, approximately 24 hours prior to treatment, confluent monolayers were washed with 3 ml of sterile PBS and media was replaced with DMEM with no added FBS; referred to as serum free-media.
Protein Isolation and Quantification

Following treatments, in order to collect total cellular proteins, cells were lysed on ice with cell lysis buffer (20 mM Tris- HCL, 150 mM NaCl, 1 mM Na$_2$EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 mM Na$_3$VO$_4$, 1 $\mu$g/ml leupeptin) (Cell Signaling) supplemented with 1% phosphatase inhibitor cocktail 2 (Sigma-Aldrich), 2 $\mu$g/ml aprotinin (Sigma-Aldrich) and 1 mM PMSF (Sigma-Aldrich). Working on ice, 150 $\mu$l of lysis buffer was added per 10 cm plate and left on ice for approximately 5 minutes and lysed cells were next detached using cell scrapers and cell lysate was then transferred to 1.5 ml microcentrifuge tubes, then centrifuged for 15 minutes at 15000 X g. Supernatant containing total cellular protein was then transferred to a new microcentrifuge tube, aliquoted and stored at -80°C until further use.

Bio-Rad protein quantification system was used for protein quantification. To quantify samples, an aliquot of 5 $\mu$l of each sample was loaded in duplicate into a 96-well plate and combined with 25 $\mu$l of 1 in 50 dilution of Reagent S with Reagent A (Bio-Rad). To this, 200 $\mu$l of Reagent B was added (Bio-Rad) and samples were incubated at room temperature for 10 minutes. Known concentrations of BSA were then used to generate a standard curve from which sample concentrations were interpolated. Absorbance was then read at 630 nm using an ELx800 Universal Microplate Reader (BIO-TEK Instruments Inc).

Western Blotting
Samples were prepared by combining 20-30 µg of total cellular lysate with Milli-Q water and 6.8 µl of 8X loading buffer containing SDS and β-mercaptoethanol. Samples were then heated at 95°C for 5 minutes to allow for denaturation of proteins. Following this, samples were briefly vortexed and lysate was loaded onto 10% SDS-polyacrylamide gel (Bio-Rad); 5 µL of PageRuler Plus Prestained Protein Ladder (Bio-Rad) was also incorporated to allow for protein size determination. Then, cell lysates were loaded onto 10% acrylamide gels and run at 120V using the BioRad Mini Protean system until the dye front reached the bottom of the gel. Next, gels were incubated in transfer buffer for 10 minutes preceding the transfer to a PVDF membrane at 100V for 2 hours using a wet transfer system (Bio-Rad). Membranes were then blocked in 5% skim milk in Tris Buffer Saline containing 1% Tween 20 (TBST) under constant agitation overnight at 4°C.

Membranes were incubated in primary antibody for 2 hours at room temperature. The concentrations of antibodies used were: 1:800 rabbit monoclonal anti-VEGFR2, 1: 1000 rabbit polyclonal anti-Notch1, 1:1000 rabbit monoclonal anti-Dll4 (all Cell Signaling Technology), 1:500 rabbit monoclonal anti-endoglin (ABBIOTEC) and 1:200,000 mouse monoclonal anti-α-tublin (Sigma-Aldrich).

Next, membranes were washed 3 times for 10 minutes each with TBST and incubated with a secondary antibody of either 1:20,000 goat anti-mouse HRP or 1:2000-1:5000 goat anti-rabbit HRP (both Sigma-Aldrich), diluted in 5% skim milk in TBST, for 30 minutes at room temperature. The secondary antibody was then discarded and membranes were washed 4 times for 15 minutes each in TBST under
constant agitation. Immunoglobulin-antigen complexes were visualized by incubating the PVDF membranes in either Luminata Classico or Luminata Forte Western HRP Substrate (Milipore) for 2 minutes and then membranes were imaged using the ChemiDoc MP Imaging System (Bio- Rad). In order to probe for a second protein of interest some of the membranes were washed in TBST for 5 minutes following imaging, and a stripping solution of 1:10 in distilled water from the stock stripping solution (Reblot Plus; Millipore) was added. Membranes were incubated for 10 minutes in the 1X stripping solution and then washed in TBST. Membranes were then blocked and probed again for the second protein of interest. Protein loading was normalized by densitometry using bands for α-tubulin.

**BAEC Dose-Response Experiments**

TGF-β1 dose-response experiments were conducted by treating serum starved BAEC with fresh serum free DMEM supplemented with 0 (vehicle), 0.1, 1, 5, 10 and 20 ng/ml of recombinant human (rh) TGF-β1 (R&D Systems) over 24 hours. The endoglin dose-response experiments were conducted by treating the BAECs with recombinant human (rh) endoglin (0.1, 0.2, 0.5 and 1 µg/ml; R&D Systems) with and without 5 ng/ml of rh-TGF-β1 over 24 hours. Following this, cell supernatant was collected and transferred to a 15 ml conical tube and centrifuged at 350 X g for 4 minutes. These samples were then aliquoted and stored at -80°C and used later in western blotting experiments.
CRC Characterization

Five CRC cell lines HCT116, SW480, LS174T, CaCo2 and DLD1 were grown in 10 cm tissue culture dishes (Sarstedt) until they reached 80% confluency. Cell monolayers were then washed with PBS and incubated in serum free media for 48 hours. Following this, cell supernatant was collected and transferred to a 15 ml conical tube and centrifuged at 350 X g for 4 minutes; conditioned media supernatants were collected and used immediately or stored at -80°C. Cell lysate was collected from the five CRC cell line plates as described above and both cell lysate and conditioned media samples were used later in western blotting experiments.

Cord Formation Assay

Dose-response experiments for both rh-TGF-β1 and rh-endoglin were performed where BAECs were plated in each well of a 24 well plate coated with growth factor-reduced Matrigel (BD Biosciences). Matrigel was thawed overnight at 4°C and was diluted to 25% in cold sterile and serum free DMEM. Working in sterile conditions on ice, 200 µl of diluted Matrigel was added per well and then left in the incubator for 30 minutes. When Matrigel coating was stable 50,000 BAECs were plated in 200 µl per well. Plates were then left in the incubator for 18 hours, following which several phase contrast photographs at 20X magnification were taken from each well. Pictures were then analyzed using the tube formation assay Wimtube (http://www.wimasis.com). This assay was used to quantitatively evaluate the generation of cord networks, and the analyzed parameters were: Total
Branching points, Tube lengths and Total Loop numbers.

**Statistical Analysis**

All statistical analysis and graphing were performed using GraphPad Prism 6 software (GraphPad Software). Means for three biological replicates were calculated and plotted with standard error bars. Data were analyzed by using the nonparametric Kruskal-Wallis test and significant differences between means were determined using the Dunn’s Multiple Comparison post-test and were considered statistically significant when the p value was less than 0.05.
RESULTS

TGFβ dose-effect on the expression of NOTCH1, Dll4 and CD105 in BAECs

Experiments were performed in order to examine the effect of varying exogenous recombinant human TGF-β1 doses on the expression of Notch1, Dll4 and endoglin (CD105) in serum starved BAECs. In this experiment BAECs were treated with 0 (vehicle), 0.1, 1, 5, 10 and 20 ng/ml of recombinant human rh-TGF-β1 and these treatments were applied for 48 hours. Three biological replicates were obtained to produce the results described in this section and a p-value of <0.05 was considered to be statistically significant. Both untreated BAECs and human umbilical vein endothelial cells (HUVEC) were used as a positive control on the western blots of Notch1 and Dll4, and only untreated BAECs were used as a positive control for the CD105 western blots.

Notch1 expression in BAEC was high in response to the lower doses of TGF-β treatments 0.1 and 1 ng/ml; it was also high in two of the higher doses of TGF-β treatments 10 and 20 ng/ml (Figure 3). The lowest expression of Notch1 was observed in the BAEC treated with 5 ng/ml TGF-β. Kruskal-Wallis test was performed on the densitometric analysis data collected from analyzed western blots of Notch1 expression and the results were statistically significant (P<0.0172). Next, Dunn’s test for multiple comparisons was conducted and the BAEC treated with the 5 ng/ml dose was significant in comparison to the control treatment.
Dll4 expression in BAEC was high in response to the lowest dose of TGF-β treatments (0.1 ng/ml) as well as in the two high doses of TGF-β treatments (10 and 20 ng/ml). The lowest expression of Dll4 was detected in the BAEC treated with 1 and 5 ng/ml doses of TGF-β. Kruskal-Wallis test was performed on the densitometric analysis data collected from analyzed western blots of the Dll4 expression and the results were statistically significant ($P<0.0276$). Next, Dunn’s test for multiple comparisons was conducted and the BAEC treated with the 1 and 5 ng/ml doses were significant in comparison to the control treatment (Figure 4).

Endoglin expression was higher in the BAEC treated with the low dose of TGF-β treatment (0.1 ng/ml), and then was slightly reduced in the BAEC treated with 1, 5 and 10 ng/ml doses of TGF-β, and slightly increased again in the BAEC treated with 20 ng/ml of TGF-β. Kruskal-Wallis test was performed on the densitometric analysis data collected from analyzed western blots of endoglin expression and the results were statistically significant ($P<0.0222$). Next, Dunn’s test for multiple comparisons was conducted and the BAEC treated with the 10 ng/ml dose was significant in comparison to the control treatment (Figure 5).
Figure 3: Changes in Notch1 expression following 48 hours of TGF-β1 treatment as indicated in serum starved BAECs. A. Representative western blot of BAEC protein lysates. B. Relative expression of Notch1 normalized to α-tubulin (n=3). Kruskal-Wallis test for treatments was significant (* P< 0.0172); Dunn’s test has a significance level of 0.05 and showed that levels of Notch 1 in 5 ng/ml treated cells were significantly lower than control.
Figure 4: Changes in Dll4 expression following 48 hours of TGF-β1 treatment as indicated in serum starved BAECs. A. Representative western blot of BAEC protein lysates. The tubulin blot is the same as for Figure 3 because this Dll4 blot was probed from the same transfer. B. Relative expression of Dll4 normalized to α-tubulin (n=3). Kruskal-Wallis test for treatments was significant (* P< 0.0276). Dunn’s test has a significance level of 0.05 and showed that levels of Dll4 in 1 and 5 ng/ml treated cells were significantly lower than control.
Figure 5: Changes in CD105 (endoglin) expression following 48 hours of TGF-β1 treatment as indicated in serum starved BAECs. A. Representative western blot of BAEC protein lysates. B. Relative expression of CD105 normalized to α-tubulin (n=3). Kruskal-Wallis test for treatments was significant (* P< 0.0222). Dunn's test has a significance level of 0.05 and showed that levels of CD105 in 10 ng/ml treated cells were significantly lower than control.
Expression of VEGFR2 and Dll4 in BAECs treated with CD105 plus and minus TGF-β1

The expression of VEGFR2 and Dll4 was examined in serum starved BAECs by performing a dose response experiment in which BAECs were incubated with varying concentrations of exogenous rh-endoglin in the absence as well as in the presence of rh-TGF-β1. In this experiment BAECs were treated with 0 (vehicle), 0.1, 0.2, 0.5 and 1 µg/ml of rh-endoglin with and without a dose of 5 ng/ml of TGF-β1, for 48 hours. Three biological replicates were obtained to produce the results described in this section and a Kruskal-Wallis test for treatments was used where a p-value of <0.05 was considered to be statistically significant. Untreated BAECs were used as a positive control on the western blots of VEGFR2 and Dll4.

VEGFR2 protein expression was notably down regulated in the BAECs treated with the varying doses of rh-endoglin in the absence of 5 ng/ml TGF-β1. On the other hand, the cells treated with both rh-endoglin and rh-TGF-β1 were found to have upregulated VEGFR2 compared to the positive control of untreated BAECs. The most significant down regulation in the expression of VEGFR2 in all treatment groups was in the cells treated with 0.1 µg/ml rh-endoglin and 0 ng/ml TGF-β1, while the strongest expression of VEGFR2 was in BAEC cells treated with 0.1 and 0.5 µg/ml rh-endoglin and 5 ng/ml TGF-β (Figure 6). Furthermore, Dll4 protein expression showed the same pattern of down regulation in the cells treated with only the rh-endoglin doses. Moreover, significant down regulation in the expression
of Dll4 in all treatment groups was observed in the cells treated with 1 μg/ml endoglin plus 0 ng/ml TGF-β and 1 μg/ml endoglin plus 0 ng/ml TGF-β, and even though there was no statistical significance, the strongest expression was also in BAECs treated with 0.1 and 0.5 μg/ml endoglin and 5 ng/ml TGF-β (Figure 7).
Figure 6: Changes in VEGFR2 expression following 48 hours CD105 (endoglin) treatment with and without TGF-β1 as indicated in BAECs. A. Representative western blot of BAEC protein lysates. B. Relative expression of VEGFR2 normalized to α-tubulin (n=3). Kruskal-Wallis test for all treatments was significant (* P< 0.0076 for treatment with TGF-β1 and P<0.0136 for treatment without TGF-β1). Dunn’s test has a significance level of 0.05 and showed that levels in 5 ng/ml TGF-β1 plus 0.5 µg/ml CD105 and 0 ng/ml TGF-β1 plus 1 µg/ml CD105 treated cells were significantly lower than control.
Figure 7: Changes in Dll4 expression following 48 hours CD105 (endoglin) treatment with and without TGF-β1 as indicated in BAECs. A. Representative western blot of BAEC protein lysates. The tubulin blot shown here is the same as in Figure 6 since the Dll4 blot shown here was probed from the same transfer. B. Relative expression of Dll4 normalized to α-tubulin (n=3). Kruskal-Wallis test for all treatments was significant (* P< 0.0143 for treatment with TGF-β1 and P<0.0098 for treatment without TGF-β1). Dunn’s test has a significance level of 0.05 and showed that levels in 5 ng/ml TGF-β1 plus 1 μg/ml CD105 and 0 ng/ml TGF-β1 plus 1 μg/ml CD105 treated cells were significantly lower than control.
Expression of NOTCH1, Dll4 and endoglin by CRC cells

The expression of Notch1, Dll4 and endoglin was measured by western blotting in the cell lysates and conditioned media collected from serum starved CRC cell lines: HCT116, SW480, LS174T, DLD1 and CaCo2 as well as in normal epithelial cells of the rat ileum (IEC18). Kruskal-Wallis test was performed on the densitometric analysis data collected from analyzed western blots where a $p$-value of <0.05 was considered to be statistically significant, followed by Dunn’s test for multiple comparisons.

All three molecules were detected in the five CRC cell lysates and only Dll4 and endoglin in the serum free media at varying levels. Notch1 expression showed no statistically significant differences between cell lines ($P > 0.05$), although the strongest expression of Notch was observed in IEC18 followed by DLD1 (Figure 8). Differences in Dll4 expression in CRC cell lysates were statistically significant between cell lines ($P < 0.0303$) compared to control BAEC. Furthermore, the highest expression of Dll4 in CRC cell lines was observed in DLD1, which was statistically significant. There were varying but similar levels of expression in the rest of the CRC cell lines (Figure 9). The expression of Dll4 in the serum free media existed in varying concentrations, but differences were not statistically significant ($P > 0.05$). The highest level was also seen in DLD1 CM and no detectable expression was found in the LS174T cell CM (Figure 10). Differences in endoglin expression in CRC cell lysates were statistically significant ($P < 0.0236$); the highest expression of endoglin in CRC cell lines was observed in SW480 and DLD1 ($P < 0.05$) with varying but similar levels of expression in the rest of the CRC cell lines (Figure 11). Endoglin
expression in CRC serum free media existed in varying concentrations with the highest concentration in SW480 and lowest in LS174T and HCT116 cell lines, but these differences were not statistically significant ($P > 0.05$) (Figure 12).
Figure 8: Differences in Notch1 expression in CRC cell line lysates. A. Representative western blot of Notch1 in: HCT116, DLD1, LS174T, CaCo2, SW480 and IEC18 protein lysates. B. Relative expression of Notch1 normalized to α-tubulin (n=3). Kruskal-Wallis test for all treatments was not significant (P > 0.05).
Figure 9: Differences in Dll4 expression in CRC cell line lysates. A. Representative western blot of Dll4 in: HCT116, DLD1, LS174T, CaCo2, SW480 and IEC18 protein lysates. This tubulin blot is the same as the one in Figure 8 because the Dll4 blot was from the same transfer. B. Relative expression of Dll4 Normalized to α-tubulin (n=3). Kruskal-Wallis test for all treatments was significant (* P< 0.0303). Dunn's test has a significance level of 0.05 and showed that levels of Dll4 were significantly higher in DLD1 cells than in BAEC.
Figure 10: Differences in Dll4 expression in CRC cell lines’ media. A. Representative western blot of Dll4 secreted by HCT116, DLD1, LS174T, CaCo2, SW480 and IEC18 cells. B. Relative densitometry expression of Dll4 (n=3). Kruskal-Wallis test for all groups was not significant (P > 0.05).
Figure 11: Differences in CD105 (endoglin) expression in CRC cell lines’ lysates. A. Representative western blot of CD105 in: HCT116, DLD1, LS174T, CaCo2, SW480 and IEC18 protein lysates. This tubulin blot is the same as the one in Figure 8 and Figure 9 because the CD105 blot was from the same transfer B. Relative expression of CD105 normalized to α-tubulin (n=3). Kruskal-Wallis test for all treatments was significant (* P< 0.0236). Dunn’s test has a significance level of 0.05 and showed that CD105 levels were significantly higher in SW480 ad DLD1 cells compared to BAEC.
Figure 12: Differences in CD105 (endoglin) expression in CRC cell lines’ media. A. Representative western blot of CD105 secreted by HCT116, DLD1, LS174T, CaCo2, SW480 and IEC18 cells. B. Relative densitometry expression of CD105 (n=3). Kruskal-Wallis test for all groups was not significant (P > 0.05).
**Matrigel cord formation assay**

In order to study the effect of TGF-β and endoglin on angiogenesis *in vitro*, a cord formation assay was performed where 50,000 BAECs were plated in each well of a 24 well plate coated with 200 µl of 25% Matrigel diluted in serum free media. For the first cord formation experiment, TGF-β treatments of 0, 0.1, 5 and 20 ng/ml were added for 18 hours, and then phase-contrast images were taken. The second experiment included treatment of varying endoglin concentrations 0, 0.1, 0.2, 0.5 and 1 µg/ml with and without a dose of 5 ng/ml of TGF-β. Three images from pre-determined positions across all wells were taken in each biological replicate and sent for quantitative analysis of newly formed cords (Wimasis image analysis) by the Wim Tube assay. The data produced by this analysis included branching points, cord length, and total loops, which were analyzed using the Kruskal-Wallis and Dunn’s tests for statistical significance.

In the first cord formation experiment, total cord length was noticeably reduced in the wells treated with TGF-β compared to the control wells (Figure 13). The most noticeable reduction was found in wells treated with 5 and 20 ng/ml doses (Figure 14.A), however these differences were not statistically significant ($P > 0.05$). As for the total branching points, their numbers decreased in treated wells compared to the control with the lowest number of branching points found in the 5 ng/ml treated wells. Statistical analyses of the data showed these results were non-significant ($P > 0.05$) (Figure 14.B). Furthermore, total loop numbers were examined and even though there was noticeable reduction of loop numbers in the
TGF-β treated wells, when compared to control, there was no statistical significance in the analyzed data ($P>0.05$) (Figure 14.C).

In the second experiment, cord formation was observed in all treatment groups, furthermore there was no correlation between cord formation and the different doses of treatments (Figure 15). The most perceptible down regulation in total cord length among treatment groups that contained TGF-β was found in the wells treated with 0.5 μg/ml endoglin and 5 ng/ml TGF-β compared to the control wells. The most evident down regulation in total cord length was in cells exposed to endoglin doses alone especially in the 1 μg/ml treated wells. The most marked increased in total cord length was found in the wells treated with 0.2 μg/ml endoglin plus 5ng/ml TGF-β. However, results from statistical analysis showed these differences were non-significant ($P > 0.05$) (Figure 15. A). The total branching point numbers exhibited no correlation between presence of treatments, and changes in their numbers were not significant ($P > 0.05$) (Figure 15.B).

Furthermore, total loop numbers were examined and even though there was noticeable reduction of loop numbers in the TGF-β treated wells, when compared to control, there was no statistical significance in the analyzed data ($P>0.05$) (Figure 15.C)
Figure 13: Sample phase-contrast images and cord formation images analysis. A. BAECs were plated on Matrigel and treated for 18 hours with (0, 0.1, 5, 20 ng/ml) of TGF-β1. B. Cord formation images were quantitatively evaluated by Wimasis tube formation assay.
Figure 14: Representative data analysis of TGF-β dose effect in cord formation assay. A. Total cord length. B. Total branching points. C. Total Loops. Kruskal-Wallis test for all groups for the three measurements was not significant ($P > 0.05$).
Figure 15: Representative data analysis of endoglin with and without TGF-β Dose effect in Tube formation assay. A. Total tube length. B. Total branching points. C. Total Loops. Kruskal-Wallis test for all groups for the three measurements was not significant (P > 0.05).
DISCUSSION

Angiogenesis plays an important role in the progression of solid tumors by supplying the tumor with the oxygen and nutrients it needs to grow beyond a limited size. More than three decades ago it was hypothesized that inhibition of tumor angiogenesis plays a critical role in the control and prevention of cancer. The findings of this group consequently led to the advancement of anti-angiogenesis research. Several clinical trials were conducted over the years in an effort to develop anti-angiogenic drugs.

Various signaling pathways that play an important role in sprouting angiogenesis influence and interact with each other. The crosstalk between VEGF, Notch and TGF-β pathways in angiogenesis has been demonstrated in several studies. VEGF affects Notch signaling in sprouting angiogenesis by inducing the increased expression of Dll4 in tip cells and in turn Dll4 signals through Notch1 on an adjacent stalk cell and down-regulates the expression of VEGFR2 and VEGFR3. This down-regulation, which forms a negative feedback loop between Dll4/Notch1 and VEGF/VEGFR2 pathways, is believed to be responsible for the maintenance of the tip and stalk cell phenotype.

Furthermore, TGF-β expression in the tumor microenvironment affects VEGFR2 expression as was demonstrated in our laboratory where TGF-β derived from CRC cells was shown to down-regulate VEGFR2 expression in endothelial cells in vitro. Moreover, the proteolytic cleavage of the extracellular domain of endoglin results in releasing a circulating soluble form of endoglin. The levels of soluble endoglin in the circulation correlate with metastasis in various types of cancer such
as: colorectal, breast and prostate cancers\textsuperscript{78-80}. Soluble endoglin contains the binding site of various members of the TGF-\(\beta\) superfamily; this in turn might hinder the binding of TGF-\(\beta\) to its functional receptor by sequestering the circulating ligands resulting in inhibition of angiogenesis\textsuperscript{43,58,81}. Therefore, it is essential to further examine these molecules' interactions and their possible effects on tip and stalk cell phenotype in sprouting angiogenesis. Hence, I hypothesized that TGF-\(\beta\), in combination with other factors, may influence the phenotype of endothelial cells into tip and stalk cells. Moreover, changes in endoglin levels may affect TGF-\(\beta\) availability and therefore alter its possible effect on endothelial cells.

The biphasic influence of TGF-\(\beta\) on the angiogenic process has been demonstrated by several \textit{in vitro} studies, for example, a dose of 0.5 ng/ml TGF-\(\beta\)1 lead to a pro-angiogenic reaction in which Smad2 reached maximal phosphorylation after 1 hour of treatment\textsuperscript{34,70}. Based on this, a range of TGF-\(\beta\)1 doses were chosen for my experiments that include known pro-angiogenic ranges of TGF-\(\beta\)1 such as 0.1 and 1 ng/ml as well as doses of TGF-\(\beta\)1 that have been demonstrated to inhibit angiogenesis which are 5, 10, and 20 ng/ml\textsuperscript{70}.

\textit{In vitro} experiments in my study suggest that TGF-\(\beta\) could have a dose dependent effect on Notch1, Dll4 and endoglin expression in bovine aortic endothelial cells. I observed that both Notch1 and Dll4 expression in BAEC were high in response to the low doses (0.1 and 1 ng/ml) of TGF-\(\beta\). This up-regulatory effect of these low TGF-\(\beta\) doses on the expression of Notch1 and Dll4 could lead to the suppression of endothelial tip cell phenotype as seen in previous studies\textsuperscript{3}. The lowest expression of both Notch1 and Dll4 was observed in the BAEC treated with
the 5 ng/ml dose of TGF-β and was statistically significant. The 5 ng/ml dose of TGF-β was shown in previous studies to have an inhibitory effect on VEGF-induced endothelial cell invasion and capillary lumen formation which in turn would lead to the inhibition of angiogenesis. This inhibitory effect could be explained by this reduction in the expression of Notch1 in endothelial stalk cells and its ligand Dll4 in endothelial tip cells, leading to inhibition of this pathway responsible for regulating tip and stalk cell formation. The importance of the balance between Notch1 and Dll4 expression was underlined in several studies done in tumors in which Dll4 inhibition resulted in compromised tumor vessel perfusion and growth, which was accompanied with excessive sprouting of endothelial cells from blood vessels. Therefore, this observed fluctuation in the expression of these molecules in our experiments indicates that TGF-β has an effect on the Notch1/Dll4 signaling pathway in endothelial cells.

Additionally, down regulation of endoglin expression in BAEC was not as pronounced as the changes that were seen in Notch1 and Dll4 expression. Endoglin expression was reduced in the BAECs treated with 10 ng/ml of TGF-β; this reduction at this dose was statistically significant. Endoglin is a type III TGF-β co-receptor that binds TGF-β1 in the presence of the signaling TGF-βRI and TGF-βRII, and it is highly expressed on endothelial cells. High expression of endoglin on endothelial cells associated with tumor angiogenesis correlated with a poor survival rate in colorectal cancers. Furthermore, soluble endoglin has been shown to exist in the circulation as a result to the actions of the shedding protease MMP-14; this soluble endoglin was demonstrated to contain the binding site for several members of the
TGF-β superfamily including TGF-β1\textsuperscript{58,81}. Therefore, since soluble endoglin contains the binding site of TGF-β1 it may sequester the circulating TGF-β1 ligand and interfere with its inhibitory function in angiogenesis.

In several clinical studies soluble endoglin levels have been examined in plasma and serum samples collected from patients with various types of cancer, and the varying ranges of soluble endoglin in these serum and plasma samples were noted\textsuperscript{46,78}. Furthermore, high concentrations of soluble endoglin in the circulation of cancer patients have been associated with metastasis and poor prognosis\textsuperscript{79,80}. A study of human umbilical vein endothelial cells (HUVECs) was conducted to examine the effect of soluble endoglin on endothelial cell function in vitro, and the study demonstrated soluble endoglin interference with TGF-β signaling\textsuperscript{58}. This study proceeded to demonstrate soluble endoglin’s inhibitory role in capillary formation by conducting an angiogenesis assay using growth factor reduced Matrigel and HUVECs in the presence of 1 μg of rh-soluble endoglin followed by quantification of endothelial tube lengths. I chose to use the range of soluble endoglin doses of 0.1, 0.2, 0.5 and 1μg/ml in order to examine the effect of these varying soluble endoglin concentrations on BAECs \textit{in vitro}.

Dll4 expression in BAEC treated with both endoglin and TGF-β was high in comparison to the Dll4 expression in BAEC treated with endoglin alone. The highest expression of Dll4 in all of the treatment groups was found in the cells treated with 0.1 and 0.5 μg/ml endoglin plus 5 ng/ml TGF-β. Moreover, the most pronounced down regulation of Dll4 in all treatment groups was observed in cells treated with 1 μg/ml endoglin in the absence of TGF-β. The up-regulated expression of the Dll4
protein in cells exposed to a dose of 5 ng/ml of TGF-β1 in the presence of varying doses of endoglin could be in response to endoglin binding TGF-β1 and therefore interfering with its negative effect on Dll4 expression. Venkatesha et al. hypothesized that soluble endoglin, a co-receptor of TGF-β1 in endothelial cells, may act by interfering with TGF-β binding to the receptors on the cell surface\textsuperscript{58}. To illustrate that soluble endoglin competes for TGF-β1 and prevents its binding to its receptors on endothelial cells, a radiolabeled TGF-β1 was pre-incubated with recombinant soluble endoglin. This pre-incubation with soluble endoglin was shown to have a significant interfering effect on the binding of TGF-β1 to TGF-βRII at both 50 and 100 pM\textsuperscript{58}.

The down regulatory effect of TGF-β1 on Dll4 expression was demonstrated previously in my study, however, this effect was negated in the presence of endoglin treatments. Additionally, I observed low expression levels of Dll4 in BAECs treated with endoglin only, which could be due to the absence of TGF-β1’s influence on the endothelial cells. In a previous study the crosstalk between Notch and TGF-β signaling pathways was shown to be complex in that Notch can synergize with the TGF-β/BMP signaling to modulate the expression of target genes, however Notch can also inhibit the TGF-β/BMP signaling pathway in endothelial cells\textsuperscript{27}. In my experiments Dll4 expression in BAEC treated with endoglin and TGF-β was high in comparison to the Dll4 expression in BAEC treated with endoglin concentrations without TGF-β. The highest expression of Dll4 in all of the treatment groups was found in the cells treated with 0.1 and 0.5 μg/ml endoglin plus TGF-β. Moreover, the most pronounced down regulation of Dll4 in all treatment groups was observed in
cells treated with 1 μg/ml endoglin in the absence of TGF-β. The interactions between the Notch and TGF-β signaling pathways could be affected by the impact of soluble endoglin sequestering the TGF-β1 ligand which would lead in turn to interference with its signaling in endothelial cells.58

A similar up-regulated expression of the VEGFR2 protein in cells exposed to a dose of 5 ng/ml of TGF-β in the presence of varying doses of endoglin was observed. It was previously demonstrated in our laboratory that TGF-β dose dependently reduced the expression of VEGFR2 protein and transcript in BAEC.5 Furthermore, a similar response was observed in BAEC exposed to serum free media collected from several CRC cell lines. VEGFR2 expression in BAEC treated with endoglin and TGF-β was high in comparison to the VEGFR2 expression in BAEC treated with endoglin concentrations without TGF-β. This up regulation could also be attributed to soluble endoglin binding TGF-β1 and consequently interfering with its previously observed down regulatory influence on VEGFR2 expression. Furthermore, observed low expression levels of VEGFR2 in cells treated with only endoglin compared to control untreated cells could indicate an indirect down regulatory role that endoglin might have on VEGFR2 expression. This observed interference of endoglin with the expression of two of the molecules that regulate sprouting angiogenesis could be utilized to enhance our understanding of the crosstalk between these pathways.27

Next, an in vitro cord formation assay was conducted to study the dose effect of endoglin with and without TGF-β treatments on angiogenesis. Cord formation was observed in all treatment groups, furthermore there was no correlation between cord formation and different treatments. However the most perceptible
down regulation in total cord length among treatment groups that contained TGF-β was found in the wells treated with 0.5 μg/ml endoglin and 5 ng/ml TGF-β compared to the control wells. This down regulation is somewhat similar to previous work done on HUVECs where it was suggested that a dose of 1 μg/ml of soluble endoglin could block the pro angiogenic effects of TGF-β1 on HUVECs in Matrigel. The most evident down regulation in our study in total cord length was when cells were exposed to 1 μg/ml endoglin alone. Furthermore, the most pronounced up regulation of total cord length was found in the wells treated with 0.2 μg/ml endoglin plus 5 ng/ml TGF-β.

These observations of changes in cord formation in the presence of these endoglin concentrations could support the idea that soluble endoglin in the circulation might modulate TGF-β effects on angiogenesis. This observed interplay between endoglin concentrations and TGF-β effect on endothelial cells could further explain the low survival rate of cancer patients with high levels of endoglin in their circulation. The possible interference of endoglin with TGF-β signaling would in turn obstruct TGF-β’s dual role in tumor angiogenesis, which in turn could hinder drug delivery to the site of malignancy.

TGF-β exhibits both inhibition and promotion of malignancy functions in cancer progression. TGF-β is especially important in colorectal carcinoma (CRC), since up to 85% of CRC cell lines showed resistance to TGF-β growth inhibitory influence. Moreover, studies in our laboratory observed the expression of TGF-β in the serum free media collected from several CRC cell lines. This observation
prompted our interest in characterization of the expression of other signaling molecules of significance for angiogenesis in this study in CRC cell lines.

Studies conducted on tissue microarrays and in situ hybridization of human CRC elucidated expression of Notch and its ligands to be active in intestinal and colonic cells both in terms of normal physiology and tumorigenesis\textsuperscript{86,87}. Soluble endoglin expression was observed in the circulation of patients with many solid tumors including colorectal carcinomas; its increased expression also correlated with metastasis and low survival rates\textsuperscript{78}.

Western blot analysis was conducted in order to examine protein expression of Notch1, Dll4 and endoglin in five different CRC cell lines: HCT116, SW480, LS174T, DLD1 and CaCo2. All three molecules were detected in the cell lysates at varying levels and only Dll4 and endoglin were detected in the serum free media at varying levels. Notch1 expression showed no statistical significance between cell lines, however the highest expression of Notch in CRC cell lysates was observed in DLD1. The highest expression of Dll4 in CRC cell lines was observed in DLD1 with varying but similar levels of expression in the rest of the CRC cell lines. The expression of Dll4 in the serum free media was also observed with varying concentrations; the highest was also in DLD1 and no detectable Dll4 in the LS174T cell conditioned medium.

Cancer cells can acquire resistance to chemotherapeutic treatments, which is a major advantage that transpires mainly by activating survival pathways or by inhibiting apoptotic pathways. Furthermore, Notch signaling is a known major regulator of survival pathways in colorectal cancer. For instance, oxaliplatin
treatment of colorectal cancer leads to the activation of the Notch signaling pathway\textsuperscript{88}. Moreover, colorectal cells were sensitized to chemotherapeutic drugs as a result of the blocking of Notch signaling under the influence of γ-secretase inhibitors (GSIs) \textsuperscript{88}. In another study, experiments were performed in order to establish the effect of soluble Dll4 on full-length Dll4’s cellular role in activating Notch signaling\textsuperscript{89}. Full-length Dll4 is known to induce genes that are regulated as a result of Notch signaling, however the addition of soluble Dll4-Fc and soluble Dll4 led to the blocking of the activity of the full-length Dll4 in inducing the activation of Notch signaling pathway target genes. Therefore, soluble Dll4 was found to have an inhibitory effect on Dll4/Notch signaling pathway in human endothelial cells\textsuperscript{89}. Since CRC cells secrete soluble Dll4 and it has been established that soluble Dll4 has an inhibitory effect on Dll4/Notch signaling pathway in human endothelial cells\textsuperscript{89}, then soluble Dll4 presents an interesting target for further research on CRC cell resistance to chemotherapy.

The connection between the expression of VEGF and Dll4 in tumor angiogenesis led to several studies that assessed the effect of blocking Dll4-Notch signaling on tumor angiogenesis. One of these studies found that inhibiting Dll4 mediated Notch signaling lead to reduction in tumor growth, however this inhibition was also associated with an increase in the number of newly formed vessels\textsuperscript{25}.

In my study, differences in endoglin expression in CRC cell lysates were statistical significant between cell lines, with the highest expression of endoglin observed in SW480 and DLD1. Endoglin expression in CRC serum free media was
also observed with varying concentrations; the highest was in SW480 and lowest in LS174T and HCT116 cell lines. Endoglin has two splice isoforms termed long endoglin (L-End) and short endoglin (S-End). L-End is mostly expressed in endothelial cells while S-End is found to be significantly expressed in the liver and lungs. Furthermore, a soluble form of endoglin can be found in the circulation of cancer patients, perhaps as a result of the actions of the shedding protease MMP-14. Soluble endoglin was demonstrated to contain the binding site for TGF-β1.

Previous studies in our laboratory examined VEGFR2 down regulation and compared it with total TGF-β1 and TGF-β2 in the conditioned media collected from some of the CRC cell lines used in this study. It was found that the level of VEGFR2 down regulation correlated with total TGF-β1 and TGF-β2 in the conditioned media collected from CaCo2, HCT116 and DLD1, but not from SW480 cells. In this study I found that endoglin is strongly expressed in the conditioned media collected from the SW480 cell line. These high levels of soluble endoglin in the SW480 cell line conditioned media could be the reason for the previously observed discrepancy between levels of TGF-β in SW480 conditioned media and the ability of SW480 conditioned media to down regulate VEGFR2 in endothelial cells.

Results from my study and similar investigations of the pathways that play a key role in regulating tumor angiogenesis could help in the development of antiangiogenic-targeted therapies. Furthermore, antiangiogenic targeted therapies have not improved survival rate of metastatic CRC patients so far, which calls for better understanding of the interactions of CRC cells with their angiogenic network.
SUMMARY AND CONCLUSIONS

During the course of this study, the effect of TGF-β on the expression of Notch1 and Dll4 was down regulatory in BAEC treated with 5 ng/ml of TGF-β. For endoglin expression, the only significant reduction was seen at 10 ng/ml TGF-β. Furthermore, expression levels of Notch1, Dll4 and endoglin were altered in the higher and lower doses 0.1, 1, 10 and 20 ng/ml of TGF-β. However this effect was negated in the endothelial cells that were treated with soluble endoglin in the presence of the TGF-β inhibitory dose. Moreover, BAECs treated with endoglin plus TGF-β showed significantly higher expression of VEGFR2 and Dll4 when compared to the BAECs treated with endoglin in the absence of TGF-β. Cord formation on Matrigel was noticeably improved in the presence of endoglin plus TGF-β as opposed to cord formation observed in the BAEC treated with endoglin only.

Assuming that endoglin was sequestering TGF-β and preventing receptor signaling, that would explain the observed higher cord formation in BAEC in the presence of endoglin with TGF-β. A previous study has shown that blocking of the Dll4/Notch signaling pathway causes a significant reduction in tumor growth accompanied by an increase in vessel formation. Therefore, the reduction in the expression of both Notch1 and Dll4 under the effect of TGF-β might inhibit Notch1/Dll4 signaling and that in turn could explain the increased expression of VEGFR2, observed in this study, since this pathway down regulates it. Moreover, the expression of Notch1, Dll4 and endoglin was examined in five different CRC cell lines HCT116, SW480, LS174T, DLD1 and CaCo2 and all three molecules were expressed
in the five CRC cell lysates at varying levels and only Dll4 and endoglin were detected in the serum free media at varying levels.

Ultimately, by broadening our understanding of the key pathways in tumor angiogenesis, we gain a better insight into how to exploit these pathways to achieve better drug delivery and even inhibit the blood supply to the tumor site. This knowledge would also be vital in understanding the pathogenesis of various vascular diseases such as diabetic retinopathy.

Additional analyses would be helpful to ascertain endoglin concentrations in the circulation of patients with different types of tumors and how different endoglin levels affect TGF-β availability. Moreover, it would be interesting to study TGF-β effects on other molecules that participate in sprouting angiogenesis or are affected by it, such as the Notch1 ligand Jagged1. The overexpression of this ligand in cancer cells was seen to promote sprouting angiogenesis in experimental tumors in mice90.

In conclusion, 5 ng/ml TGF-β inhibited the Notch1/Dll4 pathway by down regulating the expression of these proteins in endothelial cells. Chaotic formations of cords are to be expected since the main pathway that regulates their formation, (Notch1/Dll4), is inhibited at this dose of TGF-β. In the presence of both endoglin and 5 ng/ml of TGF-β the expression of VEGFR2 and Dll4 in endothelial cells was up regulated compared to the cells treated with 5 ng/ml of TGF-β alone. This would lead us to believe that the angiogenesis inhibitory dose effect of the 5 ng/ml of TGF-β was blocked in the presence of the levels of soluble endoglin used in this study.
LITERATURE CITED


Transforming growth factor-beta blocks inhibin binding to different target cell types in a context-dependent manner through dual mechanisms involving betaglycan. *Endocrinology.* 2007;148:5355-5368.


APPENDICES

APPENDIX I – Chemical List and Suppliers

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<tr>
<th>Chemical</th>
<th>Supplier</th>
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<tr>
<td>0.5 M Tris-HCl Buffer pH 6.8</td>
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<td>DMEM</td>
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<td>Tween-20</td>
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APPENDIX II – Preparation of Materials Used

0.1% TBST
10X TBS 100 mL
Tween-20 1 mL
H₂O 900 mL
Mix and store at room temperature

10% Resolving Gel
H₂O 9.7 mL
40% acrylamide-bis solution 5 mL
1.5 M Tris-HCL (pH 8.8) 5 mL
10% SDS 200 μL
10% APS 240 μL
TEMED 10 μL
Gel components are combined in the order listed above, mixed well, and then added to gel cassettes and gels are then allowed to set.

5% Stacking Gel
H₂O 3.2 mL
40% acrylamide-bis solution 500 μL
0.5 M Tris-HCL (pH 6.8) 1.26 mL
10% SDS 50 μL
10% APS 50 μL
TEMED 5 μL
Once the resolving gel (prepared above) has set the stacking gel mixture is prepared by adding components in the order listed above, and then poured on top of the resolving gel. A comb is then added to form lanes and the gel is allowed to set.

10X TBS
Tris base 24.25 g
NaCl 80 g
H₂O 600 mL
Mix all components and adjust pH to 7.6 with HCl, then top up to 1L with H₂O.

1X TBS
10X TBS 100 ml
Tween-20 1 ml
Adjust volume to 1 L with water.
**Towbin's Solution**

Tris Base 30.25g  
Glycine 141.1g  
H₂O to 1 L  
Mix and store at room temperature.

**Wet Transfer Buffer**

H₂O 720 mL  
2% SDS 200 uL  
Towbin's Solution 80 mL  
Methanol 200 mL  
Mix and store at 4°C until use.