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The Differential Expression of EGFL7 Transcripts during Angiogenesis in Human Fibrosarcoma

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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THE DIFFERENTIAL EXPRESSION OF EGFL7 TRANSCRIPTS DURING TUMOR ANGIOGENESIS IN HUMAN FIBROSARCOMA

(Spine title, The Differential Expression of EGFL7 Transcripts during Tumor Angiogenesis)

(Thesis Format: Integrated Article)

By

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Graduate Program in Medical Biophysics

Submitted in partial fulfillment of the requirements of the degree of Master of Science

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The University of Western Ontario
London, Ontario, Canada

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The thesis by

Navid Baktash

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Dr. Ravi Menon
Chair of the Thesis Examination Board
Abstract

The growth of a tumor depends on *de novo* angiogenesis, which involves multiple signaling cascades and is also a target for many anti-angiogenic therapies. Epidermal growth factor like (EGFL7), is a protein that is expressed by endothelial cells during angiogenesis and is necessary for the proper assembly of a sprout. However, EGFL7 is also upregulated in many tumors such as glioma and non-small cell lung cancer, and associated with poor patient prognosis. We have previously shown that EGFL7 expression by tumor cells inhibits tumor angiogenesis, which is contrary to its requirement for angiogenesis when expressed by endothelial cells. As a result, the objective of this research is to understand the basis of this differential regulation. We hypothesize that the differential effects of EGFL7 on angiogenesis are due to the presence of alternative transcripts in HT1080 tumor cells. One common method of regulation is transcriptional regulation which can involved the generation of multiple alternative transcripts due to the presence of different transcriptional start sites. Here, I demonstrate that endothelial cells express two families of transcripts, TSS1 and TSS2 families, which are transcribed from two different transcriptional start sites. In HT1080 tumor cells, however, only TSS2 families of transcripts are produced. Importantly, novel a novel non-coding transcript in both TSS1 and TSS2 family were identified. Non-coding transcripts are known to have important roles in global transcriptional regulation, such as chromatin remodification. Knocking down TSS2 transcripts in tumor cells resulted in enhanced angiogenesis. Result from this thesis identify novel differential EGFL7 transcripts expressed in HT1080 tumor cells, which inhibits angiogenesis.

Key words: EGFL7, Angiogenesis, Differential regulation, transcripts
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Lastly, I would like to thank my family for the encouragement they gave me to pursue my research ambitions. Also, for making me notice that that courage is not the absence of fear but the ability to conquer it.

Thank you.
Dedicated to the progress of science and medicine filled with a quest to understand the beautiful complexity of nature
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List of Abbreviations

ANG  Angiopoietin
ANOVA  Analysis of Variance
CAM  Chorioallantoic membrane
CD  Cluster of differentiation
Dll5  Delta-like 5
DMEM  Dulbecco’s modified eagle medium
EC  Endothelial cell
ECM  Extracellular Matrix
EGFL7  Epidermal Growth Factor Like-7
EPC  Endothelial Progenitor Cell
Erg  ETS related gene
EST  Expressed Sequence Tag
Ets  E-twenty-six transcription factor
FGF  Fibroblast growth factor
Fli-1  Friend leukemia integration 1
Fox  Forkhead Box
Gr1  Myeloid differentiation antigen
HGF  Hepatocyte Growth Factor
HIF  Hypoxia-inducible factor
HOTAIR  Hox-antisense intergenic RNA
HT1080  Human Fibrosarcoma cell line
HUVEC  Human umbilical vein endothelial cell
IGFBP2  Insulin-like growth factor binding protein-2
miR-126  Micro-RNA 126
MERTK  c-Mer tyrosine receptor kinase
MMP  Matrix metalloproteinase
NICD  Notch Intracellular Domain
IncRNA  Long-non coding RNA
NRP  Neuropilin
ORF  Open Reading Frame
PDGF  Platelet-derived growth factor
PHD2  Hypoxia-inducible factor prolyl-4 hydroxylase
PI3K  Phosphatidylinositol 3-kinase
PITPNC1  Phosphatidylinositol transfer protein cytoplasmic 1
siRNA  Small interfering RNA
Src kinase  Sarcoma kinase
TGF-α  Transforming growth factor α
TGF-β  Transforming growth factor β

Tie-2  Tyrosine kinase with immunoglobulin-like and EGF like domain
<table>
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<th>Abbreviation</th>
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<tr>
<td>TIMP</td>
<td>Tissue inhibitor matrix metalloproteinases</td>
</tr>
<tr>
<td>TSS</td>
<td>Transcriptional Start Site</td>
</tr>
<tr>
<td>uORF</td>
<td>Upstream open reading frame</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated Region</td>
</tr>
<tr>
<td>VEGFA</td>
<td>Vascular endothelial growth factor A</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>vSMC</td>
<td>Vascular smooth muscle cell</td>
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“Success is never final. Failure is never fatal. Courage is what counts.”

Sir Winston Churchill
Chapter 1: Introduction

1.1 Overview

The vasculature acts as a conduit for the delivery of nutrients, oxygen or other factors and is therefore necessary for homeostasis but also plays a role in tumor development. The formation of new blood vessels from pre-existing vasculature is defined as *de novo* angiogenesis [1]. The formation of blood vessels begins in development through vasculogenesis, a process in which a primitive vascular network forms due to differentiation of mesodermal precursor cells into endothelial progenitor cells (EPC) or angioblasts [2]. The vasculature eventually matures through sprouting angiogenesis or intussusception. In the adult, angiogenesis occurs primarily through sprouting due to the presence of an angiogenic stimulus such as growth factors, however, EPC’s have been documented to incorporate into a newly formed vessel. In the adult, angiogenesis occurs during physiological processes such as wound healing and in pregnancy, but in pathological states such as tumor progression, sustained angiogenesis is maintained by an abundance of pro-angiogenic signal competing with inhibitory signals [3,4]. The angiogenic microenvironment in tumors is regulated by a variety of secreted extracellular factors and autocrine or paracrine signaling amongst endothelial cells making up the vasculature and tumor cells.

Amongst the many secreted factors in the extracellular microenvironment in tumors, epidermal growth factor like-7 (EGFL7) has recently been identified as a novel player involved in the proper alignment and spatial organization of endothelial cells during sprouting angiogenesis. This protein, when exclusively expressed in endothelial cells, has been shown to be required for proper vasculogenesis and angiogenesis [5-8].
Interestingly, EGFL7 has also been upregulated in many malignant tumors such as colon cancer, hepatocellular carcinoma, non-small cell lung cancer and glioblastoma. We have previously shown that EGFL7 expression in HT1080 tumor cells acts to inhibit angiogenesis. This was observed both in vitro and in vivo in the chick chorioallantoic membrane (CAM), which is a vascular membrane that can be used to visualize angiogenesis through intravital imaging or microscopy. This consequently reduced metastasis of tumor cells. After observing sprout morphology, the endothelium was no longer a monolayer but endothelial cells were layered on top of one another.

Given that in HT1080 tumor cells EGFL7 acts to inhibit angiogenesis but when expressed by endothelial cells it is necessary for angiogenesis, I investigate how EGFL7 is being differentially regulated in HT1080 tumor cells and the subsequent impact this has on tumor angiogenesis. We hypothesize that HT1080 tumor cells express alternative transcripts in comparison to endothelial cells and this method of differential regulation mediates EGFL7’s inhibitory effect in tumor cells.

1.1.1 Overview of angiogenesis

De novo angiogenesis is defined as the formation of new blood vessels from pre-existing vessels [9]. Angiogenesis is a necessary process for the supply of oxygen, nutrients, transport of immune cells and other factors necessary for maintaining tissue physiology-physiological angiogenesis [10]. However, during pathological conditions the vasculature can be aberrantly activated to generate new blood vessels in disease states such as cancer and chronic inflammation. Angiogenesis dependent diseases results when new blood
vessels grow excessively or insufficiently as in cases of coronary artery disease, stroke and wound healing [11]. The key to dictating the angiogenic environment is a balance between angiogenic stimulators such as fibroblast growth factors (FGF) and angiogenic inhibitors, such as endostatin and maspin[12-13]. In disease states, this balance dictates the progression of the formation of new blood vessels.

Angiogenesis is mediated by two distinct mechanisms known as intussusception and sprouting [Fig1-1][14]. During sprouting angiogenesis, quiescent endothelial cells (EC) become activated by angiogenic signals binding to EC receptors, often in the form of a gradient [15-18]. This causes endothelial cells to form loose junctions, proliferate and migrate towards the gradient in a characteristic stalk-tip cell formation. This is further accompanied by the secretion of extra-cellular matrix (ECM) enzymes, which degrade the surrounding ECM. Eventually, these endothelial cells coalesce to form tubes known as tubulogenesis, and become stabilized by other cell types such as pericytes. However, during intussusive angiogenesis, transluminal tissue pillars form within capillaries as a result of endothelial cells fusing together [19-21]. This process follows precise stages where protrusion of opposite side of capillary walls precedes inter-endothelial cell contact. Collagen fibrils are then deposited leading to tissue expansion and formation of new tissues. The angiogenic process is a highly coordinated process requiring diverse regulatory pathways, multiple cell types and presence of many ECM proteins. As a result, it is an attractive therapeutic target for disease processes such as cancer.
Fig. 1-1 Angiogenesis: Sprouting and Intussusceptive Growth.

A) Sprouting angiogenesis is initiated by a stimulus in the ECM, such as growth factors, hypoxia or angiogenic factors which initially causes the loosening of endothelial cells subsequently acquiring a tip-stalk arrangement. B) The filopodia rich tip cells sense the stimulus gradient while stalk cells help elongate the newly formed sprout. C) This eventually forms into a mature network of vessels that coalesce together. D) In intussusceptive growth, endothelial cells (EC) on opposite side of the capillaries protrude into the lumen contacting each other. Once contact is established, the contact forms interendothelial junctions and the endothelial bi layer is perforates centrally. Endothelial cells are then invaded by fibroblasts and pericytes where collagen is deposited between the two vessels. Image from [14,15]
1.1.2 Overview of Vasculogenesis

The vascular plexus formation is necessary in the developing embryo to enable delivery of oxygen and nutrients and removal of metabolic waste in the developing embryo. Many factors influence the differentiation and commitment of many cells types involved in the vascular plexus formation during gastrulation phase. The gastrulation phase occurs during the early phase of development in which the blastula is re-organized into the 3 germ layers-mesoderm, ectoderm and endoderm. The mesoderm layer of the embryo is comprised of mesenchymal cells that gives rise to the circulatory system [22]. The formation and development of de novo blood vessels from EC precursors is termed vasculogenesis. Vasculogenesis occurs in the extra-embryonic yolk sac, a membranous sac that provides nourishment to the embryo. In this particular process mesenchymal cells differentiate into endothelial progenitor cells (EPC) forming characteristic aggregates known as blood islands [Fig 1-2] [23,24]. Ultimately, these blood islands fuse together which form the primitive capillary plexus. This primary capillary plexus under the control of a variety of molecular signals can undergo angiogenesis (sprouting and non-sprouting). Subsequently, vessels undergo pruning remodeling, where the vessels have a characteristic shape of a tree with large and small vessel branching. Following pruning remodeling, organ-dependent maturation remodeling occurs, where the vessels either mature or regress in the organ they supply. Furthermore, the particular organ will influence the shape of vessels based on extra-luminal factors such as platelet derived growth factor (PDGF) [25].

Vasculogenesis however is not limited to the embryo. Asahara et al (1997), determined CD34+ CD45- EPC’s (angioblast) isolated from human blood can
differentiate into endothelial cells \textit{in vitro} [26]. This cell arises from a precursor multipotent bi-potential hemangioblast originating from the bone marrow and capable of hematopoiesis or angiogenesis [27]. Growth factors, cytokines and chemokines secreted by the hypoxic environment or tissue injury induces the proliferation, differentiation and mobilization of EPC to sites of new-vessel formation [28]. How EPC home to an area undergoing angiogenesis is not well known. Once at the site of ischemia, EPC co-ordinate with pre-existing EC’s to stimulate angiogenic sprouts or EPC’s can differentiate into mature EC’s that incorporate into neo-vessels. Although this cell type has been previously identified in an angiogenic sprout, the molecular mechanisms for differentiation remain elusive [29,30]
Fig. 1-2 The process of vasculogenesis
The process of vasculogenesis begins with induction of mesodermal precursors, which differentiate into a bipotential hemangioblast, capable of giving rise to the hematopoietic and vascular system. The angioblast lineage forms blood islands giving rise to primary vascular plexus. Subsequent angiogenesis occurs through sprouting, and intussusception. Vascular remodel and maturation occurs in an organ-specific manner through the recruitment of pericytes and vascular smooth muscle cells. Green, are molecules involved in the development of processes (red). A; arteriole, V, venule. Image from [2]
1.1.3 The Molecular Mechanism of Sprouting Angiogenesis

Sprouting angiogenesis is the formation of a new sprout from a parental vessel, eventually forming a neo-vessel. The steps of angiogenic sprout formation can further be subdivided into 3 separate phases: quiescence, activation and resolution [Fig 1-3][31-33]. During quiescence endothelial cells are protected against insults from their surrounding environment through autocrine signaling of angiopoietin (ANG-1), vascular endothelial growth factor (VEGF) and fibroblast growth factors. ECs can sense oxygen levels through hypoxia-inducible factor prolyl-4 hydroxylase (PHD2) and hypoxia inducible factor-2 alpha (HIF2α). HIF’s are transcription factors that are activated when tissue oxygen levels decrease to below 6%/40mmHg and bind to hypoxia responsive cis-elements associated with vessel growth [34]. Moreover, ECs form a monolayer of phalanx cells with tight claudins and vascular endothelial-cadherin junctions. These proteins are necessary for maintaining the restrictive permeability of endothelial cells through strong cell-cell adhesion. However, other types of junctions exist such as desmoplakin, which is also involved in adhesion. The pericytes which ensheath endothelial cells produce a common basement membrane but also generate cell survival signals such as ANG-1 and VEGF-2.

However in activation phase, the sensing of angiogenic signals such as VEGF, FGF’s, ANG-2, causes endothelial cells to acquire an activated phenotype in which pericytes detach from vessel walls and begin migrating towards the gradient of sensory signals by first degrading the basement membrane by release of proteolytic enzymes such
as matrix metalloproteinases [35]. Furthermore, endothelial cells loosen their junctions and VEGF increases the permeability of vessels. This causes the extravasation of plasma proteins which create a permissive scaffold for the proper migration of endothelial cells [36]. The migration of endothelial cells is mediated by integrin signaling. Proteolytic cleavage activates inactive angiogenic factors in the ECM, such as VEGF or FGF [37]. To prevent a mass of endothelial cells moving towards the sensing signal, the nascent vessel acquires a stalk-tip phenotype where a tip cell gets selected and becomes a filopodia rich sensor in the presence of factors such as neuropilins (NRP’s) and NOTCH ligand, delta like-5 (DLL5) [38]. The tip cells respond to environmental cues such as ephrins and semaphorins. The remaining stalk endothelial cells however elongate and proliferate pushing the sprout towards a signal stimulus [39]. They also secrete epidermal growth factor like-7 (EGFL7) to maintain the spatial organization of cells.

Eventually, endothelial cells will coalesce together and pericytes aid with the fusion with another vessel branch [40-42]. Endothelial cells acquire a stable and mature phenotype as they acquire their phalanx state phenotype and signals such as TGF-B, ephrin-B2 causes cells to become covered with pericytes [43]. Once pericytes re-establish contact with endothelial cells, induction of tissue inhibitor of metalloproteinases (TIMP) halts the proteolytic nature of tip cells. TIMPs also cause the deposition of the basement membrane and tight junctions and adherens junctions are further re-established [44]. Other cell types such as smooth muscle cells stabilize the resulting blood vessel. Subsequent blood flow to the completed mature vessel raises local oxygen levels resulting in a decrease of vascular endothelial growth factor-A (VEGFA) and the induction of endothelial cell quiescence [45].
Fig.1-3 Molecular Mechanisms of Sprouting Angiogenesis.

A) The quiescent state of endothelial cells (red) is maintained by the pro-survival autocrine signaling of a variety of factors such as VEGF, NOTCH and ANG-1. B-D (Activation phase). B) Extracellular signaling of growth factors such as VEGF or hypoxia causes the activation of endothelial cells and liberation of pericytes (green). C) The release of proteolytic enzymes such as MMPs (red spheres) causes the degradation of the basement membrane (purple line) and the extracellular matrix. Additionally, VEGF increases permeability of endothelial cells allowing serum proteins (green squares) to be laid down on the provisional matrix. D) Stalk cells undergo proliferation (circular cells) while tip cells (cells with protrusions) extend filopodia to sense their surrounding and respond to growth factor gradient. E) During the resolution phase, endothelial cells coalesce together and factors such as TGF-B, Ephrin-B2 causes the recruitment of pericytes and TIMP inhibits release of proteolytic enzymes from tip cells. It is not known whether pericytes are involved in establishing the contact between tip cells. Modified from [31]
1.1.4 Overview of Tumor Growth & Tumor Angiogenesis

The growth of a tumor is considered as deregulated and uncontrollable growth of cells in any particular tissue. The development of tumor is dependent on many environmental and hereditary factors. However, the transformation of a normal cell to a cancerous type often is due to accumulation of genetic mutations [46]. These mutations can be chromosomal aberrations such as additions, deletions or inversions. Other mutations can be simple point mutation in the genetic code. The hallmarks of cancer that collectively dictate malignant growth are as a result of manifestations that are shared by all tumors: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, limitless replicative potential, sustained angiogenesis, evasion of apoptosis or programmed cell death, and tissue invasion and metastasis [Fig 1-4] [47-49]. The updated version of these manifestation also included different forms of biological stressors such as mitotic stress, oxidative and DNA damage stress. Cancer can arise when oncogenes that are involved cell growth become activated or alternatively when tumor suppressor gene become inactivated [50]. These genes can be involved in cell death due to sensing DNA damage (i.e. p53). Many humans have cancer cells but are kept in check by immune mechanisms and often can’t induce angiogenesis. These tumors are said to acquire a dormant phenotype or regress [51]. However, an angiogenic switch to a pro-angiogenic milieu can result in the recruitment of blood vessels and subsequent development of tumors.
Fig. 1-4. The Hallmarks of Cancer
There biological capabilities, which are acquired during tumor development. The purpose of these descriptions are to rationalize the complex nature of neoplastic disease. They include proliferation signals, evasion of growth suppressors, evasion of apoptosis, sustained angiogenesis, tissue invasion and metastasis, limitless proliferative ability and different forms of biological stress. From [47]
Passive diffusion in interstitial tissue provides avascular tumors oxygen and nutrient and limits the tumor size to 1-2mm³ [52]. However, tumor beyond this size trigger angiogenesis [Fig 1-5]. Most often, the angiogenic switch is turned on as a result of local hypoxia. Activation of angiogenic switch is mediated by cytokines, oncogene activation and inactivation of tumor suppressor genes. Transcription of hypoxia related genes induced by HIFs and their over-expression has been previously correlated with poor prognosis [53-55]. The cascade of tumor angiogenesis resembles normal angiogenesis, however with some key differences. Tumor cells themselves tend to overexpress a variety of angiogenic proteins such as FGF, VEGF and ephrins [56]. The overexpression of these factors often deregulates the proper formation of blood vessels surrounding tumors causing tumor vasculature to be tortuous/leaky due to affecting the stability and proper arrangement of endothelial cells. This, however, further leads to poor delivery of oxygen and nutrients. Activated platelets further release angiogenic and permeability factors in the local environment [57,58].

In response to hypoxia, tumors tend to release a variety of ECM proteins to which foster tumor expansion and accommodates tumor growth. Tumor cells also release a range of soluble factors such as myeloid differentiation antigen-1 (Gr1), phosphatidylinositol-3 kinase (PI3K) that are involved with the mobilization of myeloid cells from bone marrow and subsequent differentiation into tumor associated macrophages [59-61]. In the context of PI3K’s, they phosphorylate various phosphoinositides which recruit various proteins to the cellular membrane involved in
cellular proliferation, differentiation and cell survival. The interaction between endothelial cells with other cell types and ECM proteins leads to their migration, survival and proliferation. Importantly, interactions between immune cells and endothelial cells or cross-talk pathways between different cell types can suppress or augment endothelial activation [62].
Fig.1-5 The Development of Tumor Angiogenesis.
Tumor angiogenesis occurs through the interaction of a variety of cell types. Tumor cells abundantly express VEGF which binds to VEGFRs. Binding to VEGFR1,2,3 causes lymphoangiogenesis, proliferation of stalk endothelial cells and recruitment of EPCs, respectively. This ultimately establishes an angiogenic environment of tumor growth and metastasis. From [60]
Eventually after the tumor grows the angiogenic environment allows tumor cells to proliferate, migrate and intravasate into the vasculature [63,64]. After intravasation, tumor cells can be suspended in the vasculature or metastasize to another distant tissue [Fig 1-6]. During this process, tumor cells extravasate into tissues to form a secondary tumor. However, it is important to note that out of all the cancer cells that metastasize only a small number of cells survive as metastasis is an inefficient process [65]. This concept suggests that there are only a few cancer cells that have the potential to make it to a secondary site. Most other cells die during the process or at the secondary site. Each phase of tumor development originating from primary tumor growth to secondary growth involves changes in the genetic program of tumor cells, potentially making them resistant to targeted therapies [66]. Metastasis is 90% of human cancer related deaths and consequently targeting the tumor vasculature is a favorable approach.
Fig 1-6. The Metastatic Process.
The metastatic process involves the growth of a primary tumor, invasion of the tumor through the stroma and ultimately intravasation into a vessel. Upon intravasation, tumor cells can travel to distant sites extravasate to another tissue. The process of a secondary site tumor formation inefficient due to cell death but cells can also remain dormant and re-activated. From [65]
1.2 EGFL7

1.2.1 EGFL7 expression by endothelial cells

Epidermal growth factor like-7 (EGFL7) also known as vascular endothelial statin was identified by 3 laboratories as a endothelial specific gene expressed during embryogenesis [67-70]. EGFL7 is a 30kDa protein that is abundantly expressed by proliferating endothelial cells during embryogenesis, physiological and pathological angiogenesis. EGFL7 transcripts are first observed in endothelial cells at the 8-cell morula stage, which are undifferentiated ball of cells contained in the zona pellucida. EGFL7 transcripts can also be observed both in the extra-embryonic and embryonic mesodermal progenitors which are precursors for endothelial cells [69]. After birth however, EGFL7 expression levels decrease but expressed in a subset of vessels within the heart, spleen, liver, lungs and heart. EGFL7 expression is also expressed in the developing post-natal retinal vascular plexus, in which sprouting angiogenesis dominates. EGFL7 in this case is localized to the basal side of stalk cells showing patchy expression in tip cells. During physiological angiogenesis however, EGFL7 becomes elevated. EGFL7 expression is elevated in the endothelium of the pregnant uterus and increased levels are observed after vascular injury including hypoxic insults[70], arterial injury [69] in coronary artery disease, solid tumors[4] or other cases of pathological angiogenesis [71-72]. EGFL7 expression has also been observed in neurons of the adult mice and primordial germs cells suggesting alternate roles in different tissues [73]. In summary, the spatial and temporal profile expression pattern of EGFL7 indicates a function of this protein in vascular formation and remodeling.
With the exception of stem cell population and neurons, EGFL7 expression remains restricted to the endothelium. All other angiogenic molecules are secreted by a diversity of cell types in the angiogenic environment. VEGF for instance is expressed by a variety of cell types such as macrophages [74], aortic smooth muscle cells [75] and epithelial cells [76]. As a matter of fact, the endothelium is a minor source of VEGF. EGFL7 however, is uniquely and abundantly secreted by endothelial cells and for this reason is an excellent marker for endothelial cells.

1.2.2 EGFL7 Gene Structure and Regulation

The mouse and human Egfl7 genes are located on chromosome 2 and 9 respectively [77]. Both human and mouse genes span a size of approximately 10kb. In humans, the Egfl7 gene comprises 10 exons, in which exons 3 to 10 are the open reading frame of the EGFL7 protein [Fig1-7]. The only ATG site of the Egfl7 gene is located at exon 3. Between exons 7-8 or alternatively intron 7, the gene houses microRNA-126. This microRNA is implicated in vascular integrity and angiogenesis [80]. miR-126 has also been implicated in cardiovascular diseases and development of tumors [78-79]. The 5.4 kb promoter fragment positioned directly upstream exon 3 contains two evolutionary conserved transcriptional start sites, TSS1 and TSS2. The upstream promoter also houses 2 non-coding exon and a partial non-coding region on exon 3. The TSS1 is situated in non-coding exon 1 and TSS2 is located just upstream of exon 3, between non-coding exon 2 and exon 3. It has been previously documented that EGFL7 expresses multiple transcripts. The promoter region contains Ets (E-twenty six) transcription factor binding sites for Erg (Ets related gene), Fli-1 (Friend leukemia integration 1 transcription factor), which have been shown to be necessary for expression of EGFL7 reporter gene in the
endothelium in adult tissues and during embryogenesis [80]. However, Ets binding sites do not contain FOX/ETS motifs, which is a composite cis-acting element that can bind to FOX:ETS transcription factors synergistically [81]. These factors transcribe VEGFR2, Notch4, tie2 genes necessary for angiogenesis. Through site-directed mutagenesis studies, it has been observed the EGFL7 promoter region also contains a GATA consensus sequence suggesting that GATA transcription factors, like GATA-2, are also involved in modulating EGFL7 expression [82]. Both ETS and GATA sites are important for endothelial development by promoting differentiation of these cells. Furthermore, Le Bras et al (2010), concluded that targeting Fli-1, Erg and GATA-2 transcription factors using siRNA reduces EGFL7 protein levels [82].
The EGFL7 Locus
The EGFL7 gene locus comprises 10 exons. The 5’end contains 2 untranslated exons (white) and two transcriptional start sites (TSS1, TSS2). The ORF of the gene is from exon 3 to exon 10 (red). Intron 7 contains the sequence for miR-126 processing.
1.2.3 EGFL7 Protein

The EGFL7 protein is a highly conserved protein among species. Recombinant and native EGFL7 protein has a mass of approximately 41Kda, after post-translation modifications such as several potential N- and O-linked glycosylation at several sites. These are sites located on the protein where sugar moieties can bind to and potentially alter their interaction in the extracellular matrix. The EGFL7 protein sequence consists of amino signal peptide, an EMI domain and 2 EGF-like domains [Fig1-8] [6]. These motifs are commonly found in many proteins secreted in the ECM, as they are often used with interaction to ECM proteins [83,84]. This is suggestive that EGFL7 has an extracellular role. One of the EGF-like domains contains a Delta-Serrate, LAG-2 (DSL) domain that binds to Notch, which is also a key regulator of angiogenesis [85,86]. However, the second EGF-like domain binds to calcium. The EMI domain of EGFL7 is found commonly in many ECM proteins and associated with regulating cell adhesion and multimerization[87]. Supporting the idea that EGFL7 is a ECM protein, immunohistochemistry of EGFL7 reveals it is a localized to organelles of the secretory pathway in over-expressing cell cultures [67, Soncin]. EGFL7 has also been found to be secreted in the supernatant of cultured cells [6, 67, 88].
Fig.1-8 The EGFL7 Protein
The EGFL7 protein comprises 4 domains. A signal peptide in the N-terminus which dictates extracellular release of the protein, a EMI domain and 2 EGF-like domains, of which the distal domain belongs to Ca²⁺ binding EGF-like domains. The presence of EGF domains further elucidate a extracellular role for EGFL7, as they are commonly found in ECM proteins.
1.2.4 Role of EGFL7 expression by endothelial cells during sprouting angiogenesis

In contrast to vasculogenesis, angiogenesis involves the formation of new blood vessels from pre-existing vasculature. The angiogenic process is a highly co-ordinated process involving multiple steps: vasodilation, endothelial cell activation and permeability, proliferation and migration, lumenization and endothelial cell survival and vascular maintenance [89]. Based on EGFL7’s role during tubulogenesis, it is safe to hypothesize a similar role during sprouting angiogenesis. To study the function of EGFL7 during sprouting angiogenesis, Schmidt et al (2007) generated two mouse models with EGFL7 deletions [88]. The first model was a retroviral gene trap inserted in intron 2, upstream of the translation initiation codon in exon 3. The second mouse line was generated by homologous recombination resulting in the removal of exon 5 to exon 7. Approximately 50% of EGFL7−/− mice died in utero and the rest of the mice developed compromised lumens with severe vascular defects. A delay in the development of the vasculature was observed in cranial, coronary and retinal areas. These phenotypes however were later resolved in the later developmental stages. This could be explained by the fact that EGFL7, an orthologue of EGFL7 was upregulated in these tissues. EGFL8 and EGFL7 both share similar domain structure and similar expression pattern with 35% protein homology [90]. The fact that zebrafish embryos never did recover, but mice did recover from EGFL7 deletions could be explained by the fact that zebrafish lack the EGFL8 paralogue [4]. To examine the cellular basis of vascular defect observed, they further examined the angiogenic sprout morphology.

During angiogenesis, tip cells are selected and sense a gradient of angiogenic signal leading the sprouting vessel. The stalk cells however, proliferate and further elongate the
sprouting tip. In wild type animals, tip and stalk cells organize themselves in a single cell layer [91]. However, EGFL7−/− mice arrange themselves in multiple cell layers, forming aggregates [88]. Immunohistochemical staining of collagen IV indicated that they were localized within the sprouting vessels in comparison to between vessels [88, 4]. This concludes that endothelial cells lacking EGFL7 cannot detect sprouting boundaries, suggestive of EGFL7 role as a boundary organizing ECM protein dictating proper organization of endothelial cells [Fig 1-9]. In support of this observation, it has been previously been observed that EGFL7 supports the adhesion of endothelial cells to the ECM  [69]. More specifically, it creates an environment where cells can attach and detach until positioned properly. When EGFL7 is missing the cells clump together endothelial cells cannot migrate and thus sprouting angiogenesis is delayed.

The Notch pathway is a highly conserved signaling pathway conserved in most multicellular organisms [92-95]. Mammals possess four different Notch receptors (NOTCH1, NOTCH2, NOTCH3 and NOTCH4). The receptor is a single pass transmembrane protein with a large extracellular portion, a single transmembrane pass, and a small intracellular region known as notch intracellular domain (NICD). The binding of notch to its five canonical ligands, Delta 1, 3, 4, or jagged1 and 2, which are also single pass transmembrane proteins, promotes proliferative signaling and angiogenesis. The specific mechanism is the translocation of the NCID into the nucleus promoting transcription of many target genes. It has been shown that EGFL7 acts to inhibit notch signaling by binding to Notch on tip cells and competing with jagged type ligands [96]. This can have important implications for the vascular system since Notch has an important role in tip-stalk cell fate determination [97-98]. High levels of Dll4 on
tip cells activate notch receptors present on adjacent stalk cells. This suppresses the
tip cell phenotype by mechanisms that remain unclear. The presence of EGFL7 therefore
acts to promote the tip cell phenotype.
**Fig1-9. EGFL7 expression by endothelial cells during sprouting angiogenesis.**
EGFL7 is expressed by endothelial tip and stalk cells during the development of a vessel. Through fluorescence studies, it has been shown that EGFL7 is localized to the surroundings of a sprout. EGFL7 seems to dictate the spatial arrangement and boundary of a sprout.
1.2.5 EGFL7 during Vasculogenesis

Evidence of the role of EGFL7 in the vasculature was presented by Parker et al (2004), in which they were interested to understand the role of EGFL7 during vascular tube formation during vasculogenesis [4]. During the process of primitive plexus formation cells do not simply assemble into tubes but form aggregates attached to each other eventually forming cord like structures, which then form a vascular lumen, also known as tubulogenesis. Over-time angioblasts gradually separate from the cords during the cord-tube transition as a result of loosening of junctions between endothelial cells [99-100]. However, in zebrafish that lack EGFL7 the process of angioblast separation is impaired and tubes cannot be formed. Thus, EGFL7 is necessary for tubulogenesis but the mechanism remains unclear. A further study by Durrans et al (2010), showed that the deletion of EGFL7 in the embryoid body model, which is a cluster of cells stem cells representing the three germ layers, resulted in the formation of abnormal endothelial cell aggregations lacking basement membrane and cell junctions [101]. In summary, EGFL7 is a key protein necessary for vasculogenesis.

1.2.6 EGFL7 and miR-126 relationship in endothelial cells

miRNAs are a class of 22 nucleotide long, non-coding RNA molecules that are important regulators of protein expression. Importantly, a single RNA molecule can regulate a repertoire of proteins globally [102]. These RNA molecules bind to target messenger RNA molecules to repress translation or to a lesser degree cause protein degradation. In recent studies, of paramount importance is the role of miR-126 in angiogenesis and tumor progression. Interestingly, miR-126 is located in intron 7 of the EGFL7 gene and is specifically expressed in the endothelial cell lineage, hematopoietic
progenitors and a number of tumors. There have been two papers studying the role of miR-126 expression in endothelial cells of zebrafish and mouse [80,103]. Loss of miR-126 causes compromised vessels with collapsed vessel lumens and impaired endothelial organization. At the molecular level, miR-126 represses (Sprouty-related, EVH1 domain-containing protein) SPRED1 and p85 (subunit of PI3K) which negatively regulate VEGF and inhibit angiogenesis. Thus, miR-126 indirectly promotes angiogenesis. The vascular effects seen in miR-126–/– mice is reminiscent of loss of EGFL7 described earlier by Schmidt et al (2010). The mice were born with 50% embryonic lethality and vascular defects with hemorrhaging, thickening or aggregated sprout morphology and delayed angiogenesis. This evidence suggests that the vascular phenotype observed with EGFL7–/– may be likely due to the loss of miR-126. To resolve this, Parker et al (2004), used a knockdown approach to lower both miR-126 and EGFL7 and compared the phenotypes. In both cases, the vessels had compromised lumens and hemorrhage was apparent. Certain distinct differences however were noted: In EGFL7 knockdown angioblasts fail to separate to form lumens but in miR-126 knockdowns lumens form but collapse later in development. The role of EGFL7 in tubulogenesis however does not reflect the function of miR-126. Two morpholinos were previously used to knockdown EGFL7 in zebrafish preventing EGFL7 mRNA translation or caused termination of translation [104]. Levels of miR-126 were not affected. The phenotypes associated with the loss of EGFL7 were rescued with injection of EGFL7 mRNA. This suggests the EGFL7 is the major player of tubulogenesis. However, potential interference with pre-mRNA formation and miR-126 maturation can occur using a morpholino. In summary, mir-126 is essential for enabling proper angiogenesis during the formation of vessel. The transcription of miR-126 seems less likely to be achieved by its own
promoter but rather be mediated by the promoter of its host gene-EGFL7 [80]. Although it has not yet been observed, it is likely that miR-126 arises from one of EGFL7's pre-mRNA transcripts. A study has shown that miR-126 expression depends on two regions with ETS binding sites located in the 5’end of the EGFL7 gene [105]. However, tissue expression of miR-126 and EGFL7 are not strictly linked in many tissue, so they may not be co-regulated [77]. Whether miR-126 expression in addition to expression of EGFL7 is connected has yet to be investigated.

1.2.7 EGFL7 expression and tumorigenesis

EGFL7 is not only expressed in endothelial cells but also abundantly expressed in a number of cancer cell lines. It’s expression is elevated in human tumors such as glioblastoma [109], hepatocellular carcinomas (HCC’s)[108], colon cancers[107], and non-small cell lung cancer[110]. In gliomas, EGFL7 expression has been correlated with increased microvascular density and cell proliferation [107]. Particularly in gliomas, EGFL7 expression is localized to tumor cells but also the vasculature surrounding it. This suggests that EGFL7 may indeed act in an autocrine, paracrine manner or through cross talk between endothelial cells. The exact mechanisms of EGFL7 cross talk between endothelial cells and tumor cells however remain to be investigated. In the HCC model however, EGFL7 knockdown resulted in decreased metastasis with reduced vascular density in comparison to control tumors, which were injected with normal cells [108]. This further supports the idea that tumor angiogenesis is regulated by EGFL7. Expression of EGFL7 in this cell line in in vitro studies further showed that down regulation of EGFL7 affected migration without changing proliferation. In another study, EGFL7 has
been positively correlated with tumor stage in colorectal cancer [109]. Specifically, EGFL7 expression was found to be highest in stage III and stage IV with increased lymph node metastasis. In glioma’s, high expression of EGFL7 has also been correlated with increased Ki-67, a marker for prognostic indicator of gliomas [109]. All together these data suggests that EGFL7 expression in tumors is correlated with poor prognosis and invasiveness of many cancer types, adding value to its potential for a therapeutic target and prognostic marker in patients.

The mechanisms by which tumor EGFL7 exerts its effect are not clearly undertood. In the case of HCC, since EGFL7 is localized to the tumor it is hypothesized that it may act in a paracrine fashion. One mechanism this may occur is through activation of Notch signaling. Inhibiting notch signaling in tumors by using a Notch 1 decoy, soluble form of notch receptor results in a decreased angiogenesis[111]. Reminiscent to this phenotype is inhibiting EGFL7, also resulting in reduced tumor angiogenesis. However, the benefits of inhibition of the Notch pathway in comparison to EGFL7 inhibition in tumor models over long-term needs to be investigated because Notch inhibition has been associated with adverse side affects [112,113]. These side affects include disorders of multiple organs and developments of vascular tumors. Given that EGFL7 over-expression in mice endothelial cells results in abnormal vessel patterning and remodeling, it is possible that abundant EGFL7 secretion by tumors in the micro-environment contributes to the irregular, leaky and tortuous vessels characterized in most tumors.

In another study by Fung et al [125], we have showed that EGFL7 however acts to inhibit tumor angiogenesis (Fig1-10). In this particular study, EGFL7 constructs were
over-expressed in an aggressive fibrosarcoma cell line, HT1080. Expression of EGFL7 resulted in inhibition of tumor growth in \textit{vivo} and had a negative effect on tumor progression from a primary site to a secondary site. After performing \textit{in vitro} and \textit{in vivo} angiogenesis assays it was observed that EGFL7 decreases angiogenesis. Interestingly, over-expression of EGFL7 increased permeability. A closer analysis of sprout morphology revealed vessels consisting of a thickened multi-layered endothelium. These data suggest that EGFL7 can also act as an inhibitor of angiogenesis in tumors, contrary to its requirement in endothelial cells during sprouting angiogenesis. The mechanism for this alternative function which is cell-context dependent seems unclear.
Fig 1-10. EGFL7 Reduces Angiogenesis in vivo
When HT1080 fibrosarcoma cells were transfected with an EGFL7 construct, HT1080 EGFL7 GFP, reduced angiogenesis was observed in the chick chorioallantoic membrane compared to control HT1080 cells and baseline untransfected HT1080 cells. This is indicative that EGFL7 acts to inhibits HT1080 tumor angiogenesis. Data was processed using Graphpad Prism software. Statistical significance was determined with the Tukey’s test, ***, p<0.001. Columns, mean; bars, SEM. The data are representative of three consecutive experiments. Study by Fung et al [125]
A recent study shows that EGFL7 is also involved in tumor growth through preventing the evasion of host immune cells to the site of the primary tumor [114]. EGFL7 overexpression in lung and breast cancer in immunocompetent mice reduced the expression of leukocyte adhesion molecules vascular adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1). This ultimately represses immune cell infiltration to the site of the primary tumor, thus escaping immune evasion.

1.2.8 EGFL7 as a therapeutic target

Given that EGFL7 is implicated in tumor angiogenesis, invasiveness and metastatic progression, EGFL7 is an ideal therapeutic target. Human clinical trials have been underway combining avastin, an anti-VEGF treatment, with anti-EGFL7 antibodies. This study has shown that human cancer xenografts treated with anti-VEGF and anti-EGFL7 results in an additional inhibition of tumor growth than anti-VEGF alone. This is expected, considering that both VEGF and EGFL7 are important players in tumor angiogenesis. It is possible that the use of anti-EGFL7 will contribute to the normalization of vasculature, given its role in vessel remodeling, and thus can extend the therapeutic window for drug delivery of avastin or other drugs. Although this study is promising, the overall long-term outcome and side effects of anti-EGFL7 treatment needs to be further investigated.
1.2.9 The role of miR-126 in tumorigenesis

In the recent past, miRNAs have been identified through transcriptional profiling and micro-array analysis as key regulators of various pathways during tumorigenesis. These microarrays reveal significant difference in expression when comparing tumor burdened tissue with non-cancerous tissue \[115\]. Recently, miR-126 has been shown to have a tumor suppressive role in a variety of cancers. In breast cancer, miR-126 is lost and patients with primary tumors characterized by low miR-126 levels displayed poor overall-metastasis free survival in comparison to patients with high levels of miR-126 \[116\]. This data suggests that miR-126 is associated with metastasis free survival, a clinical correlation that can be used to assess treatment approaches. Down-regulation of miR-126 has further been observed in cancers of stomach, lung, bladder, prostate, colon \[118-121\]. It has been reported by Liu et al (2009) that miR-126 has a role in downregulating VEGF-A, after inoculation of lung carcinoma cell lines with a lentivirus containing miR-126 \[122\]. The resulting observations were inhibition of tumors due to cell cycle arrest. Furthermore, treatment of breast cancer lines with a Src kinase inhibitor protein phosphatase 2 (PP2) caused increased levels of miR-126 with subsequently decreased migration \[123\]. In another study, Guo et al (2008). showed loss of miR-126 resulted in impaired signaling in the phosphatidylinositol (PI3K) pathway, which enabled enhanced migration of colon cancer cells \[106\]. In particular, gastric cancers with high levels of miR-126 inhibited tumor metastasis both \textit{in vitro} and \textit{in vivo}.

A recent study by Png et al (2011), showed that miR-126 expression in metastatic breast cancer cell line mediates endothelial cell recruitment \[124\]. Specifically, miR-126
expression suppresses metastatic colonization, angiogenesis and endothelial recruitment by targeting insulin-like growth factor binding protein 2 (IGFBP2), phosphatidylinositol transfer protein cytoplasmic 1 (PITPNC1) and c-Mer tyrosine receptor kinase (MERTK). These are genes associated with metastasis and are often used as a biomarker for metastasis.

The transcriptional regulation of miR-126, however with respect to EGFL7 is not completely understood. Tumors that normally have elevated levels of EGFL7 expression have low levels of miR-126. Given that miR-126 is part of EGFL7, it is likely in this case a separate mechanism drives miR-126 expression and EGFL7 expression in the 5’end promoter region of EGFL7. How mir-126 is regulated independently of EGFL7 is yet to be understood.
1.2.10 Research Motivation

The expression of EGFL7 and its role in tumor angiogenesis still remains unclear. It has been shown that EGFL7 expression is upregulated in the tumor microenvironment and associated with invasiveness, however, our group has shown that the expression of EGFL7 by aggressive fibrosarcoma tumor cells is inhibitory on angiogenesis both in vitro and in vivo. In contrast to its inhibitory effect on angiogenesis, endothelial expression of EGFL7 is rather necessary and promotes the proper orientation and spatial organization of a developing sprout during angiogenesis. This suggests that the opposing function of EGFL7 on tumor angiogenesis is cell-context dependent. Given that EGFL7 exhibits opposing functions during angiogenesis based on whether it is expressed by endothelial cells or tumor cells, I investigated how EGFL7 is differentially regulated in tumor cells in comparison to endothelial cells during tumor angiogenesis.
1.2.11 Hypothesis

The production of alternative transcripts from the EGFL7 locus in tumors mediates an inhibitory impact on tumor angiogenesis.

1.2.12 Objectives

The main objective of this work is: Determine the basis of differential effects of EGFL7 on tumor angiogenesis.

The specific objectives of this work are:

1. Characterize and map the transcripts that are produced in human endothelial cells and HT1080 tumor cells
2. Establish a knockdown model of individual transcripts produced from the EGFL7 locus in HT1080 tumor cells
3. Assess the function of EGFL7 transcripts produced in tumor cells on tumor angiogenesis
1.2.13 References


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Chapter 2

Title: The Differential Expression of EGFL7 Transcripts during Tumor Angiogenesis in Human Fibrosarcoma

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2.1 Introduction

*De novo* angiogenesis is an essential process necessary for tumor growth, its progression and a mechanism for cancer metastasis. The growth of a large tumor is often accompanied by a sustained release of angiogenic factors in the tumor microenvironment, switching the angiogenic balance favoring blood vessel formation [1,2]. This is often initiated by a hypoxic tumor microenvironment and subsequent release of growth factors that bind to quiescent endothelial cell receptors through paracrine mechanisms [3]. Upon activation, endothelial cells release MMP’s that degrade the basement membrane and surrounding extracellular membrane. Other factors such as VEGF contribute to increasing the permeability of vessels as endothelial cell loosen their junctions [4]. The release of ECM proteins by endothelial cells and tumor cells, in addition to plasma proteins, creates a permissive microenvironment for sprouting angiogenesis to occur. Endothelial cells acquire a tip-stalk phenotype, where the tip cells are filopodia rich cells directing the sprout towards the tumor and stalk cells proliferate to elongate the sprout [5]. There are many signaling pathways that are activated and coordinated in physiological angiogenesis but in the context of tumors many pathways are deregulated. More specifically, tumor cells increase VEGF and FGF secretion, contributing to sustained angiogenesis but also tortuous, leaky and irregular vasculature [6]. In the tumor microenvironment the source of ECM factors are not only restricted to tumor cells but endothelial cells and other cell types. Some of these ECM factors act in paracrine and autocrine mechanisms.
EGFL7, also known as epidermal growth factor like-7 protein, is a secreted ECM protein with an apparent mass of 30kda. EGFL7 is unique in that endothelial cells express EGFL7 and is normally not expressed in the adult vasculature [7]. However, EGFL7 becomes upregulated during angiogenesis, such as in pregnancy [8], wound healing and tumor growth [7]. In zebrafish, loss of EGFL7 has resulted in impaired tube formation with hemorrhaging [7]. Similarly, down regulation of EGFL7 in mice caused abnormal vasculature with 50% deaths in utero [9]. Furthermore, it was observed that EGFL7 functions by coordinating the proper spatial organization and adhesion of endothelial cells during sprouting [8,10]. It is therefore plausible to conclude that EGFL7 expression by endothelial cell is a requirement for proper angiogenesis.

In tumors where leaky, irregular vasculature is apparent, EGFL7 is upregulated by tumor cells. Elevated EGFL7 levels have been observed in glioma [11], hepatocellular carcinoma [12] and non-small cell lung cancer [13,14]. Increased expression of EGFL7 has been correlated with poor prognosis, increased metastasis and invasiveness of cancer cells. However, the function of EGFL7 during tumor angiogenesis remains elusive. In an in depth analysis by our group we have shown that EGFL7 expression in the human fibrosarcoma tumor inhibits angiogenesis both in vitro and in vivo using the chick chorioallantoic membrane. Importantly, we observed reduced metastasis and increased vascular permeability. After analyzing the sprout morphology through electron microscopy we were able to conclude that EGFL7 over-expression results in thickened, aggregated sprout morphology (unpublished data). Thus, we concluded that EGFL7 has an inhibitory effect on angiogenesis. EGFL7 is necessary for angiogenesis or proper sprout formation when expressed by endothelial cells, however when expressed by tumor
cells it is inhibiting angiogenesis, which interestingly is contrary to the need of vasculature for tumor growth. It seems that the location of EGFL7 expression is critical to its function. In this context, expression of EGFL7 by tumor cells is inhibiting angiogenesis, however when expressed by endothelial cells it promotes angiogenesis. Given the fact that EGFL7 in tumor cells and endothelial cells has opposing effects on angiogenesis, the question is how EGFL7 is being differentially regulated in these cell types and its impact on angiogenesis. We hypothesized the opposing phenotypes are due to the differential regulation of EGFL7. Specifically, we investigated whether there are alternative EGFL7 transcripts in fibrosarcoma tumor cells as compared to endothelial cells and its subsequent impact on tumor angiogenesis.

The EGFL7 locus comprises 10 exons spanning just over 10kb. Exon 3 through to Exon 10 is the open reading frame of the EGFL7 gene, where Exon 3 is the translation initiation site ATG [15,16]. The 5’end region upstream of exon 3 has two untranslated exons and two transcriptional start sites. It is not known what transcripts are produced from these transcriptional start sites or how these transcripts impact angiogenesis. Previous studies have shown that the 5’end regulatory region contains GATA and ETS binding sites that modulate endothelial EGFL7 expression. Importantly, miR-126, a microRNA located in intron 7 of EGFL7 is likely also regulated from the 5’end regulatory site [16]. Investigating what alternative transcripts are produced from the EGFL7 locus in tumor cells as compared to endothelial cells may also reveal whether miR-126 is processed from any of its transcripts.
2.2 Materials and Methods

Cell Culture

The human HT1080 fibrosarcoma cell line is an aggressive tumor cell line that was extracted through serial passaging through the avian embryo. These cells were maintained in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% FBS with a rate of doubling every two days. Cell passages used for experiments were between passages 10-20. Human umbilical vein endothelial cells (HUVECs) were collected as previously described and grown in endothelial growth medium (EGM) up to 4 passages and supplemented with Brain Bovine Extract (BBE). All cells were cultured at 37 °C in 5% CO₂/95% O₂. HT1080 EGFL7 over-expressing cells were generated as previously described [].

siRNA Transfections

Cells were seeded into 6 well plates at a density of 1.5 X 10⁵ cells/well and supplemented with DMEM containing 10% FBS. After one day of adhesion cells were transfected using silentfect (biorad) with 20nM of siRNA Ex5 5’CCAUCUAUAGGACCACCUAUU’3, siRNA Non-cod (targets the non-coding transcript) 5’AUCCGGCGCCGUAGGGUGUUU’3 or scramble siRNA 5’GAAGUAACACCCGCACCUAUU’3. HT1080 cells were treated for 48hrs or 72hrs after transfection with siRNA Ex5 and siRNA non-coding respectively.
**Western Blot**

Cells were lysed with lysis buffer (150mM NaCl, 0.01% NP-40, pH 7.4, 50mM Tris). Cell pellets were then re-suspended in urea buffer (10mM NaCl, 1mM DTT, 20mM Tris-HCL, 4 M urea, pH 6.0) with protease cocktail inhibitor. Protein concentrations were then measured using the Bradford assay (Bio-Rad). Primary antibodies were anti-human EGFL7 antibody (1:500, R&D Systems) and tubulin (1:20 000). Secondary horseradish peroxidase-conjugated mouse anti-goat IgG (1:10 000; Sigma) or anti-mouse (1: 20 000; GE Healthcare) were also used. To detect the presence of protein Amersham ECL plus western blotting detection system was used (GE Health Care). Positive control for experiments was HT1080 over-expressing EGFL7 cell line and negative control was scramble/negative siRNA.

**RT-PCR and qPCR**

After transfection, RNA was collected using trizol-chloroform extraction according to manufacturers instructions (Invitrogen). RNA was DNase treated and subsequently was primed with oligo-dT or random hexamers for cDNA synthesis (Invitrogen). RT-PCR cDNA was used as a template for either real-time qPCR or standard PCR (BioRad). Primers used to pickup transcripts included

Fwd TSS1: 5’GCTGCAAGGGAGGCTCTGTGT’3

Fwd TSS2: 5’GCGCGTGCGCGCCCCGGATCG’3,

Rev Ex2-4:5’AGCCCGCTGTGCCTCTTTCA’3,

Fwd TSS2Ex4: 5’GGATCCCGCGCGCGTAGGGGTG’3

Rev Ex3: 5’CCGCCCCACTGCAACACCCAGA
Real-time PCR template levels were detected using a SYBR green reagent and measured relative to GAPDH levels. Bands were gel-extracted, cloned into a pGEM-T vector (provided by Promega) and sequencing at Robarts research facility, Western university.

**Quantitative analysis of miR-126 expression**

Total RNA of cells was extracted using trizol-chloroform extraction according to manufacturer’s instruction (Invitrogen). Expression of miR-126 was analyzed using Taqman microRNA assay. RNA was reverse transcribed using a looped RT primer specific for mature miR-126 and standardized to miR-16 expression levels. Real-time PCR was then used to detect the levels of miR-126. The assay was performed in triplicate and results are of 3 independent experiments.

**In Vitro Angiogenesis assay**

*In vitro* angiogenesis/morphogenesis assay was performed as previously described (Becton Dickinson, Mississauga). Briefly, matrigel was diluted with chilled endothelial growth medium (EGM; Gibco) in a 1:2 ratio and coated onto a 96 well plate (50uL). Matrigel was then left to polymerize for 30-40 minutes at 37°C. HUVECs less then 4 passages were cultured in EGM with 5% FBS (Lonza) and plated onto the Matrigel at a density of 1.5 X 10^4 cells/well in EGM supplemented with 0.5% FBS. HT0180 transfected cells were dislodged using trypsin and co-cultured with HUVECs at a density of 7500 cells/well. Cells were incubated at 37°C in 5% CO₂/95% O₂. Wells were imaged at 2hr, 6hr and 11hr time points using an inverted phase contrast microscope at 10X magnification. Angiogenesis or endothelial tube formation was assessed by counting number of branch points in at least 10 random fields of view using Image J software.
HT1080 and HT1080 EGFL7 cells were used as positive and negative control, respectively. Each treatment was performed in quadruplets and results represent at least three independent experiments.

**Statistical Analysis**

Data was analyzed using Graphpad Prism software (Graphpad software Inc. San Diego). One way Anova with post-hoc Tukey test or student T-test using post-hoc Mann-Whitney test was used to determine significance. All columns and corresponding error bars represent the mean or standard error of the mean (SEM), respectively.

2.3 Results

**Endothelial cells express both a long and short family of transcripts originating from TSS1 and TSS2 respectively**

To determine what transcripts are produced from the EGFL7 locus, we used HUVECs, because EGFL7 expression is abundant in these cells and required for angiogenesis. Primers were designed along different regions of the EGFL7 gene according to ensemble based EST database. Here we show that endothelial cells express two families of transcripts (Fig 2-1), either a long (Fig 2-1 A-D) or short family of transcripts (Fig 2-1E,F), which originate from transcription start site 1 (TSS1) or TSS2, respectively. TSS1 based transcripts comprise a coding and non-coding transcript. Based on sequence analysis of gel-extracted bands the non-coding splice variant lacks exons 3 which contains the ATG element needed for translation. Similarly, the TSS2 family of
transcripts also comprises a coding and non-coding transcript, which lacks exon 3 and was uniquely identified using specific primers.

**Tumor cells express only TSS2 short family of transcripts and not TSS1 transcripts**

Knowing that EGFL7 is also secreted by a variety of cancer cells [11,12,13,14], I then investigated what EGFL7 transcripts are produced in a metastatic tumor cell line, human fibrosarcoma (HT1080), in comparison to endothelial cells. Using a fwd TSS2 primer with different reverse primers, showed that HT1080 cells express TSS2 originating short family of transcripts (Fig 2-2A). Interestingly, HT1080 cells do not express any long family of transcripts originating from TSS1 in comparison to endothelial cells (Fig 2-2B). After sequencing analysis and unique amplification, the short family of transcripts comprise a coding and non-coding sequence, which lacks exon 3 necessary from EGFL7 translation (Fig 2-2 C-E). The levels of coding and non-coding transcripts produced in HT1080 cells were similar, however, the overall level of EGFL7 protein produced endogenously in HT1080 cells was 2-fold less then HT1080 EGFL7 over-expressing cells (Fig 2-3).
Fig 2-1. Endothelial cells express two families of transcripts either from TSS1 or TSS2, both comprising a coding and non-coding sequence.

A) RT-PCR of transcripts in human endothelial cells originating from TSS1 with a common TSS1 forward primers (FwdTSS1) and reverse primers at exons 4 (Rev4) and 8 (Rev8). The upper band indicates a coding transcript where exon 3 is intact, whereas the lower band is representative of a non-coding transcript lacking exon 3. Transcripts were gel extracted and sequence verified using a pGEM-T vector.

B) Schematic of transcripts produced from TSS1.

C) RT-PCR that uniquely identifies the non-coding transcript in endothelial cells as part of TSS1 family of transcripts. The primers used are a Fwd TSS1 primers and a reverse primer that overlaps exon 2 and exon 4 (RevEx2-4).

D) Schematic figure of the non-coding transcript in endothelial cells with a unique reverse primer (RevEx2-4).

E) RT-PCR of transcripts originating from TSS2 using a primer set that is either specific to the coding transcript (TSS2Rev3) or the non-coding transcript (TSS2Ex4Rev5). Unique identification of transcripts are due to the presence (coding) or absence of exon 3 (non-coding).

F) Schematic of TSS2 transcripts produced from endothelial cells. All transcripts were gel extracted and sequence verified using a pGEM-T vector. Colored boxes indicate ORF exons and uncolored boxes are representative of the 5’end untranslated exons.
Fig 2-2. HT1080 tumor cells only express short family of transcripts originating from TSS2.

A) RT-PCR of TSS2 family of transcripts produced in HT1080 tumor cells using primers specific for coding (TSS2Rev3) or both coding and non-coding (TSS2Rev4). The lower band in TSS2Rev4 is indicative of the non-coding transcript based on sequencing analysis whereas the coding is of a higher molecular weight. B) RT-PCR using a fwd TSS1 primer didn’t show the presence of any transcripts in HT1080 tumor cells. C) Schematic of the TSS2 family of transcript expressed in tumor cells with fwd and reverse primers. D) RT-PCR of the non-coding transcript expressed in HT1080 tumor cells. E) Schematic of the non-coding transcript with a unique fwd primer (FwdTSS2Ex4) and a reverse primer (Rev5). All transcripts were gel extracted and sequence verified using a pGEM-T vector. Colored boxes indicate ORF exons.
Fig 2-3. Endogenous levels of transcripts produced in HT1080 cells.

A) Quantification of endogenous levels of transcripts produced in HT1080 tumor cells using qPCR. The relative expression of transcripts was detected using primers specific for each transcript and normalized to GAPDH. Results are in arbitrary units. No significant difference between coding transcript and non-coding transcript levels were detected (p=0.46). Columns, mean of three replicates, bars, SEM. Expression normalized to GAPDH. B) Western blot data of endogenous EGFL7 levels in HT1080 cells relative to HT1080 EGFL7 over-expressing cell line. Tubulin was used as a loading control.
Transcripts knockdown validation data

Once the different type of transcripts produced by tumor cells were known, I designed siRNA Ex 5 that targets exon 5 of both coding and non-coding transcripts or siRNA non-coding that uniquely targets the non-coding transcript. Knockdown is achieved after transfection of cells using a lipid-reagent. Ultimately, the transcript is repressed or degraded. siRNA Ex 5 reduced both coding and non-coding transcript levels relative to scramble siRNA (p<0.05)(Fig.2-4,A). Western blot analysis also shows that siRNA Ex 5 affects EGFL7 production due to targeting EGFL7 coding transcript, however, the non-coding siRNA doesn’t affect EGFL7 protein levels as expected (Fig2-4,B). This was further validated through qRT, which shows that the coding transcript isn’t affected by siRNA non-cod but significantly reduced by siRNA Ex 5 (p<0.05)(Fig 2-4C). The non-cod siRNA, however, significantly reduced non-coding transcript levels relative to negative siRNA (p<0.001) (Fig 2-4D).

miR-126 expression is not affected after knockdown of transcripts

Since miR-126 regulation has previously been affected by EGFL7 knockdown [125], as its regulation remains unknown despite its important role in mediating angiogenesis, I investigated whether transcripts produced from HT1080 tumor cells can affect miR-126 regulation. Briefly, mature miR-126 was reverse transcribed using stem looped a RT-primer and levels were standardized to miR-16 levels, which is a constitutively expressed gene. The expression of miR-126 is not affected knockdown of both coding and non-coding transcripts.(Fig. 2-5).
Fig 2-4. Knockdown validation of both coding and non-coding transcripts in HT080 tumor cells.

A) Real time quantification showing siRNA Ex 5 significantly lowers both non-coding and coding transcript levels compared to a negative control siRNA negative. Expression is normalized to GAPDH. * p<0.05, **p<0.01. Columns, mean of 3 replicates; bars, SEM.

B) Western blot & C) real time analysis showing siRNA Ex.5 knockdown of EGFL7 relative to siRNA Non-cod, targeting the non-coding transcript or control siRNA. Non-coding siRNA does not affect EGFL7 levels or alternatively protein coding transcript levels. **p<0.01. Columns, mean of 3 replicates; bars, SEM. Expression normalized to GAPDH.

D) siRNA non-cod significantly knocked down non-coding transcript relative to scramble. ***p<0.001. Columns, mean of 3 replicates; bars, SEM.
Fig 2-5. miR-126 levels are not affected by knockdown of both transcripts in HT1080 tumor cells.

Levels of miR-126 was quantified by qPCR after knockdown of both transcripts with siRNA Ex 5 and non-coding transcript using siRNA Non-cod. The relative expression of miR-126 was detected using a looped-RT primer which specifically detects mature miR-126 levels. Data was normalized to miR-16 standard. No significant difference between negative siRNA and siRNA Ex 5 was observed (p=0.100). Columns, mean of 3 replicates; bars, SEM. Data is representative of 3 independent experiment.
EGFL7 transcripts play a role in inhibiting tumor angiogenesis

Knowing what alternative transcripts are produced in tumor cells the next question was to understand the functional role of these transcripts during tumor angiogenesis. An in vitro angiogenesis assay was done co-culturing human endothelial cells (HUVECs) with HT1080 cells containing transcript knockdowns. Lowering levels of both transcripts in HT1080 cells resulted in significantly enhanced angiogenesis relative to HT1080 cells alone cultured with endothelial cells (p<0.05) (Fig. 2-6). As a negative control we further confirm that EGFL7 over-expression reduces angiogenesis relative to control HT1080 (p<0.05), based on previous preliminary data. In summary, this data suggests a novel role for both coding and non-coding short family of transcripts produced from TSS2 of the EGFL7 locus within HT1080 tumor, which act to inhibit angiogenesis in the tumor microenvironment.
Fig. 2-6. HT1080 TSS2 family transcripts inhibit angiogenesis *in vitro*

HUVECs (1.5 $\times$ 10$^4$ cells/well) were seeded into a 96-well plate pre-coated with matrigel and co-cultured with treated or control HT1080 cells (1.5X10$^3$ cells/well). Controls included HT1080 cells, cells over-expressing EGFL7 and negative siRNA treated cells. Treatment groups were knockdown of either both transcripts or specifically the non-coding transcript using siRNA Ex 5 or siRNA non-coding, respectively. Knockdown of both transcripts or non-coding transcripts significantly enhances angiogenesis, whereas over-expression reduced angiogenesis relative to control HT1080 cells. Below are representative phase-contrast images of endothelial tube formation on matrigel. *p<0.05 relative to HT1080 control. Columns, mean; bars, SEM. Scale Bar, (250μm). The data is representative of at least 2 independent experiments.
2.4 Discussion

In this study I show that EGFL7 is differentially regulated in tumor cells as compared to endothelial cells by producing only TSS2 family of transcripts. Importantly, this family of transcripts in tumor cells inhibits tumor angiogenesis. In endothelial cells however, both TSS1 and TSS2 families of transcripts are produced in which EGFL7 in this context is a requirement for angiogenesis. Interestingly, both families of transcripts produced 2 alternative variants of EGFL7-a coding transcript in which exon 3 is intact and a non-coding transcript lacking exon 3. The differential regulation of transcripts may be a potential mechanism for EGFL7s opposing function on angiogenesis.

EGFL7 produces 4 alternative transcripts that are different by family based on the origin of transcription at the 5’ end or intra-family variability is observed by the splicing or inclusion of exon 3. In the context of intra family variability, tumor cells produce TSS2 family of transcripts, one transcript that is a protein coding sequence and the other a non-coding processed transcript. Factors determining what exons are spliced or included during splicing, can result in a repertoire of proteins from a single gene with multiple functions[18,19]. Moreover, tissue dependent splicing patterns are regulated by many trans-acting factors or cis-acting RNA sequence motifs [17]. In the context of splicing regulations, EGFL7 exon3 exclusion results in a non-coding sequence with no protein being produced, since it is the ORF of EGFL7. The generation of a non-coding RNA, which are often greater than 200 nucleotides, are crucial in global gene regulation and protein function [20,21,22,23]. For example, HOTAIR long-noncoding RNA
(lncRNA) expression is increased in breast tumors and its over-expression in non-invasive cell lines enhanced metastasis and invasiveness [24,25]. This is due to its ability to cause genome-wide changes in methylation patterns in many tumor suppressor genes. It is therefore suggested that HOTAIR can serve as a diagnostic bio-marker for cancer patients. However, this is the first study to show a role of lncRNA expressed in tumor cells that inhibits angiogenesis. The mechanism of anti-angiogenic activity of TSS2-non-coding transcript in tumor cells thus, may be mediated by causing chromatin modifications of pro-angiogenic genes, repressing transcriptional activity. This can be further studied using chromatin immunoprecipitation studies.

EGFL7 transcripts produced in HT1080 tumor cells only are TSS2 family of transcripts and lack TSS1 transcripts, whereas endothelial cells express both families. These families of transcripts are different because there are transcribed from different transcriptional start sites, thus they have different 5’UTRs, where TSS1 is a longer transcript than TSS2. However, all transcripts contain the same ORF. Given this finding, it is possible that the opposing role of EGFL7 in these cell types stems from differential expression of these transcripts due a novel method of 5’UTR regulation. For instance, it is possible that TSS2 transcripts have naturally an inhibitory role on angiogenesis as was the case in tumors, but in endothelial cells this inhibitory function is repressed. The structure of the 5’ UTR in endothelial cells may be different, docking endothelial specific inhibitory molecules that repress translation[26]. For instance, the cytochrome P450 1B1 mRNA involved in metabolism is not abundantly expressed in tumor tissue as compared to normal hepatic tissue [27]. After molecular analysis, it was shown that cytochrome
P450 1B1 in tumors is repressed due to the nature of its complex 5’UTR. The 5’UTR was a site for miRNA-27b to bind, repressing translation. In addition, the 5’UTR contained a uORF and mutations in this site promoted the translation of the mRNA due to lack of inhibition by 5’UTR proteins. In the context of endothelial cells, TSS1 may house upstream open reading frames (uORFs) in the 5’UTR that may produce a protein with an angiogenic promoting function. For instance, the transforming growth factor beta-3 gene (TGF-B3), which plays an important role in tumorigenesis and other diverse processes, produced two transcripts, 2.6kb and 3.5kb in length [25]. The 3.6 kb transcript has a longer 5’UTR with 11 uORFs, whereas the 2.6 kb has 2 uORFs. The longer transcript is translationally inhibited in breast cancer cell lines and other cancerous tissue but the shorter one is translationally active in breast cancer cell lines. Importantly, Signori et al (2010) showed that decreased expression of breast cancer 1 (BRCA1) associated with breast cancer is as a result of a point mutation decreasing translation efficiency [25]. It is possible, that TSS2 family of transcripts harbor 5’UTR mutations in HT1080 tumor cells making them more efficient for translation and subsequent inhibition of angiogenesis, where they should normally be repressed. Understanding the precise mechanism of this differential expression of transcripts and the reason for exclusive expression of TSS2 transcripts in tumor cells may provide insights on tumor development in the context of angiogenesis.

The knockdown of TSS2 based coding transcript, which is translated to EGFL7 protein, also resulted in enhanced angiogenesis, suggesting an inhibitory role for TSS2 transcripts in HT1080 cells. This begs the question as to why HT1080 cancer cells, which are highly metastatic, express EGFL7 transcripts that inhibits angiogenesis-a process that
favors tumor growth. One potential explanation for this is that tumor cells may want to
generate a hypoxic microenvironment which permits cancer cell to become more
invasive, enabling migration and potential metastasis to a secondary site [28,29]. In
contrast, a reduction in vasculature in tumors can also permit a dormancy of tumor cells,
ensuring growth advantage during more ideal conditions [30]. Competing suppression of
antiangiogenic factors with pro-angiogenic factors in primary and metastatic tumors
maintains a balance between apoptosis and proliferation. In mice, the expression of anti-
angiogenic inhibitor, angiostatin, resulted in a dormant state of fibrosarcoma tumor cells
but once the inhibitor was removed, tumors experienced rapid growth [31]. According to
Fung et al. [32] another possible explanation of reduced angiogenesis due to EGFL7 is
that too much EGFL7 in the micro-environment produced by endothelial cells and tumor
cells can deregulate proper assembly of endothelial cells, therefore inhibiting
angiogenesis. Too much EGFL7 can potentially cause endothelial cells to improperly
detect sprouting boundaries and cause cells to clump on one another, resulting in a
compromised vessel.

Due to previous attempts to knockdown or knockout EGFL7 resulted in changes
in miR-126 levels [9], it is important to confirm that the results are not due to major
alteration in miR-126 levels. There was no change in expression of miR-126 after
knockdown of both TSS2 transcripts in tumors as expected, since miR-126 is generated
from a pre-mRNA and thus alterations in mature mRNA shouldn’t affect levels. This
said, it might be possible that EGFL7 transcripts are associated with miR-126 processing
at the pre-mRNA level.
2.5 Conclusion

In summary, this data identifies novel transcripts produced from the EGFL7 locus that are differentially expressed in tumor cells as compared to endothelial cells. It is possible that this differential expression stems from differences in regulation at the 5’UTR of TSS1 and TSS2 transcripts. Importantly, we identify TSS2 coding and non-coding transcripts as inhibitors of angiogenesis in tumor cells. Further insight as to the mechanism of this differential regulation may shed light on understanding EGFL7 function and regulation during angiogenesis.

2.6 Acknowledgements

Laura Fung assisted with teaching the in vitro angiogenesis and miR-assay, in addition to performing in vivo angiogenesis assay (Fig 1-10). This work was supported by CIHR-STP, TBCRU and OGS funding sources.
2.7 References


32. Fung Laura, Amber Ablack, Desmond Pink, John D. Lewis. EGFL7 is a potent endogenous inhibitor of tumor angiogenesis [abstract]. In proceedings of the 102 Annual Meeting of the American Association of Cancer Research; 2011 April 2-6; Orlando, Florida. AACR;2011 p.455 Abstract nr 5146
Chapter 3: Conclusions, Strengths and Limitations

3.1 Summary

Tumor angiogenesis is a necessary process for tumor growth and a mechanism for cancer cells to metastasize. There are many signaling cascades in the angiogenic environment. Tumor cells secrete factors that act in autocrine fashion or paracrine mechanisms, interacting with endothelial cells of the vasculature. Importantly, in tumors however, the tight regulation of proper angiogenesis seems to be lost due to abundant expression of factors such as VEGF or inhibitory molecules. This accounts for why the tumor vasculature appear irregular and tortuous. Understanding how different signaling pathways are regulated may shed light on therapeutic possibilities and a better grasp of tumor biology. Regulation of signaling molecules can be mediated at many levels such as transcriptionally, translationally or even modifications in the protein structure in the ECM.

In this study we have identified a novel method of differential regulation for EGFL7 through the production of alternative transcripts that may explain why EGFL7 is inhibitory in tumor cells. Interestingly, the TSS2 family of transcripts produced in tumor cells, including the non-coding transcript, has an inhibitory role on tumor angiogenesis. Understanding how both non-coding and coding transcripts mediates their effects on inhibiting angiogenesis will provide further insight into EGFL7 biology, transcriptional regulation and presumably miR-126 processing.
**Objective 1:** Characterize and map the specific transcripts that are produced in endothelial and HT1080 tumor cells:

In Chapter 2, I demonstrated that there is differential expression of transcripts in HT1080 tumor cells in comparison to endothelial cells. It was determined that in endothelial cells, the EGFL7 locus transcribes 2 families of transcripts-TSS1 and TSS2. Both families comprise a non-coding and coding transcript, based on the splicing or inclusion of exon 3, respectively. I further show that in tumor cells, however, only TSS2 transcripts are transcribed. The differential regulation of EGFL7 is therefore cell-context dependent and could be potential reason as to why EGFL7 is inhibitory in tumor cell, whereas it promotes angiogenesis in endothelial cells.

**Objective 2:** Establish a knockdown model for individual transcripts transcribed from the EGFL7 locus in HT1080 tumor cells

I also further show knockdown of TSS2 transcripts in tumor cells. After cloning transcripts into a pGEM-T vector for sequencing, siRNAs were designed to effectively knockdown transcripts. I showed the successful knockdown of the non-coding transcript and that EGFL7 protein levels are not affected. Moreover, siRNA mutually present exon 5 in both transcripts resulted in lowered EGFL7 levels. This is as a result of knocking down the coding version which translates to EGFL7 protein.
Objective 3: Assess the function of EGFL7 transcripts produced in HT1080 cells on tumor angiogenesis

In this *in vitro* angiogenesis assay, knockdown of both TSS2 transcripts, coding and non-coding, results in enhanced tumor angiogenesis. This suggests that endogenous TSS2 transcripts produced by HT1080 cells is inhibitory in nature. Based on sprout morphology, there was no striking difference observed in transcript knockdowns. This study further concludes that the non-coding transcript has an inhibitory role on angiogenesis. Exactly how a lncRNA mediates this affect remains to be investigated.

3.2 Strengths

The study is the first to examine what EGFL7 transcripts are produced and how these transcripts affect tumor angiogenesis. The role of EGFL7 protein in tumor angiogenesis remains elusive to date but this study has opened the window to understand EGFL7 biology further in terms of transcriptional regulation and how this may affect its function. Specifically, it would be interesting to understand how TSS2 family of transcripts presumes an inhibitory role in tumor cells, yet in endothelial cell where EGFL7 induces angiogenesis, TSS2 is still transcribed. One way to explore this is to over-express TSS1 transcripts in HT1080 cells and observe how TSS2 transcripts are affected or alternatively, whether a pro-angiogenic phenotype is observed during angiogenesis. Moreover, this study examines the identification of a lncRNA involved in angiogenesis. These families of non-coding RNA have global roles in gene and protein
regulation and are becoming increasingly important in tumor biology. Understanding how TSS2 lncRNA is mediating an inhibitory function on angiogenesis can enable a better understanding of the regulatory network of lncRNA on tumor angiogenesis. The role TSS2 lncRNA on inhibition of angiogenesis can be mediated by regulating other pro-angiogenic genes through chromatin modification such as deacetylation methylation or transcriptional interference. It is also possible that this lncRNA may directly bind to proteins necessary for angiogenesis.

### 3.3 Limitations

One limitation in this study is the lack of understanding of the role of TSS2 transcripts on tumor angiogenesis in vivo, using the chick CAM. The HT1080 tumor cells on the chick CAM need to be incubated for approximately 4 days to observe its effect on angiogenesis. Using an siRNA for this period of time may be ineffective, as transcript levels may rise to normal levels. However, a future in vivo study would be to use shRNA to have stable knockdowns of transcripts rather than transient knockdowns. Furthermore, in order to better elucidate the role of TSS2 transcripts as inhibitory, it would have been interesting to over-express these transcripts in endothelial cells to see whether an inhibitory function on angiogenesis is acquired. A good future study would be to design constructs harboring TSS2-family transcripts and transfecting these transcripts in HUVECs or a cell-type that induces angiogenesis. Additionally, due to siRNA optimization or design issues, it wasn’t possible to knockdown the TSS2 coding transcript specifically without affecting the non-coding transcript. Putative siRNA’s targeting exon 3, unique to the coding transcript, resulted in no knockdown. Therefore,
optimal designs of siRNAs targeting exon 3 of the coding transcript would be ideal to further prove that TSS2 coding transcript expressed in tumor cells is inhibitory.

Additionally, this study saw no changes in miR-126 levels because of using siRNAs which knocks down mature mRNA not pre-mRNA, where most miRNAs are processed from. Examining the effect of TSS2 family transcript knockdowns at the pre-mRNA level may elucidate whether TSS2 transcripts regulate miR-126 processing.

A major limitation to this study also includes the use of one cell line. Although we observed an anti-angiogenic phenotype in HT1080 fibrosarcoma cell line, it is possible that other cell types may result in a pro-angiogenic phenotype. This could be due to the cell-specific nature of transcriptional regulation of EGFL7 or specific mechanism of alternative splicing of EGFL7.

3.4 Future Considerations

To understand the differential basis of EGFL7 expression of transcripts biochemically, future studies understanding what transcription factors mediate alternative transcription will be important. For instance, over-expression of transcription factors and association with production of transcripts will be necessary in the HT1080 fibrosarcoma cell line. Importantly, the significance of non-coding EGFL7 transcripts on as an inhibitor or angiogenesis needs to be elucidated through future chromatin immunoprecipitation studies. The role of TSS1 transcripts in promoting angiogenesis can further be identified by its over-expression in HT1080 cell line, since it is not expressed. It would be interesting to see whether over-expression of this transcripts results in enhanced angiogenesis. Overall, differential transcript expression can vary from cell to cell, so
understanding the relevance of transcripts on angiogenesis in different tumor cell lines can pave the way for diagnostic and therapeutic approaches.

3.5 Concluding Remarks

The role of EGFL7 during tumor angiogenesis is yet not completely known. EGFL7 can act as an inhibitory protein when expressed by HT1080 cells, yet it promotes angiogenesis when expressed by endothelial cells. In our case, the differential regulation of EGFL7 mediating opposing effects on angiogenesis is cell-context dependent. Specifically, the presence of alternative transcripts present in tumor cells may dictate inhibitory function of EGFL7 on tumor angiogenesis. Precisely, understanding how EGFL7 transcription in tumor cells meditate an inhibitory effect on angiogenesis during tumor development, may yield insight in the transcriptional regulation of EGFL7 and its potential use as a diagnostic and therapeutic target.
Navid Baktash

Education:

Sep 2010-Present  -University of Western Ontario, London, Ontario
- MSc in Medical Biophysics, Molecular Imaging
to be earned on August 2012
- Thesis: Differential regulation of EGFL7 and miR-126 during tumor
angiogenesis (Publishing at end of MSc)

Sep 2005- April 2010  -University of Western Ontario, London, Ontario
- Bachelor’s of Medical Science (BMSc), Honors Basic Medical Science
  earned June 2010
- Thesis: Characterizing the effects of statins on the rat fed with a high
  cholesterol diet

Research Experience

May 2010-August 2010
Summer student, London Regional Cancer Program, London, Ontario

- Use of short hairpin RNA’s to knockdown proteins involved in angiogenesis in
  fibrosarcoma cancer cells
- Learned experimental methodology, such as, tissue culturing, imaging, cloning,
  angiogenesis and scratch-wound assays
- Assisted a MSc student with determining the role of confluency on specific mRNA
  expression profiles in fibrosarcoma cells

September 2008-April 2009
Honors Research Project, Department of Medical Science’s, London, Ontario

- Seminar classes on atherosclerosis and aortic valve stenosis from an integrative and
  cross-disciplinary perspective
- Induced atherosclerosis through a high cholesterol diet and examined the efficacy of
  statins on cholesterol reduction
- Conducted imaging, biochemical and histological analysis of the liver, aorta and blood
May 2007- August 2007  
Summer Research Project, Robarts Research Institute, University of Western Ontario, London, Ontario  
- Investigated the role of FRS-2 in the regulation of proliferation versus differentiation in neurons  
- Learned a variety of techniques in cell biology and biochemistry such as protein assays

May 2006-August 2006  
Summer Research Project, The Hospital for Sick Children, Toronto, Ontario  
- Project involved differentiating teratocarcinoma cancer cells into a neuronal phenotype using various compounds to test the efficacy of differentiation therapy in cancer  
- Investigated neuroblastoma angiogenesis and growth factors that contribute to angiogenesis

Jan 2003- August 2003  
Co-op student at Mount Sinai Hospital Pathology Laboratories, Toronto, Ontario  
- Obtained specimens from the operating room and assisted the pathologist assistant with making cassettes for specimens  
- Explored the gross anatomy of diseased tissue, how they present and their morphological appearance  
- Observed how biopsies of such specimens are examined both in the laboratory and histologically for diagnostics  
- Trained on how cancer grading and staging are assigned

Oral Presentations

Feb/3/2011  
Medical Biophysics Seminar, UWO  
- Baktash, N., Ablack, A., Lewis, J. “Characterizing the role of Epidermal Growth Factor like 7 during tumor angiogenesis”

March/6/2010  
Translational Models of Cancer Course, University of Western Ontario, London, Ontario  

Feb/8/2009  
Cardiovascular Physiology Course, University of Western Ontario, London, Ontario  
connexin43 during electrical uncoupling induced by ischemia. *Circulation Research*, 87(8), 656-662.

**Poster Presentations**

**Feb/2011**
- Baktash, N., Ablack, A., Lewis, J. Elucidating the function of EGFL7 independent of miR126 during tumor angiogenesis. “Elucidating the function of EGFL7 independent of miR-126 during tumor angiogenesis.” *Oncology Research and Education Day* (Submitted and accepted for presentation)

**April/2011**
- Baktash, N., Fung, L., Pink D., Ablack A., Lewis. J. "EGL7 is potent endogenous inhibitor of tumor angiogenesis". *Annual Association of Cancer Research Conference, Orlando Florida (AACR)*

**Community Volunteer Experience**

**Sep 2009-Present**
Women’s Community Shelter
- Entertain and organize activities for children living at a shelter
- Take initiative in being a positive male role model for the children who may have been part of male dominated family abuse

**Oct 2007-Present**
London Regional District Distress Center Helpline, London, Ontario
- Assist callers facing various forms of stress, ranging from depression, loneliness, suicidal ideation and abuse in London or surrounding areas
- Train new volunteers in handling phone conversations
- Participate in various information sessions with community services that collaborate with the Distress Center
- Usually a 16hrs/month minimum requirement is necessary

**Aug 2011-Sep 2011**
TBCRU Paddling for a Cure
- A participant in a one day event raise funds and awareness for breast cancer through dragon boat racing competition
- Also a chance to meet breast cancer survivor’s
May 2009-August 2009
Sick Children’s Hospital Cancer Ward

- Assisted children and families at the cancer ward during my summer research position to facilitate my understanding of the importance of research to the clinical and bedside setting

May 2007-2009
University Hospital Intensive Care Unit & Emergency Department

- Provided a caring and guiding environment between patients and families
- Took leadership in keeping families updated with patient status and communicating information to physicians and nurses

Professional Associations

April 2011-Present
CIHR-STP Speaker Committee
- Member of the selection committee for selecting CaRTT speakers for talks at LRCP

Feb 2011- Present
Molecular Imaging Member
- Gained Acceptance into the program in september
- The molecular imaging program comprises of teaching students about applying science principles to molecular imaging techniques
- Mandatory to take a molecular imaging course, attend presentations and monthly talks

Aug 2005-June 2010
Physicians for Social Responsibility Member
- Participate in this national committee by writing letters to government authorities regarding policies that may effect or benefit health which may include pollution policies or environmental bills

Certifications

2010
Applied Suicide Intervention Skills Training (ASIST)
- Certificate of a two day training courses in suicide intervention

2010
Global Appraisal of Individual Needs (GAIN) Short Screener
• Participated in the evaluation of a new clinical short screener at the developed from a research article on screening individuals facing addiction

2009
Distress Centre appreciation Certificate for 358 hours of service by the end of 2009

2009
Lab Animal Monitoring, Assessment and Intervention Advanced Rat Training

2000-2003
Alliance Francais School of French Completion Certification

Honors and Awards

2011
Ontario Graduate Scholarship (OGS) Recipient
• OGS is given to student who demonstrate strong academic background and a strong potential for research during graduate school $5,000 per term

2011
Molecular Imaging Travel Grant
• Travel grant for students in the molecular imaging program $1000

2010
Recipient of CIHR Cancer Research Technology Transfer (CaRTT) Scholarship $25,000
• CIHR award given to students to broaden their cancer research experience by providing a training program in cancer biology and method of scientific communication transfer to the private/public sectors

2010
Recipient of Translational Breast Cancer Research $22,138
• To encourage trainees to conduct research on breast cancer and encourage participation in seminars, conferences and public presentations

2010
Schulich School of Medicine and Dentistry Graduate Scholarship $7,140
• Given to students with a strong academic average

2010
Outstanding Community Contribution Award
• Awards given to students who have outstanding leadership roles in the London community

2010
Dean’s Honor Roll
• Award for academic excellence

2009
Dean’s Honor Roll
• Award for academic excellence

References available upon request