EGFL7 inhibits tumor angiogenesis via its Emilin-like domain

by

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Abstract

Epidermal Growth Factor-like domain 7 (EGFL7) is a secreted angiogenic factor that is almost exclusively expressed by endothelial cells. High EGFL7 expression occurs during physiological angiogenesis, under pathological conditions, and in solid tumor angiogenesis. Evidence suggests that EGFL7 expression is elevated in several human tumors, such as glioma, hepatocellular carcinoma, and colon cancer. EGFL7 is composed of three domains: an Emilin-like (EMI) domain, two Epidermal Growth Factor-like (EGF) repeats and a Matrilin-like (MAT) domain. While EGFL7 is a known regulator of physiological angiogenesis, little is known about its function in tumors. In this study, we attempted to clarify EGFL7's role in the regulation of tumor angiogenesis and its mechanism of action.

EGFL7 was stably expressed in HT1080 fibrosarcoma cells as either full length protein or as a mutant with one domain deleted per cell line (HT1080 EGFL7ΔEMI, HT1080 EGFL7ΔEGF and HT1080 EGFL7ΔMat). Cell proliferation was assessed using an MTT-based proliferation assay. A tumor growth assay and two angiogenesis assays were carried out to elucidate the effect of EGFL7 and its domains on angiogenic sprouting in tumors. Contrary to expectation, the cancer-specific expression of EGFL7 in fibrosarcoma cells led to the suppression of tumor growth *in vivo*. Importantly, there was no significant difference in their ability to proliferate *in vitro*. Both *in vitro* and *ex ovo* angiogenesis assay showed significant reduction in the vascular density for the

EGFL7 expressing cells. Interestingly, both the reduction in tumor size and inhibition of angiogenesis were dependent upon the presence of the Emilin-like domain in EGFL7.

To study EGFL7's mechanism of action, EGFL7 from tumor cell lysates was immunoprecipitated using antibody coated protein-A beads and co-precipitated proteins were identified using western blotting. Integrin β_1 and Thrombospondin-1 were discovered to be interacting partners for EGFL7 using this method.

Finally, extracellular vesicles (EVs) were isolated from enriched medium of HT1080 cells (Control, EGFL7 and deletion mutants) and analysed using flow cytometry, western blotting, TEM, and confocal imaging. EGFL7 was observed to be secreted via EVs from EGFL7-expressing cancer cells, as confirmed by the TEM imaging. Interestingly, deleting the EGF-like domain significantly reduced the number of EGFL7-expressing EVs that were released from cancer cells.

To summarize, EGFL7 is a potent inhibitor of tumor angiogenesis and its anti-angiogenic phenotype is medium ted via the Emilin-like domain. The EGF-like domain is also functional as it regulates the EV-mediated secretion of EGFL7 from cancer cells. Additionally, two important angiogenic mediators, Integrin β_1 and Thrombospondin-1, were identified as protein interactors of the EGFL7 protein and may be involved in EGFL7's inhibition of tumor angiogenesis.

Preface

Some of the research conducted for this thesis forms part of a research collaboration led by Dr. John Lewis at the University of Alberta, with Dr. Len Luyt at the University of Western Ontario and Dr. Nicole Steinmetz at Case Western Reserve University.

Figure 3.9 from chapter 3 of this thesis has been published in Cho CF, Yu L, Nsiama TK, Kadam AN, Raturi A, Shukla S, Amadei GA, Steinmetz NF, Luyt LG, Lewis JD, “Viral nanoparticles decorated with novel EGFL7 ligands enable intravital imaging of tumor neovasculature,” *Nanoscale*. 2017 Aug 24;9(33):12096-12109.

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Finally, I thank my parents, family and friends for the continuous love and support that they have shown me throughout my academic journey. My mum and dad sacrificed a lot to be able to support me, and I am forever grateful for that.

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LIST OF ABBREVIATIONS

µL	microliter
µM	micromolar
3D	3 Dimensional
AF-647	Alexa Fleur 647
ANG	Angiopoeitin
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
CAM	Chorioallantoic Membrane
CD	Cluster of Differentiation
CO_2	Carbon Dioxide
DMEM	Dulbecco's Minimum Essential Medium
DNA	Deoxyribonucleic Acid
DSL2	Delta/Serrate/LAG-2
DTSSP	3,3'-Dithiobis(sulfosuccinimidylpropionate)
E	Embryonic
EC	Endothelial Cells
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFL7	Epidermal Growth Factor-like 7
EGFR	Epidermal Growth Factor Receptor
EMI	Emilin
EpCAM	Epithelial Cell Adhesion Molecule
ESCRT	Endosomal Sorting Complexes Required for Transport
EV	Extracellular Vesicles
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor

FITC	Fluorescein Isothiocyanate
HCC	Hepatocellular Carcinoma
HIF	Hypoxia Inducible Factor
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM	Intercellular Adhesion Molecular
IL	Interleukin
INF	Interferons
IP	Immunoprecipitation
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISEV	International Society of Extracellular Vesicles
KD	Knocked Down
LOX	Lysyl Oxidase
MAT	Matrilin
mFOLFOX6	Modified 5-Fluorouracil, Leucovorin, and Oxaliplatin
MHC	Major Histocompatibility Complex
miRNA	Micro Ribonucleic Acid
MMP	Matrix Metalloproteinase
mM	milimolar
mRNA	Messenger Ribonucleic Acid
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
MVB	Multivesicular Bodies
NK cells	Natural Killer cells
nM	nanomolar
NRP	Neutropilin
PBS	Phosphate Buffered Saline
PC	Pericyte
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PlGF	Placental Growth Factor

RGD	L-arginine, glycine, and L-aspartic acid
RNA	Ribonucleic acid
SCID	Severe Combined Immunodeficiency
SDS	Sodium Dodecyl Sulphate
SF21	*Spodoptera Frugiperda* 21
shRNA	Short Hairpin Ribonucleic Acid
siRNA	Small Interfering Ribonucleic Acid
SMC	Smooth Muscle Cells
TBS	Tris Buffered Saline
TEM	Transmission Electron Microscopy
TIMP	Tissue Inhibitor of Metalloproteinases
TGF	Transforming Growth Factor
TKI	Tyrosine Kinase Inhibitor
TNF	Tumor Necrosis Factor
TSP-1/2	Thrombospondin-1/2
VEGF	Vascular Endothelial Growth Factor

Chapter 1: Introduction

1.1 Vascular System

The vascular system is composed of vessels that circulate blood and lymph throughout the body, its importance is demonstrated by its early appearance in vertebrate development. Diffusion by itself, as seen in invertebrates, is not adequate for providing oxygen and nutrient delivery that is required in the complex vertebrate morphology[1].

The blood vessels of the vertebrate vascular system transport nutrients, oxygen, carbon dioxide, hormones and waste matter to and from all the cells in the body[2,3]. This network of blood vessels is required for the development and maintenance of all tissues and organs. The process of vascular development begins in the embryo and consists of two methods: Vasculogenesis and Angiogenesis[4] (Fig 1.1).

In the embryo, mesoderm-derived angioblasts, the precursors of endothelial cell and smooth muscle cells, assemble to form new vessels *de novo* that differentiate into a primitive vascular plexus. This process is known as vasculogenesis[3,4]. Vasculogenesis also involves the elongation, division and maturation of the primitive vascular plexus into a three-dimensional structure containing arterial and venous axes[5]. The sprouting of new blood vessels from this pre-existing labyrinth into a network of mature arteries and veins is known as angiogenesis[6–8].

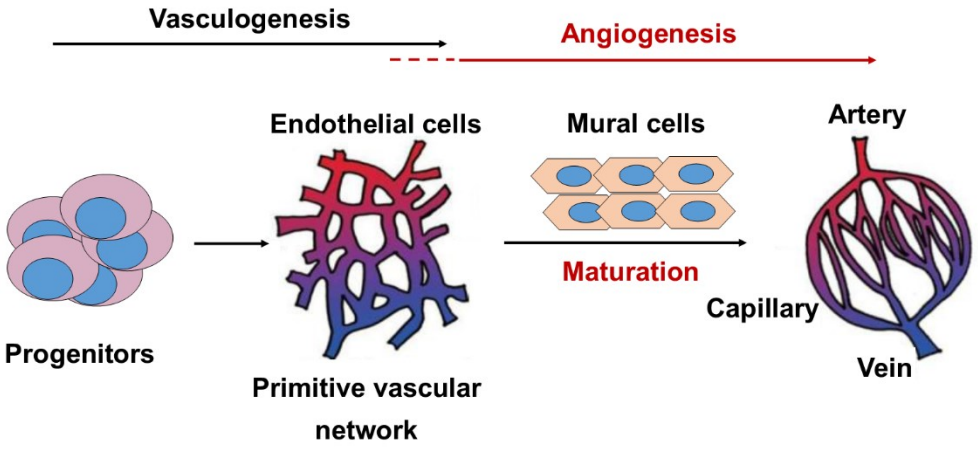


Figure 1.1: Schematic representation of vascular network formation. The process of vascular growth is regulated by vasculogenesis and angiogenesis. During vasculogenesis, angioblasts assemble to form new vessels *de novo* that differentiate into a primitive vascular plexus. New sprouts emerge and are strengthened and remodelled during angiogenesis to form a complex, tightly regulated vascular network. Adapted from Takuwa *et al*, 2010

1.1.1 Overview of vasculogenesis

Risau *et al* first coined the term 'vasculogenesis' for the process of blood vessel formation at its early developmental stage[9].

The embryonic mesoderm, allantois, and placenta are sources of vascular endothelial progenitor cells. The progenitor cells, termed angioblasts, are first observed in the extra-embryonic tissues followed by development within the embryos at embryonic day 6.5-7[2,9]. Angioblasts associate to form primitive tube-like vessels after multiple rounds of proliferation and migration (Fig 1.1).

Angioblasts eventually differentiate into endothelial cells that form a vascular lumen and deposit a basal lamina. In the embryo, the endothelial cells differentiate to form a primitive vascular plexus, with capillaries forming in the endocardium and head mesenchyme. Before the initiation of a heartbeat, the primitive vascular plexus attaches to the developing heart tube[2].

The molecular mechanisms of vasculogenesis are not as well understood as angiogenesis, but a number of growth factors such as members of the Fibroblast growth factor (FGF) family, the hedgehog family, and the vascular endothelial growth factor (VEGF) family are known to play significant roles[5].

Vasculogenesis not only establishes the primary vascular plexus, but also mediates vascularization in several organs (e.g. liver and lungs)[10]. Vasculogenesis also plays a role in capillary formation following ischemic injury in adults[11,12].

1.1.2 Overview of angiogenesis

De novo angiogenesis is defined as the formation of new blood vessels from pre-existing blood vessels[7,13]. in the embryo, the process of vasculogenesis establishes a primitive vascular labyrinth that is expanded and remodelled during angiogenesis[13]. Angiogenesis leads to the formation of a complex network of larger arteries and veins branching into smaller capillaries.

The process of angiogenesis involves two distinct mechanisms known as intussusception (or splitting, Fig 1.3)) and sprouting[14] (Fig 1.2). Quiescent endothelial cells (EC) become activated when angiogenic signals bind to the EC receptors during sprouting angiogenesis. These angiogenic signals destabilize the contact between ECs and the ECM causing the endothelial cells to proliferate and migrate towards the signal gradient in a characteristic stalk-tip cell formation[15,16] (Figure 1.1). Along the way, ECs also secrete proteolytic enzymes to degrade the surrounding ECM[17]. ECM degradation also releases matrix-bound angiogenic growth factors, such as VEGF and transforming growth factor-β (TGF-β)[18]. The migrating ECs eventually merge to form tubes in a process known as tubulogenesis. Pericytes (PC) and smooth muscle cells (SMC) are recruited to stabilize these endothelial cell tubes[15–17] (Fig 1.2).

During intussusceptive angiogenesis, endothelial cells within a capillary fuse together to form a transluminal tissue pillar splitting the lumen into two. Intussusception allows for expansion of the vasculature via the reorganization of existing cells. This is critical in embryonic development as there are limited resources available[15,19] (Fig 1.3).

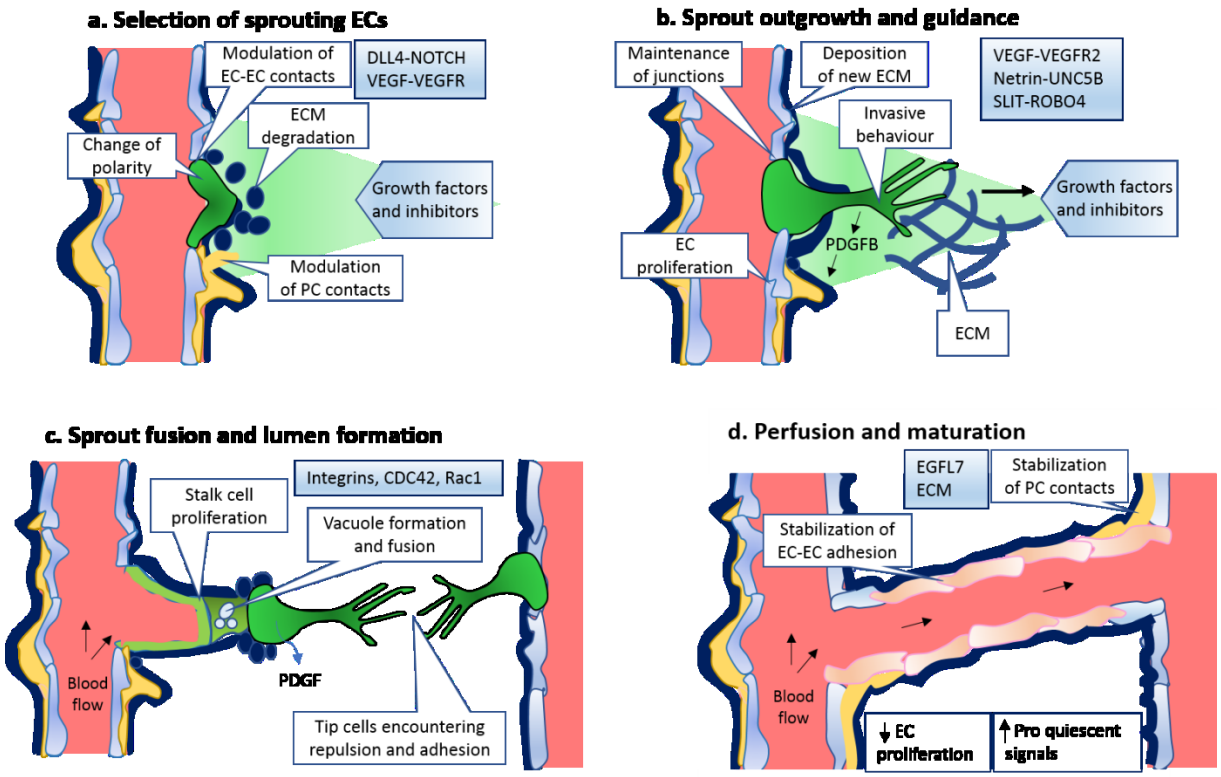


Figure 1.2: Steps in sprouting angiogenesis. ECs loosen their contacts with adjacent endothelial and mural cells and degrade the surrounding matrix. One EC is selected as the tip cell to lead the sprout in the direction of the angiogenic signal gradient. Myeloid bridge cells help in the fusion with another vessel leading to the initiation of blood flow in the final resolution stage. Pericytes form a layer around the new vessel to stabilize it.

Adapted from Adams and Alitalo, 2015

Although the vascular plexus is expanded via both sprouting angiogenesis and intussusceptive angiogenesis, most remodelling is achieved only via intussusceptive angiogenesis[20] (Fig 1.3).

1.1.3 Molecular mechanisms of sprouting angiogenesis

There are three separate steps leading to the formation of a new angiogenic sprout from a parental vessel: quiescence, activation and resolution. Endothelial cell migration and sprouting is a complex process involving numerous stimulators and inhibitors[3,10] (Fig 1.2).

In normal vasculature, most endothelial cells are quiescent. They are protected from maintenance signals by a layer of pericytes and smooth muscle cells and autocrine signaling of angiopoietin (ANG-1), VEGF and FGFs[21]. Endothelial cells form a layer of phalanx cells with tight junctions between cells that provide mechanical support. Moreover, endothelial cells maintain a strong connection with the extracellular matrix via the expression of cell adhesion molecules, such as vascular endothelial-cadherin (VE-cadherin)[21]. The mural cells suppress endothelial cell proliferation and release cell survival signals[5].

Blood vessels are responsible for the supple of oxygen to all tissues, hence endothelial cells abundantly express oxygen sensors and hypoxia-inducible factors (such as prolyl-4 hydroxylase and HIF2α). HIFs are activated when oxygen levels

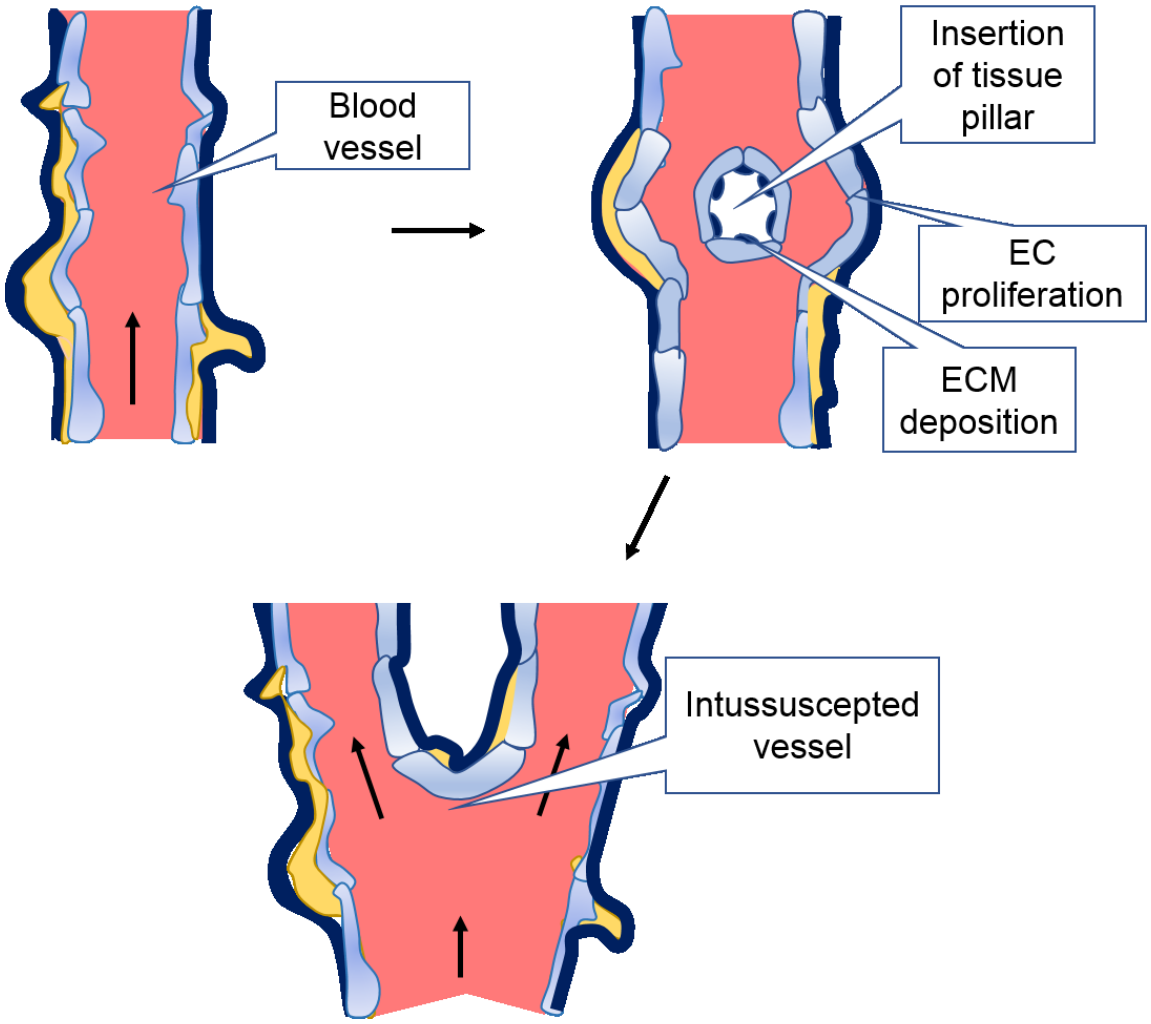


Figure 1.3: Process of intussusceptive angiogenesis. Intussusception is the splitting of one blood vessel into two via the insertion of a transluminal tissue pillar. Intussusceptive angiogenesis leads to the expansion of the vascular network using limited resources.

Adapted from Adams and Alitalo, 2015

in a tissue drop below 6%/40mmHg and bind to hypoxia responsive cis-elements associated with vessel growth[22].

When a quiescent blood vessel senses an angiogenic signal (like VEGF or FGF) from another cell in distress, it becomes activated. The mural cells first detach from the basal lamina. This is mediated via proteolytic degradation of the extracellular matrix by matrix metalloproteinases (MMPs)[23]. To migrate from the vessel, ECs loosen their contacts with surrounding endothelial and mural cells and degrade the surrounding matrix via plasminogen activators and MMPs. Proteinases release matrix-bound angiogenic molecules like FGF and VEGF and remodel the matrix to support angiogenic sprouting[9].

Angiogenic sprouting begins when one endothelial cell, known as the tip cell, is selected to lead the sprout in the direction of the angiogenic signal gradient. The signal could be the presence of factors such as neutropilins (NRPs), VEGF receptors and the NOTCH ligands JAGGED1 or DLL4. The adjacent ECs assume the roles of stalk cells, proliferating behind the tip cell to elongate the sprout and form a vascular lumen. The proliferation and migration of the stalk cells is stimulated by NOTCH, FGFs, placental growth factor (PlGF) and WNTs; the lumen formation is mediated by CD34, VEGF, hedgehog and VE-cadherin. The tip cells have a filopodia sensor to enable migration in the direction of angiogenic guidance signals. The stalk cells secrete molecules such as EGFL7 onto the ECM to communicate their spatial information to the following ECs[5,21] (Fig 1.2).

Finally, myeloid bridge cells help in the fusion with another vessel leading to the initiation of blood flow in the final resolution stage. Pericytes form a layer around the new vessel to stabilize it. They also inhibit MMP-mediated ECM degradation via the endogenous production of tissue inhibitor of metalloproteinases (TIMP). TIMPs enable the ECs and pericytes to deposit a basement membrane and rebuild the adherens junctions[24]. Smooth muscle cells are also recruited for additional maturation of the new blood vessel. By this stage, ECs reassume their quiescent phalanx formation. Continuous blood flow via the new mature vessel raises the surrounding oxygen levels leading to a decrease in VEGF signalling[21,25] (Fig 1.2). The molecules involved in angiogenesis are described in greater detail in Table 1.

1.1.4 Tumor angiogenesis

Uncontrolled growth of abnormal cells in any tissue of the body is known as a tumor. Tumor growth is dependent on and influenced by a variety of genetic and environmental factors. The transformation of a healthy cell to a cancerous cell is often attributed to the accumulation of multiple genetic mutations. The mutated genes are either categorized as oncogenes or tumor suppressor genes based on whether they stimulate or inhibit cellular proliferation, respectively. The genetic mutations can be chromosomal aberrations like additions, deletions or inversions or simple point mutations in the genetic code[25,26].

Factor	Role in angiogenesis
	Increases endothelial cell permeability[27,28]
	Stimulates endothelial cell proliferation and migration[29]
	Inhibits endothelial cell apoptosis[30]
	Stimulates in vivo angiogenesis[31,32]
	Stimulates in vitro endothelial cell sprout formation[33]
	Increases girth and stability of endothelium[34]
	Stimulates endothelial cell proliferation and migration[35]
	Stimulates endothelial tube formation[36]
	Stimulates in vivo angiogenesis[37,38]
	Stimulates endothelial cell proliferation, sprouting and migration[39,40]
	Regulates tubulogenesis in zebrafish[41]
	Stimulates DNA synthesis in endothelial cells[42]
	Stimulates endothelial cells to form cords in vitro[43,44]
	Stimulates proliferation of smooth muscle cells and pericytes[45]
TGF-β	Inhibits proliferation and migration of endothelial cells[46]
TNF-α	Stimulates angiogenesis in vivo and formation of endothelial cell tubes in vitro[47,48]
$a_v\beta_3$ Integrin	Mediates endothelial cell attachment, spreading and migration[49]
$a_5\beta_1$ Integrin	Required for non VEGF-stimulated angiogenesis in vivo[50]
	Required for in vivo angiogenesis[51,52]
	Prevents endothelial cell apoptosis[51]

Table 1.1: Factors involved in angiogenesis. Adapted from Papetti *et al*, 2002.

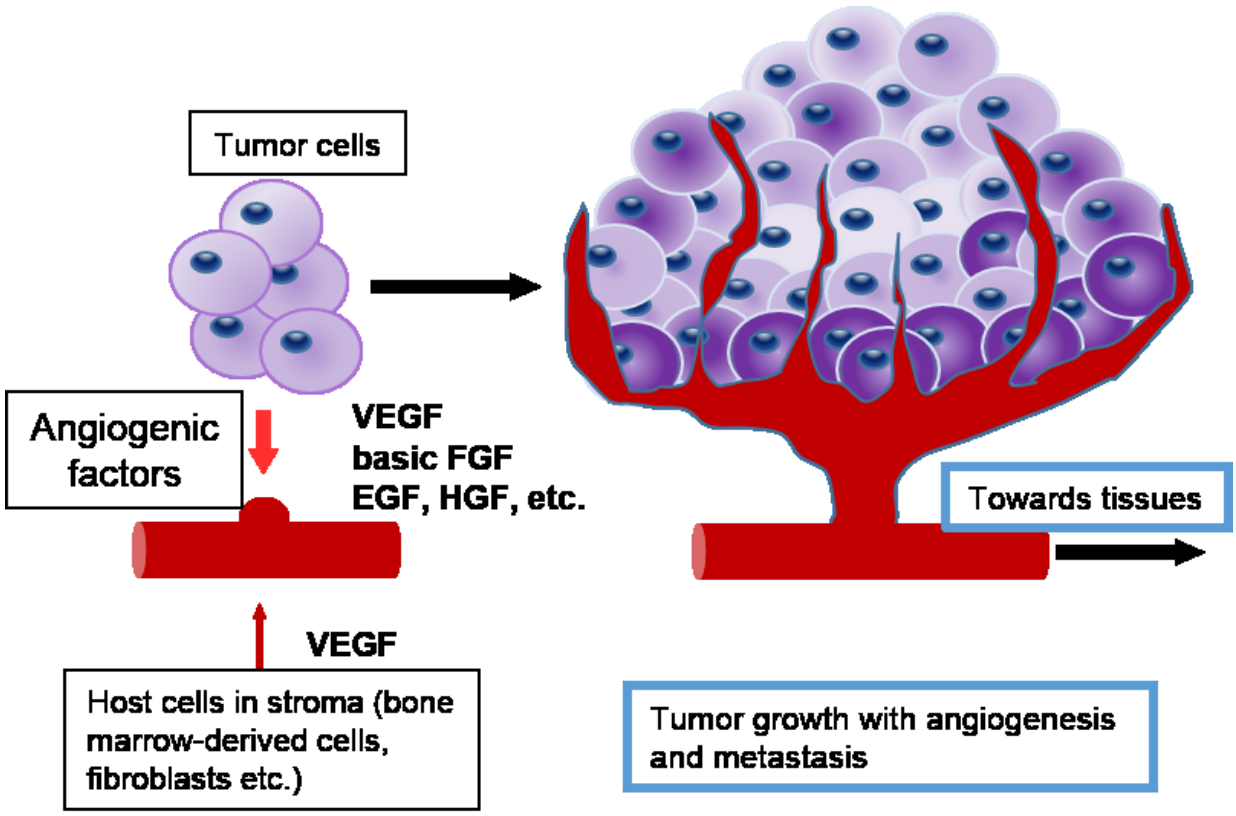


Figure 1.4: Angiogenesis in a tumor. The process of tumor angiogenesis is similar to the process of angiogenesis in normal physiology, except that tumor cells overexpress angiogenic growth factors such as VEGF, ephrins and FGF. Passive diffusion of oxygen and nutrients is unable to reach the innermost cells (tumor core) leading to a hypoxic environment. Hence, larger tumors need to recruit angiogenic vessels for their survival and growth.

Adapted from Shibuya *et al,* 2011

Hanahan and Weinberg proposed the six hallmarks of cancer to be the sustenance of proliferative signalling, evasion of growth suppressors, resistance of cell death, replicative immortality, induction of angiogenesis, and metastasis[53]. In this study, we will be focusing on the process of tumor-mediated angiogenic growth and its inhibition.

When a tumor grows beyond the size of 1-2 mm^3, passive diffusion of oxygen and nutrients is unable to reach the innermost cells (tumor core) leading to a hypoxic environment. Hence, larger tumors need to recruit angiogenic vessels for their survival and growth. An angiogenic switch is turned on because of the hypoxic environment and HIFs are expressed by the cancer cells. The expression of HIFs has been identified as one of the markers of poor prognosis in past studies[26,53].

The process of tumor angiogenesis is very similar to the process of angiogenesis in normal physiology, expect that cancer cells overexpress angiogenic growth factors such as VEGF, ephrins and FGF. The elevated expression of these growth factors leads to the formation of a disorganized vasculature with tortuous and leaky blood vessels. Moreover, many blood vessels lack a layer of functional pericytes and a complete basement membrane[54,55]. However, the misshapen vessels are unable to meet the metabolic requirements of aggressively expanding tumors leading to further expression of angiogenic growth and permeability factors in the tumor microenvironment[56,57] (Fig 1.4). The factors involved in tumor angiogenesis are described in Table 2 in detail.

Factor	Role in tumor angiogenesis
	Highly upregulated in most human cancers[58]
	Expression correlates with intratumoral microvessel density and poor prognosis in cancer patients[59]
	Inhibition decreases tumor vessel density and tumor growth[60,61]
	Inhibition suppresses generation of tumor vessels *in vitro* and *in vivo* and tumor growth i*n vivo*[62]
	Important for maintenance, vs. induction, of tumor angiogenesis[62]
	Synergizes with VEGF to promote angiogenesis *in vitro* and *in vivo*[63]
	Induces VEGF expression in cancer cells and VEGF receptor expression in endothelial cells[64]
Ang 2	Induced in endothelial cells of newly formed vessels of a tumor, leading to vessel plasticity and VEGF-mediated growth[65]
	Stimulates angiogenesis *in vivo*[66]
	Overexpression increases invasiveness, tumorigenicity, neovascularization, and metastatic potential of cancer cells[67,68]
	Directly modulates melanoma cell adhesion and spreading on ECM[69]
	Mediates tumor growth and neovascularization in CAM[70]

Table 1.2: Factors involved in tumor angiogenesis. Adapted from Papetti *et al*, 2002.

1.1.5 Inhibitors of tumor angiogenesis

Along with the various factors that support angiogenesis, more than 40 endogenous inhibitors of angiogenesis have also been characterized. The four types of angiogenesis inhibitors are interferons, proteolytic fragments, interleukins and TIMPS[71].

Interferons (INF-α, -β, and -γ) are glycoproteins that were first identified for their antiviral activity. Interferons have been shown to inhibit endothelial cell motility *in vitro* and angiogenesis *in vivo*[72]. Leukocytes secrete interleukins to mediate lymphocyte activation and proliferation. Fascinatingly, IL-4 has been observed to suppress blood vessel growth and also inhibit tumor cell proliferation[73,74].

TIMPS inhibit angiogenesis indirectly by remodelling the ECM to arrest endothelial cell motility. TIMPs have been shown to specifically inhibit the activity of gelatinases and MT1-MMP, which play a crucial role in endothelial cell migration and capillary formation[75,76].

Lastly, proteolytic fragments, which arise when larger proteins are cleaved, have also been known to be anti-angiogenic. Angiostatin (formed as a result of plasminogen cleavage) and endostatin (formed as a result of Collagen XVIII cleavage) are two such proteolytic fragments that inhibit endothelial cell proliferation and migration[77].

Many of these angiogenesis inhibitors have been tested or are currently being tested in clinical trials for anti-angiogenic therapy.

1.1.6 Anti-angiogenic therapy

Metastasis is responsible for nearly 90% of cancer-related deaths[78]. Tumor angiogenesis can facilitate the metastatic dissemination of a cancer cell. Thus, many cancer researchers have suggested the inhibition of angiogenesis as an effective method of suppressing tumor growth[78].

Over the last two decades, many agents have been tested for their anti-angiogenic activity *in vitro* and in clinical trials. Currently, over forty drugs are being tested in clinical trials for the inhibition of angiogenesis[79].

Three main types of anti-angiogenesis drugs (based on functionality) have currently been approved by the Food and Drug Administration (FDA) in the United States. These are drugs that block angiogenesis signaling (like anti-VEGF antibodies), drugs that inhibit growth of endothelial cells (like endostatin), and drugs that block ECM breakdown (like MMP inhibitors)[80–84]. These US-FDA approved drugs can also be categorized as either monoclonal antibody therapies or small molecule tyrosine kinase inhibitors (TKIs). More information about current anti-angiogenic therapies is mentioned in Table 3.

Despite the effort invested in developing anti-angiogenic therapies, the outcomes have been inadequate. In some cases, anti-angiogenic therapies enabled the proliferation of aggressive hypoxia-resistant cells *in vivo* and did not lead to an improvement in long-term patient survival[79,85–87].

Drug	Target	Class
Bevacizumab	VEGF	Monoclonal antibody
Cetuximab	EGFR	Monoclonal antibody
Panitumumab	EGFR	Monoclonal antibody
Trastuzumab	HER-2	Monoclonal antibody
Erlotinib	EGFR (ErbB1)	Tyrosine kinase inhibitor
Sorafenib	VEGFR-1, VEGFR-2, VEGFR-3, PEGFR-β	Tyrosine kinase inhibitor
Sunitinib	VEGFR-1, VEGFR-2, VEGFR-3, PEGFR-β	Tyrosine kinase inhibitor

Table 1.3: US-FDA approved anti-cancer agents that directly or indirectly inhibit tumor angiogenesis. Adapted from Samant R., 2011

The VEGF family is the most studied and targeted protein family in embryonic and tumor angiogenesis. VEGF- A was first identified as a vascular permeability factor in 1983 by Senger and later characterized by Ferrara *et al* in 1989[29,88]. The whole VEGF family has since been identified to be involved in the formation, development and maintenance of the vascular system. During embryogenesis, VEGF-A is detected early (embryonic day 7) in the extra-embryonic endoderm, signifying its importance in embryonic development[89]. VEGF-A gene inactivation in embryos leads to lethal haemorrhaging and ischemic cardiomyopathy in newborn mice[90]. Upon the blockade of VEGF signalling, other endothelial growth factors such as FGFs, PDGFs etc continue to promote tumor growth angiogenesis[91].

Although targeting VEGF and other pro-angiogenic signalling molecules has been beneficial, cells in the tumor microenvironment can eventually develop resistance to these therapies. This resistance can be attributed to the autotaxin-lysophosphatidate axis.Lysophosphatidate (LPA) is another growth factor that is generated outside cells by autotaxin (ATX), whose expression is associated with increased tumor progression, angiogenesis and metastasis[92,93]. Brindley et al show that increases expression of ATX and LPA signaling in cancer cells leads to the development of resistance to several chemotherapeutic agents[92].

1.2 EGFL7

Epidermal growth factor like-7 (EGFL7) or Vascular endothelial statin (VE-statin) was identified by three laboratories as a unique endothelial specific gene expressed during embryogenesis[40,94,95]. The human and mouse *Egfl7* genes are located on chromosome 9 and 2, respectively[95]. EGFL7 is highly conserved among species and is an approximately 30kDa secreted protein that is almost exclusively expressed in proliferating endothelial cells[40,41].

1.2.1 EGFL7 protein structure

The EGFL7 protein is 278 amino acids in length and contains a putative amino-terminal signal peptide, an Emilin-like (EMI) domain and two EGF-like (EGF) domain repeats at the centre[94,95]. Through sequence similarity analysis, we termed the C-terminal region in EGFL7 as the Matrilin-like (MAT) domain (Fig 1.5).

The first EGF-like domain contains a region similar to the Delta-Serrate-LAG-2 domain, which is a sequence conserved in Notch ligands[96]. The second EGF-like domain interacts with calcium ions[95]. The Emilin-like domain is typically observed in the ECM associated Emilin and Multimerin (EMU) protein family and is suggested to play a role in protein-protein interactions[97,98]. The domains of EGFL7 are detected in other secreted and ECM associated proteins as well, further solidifying its localization to the ECM[99]

1.2.2 EGFL7 expression in endothelial cells

The genetic expression of *Egfl7* is mostly restricted to the proliferating endothelium during embryogenesis and pathologic angiogenesis[41,95]. Transcripts of EGFL7 are first detected at the 8-cell morula stage during embryogenesis[39]. EGFL7 mRNA is observed at both embryonic and extra-embryonic mesodermal progenitor sites and in the primitive vascular plexus during vasculogenesis[94,95]. At later stages of embryogenesis, EGFL7 expression is limited to cardiovascular structures and sprouting vessels[100,101]. EGFL7 displays co-expression with collagen type IV at this stage, suggesting its localization in the ECM[100]. In the tip-stalk cell arrangement of an angiogenic sprout, EGFL7 expression is mainly observed at the base of stalk cells with patchy expression also seen in the tip cells[101] (Fig 1.6).

In adult tissues, very low levels of EGFL7 are observed in quiescent vessels compared to the proliferating vasculature of an embryo[39]. However, expression of EGFL7 is elevated at sites of vascular injury and in the endothelium of a pregnant uterus. This suggests that EGFL7 plays a role during physiological and pathological angiogenesis[39,40].

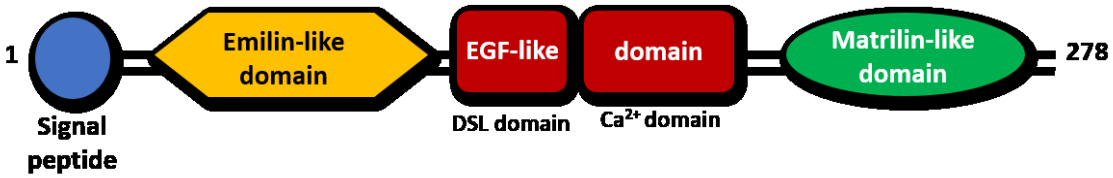


Figure 1.5: Protein structure of EGFL7. EGFL7 is composed of three domains: an Emilin-like (EMI) domain, two Epidermal Growth Factor-like (EGF) repeats and a Matrilin-like (MAT) domain. The first EGF-like domain contains a region similar to the Delta-Serrate-LAG-2 domain, which is a sequence conserved in Notch ligands. The second EGF-like domain interacts with calcium ions.

Adapted from Nichol and Stuhlmann, 2012

1.2.3 EGFL7 in vascular development

Unlike other angiogenic signalling molecules that are expressed by both endothelial cells and other tissues, EGFL7 is only expressed in the endothelium in normal physiology[41].

Parker *et al* first reported EGFL7's role in vascular tube formation during vasculogenesis when knocking down EGFL7 in zebrafish impaired tubulogenesis. Another study showed that a lack of EGFL7 resulted in the formation of abnormal aggregations of endothelial cells that were missing a basement membrane[102].

EGFL7 promotes endothelial sprout formation and invasion during angiogenesis by positively regulating endothelial cell proliferation and migration[39,101,103]. Knockdown of EGFL7 in HUVEC cultures supresses their ability to proliferate, migrate and form capillary sprouts. Additionally, EGFL7 increases endothelial cell adhesion[40]. Compared to other cell adhesion molecules like fibronectin and vitronectin, EGFL7's capacity for binding endothelial cells is relatively weak. This implies that EGFL7 favors endothelial cell motility over adhesion[40].

EGFL7 has been shown to inhibit the deposition of mature elastic fibres by blocking the LOX (lysyl oxidase)- mediated conversion of tropoelastin into elastin[103]; a less rigid ECM promotes endothelial cell migration and invasion. Thus, EGFL7 appears to promote angiogenesis by influencing endothelial cell behaviour and creating a permissive ECM environment.

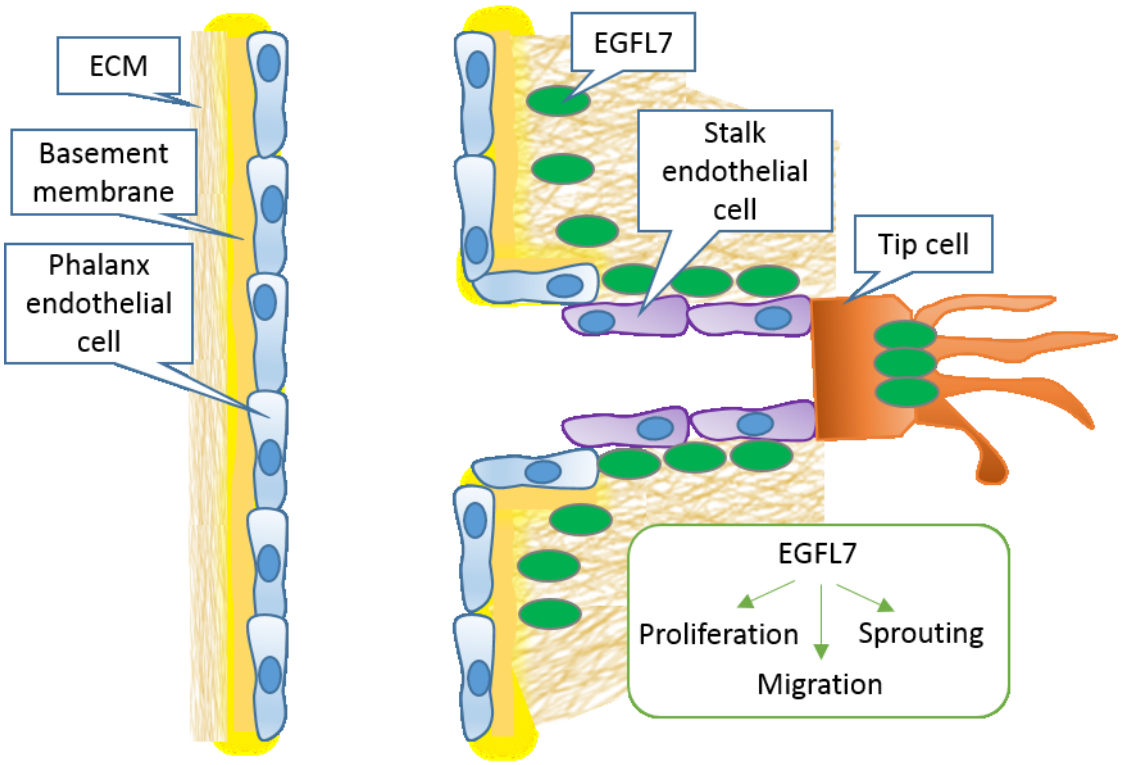


Figure 1.6: EGFL7 in angiogenesis. EGFL7 was first identified as a protein that is involved in embryonic angiogenesis. EGFL7 promotes endothelial sprout formation and invasion during angiogenesis by positively regulating endothelial cell proliferation and migration.

Adapted from Nichol and Stuhlmann, 2012

1.2.4 EGFL7 in vascular injury and disease

In adults, EGFL7 expression is limited to sites of vascular injury or in the pregnant uterus[40]. EGFL7 appears to play a role in repairing vasculature as *Egfl7* is briefly expressed in the redeveloping endothelium of mice at sites of vascular insult[39].

Sites of vascular injury are often hypoxic environments and *Egfl7* expression correlates with hypoxia both *in vitro* and *in vivo*. *Egfl7* expression was observed to stimulate an angiogenic response in the neonatal rat brain under hypoxic conditions[104,105]. Unfortunately, the mechanism of *Egfl7* expression during hypoxia is unknown. Nichol and Stuhlmann reported the presence of nine potential hypoxia-inducible factor-1α binding sites in the *Egfl7* promoter, suggesting that the induction of *Egfl7* expression might be facilitated by the direct binding of this transcription factor to the *Egfl7* promoter[41].

Conversely, EGFL7 expression is suppressed in hyperoxic environments in both neonatal lungs *in vivo* and in endothelial cells *in vitro*. Furthermore, lower levels of EGFL7 are associated with endothelial cell death[106]. These studies indicate that EGFL7 may potentially be used as a therapeutic target against diseases associated with hypoxic-ischemic injury.

1.2.5 EGFL7 expression in tumors

In the past few years, EGFL7 expression has been shown to be highly elevated in several cancer cell lines and clinical tumor samples. EGFL7 expression is especially elevated in human tumors like malignant gliomas, hepatocellular carcinomas, and colon cancers[40,107,108]. Endothelial EGFL7 overexpression in mice leads to the formation of abnormal vessel patterning and remodeling, suggesting that EGFL7 may contribute to the leaky and tortuous vessels observed in tumor angiogenesis[100]; VEGF shows a similar phenotype[109].

In gliomas and hepatocellular carcinomas, EGFL7 expression is localized to both cancer cells and the vasculature surrounding it[110,111]. This implies that EGFL7 may function in an autocrine or paracrine manner, or through cross talk between endothelial cells. In gliomas particularly, EGFL7 expression shows correlation with a higher microvascular density and cell proliferation[112]. Hepatocellular carcinoma (HCC) cells with EGFL7 KD (knocked down) formed smaller tumors compared to the control cells in mice[108], further supporting EGFL7's involvement in tumor progression. In this study, EGFL7 seems to be acting on the vasculature in a paracrine manner, as the EGFL7 expression is mostly restricted to the HCC cells[108].

Although the mechanisms of tumor-derived EGFL7 are not fully understood, one possible explanation is that EGFL7 activates Notch signaling. Blocking Notch signaling has shown a very promising reduction in tumorigenesis in a number of models[113–115]. Moreover, using a Notch1 decoy to block Notch signalling disrupts the growth of human and mouse tumor xenografts in mice[116].

Therefore, it is suggested that EGFL7 functions as a Notch agonist to regulate tumor angiogenesis.

Delfortrie *et al* also observed that EGFL7 expression in breast and lung carcinoma cells increased tumor growth and metastasis in immunocompetent mice, but not in immunodeficient mice[117]. Relatively fewer immune cells infiltrated tumors that expressed EGFL7; tumors expressed reduced levels of interleukin-12, immunostimulatory cytokines and fewer endothelial cell adhesion molecules (like ICAM-1)[117]. A recent study confirmed this phenotype and found that EGFL7 regulated ICAM-1 expression in breast carcinomas. They concluded that EGFL7 assists in endothelial cell activation in relation to immune infiltration[118].

On the contrary, a study was published recently that correlated the tumor-specific expression of EGFL7 with low-grade invasive lesions in breast cancer. Philippin-Lauridant *et al* observed that high expression of the *Egfl7* transcript and elevated levels of EGFL7 protein significantly correlated with the absence of axillary lymph node invasion in human breast cancer[119].

Although the expression of EGFL7 has been linked to high-grade tumors, the exact function of EGFL7 in the tumor microenvironment is unclear.

1.2.6 EGFL7 inhibits tumor growth and disrupts tumor neovasculature

Contrary to the published literature on EGFL7, Cho CF observed that EGFL7 expression in fibrosarcoma and breast carcinoma significantly decreased tumor growth *in vivo*[120].

Cho generated stable EGFL7-expressing HT1080 and MDA-MB-231 cancer cell lines to analyse the effect of EGFL7 expression on tumor growth and angiogenesis. The results from the *ex ovo* tumor growth assay showed that EGFL7-expressing tumors were significantly smaller (~50% decrease in tumor weight) than the control tumors (empty vector) for both cancer cell lines (Fig 1.7)[120]. Next, they tested EGFL7's overall effect on tumor angiogenesis by measuring the tumor microvascular density (MVD), angiogenic sprouting, vascular leak and the ultrastructure of angiogenic vessels.

To perform a more detailed comparison between the microvasculature structure of HT1080 and HT1080-EGFL7 tumors, the CAM vessels were perfused with rhodamine *lens culinaris* agglutinin (LCA), a fluorescent lectin that allows visualization of the neovasculature. Surprisingly, there was no significant difference in the microvessel density (MVD) between both groups However, they observed substantially more angiogenic sprouts in control tumors compared with the EGFL7-overexpressing tumors (Fig 1.7.1 a-c)[120]. To perform a closer examination, Cho used transmission electron microscopy (TEM) to visualize the ultrastructure of these vessels. They found that the vessel walls in the control HT1080 tumor were lined with a monolayer of endothelial cells, each connected to one another via electron dense tight junctions (Fig 1.7.1 d). Interestingly, the

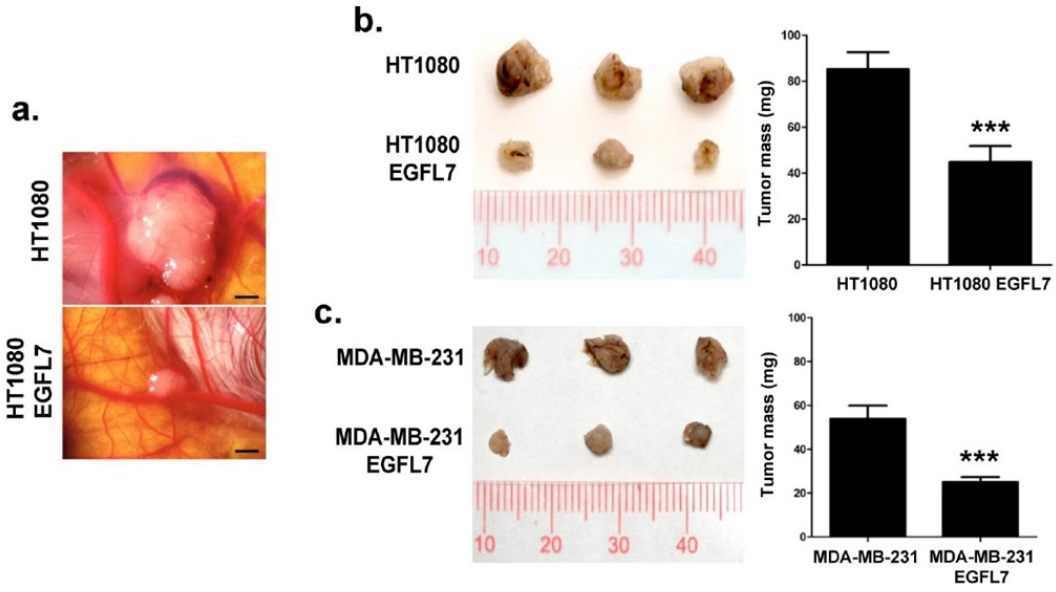


Figure 1.7: EGFL7 expression results in decreased tumor growth. A] *Ex ovo* tumor growth 7 days post-implantation in the CAM with equivalent number of control HT1080 and HT1080-EGFL7-eGFP tumor cells. Scale bar, 1mm. B] Left: Image of excised HT1080 or HT1080-EGFL7-myc tumors established in the CAM *in ovo* for 7 days. Right: Graph showing the mass of excised HT1080 tumors. C] Left: Image of excised MDA-MB-231 or MDA-MB-231-EGFL7-myc tumors established in the CAM *in ovo* for 7 days. Right: Graph showing the mass of excised MDA-MB-231 tumors.

Adapted from Cho, 2012

vessels in HT1080-EGFL7 tumors were bordered by multiple layers of interdigitated endothelial cells, with discontinuous small tracks of electron dense tight junctions (Fig 1.7.1 d)[120].

Cho also performed a vascular leak assay to investigate if EGFL7 expression in tumors would further exacerbate the disruption of endothelial function. To test this, they performed a real-time fluorescence vascular leak assay. This method measures the kinetics of intravenously injected fluorescent dextrans to simultaneously measure vascular integrity integrity (2 MDa FITC-dextran) and vascular permeability (160 kDa TRITC-dextran). They found that for both the HT1080 and HT1080-EGFL7 tumors, majority of the large 2 MDa dextran remained within the vasculature over 3 hours, indicating structurally intact vasculature in both tumors (Figure 1.7.2 a-c). When a 160 kDa dextran was used, they observed a steady and progressive leak from the vasculature into the surrounding stroma in HT1080 tumors over a 3 hour period. In contrast, they saw an immediate and pronounced leak of the 160 kDa dextran in HT1080-EGFL7 tumors, with stroma accumulation of almost 3-fold higher than that observed in HT1080 control tumors (Figure 1.7.2 a-c)[120]. Altogether, these data suggest that EGFL7 overexpression in a tumor inhibits angiogenesis through the disruption of the function and structural organization of its endothelium.

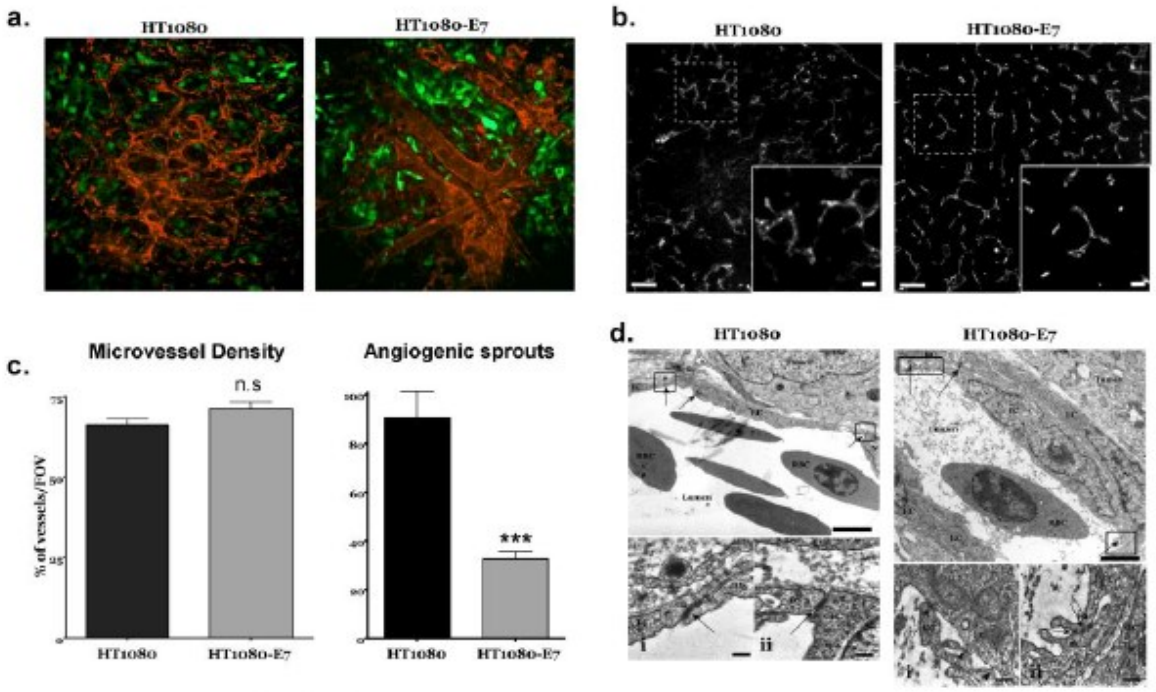


Figure 1.7.1: Tumor EGFL7 expression leads to the disruption of neovasculature organization. A] Fluorescence images of HT1080 or HT1080-EGFL7 tumor (green) established in the CAM and their associated neovasculature. Blood vessels were perfused with rhodamin lectin (red), and confocal microscopy was performed. B] Fluorescence images of tumor sections showing the neovasculature (perfused with rhodamine lectin (red)) of HT1080 or HT1080-EGFL7 tumor. Scale bar, 100μm. Insert shows truncated vessels in EGFL7-overexpressing tumors. Scale bar, 25μm. C] Quantification of microvessel density from (b), ***, $p<0.001$. D] (Left) Transmission electron micrograph of a HT1080 blood vessel showing a monolayer of endothelial cells adjoined by a continuous area of electron dense material (i) and (ii). Scale bar, 5μM; Inset scale bar, 200nm. (Right) Transmission electron micrograph of EGFL7-overexpressing tumor neovasculature showing multiple layers of interdigitated endothelial cells adjoined by discontinuous area of electron dense material (i) and (ii). Scale Bar, 2.33μm; Inset scale bar, 200nm.

Adapted from Cho, 2012

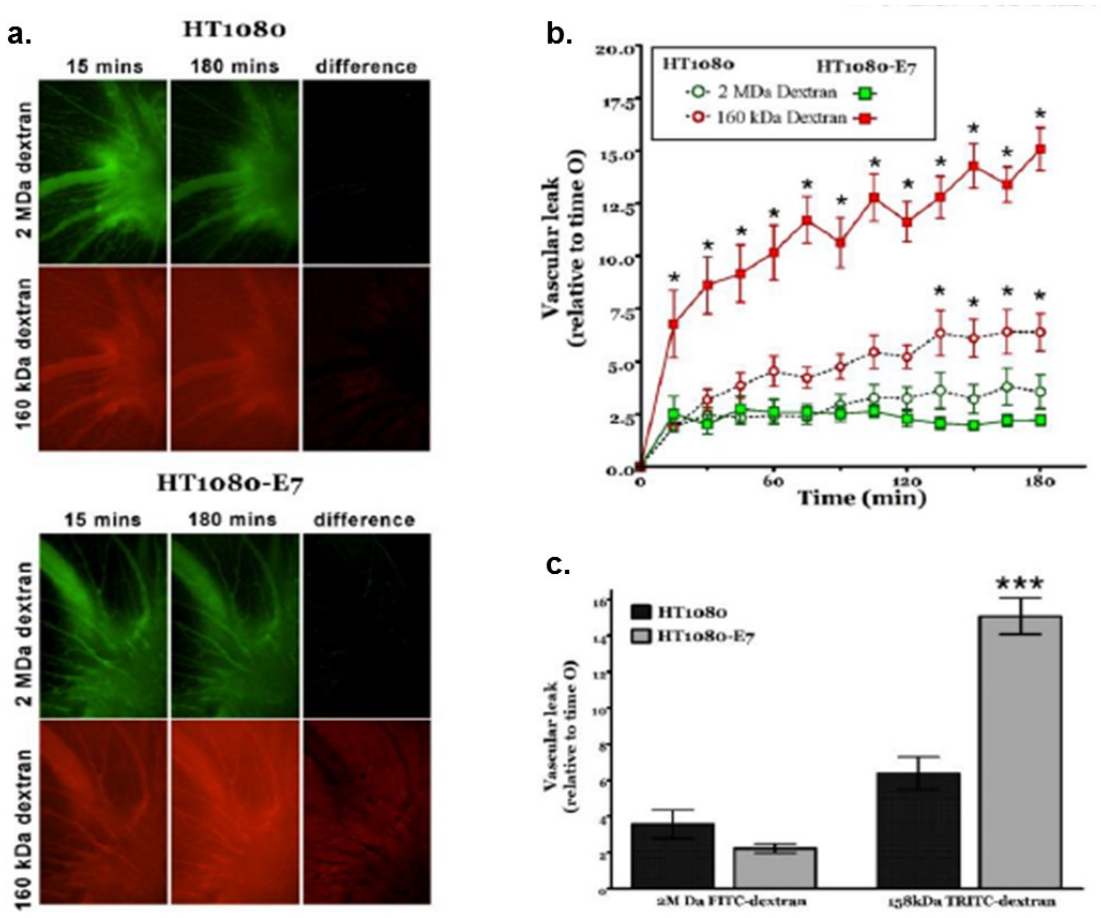


Figure 1.7.2: Tumor EGFL7 expression leads to leaky vasculature. A] Vascular leak assay demonstrating that EGFL7-expressing tumors have leakier vessels than regular HT1080 tumors. B] Quantification of fluorescence level over time showing no difference in leak of the high-molecular weight FITC-dextran between both tumor types (p=0.1037). There is significant vascular leak of the low-molecular weight TRITC-dextran after 2 hrs in control HT1080 tumors, as expected. However, vasculature in HT1080-EGFL7 tumors had significant leak after 15 mins, and this continued over 180 minutes. C] Quantification of vascular leak at 180 mins showing neovasculature of HT1080-EGFL7 tumors had significant increase in leak of low-molecular weight TRITC-dextran compared to control HT1080 tumors

Adapted from Cho, 2012

1.2.7 EGFL7 as a therapeutic agent

EGFL7 is proposed to be an ideal therapeutic target in many literature reviews as it is implicated in tumor angiogenesis, invasiveness and metastatic progression[41,117].

A few studies have attempted to therapeutically target EGFL7 to disrupt tumor angiogenesis *in vitro*[111,121–125]. For instance, Li *et al* simulated glioma cell and endothelial cell interactions *in vitro* via a transwell co-culture. EGFL7 expression was silenced in both cell types using siRNA and it led to a reduction in angiogenic sprouting[121]. Similarly, shRNA targeting of EGFL7 showed suppressed proliferation of laryngocarcinoma cells[125].

In clinical trials, there has been some success targeting tumor angiogenesis using Bevacizumab and other small molecule tyrosine kinase inhibitors of angiogenesis[126]. However, a phase II clinical trial adding a humanized monoclonal anti-EGFL7 antibody (Parsatuzumab) to a combination of mFOLFOX6 and anti-VEGF-A antibody (Bevacizumab) therapy to a cohort of 127 patients showed no improvement compared to the placebo results[127]. The overall response rate was 59% in the parsatuzumab (anti-EGFL7) arm and 64% in the placebo arm[127]. The results of the phase II clinical trial prove that targeting and inactivating EGFL7 using an antibody-based approach may not be the ideal method to inhibit tumor progression and angiogenesis. More understanding about EGFL7's mechanism of action is required before it can be used as an anti-cancer agent.

1.3 Extracellular vesicles

Cells in multicellular organisms have been known to communicate through either a transfer of secreted molecules or through direct cell-cell contact[128]. However, in the past two decades, extracellular vesicles (EVs) have been identified as a third mechanism for intracellular communication[128]. In literature, EVs are also refereed to as microvesicles or microparticles among other names[129–131]. The release of apoptotic bodies from cells during apoptosis has been know for decades, but the shedding of EVs from healthy cells has only recently been studied[132]. EVs have been detected in the medium supernatant of most cell cultures and also in most biological fluids such as blood, urine, breast milk and saliva[133–136].

EVs is a broad term for nano-sized structures surrounded by lipid bilayers that are released by healthy or apoptotic cells[137]. The International Society of Extracellular Vesicles (ISEV) recently defined three main subgroups of EVs as exosomes, microvesicles (MVs) and apoptotic bodies (Abs)[138] (Fig 1.8). Exosomes are small EVs that fall in the 30-100 nm diameter range. Microvesicles are the mid-sized EVs ranging from 100 nm to 500 nm. Apoptotic bodies are the largest and most variable EVs and can range from 500-2000 nm in diameter; they are comparable to platelets[139–141] (Table 4). In this study, we are interested in MVs and exosomes and any information from this point on will be referring to those subgroups only.

Type of EV	Properties	Biogenesis
	Homogenous population with a size of 30 – 100 nm in diameter.	End of endocytic pathway, released from cells when mutivesicular bodies fuse with the plasma membrane
	Exosomal markers include CD9, CD63, Alix, flotillin-1 and Tsg101	ESCRT is involved
	Heterogeneous population with a size of 100-500 nm in diameter	
	Plasma membrane associated proteins are commonly observed in MVs	
	Variable size, 500-2000 nm in diameter	
	Consists of cytoplasm with tightly packed organelles	

Table 1.4: The subgroups of Extracellular vesicles and their properties

Adapted from Lee Yi, 2012

1.3.1 Extracellular vesicle composition.

EVs are derived from the plasma membrane and hence membrane proteins are often enriched on the EV surface. Both MVs and exosomes expresses abundant cytoskeletal- and heatshock proteins, and other proteins involved in vesicle trafficking[142]. Studies have confirmed the expression of tetraspanins such as CD81, CD62 and CD37 in EVs[143]. Apart from these proteins, EVs also expresses parental-cell specific proteins. For example, EVs derived from epithelial cells express the epithelial cell adhesion molecule EpCAM[144].

Extracellular vesicles have an aqueous core that can carry cargo to nearby or distal sites[145]. EVs have been observed to carry diverse cargo such as proteins, nucleic acids (DNA, mRNA, microRNA) and lipids, enabling cell-cell communications[146] (Figure 1.8).

1.3.2 Extracellular vesicles in normal physiology

EVs have been suggested to play a role in the maintenance of physiological homeostasis by regulating processes such as immune signalling, angiogenesis and the detoxification of bacterial products. Any physiological functions of EVs are dependant on them successfully delivering cargo to the recipient cells or the extracellular matrix[141,147]. Denzer *et al* found that isolated B cell EVs selectively target and bind follicular dendritic cells in lymphoid follicles[148].

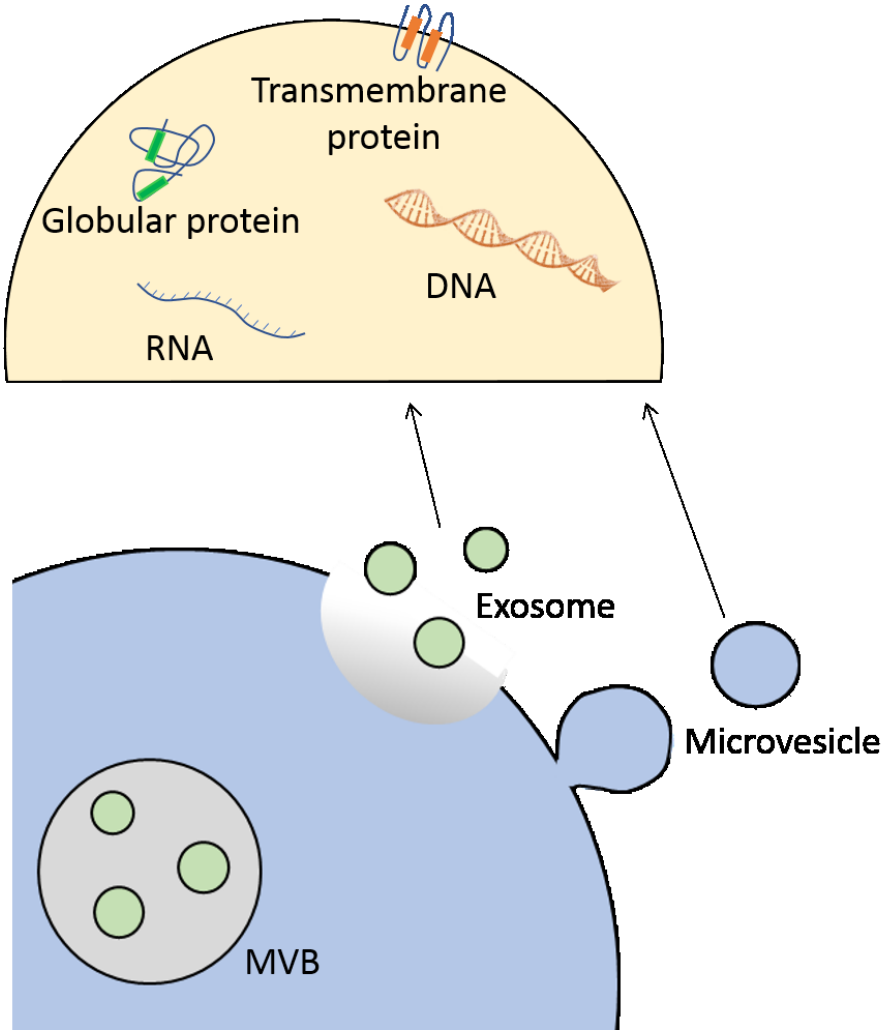


Figure 1.8: Extracellular vesicle secretion from cells. EVs is a broad term for nano-sized structures surrounded by lipid bilayers that are released by healthy or apoptotic cells. Extracellular vesicles have an aqueous core that can carry cargo to nearby or distal sites. EVs have been observed to carry diverse cargo such as proteins, nucleic acids (DNA, mRNA, microRNA) and lipids, enabling cell-cell communication.

Adapted from Kaselowitz B, 2014

The phenotype of recipient cells can be altered after an EV-mediated transfer of information (in the form of cargo). The Sharkis laboratory first demonstrated this idea when they discovered that bone marrow cells co-cultured with liver cells had started expressing mRNA that encoded albumin[149]. Interestingly, this EV-mediated phenotype change was also observed when brain, heart and liver tissue specific mRNAs were also expressed in bone marrow cells upon co-culture with those cell types, respectively[150,151].

EVs derived from cells of the immune system can carry immunologically relevant molecules such as CD86, MHC class II and intercellular adhesion molecules that influence immunological functions. T cell activation, dendritic cell maturation etc. can be regulated by this method[152–154]. Moreover, recent discoveries have found that EVs in breast milk are highly enriched in immune and development related miRNAs like miR-148a-30 and let-7 family. It is likely that breast milk-EVs play a role in immune system development of the cells in the newborn infant's gastrointestinal tract[135,155]. Similarly, EVs derived from neurons have also been shown to transfer information (via proteins) to regulate neural circuit function[156].

Interestingly, EVs isolated from endothelial cell progenitors (angioblasts) stimulated blood vessel formation both *in vitro* and in a SCID mouse model[157].

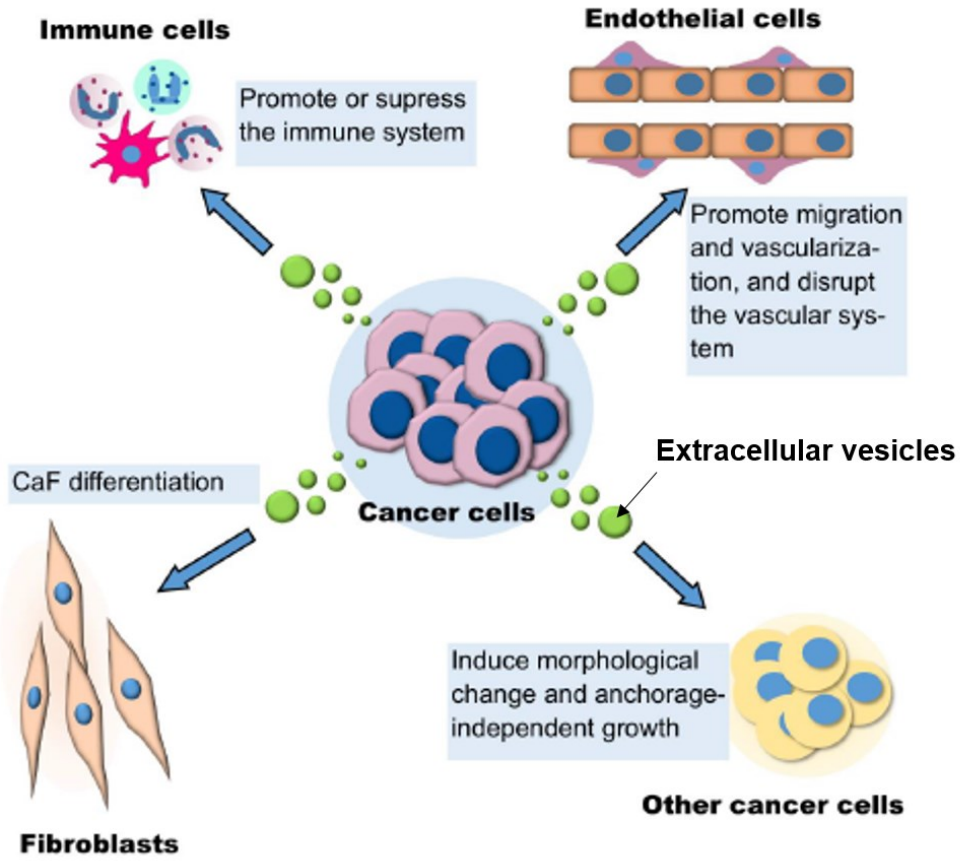


Figure 1.9: Tumor-EVs have a physiological effect on their surrounding environment. The tumor microenvironment is composed of various cell types such as fibroblasts, immune cells, endothelial cells, in addition to cancer cells. Cancer cells use EVs to modify cells within the tumor microenvironment. EVs can contain cargo such as miRNA and proteins to produce a physiological effect on recipient cells.

Adapted from Naito Y, 2006

1.3.3 Extracellular vesicles in tumorigenesis

Not surprisingly, EVs also play a role in both tumor progression and inhibition. For a cell to become cancerous, it must undergo a transformation (acquire mutations) to become malignant. EVs from malignant cancer cells have been shown to bring about this transformation in non-cancerous cells[150]. EVs can manipulate the tumor microenvironment to enable tumor growth. But, EVs can also program the immune system to provoke an anti-tumor response[158].

Information (cargo) from EVs is taken up by recipient cells using either of three uptake mechanisms: 1) direct fusion with the plasma membrane, 2) receptor-ligand interaction, or 3) endocytosis by phagocytosis[152,153]. Cancer cells also exchange oncogenic proteins in this manner. For example, aggressive glioma cells express a mutant oncogenic form of an EGF receptor (EGFRvIII) that is also found on the EVs they secrete. Although very few cells in the tumor actually express this receptor, all the cells in the tumor seem to share the transformed phenotype. Al-Nedawi *et al* observed that the transformed cells delivered the mutant oncogenic protein to surrounding cells via an EV-mediated transfer. The incorporation of the mutant EGFR in the cells led to an increased capacity for anchorage independent growth[159]. Some preliminary studies have also shown that EV-mediated transfer of proteins can lead to chemoresistance in cancer cells[160–162] (Fig 1.9).

Cancer cells selectively uptake pro-growth and pro-angiogenesis signals from circulating EVs. Proteomic analysis showed that EVs in the tumor microenvironment are enriched with many tumorigenic factors[152].

Additionally, cancer cells also recruit immune cells via EV-mediated communication to enhance tumor invasion, angiogenesis and metastasis[163]. In an *in vivo* breast cancer model, blockade of EV secretion by 4T1 cancer cells (in a Rab27a-dependant manner) led to a decreased infiltration by neutrophils. This resulted in a decrease in tumor growth and metastasis[164]. In addition to other such effects, cancer cells are also able to stimulate the expansion of regulatory T (Treg) cells leading to immunosuppression in the tumor microenvironment[165,166].

Conversely, some studies have shown that EVs can transport antigens from cancer cells to antigen presenting dendritic cells[167–169]. Moreover, EVs from pancreatic cancer cells and HCC cells express heat shock protein 70 which can directly activate natural killer cells (NK)[170,171]. In this way, EVs can also recruit immune cells to suppress tumor growth.

In the past few years, EVs have been utilized as tools for diagnosis and therapy. EVs are ideal cancer biomarkers to predict cancer burden at an early stage[172]. Research is also being done to block the release of EVs to control tumor growth[173,174].

1.4 Hypothesis

Tumor EGFL7 expression significantly inhibits angiogenesis in the tumor microenvironment via its Emilin and EGF-like domains.

1.4.1 Objectives

The main objective of this study is to clarify the role of EGFL7 and its domains in tumor angiogenesis and identify its mechanism of action.

The specific objectives are:

1. Identify the domain within EGFL7 that regulates endothelial cell sprouting and adhesion

2. Show that functional EGFL7 is secreted from cancer cells via extracellular vesicles

3. Identify the binding partners for EGFL7 within the tumor microenvironment

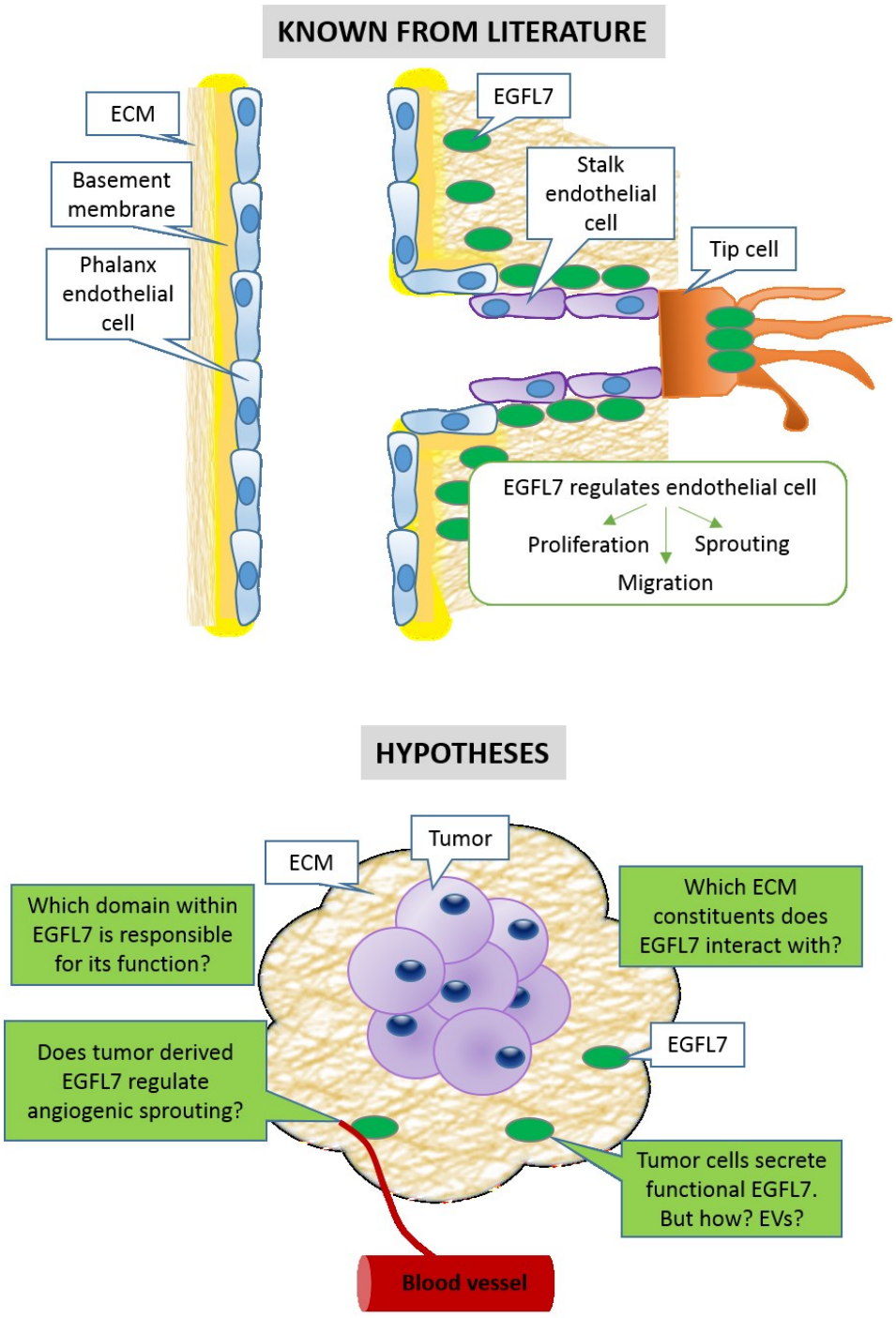


Figure 1.10: Hypothesis of this study. Endothelial cell-derived EGFL7 is known to promote endothelial cell sprouting, migration and proliferation from published literature. In this study, we study the role of EGFL7 specifically secreted by tumor cells and its effect on the components of the tumor microenvironment.

Chapter 2: Materials and Methods

2.1 Construction of EGFL7 Expression Vectors

The open reading frame of human EGFL7 was cloned into the pcDNA3.1 expression vector (Invitrogen) with V5 tag/His from pcDNA3.1 V5 tag/His B+ (Invitrogen) at the 3' end. Inverse PCR was used to generate domain-deleted mutants using the following primers:

Primer	**Sequence**
EMI deletion Reverse	5' CTACAGGGCTGTGAGCCCGGACAGCACCCTACG '3
EMI deletion Forward	5' AGCCCTGTAGCGAGGCCTGGCAGGGGCCAGCCCGCG '3
EGF deletion Reverse	5' GCTGTGCCCCGCGGGCTGGCCCCTGCCAGGCCTCGC '3
EGF deletion Forward	5' GGGCACAGCCTGTCTGCAGACGGTACACTCTGTGTG CCC '3
Matrilin deletion Reverse	5' TGCAGATATGAATTCCTACGAGTCTTTCTTGCAGGAG CCCAGC '3
Matrilin deletion Forward	5' TGCAGATATGAATTCCTAGGAGTGGACCACCCTGGG GGGCC '3

Table 2.1: Primer design for the construction of EGFL7 plasmids.

EGFL7-V5 tag/His, domain deletions or empty vector were transfected into HT1080 fibrosarcoma cells using SuperFect Transfection Reagent (Qiagen) following manufacturer's instructions. Stable HT1080-EGFL7, HT1080-EGFL7ΔEMI, HT1080-EGFL7ΔEGF and HT1080-EGFL7ΔMAT cell lines were generated by selection with Neomycin (G418, 500µg/ml) (GIBCO) for three weeks.

2.2 Cell lines and reagents

The HT1080 and EA.hy926 cell lines were obtained from American Type Culture Collection (ATCC). All cells were cultured in high glucose DMEM supplemented with 10% FBS at 37°C and 5% CO_2. Full-length and modified EGFL7 was stably overexpressed in HT1080 cells using Neomycin resistance to create the HT1080-EGFL7, HT1080-EGFL7ΔEMI, HT1080-EGFL7ΔEGF and HT1080-EGFL7ΔMAT cell lines. The medium was additionally supplemented with Neomycin at 100ug/mL to main a positive selection pressure. Neomycin (G418) was purchased from Thermo Fischer scientific.

SF21 (Army worm ovary, Thermo Fischer) cells were grown in suspension in Sf-900 II medium (Gibco) supplemented with 10% FBS, 1% Penicillin, and 1% Streptomycin. Culture flasks were incubated at 27°C in a shaking incubator.

2.3 Cell proliferation assay

HT1080-control, HT1080-EGFL7, HT1080-EGFL7ΔEMI, HT1080-EGFL7ΔEGF and HT1080-EGFL7ΔMAT cancer cells were seeded in seven 96-well plates at 200 cells per well (100ul aliquots) and cultured for 7 days. Viable cells were determined every 24 hours by 5 µL of MTT (5 mg/ml; Sigma) added to each well with 100 µL of medium for 4 h at 37°C. After incubation, supernatant was removed, 100 µL of precipitation solution (1M HCL, 10% Triton-X in PBS) was added to each well. After 30 minutes of incubation at room temperature, the absorbance was read at a test wavelength of 570 nm.

2.4 *In vivo* xenograft assay on the chorioallantoic membrane (CAM) model.

Fertilised leg-horn chicken eggs from the Rochester Hatchery in Edmonton were deposited onto sterile plates and avian embryos were developed for 9 days in 37°C incubators with 50% humidity. On embryonic day 9, a small abrasion was made on the CAM using a sterile Q-tip and 1 x 10^6 cancer cells (HT1080-con, HT1080-EGFL7, HT1080-EGFL7ΔEMI, HT1080-EGFL7ΔEGF or HT1080-EGFL7ΔMAT) were pipetted onto the abrasion. The tumors were monitored daily until embryonic day 15; all tumors were harvested at the same time and the tumor mass was measured.

2.5 *In vitro* 3D bead angiogenesis assay

EA.hy926 endothelial cells were detached from culture flasks using Trypsin-EDTA (Sigma) and resuspended in DMEM- high glucose medium containing 1% FBS. Approximately 10,000 114-198 µm sterile cytodex microcarrier beads (Sigma) were added to EA.hy926 cell suspension (4 million cells/ 4 ml) and incubated at 37°C for 4 hours; gentle manual shaking was applied every 10 minutes.

Fibrinogen (Sigma) was dissolved in PBS to a final concentration of 2.0 mg/ml. 0.15 Units/ml of aprotinin (Sigma) was added to the fibrinogen solution. The endothelial cell-coated beads were allowed to settle without centrifugation and the supernatant was removed. The beads were mixed with the fibrinogen solution at a concentration of 500 beads/ml. Next, 0.625 units of thrombin (Sigma) were deposited at the centre of each well of a 24-well plate and 0.5 ml of the bead-fibrinogen solution was pipetted into each well. The clots were allowed to solidify for 15 minutes at 37°C. A single layer (25×10^3) of either HT1080-con, HT1080-EGFL7, HT1080-EGFL7ΔEMI, HT1080-EGFL7ΔEGF or HT1080-EGFL7ΔMAT cancer cells were coated on top of the fibrin clots in low serum (1%) DMEM-high glucose media. The endothelial cell-coated beads were monitored over 7 days and bright-field images were captured at 10x magnification using the Evos FL cell imaging system (Invitrogen). The average number of angiogenic sprouts per bead were calculated for each tumor cell type using ImageJ.

2.6 *Ex ovo* angiogenesis assay

Nylon gridded meshes were cut into squares of 2x2 mm and 4x4 mm and autoclaved. Small meshes were overlaid on top of large meshes at a 45° angle. HT1080-con, HT1080-EGFL7, HT1080-EGFL7ΔEMI, HT1080-EGFL7ΔEGF and HT1080-EGFL7ΔMAT cancer cells were detached using Trypsin-EDTA and resuspended in 200 µl of PBS at a concentration of $20x10^6$ cells/ml. The cell suspension was mixed with collagen and 30 µl of this mixture was pipetted onto each mesh. These collagen meshes onplants were allowed to solidify at 37° C for 2 hours. Healthy shell-less chicken embryos at embryonic day 10 were divided into 5 groups; 12 for each cell type. 4 onplants were placed on the chorioallantoic membrane (CAM) of each chicken embryo and the embryos were incubated in at 37° C in 50% humidity. Onplants were imaged on day 3 (embryonic day 13) using Volocity (Improvision, UK) at 6.3x magnification using the Lumar V.12 stereo-microscope (Carl Zeiss, Germany). The angiogenic index of the onplant tissue was determined as the percentage of grids that contained newly formed blood vessels out of the total number of grids in the upper mesh.

$$\frac{Number\ of\ grids\ containing\ blood\ vessels}{Total\ number\ of\ grids} x\ 100 = Angiogenic\ Index$$

2.7 Immunoprecipitation of EGFL7

HT1080 control, HT1080-EGFL7 and HT1080-EGFL7ΔEMI cells were cultured to 80% confluency in 15 cm plates. Membrane impermeable DTSSP protein

crosslinker (Sigma) was dissolved in PBS at a 2mM concentration;10 mls of this solution was added to each plate and incubated for 30 minutes at room temperature. At 30 minutes, cells were washed with 10 mls of stop solution (1 M Tris). Lysates were prepared using lysis buffer (150mM NaCl, 0.01% NP-40, pH 7.4, 50mM Tris) with 1% protease inhibitor (Sigma) and 5 µl of anti-V5 tag antibody (1 mg/ml, Abcam) was added to each sample and incubated for 1 hour at room temperature. Protein A magnetic beads (Bio-rad) were added to each lysate and incubated for another hour at room temperature. Magnetic beads were precipitated and heated in Laemmli loading buffer for 5 minutes at 90° C. Samples were analysed via SDS polyacrylamide gel electrophoresis and Western blotting.

2.8 Western Blot Analysis

Cells were grown to subconfluency in 15 cm culture plates. Lysates were prepared using ice-cold lysis buffer (150mM NaCl, 0.01% NP-40, pH 7.4, 50mM Tris) with 1% protease inhibitor purchased from Sigma-Aldrich. Samples were heated in Laemmli loading buffer and separated by electrophoresis in SDS on 10% polyacrylamide gels at 120 Volts for 1.5 hours. One lane was loaded with Bio-rad Kaleidoscope protein marker to allow for protein mass determination. Protein was transferred from acrylamide gels to 0.2-micron nitrocellulose transfer membranes (Bio-rad) using the blotting apparatus (Biorad) in transfer buffer (25 mM Tris, 192 mM Glycine,20% Methanol) for 1 hour at 80 Volts.

Nitrocellulose membranes were blocked for 1 hour at room temperature in Rockland blocking buffer for fluorescent Western blotting.

Western blots were incubated with primary antibody for 1 hour at room temperature followed by the appropriate secondary antibody at room temperature after 3x washes with TBS-0.1% Tween.20. Finally, blots we imaged and analysed using the Odyssey LI-COR imaging system.

Western blot analyses were carried out using the appropriate dilution for the following primary antibodies:

Antibody	**Supplier**	**Final concentration**
anti- VE statin (EGFL7) (goat polyclonal)	R & D systems	1:500
anti-V5 tag (mouse polyclonal)	Abcam	1:1000
anti-β-tubulin (mouse monoclonal)	Abcam	1:2000
anti- Integrin β_3 (rabbit monoclonal)	Abcam	1:250
anti-Thrombospondin-1 (rabbit monoclonal)	Sigma-Aldrich	1:500
anti-Integrin β_1 (rat monoclonal)	Developmental Studies Hybridoma Bank (DSHB)	1:250

Table 2.2: Antibodies used for Western blotting.

All secondary fluorescent antibodies were Alexa Fluor conjugates purchased from Thermo Fischer and were used at a 1:10000 dilution.

2.9 EGFL7 baculovirus expression system

Gene of interest (EGFL7 and EGFL7-ΔEMI) were isolated from pre-existing pcdna3.1-EGFL7 and pcdna3.1- EGFL7-ΔEMI plasmids using the following primers:

Fwd: 5’ GCGCGGATCCACCATGAGGGGCTCTCAGGAGGTGCT

Rev:3’ATGCCTCGAGTTAGTGATGGTGATGGTGATGCGAGTCTTTCTTGCAG GAGCAG

The gene of interest was ligated at the multiple cloning site (specifically BamH1 and Xho1) of pFastBac recipient plasmid from Thermo Fischer. *One shot DH5α E. coli* cells (Thermo Fischer) were transformed with either pFastBsc-EGFL7 or pFastBac-EGFL7- ΔEMI and colonies were grown on agar plates supplemented with Ampicillin (Sigma Aldrich). Six colonies each were picked and expanded in liquid LB agar culture overnight. DNA was extracted from these cultures using the Qiagen Mini-prep kit; length of the insert was confirmed via agarose gel electrophoresis.

DH10Bac E. coli cells were transformed from the plasmid DNA purified from the previous steps. Colonies were grown on agar plates supplemented with 50 μg/mL kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracycline, 100 μg/mL Bluo-

gal, and 40 μg/mL IPTG to select for positively transformed clones. White colonies for each (EGFL7 or EGFL7- ΔEMI) were picked and grown in liquid LB agar culture overnight. Bacterial cells were centrifuged and bacmid DNA was purified using the Qiagen large construct kit. The following primers were used to verify correct gene of interest expression by PCR:

M13 Forward 5′-CCCAGTCACGACGTTGTAAAACG-3′

M13 Reverse 5′-AGCGGATAACAATTTCACACAGG-3′

Finally, SF21 insect cells were transfected by Cellfectin II reagent (Thermo Fischer) supplied with either EGFL7 bacmid or EGFL7- ΔEMI bacmid. Insect cells were monitored every day and baculovirus-enriched medium was collected once cells showed signs of viral infection (e.g. increased diameter). Baculovirus-enriched medium was spun down at 300xg to remove large cell debris and supernatant was saved as EGFL7-his tag and EGFL7-ΔEMI-his tag viral stock.

2.10 Purification of recombinant EGFL7, EGFL7-ΔEMI and EGFL7-FITC protein

SF21 cells were transfected with EGFL7-his tag or EGFL7-ΔEMI-his tag baculovirus and allowed to reach 20-30% cell death. The cell suspension was lysed in pH 8.0 PBS with 1% Triton x and 1% protease inhibitor. The resulting pellet was again lysed using a pH 10.0 50 mM dibasic sodium phosphate buffer (with 0.5% SDS). The pH of the supernatant was adjusted to pH 8.0 and incubated with 2 mls of pre-washed cobalt resin for 30 minutes at room

temperature. The mixture was centrifuged at 500xg and the supernatant was discarded. The cobalt resin was washed 3x with pH 8.0 50 nM dibasic sodium phosphate buffer (with no SDS). The protein was eluted from the cobalt resin 3x using 500 mM imidazole elution buffer.

Purified EGFL7 protein was conjugated with FITC (Fluorescein isothiocyanate) by incubating 1 ml of concentrated EGFL7 protein (0.5 mg/ml) with 25ul of FITC solution (1 mg/ml in DMSO) for 1 hour at room temperature. Conjugated product was separated from excess FITC by passing through G-25 gel filtration medium (GE Healthcare).

The size, purity and concentration of the EGFL7, EGFL7-FITC and EGFL7-ΔEMI protein was validated via measuring absorbance, Western blotting and silver staining.

2.11 Extracellular vesicle isolation

HT1080-con, HT1080-EGFL7, and HT1080-EGFL7 deletion mutant cells were cultured to 80% confluency and incubated in DMEM High glucose medium without FBS for 12 hours to induce extracellular vesicle secretion. Enriched medium was centrifuged at 500xg to remove live cells, followed by 5000xg to remove cell debris and the supernatant was ultra-centrifuged at 100,000xg for 1 hour to pellet the extracellular vesicles. The supernatant was pipetted into a separate tube and the extracellular vesicle pellet was resuspended in 100 µl of

PBS. Extracellular vesicles (EVs) were analysed using the Apogee Micro Flow Cytometer (Apogee, UK) and western blotting.

2.12 TEM imaging

Isolated EVs were fixed for 1.5 hours in ice-cold 4% paraformaldehyde followed by 0.025% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4. Following fixation, free aldehydes were quenched in 50 mM glycine in 0.1 M phosphate buffer. The fixed EVs were then resuspended in warm 7.5% gelatin in PBS for 10 minutes and then placed on ice to solidify. Thin sections were cut using glass knives and placed directly onto carbon-coated 200 mesh nickel grids (Sigma Aldrich). Sections were blocked with 50nM Glycine, 2% BSA solution for 1 hour, followed by incubation with anti-EGFL7 (goat IgG, 1:10) antibody for 2 hours at room temperature. Grids were rinsed with PBS and incubated with biotinylated anti-goat secondary antibody (1:50, Sigma Aldrich) for 1 hour at room temperature. Grids were incubated with gold conjugated streptavidin (1:20, Sigma Aldrich) in TBS for 1 hour at room temperature, followed by PBS washes and post-fixation in 1% glutaraldehyde in PBS. Individual grids were washed in distilled water and contrasted in uranyl oxalate for 10 minutes before being blotted with filter paper to remove excess solution. The grids were then allowed to dry overnight and imaged using a Hitachi H-7650 TEM microscope.

2.13 Endothelial cell adhesion assay

EGFL7, EGFL7ΔEMI or BSA was coated onto 6-well plates at 2 µg/ml at 4° C for 12 hours. EA.hy 926 endothelial cells were plated at 50,000 cells/well and allowed to adhere for 3 hours at 37° C. All wells were washed 5x with PBS and fixed with methanol. Cell nuclei were stained with Hoechst 33342 and imaged at 10x magnification using the Evos FL cell imaging system (Invitrogen) and analysed using ImageJ.

The same cell adhesion protocol was repeated using control-EVs and EGFL7-EVs.

2.14 Immunofluorescence imaging

Fibronectin was coated onto 18 mm glass coverslips at a concentration of 2 nM overnight at 4° C. HT1080-con, HT1080-EGFL7, HT1080-EGFL7ΔEMI, HT1080-EGFL7ΔEGF and HT1080-EGFL7ΔMAT cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) in 10% FBS onto the glass coverslips at approximately 50% confluency. 48 hours later, cells were fixed with 4% paraformaldehyde and stained with antibodies against EGFL7 for 1 hour at room temperature followed by the relevant secondary antibody for another hour at room temperature. Cell nuclei and actin were stained with Hoechst 33342 and Phalloidin-Alexa 647 respectively for 10 minutes prior to mounting with ProLong Gold (Invitrogen). The Nikon A1+ confocal microscope was used to visualize the

slides. 3D Z-stacked images were captured and images were generated using the NIS Elements AR software.

2.15 siRNA Transfections

Endothelial cells were seeded into 6-well culture plates at a density of 1 x 10^5 cells/well in DMEM supplemented with 10% FBS. The next day (16 hours later) cells were transfected using Lipofectamine 2000 reagent (Invitrogen) with 20nM of Integrin β3 siRNA (sense: 5` GAAGAAUUUCUCCAUCCAAdTdT3`, antisense: 5` UUGGAUGGAGAAAUUCUUCdGdA3`) (Sigma) and scramble siRNA (negative control) 5'GAAGUAACACCCGCACCUAU'3 (Sigma) was added to wells in Opti-MEM medium (Thermo Fischer Scientific). Endothelial cells were treated for 72 hours with both siRNAs. After treatment, cell lysates were collected for Western blot analysis to confirm knockdown (KD).

2.16 Statistical Analysis

Data was processed using Graphpad Prism version 6.0 software (Graphpad Software Inc.). Unpaired T-test with post-hoc Mann-Whitney test, linear regression or one way Anova with post-hoc Fischer's LSD test were used to determine significance. All columns and error bars represent the mean and standard deviation (STDEV), respectively.

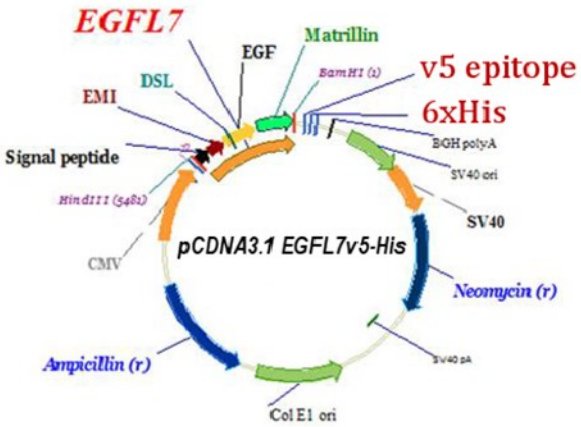
Chapter 3: Results

3.1 Stable EGFL7 expression in HT1080 fibrosarcoma cells

Elevated levels of EGFL7 were observed in high-grade human tumors such as glioblastoma[110], hepatocellular carcinoma[175], and colon cancers[107] in previous studies. The expression of EGFL7 was localized not only to the cancer cells, but also the vasculature surrounding it. In order to elucidate the role of EGFL7 in the tumor microenvironment, stable HT1080 mutant cells lines were generated that overexpressed V5-tagged EGFL7 with one of its domains deleted (HT1080 EGFL7-ΔEMI, HT1080 EGFL7-ΔEGF and HT1080 EGFL7-ΔMat) per cell line. These cell lines were in addition to the HT1080-EGFL7 full-length and HT1080-empty vector control cells. (Fig 3.1). The cells were selected using Neomycin for three weeks. The HT1080 fibrosarcoma cell line was used in this study as it is a well-established highly tumorigenic cell line that has a high transfection effeciency[176].

The rate of proliferation of transfected cells is often affected during and after the process of transfection[177]. To observe the effect of EGFL7 and domain deletion transfections on HT1080 cells, an MTT based proliferation assay was performed over 168 hours; the cell viability was measured every 24 hours. Although the MTT assay measures active cellular metabolism, it was used in this study to estimate the number of viable cells at each time point[178]. The rate of proliferation of the HT1080 variants was not significantly altered throughout the 168 hours (Fig 3.2). The EGFL7-expressing (full-length and deletions) HT1080 cells proliferated at a similar rate as the control HT1080 cells.

A]



B]

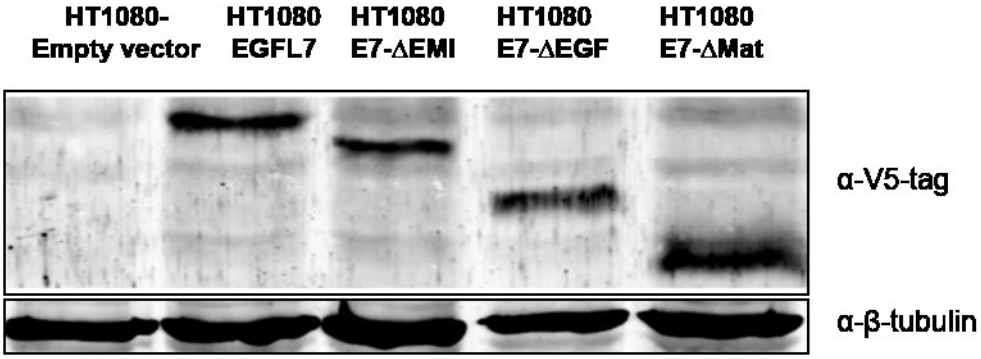


Figure 3.1: EGFL7 and domain deleted mutants were stably expressed in HT1080 cells. A] EGFL7, EGFL7ΔEMI, EGFL7ΔEGF and EGFL7ΔMAT pcdna3.1 vectors were constructed with a V5+His tag at the C-terminal. B] Western blot analysis of HT1080 cell lysates expressing full-length EGFL7 protein and its deletion variants.

3.2 EGFL7 inhibits tumor growth *in vivo*

The rate of proliferation of the HT1080 cells was unaffected by the stable expression of EGFL7 and its domain deletions, as seen from the previous experiment. In the published literature thus far, EGFL7 is associated with high grade tumors and is assumed to play a role in cancer progression[108,112,179]. Hence, we hypothesized that HT1080-EGFL7 expressing tumors will be larger than control HT1080 tumors in the chicken embryo model.

To test this, approximately 1 x 10^6 HT1080-empty vector, HT1080-EGFL7, HT1080 EGFL7-ΔEMI, HT1080 EGFL7-ΔEGF or HT1080 EGFL7-ΔMat cells were topically applied to the chorioallantoic membrane (CAM) of chicken embryos on embryonic day 9. The embryos were monitored over the next 6 days and all tumors were harvested on embryonic day 15 and measured. However, contrary to published literature, the HT1080-EGFL7 tumors were significantly smaller than the HT1080-empty vector (control) tumors (Fig 3.3). On average, the HT1080-EGFL7 tumors were 1.8x smaller than the control HT1080 tumors.

Interestingly, both the HT1080 EGFL7-ΔEMI and HT1080 EGFL7-ΔEGF tumors did not share EGFL7's phenotype of tumor growth inhibition (Fig 3.3); both sets of tumors were significantly larger than full-length EGFL7 tumors. On the contrary, the HT1080 EGFL7-ΔMat tumors were significantly smaller than the control tumors, similar to the full-length EGFL7 tumors. This suggested that both the Emilin-like and EGF-like domains could play a role in the tumor inhibitory phenotype of EGFL7, as deleting them led to a larger tumor mass.

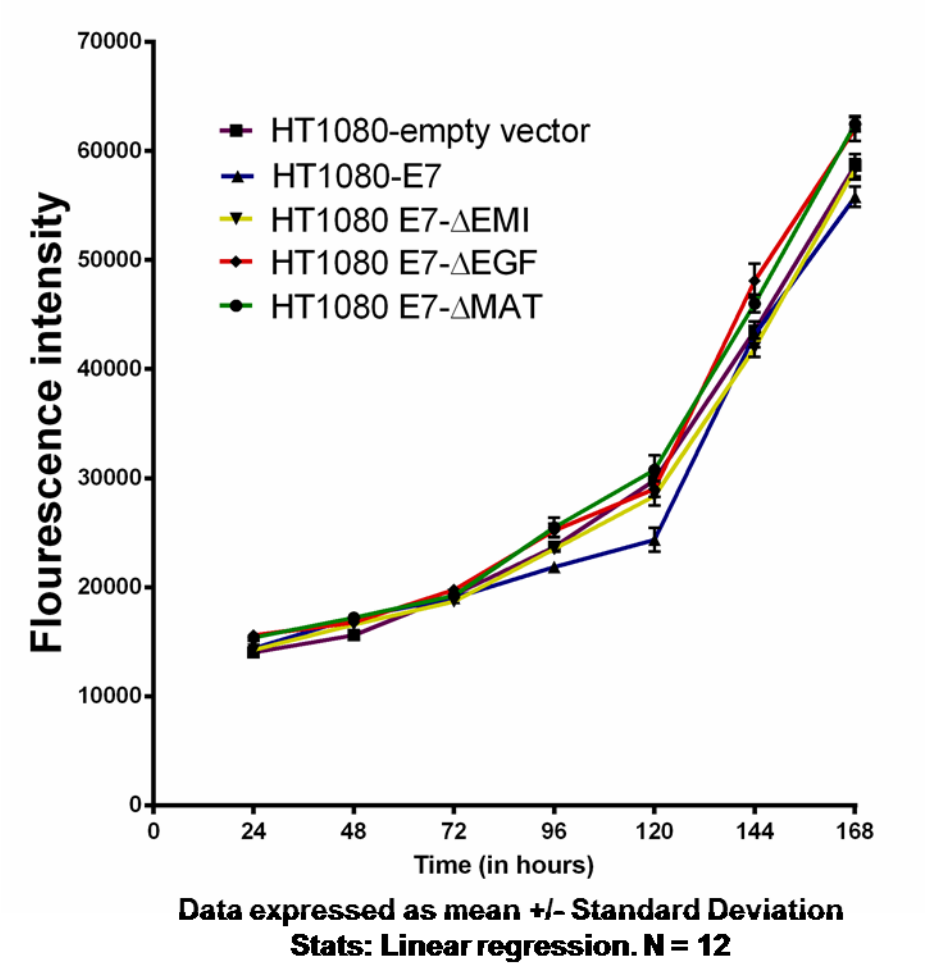


Figure 3.2: Stable EGFL7 expression in HT1080 cells does not alter its rate of proliferation. V5-tagged EGFL7, EGFL7ΔEMI, EGFL7ΔEGF, and EGFL7ΔMAT expression in HT1080 cells did not alter the proliferation rate over 7 days, (N = 12).

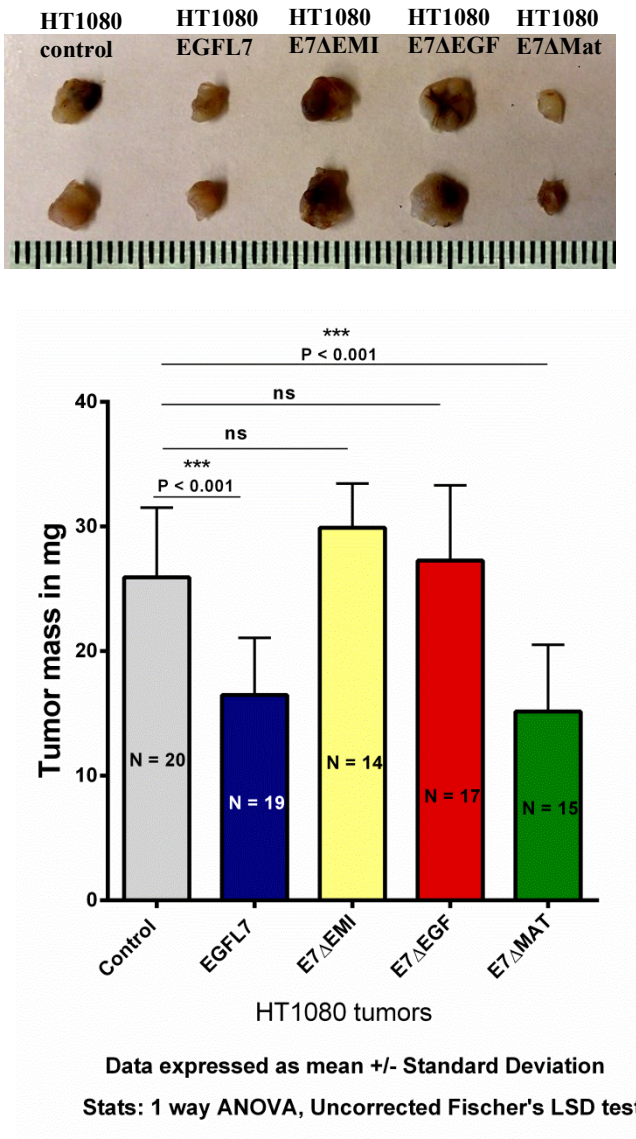
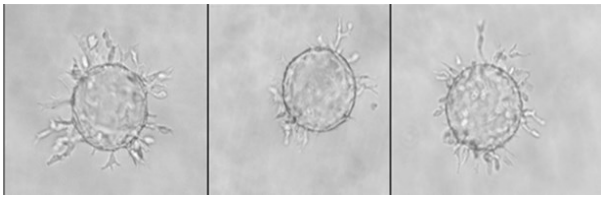


Figure 3.3: EGFL7 inhibits tumor growth *in vivo*. Stable HT1090-empty vector, HT1080-EGFL7, HT1080-EGFL7ΔEMI, HT1080-EGFL7ΔEGF and HT1080-EGFL7ΔMAT cell lines were topically applied to the chorioallantoic membrane (CAM) of avian embryos. The HT1080-EGFL7 tumors were significantly smaller ($P < 0.001$) than the empty-vector HT1080 tumors. Deleting the Emilin-like and EGF-like domains did not show a reduction in tumor size. ($N \geq 14$, $p<0.001$).

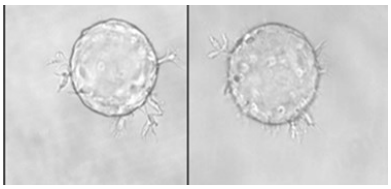
3.3 EGFL7 inhibits tumor angiogenesis *in vivo* and *in vitro* via its Emilin-like domain

In the CAM chicken embryo model, EGFL7 inhibited tumor growth via its Emilin-like and EGF-like domains. Since EGFL7 is a protein that is known to regulate endothelial cell behaviour, the function of each of the domains on the process tumor angiogenesis was observed via two assays: an *in vitro* 3D beads angiogenesis assay and an *ex ovo* CAM angiogenesis assay in the chicken embryo model.

The 3D beads *in vitro* angiogenesis assay was developed to recapitulate the important early stages of angiogenic sprouting[180]. We utilized this assay to observe the effect of EGFL7 on the quantity of endothelial cell sprouting without the interference of VEGF or other growth factors. EA.hy926 endothelial cells were cultured on cytodex microcarrier beads and embedded in a fibrin clot. HT1080 cancer cells expressing EGFL7 (full length and with deleted domains) were grown on top of the fibrin clot and allowed to secrete EGFL7 protein into the medium and the fibrin clot. The endothelial cell-coated beads in contact with EGFL7-expressing cancer cells displayed significantly fewer sprouts compared to the beads in contact with control HT1080 cells. Beads in contact with EGFL7-ΔEGF also showed fewer angiogenic sprouts. In contrast, beads in contact with the EGFL7-ΔEMI cells did not lead to a reduced number of angiogenic sprouts; the number of sprouts per bead were comparable to control (Fig 3.4).



HT1080-Control HT1080-EGFL7 HT1080 E7-ΔEMI



HT1080 E7-ΔEGF HT1080 E7-ΔMAT

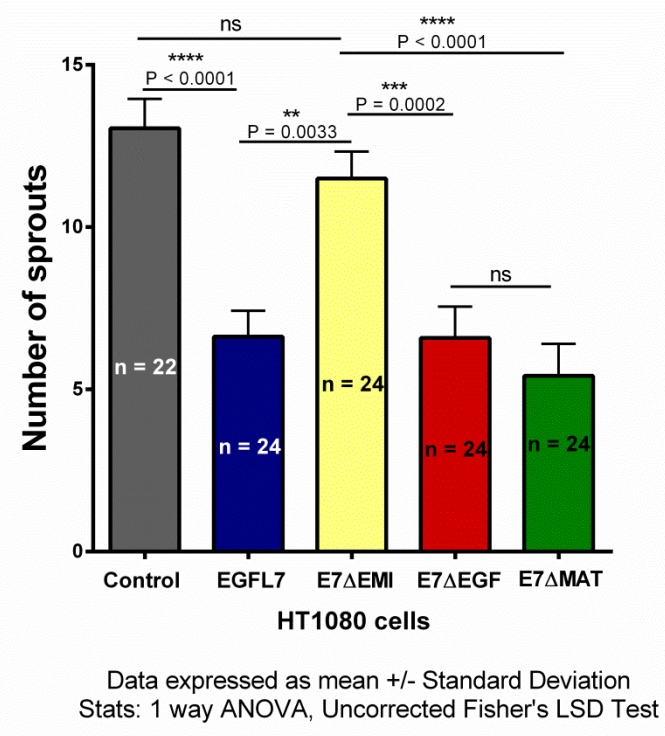


Figure 3.4: EGFL7 inhibits tumor angiogenesis *in vitro* via its Emilin-like domain. A 3D in vitro angiogenesis assay was utilized to study the effect of EGFL7 and deletion variants on early angiogenic sprouting. EGFL7 significantly reduced the endothelial cell sprouting, but deleting the Emilin-like domain negated the anti-angiogenic phenotype. ($N \geq 22$, $p<0.0001$).

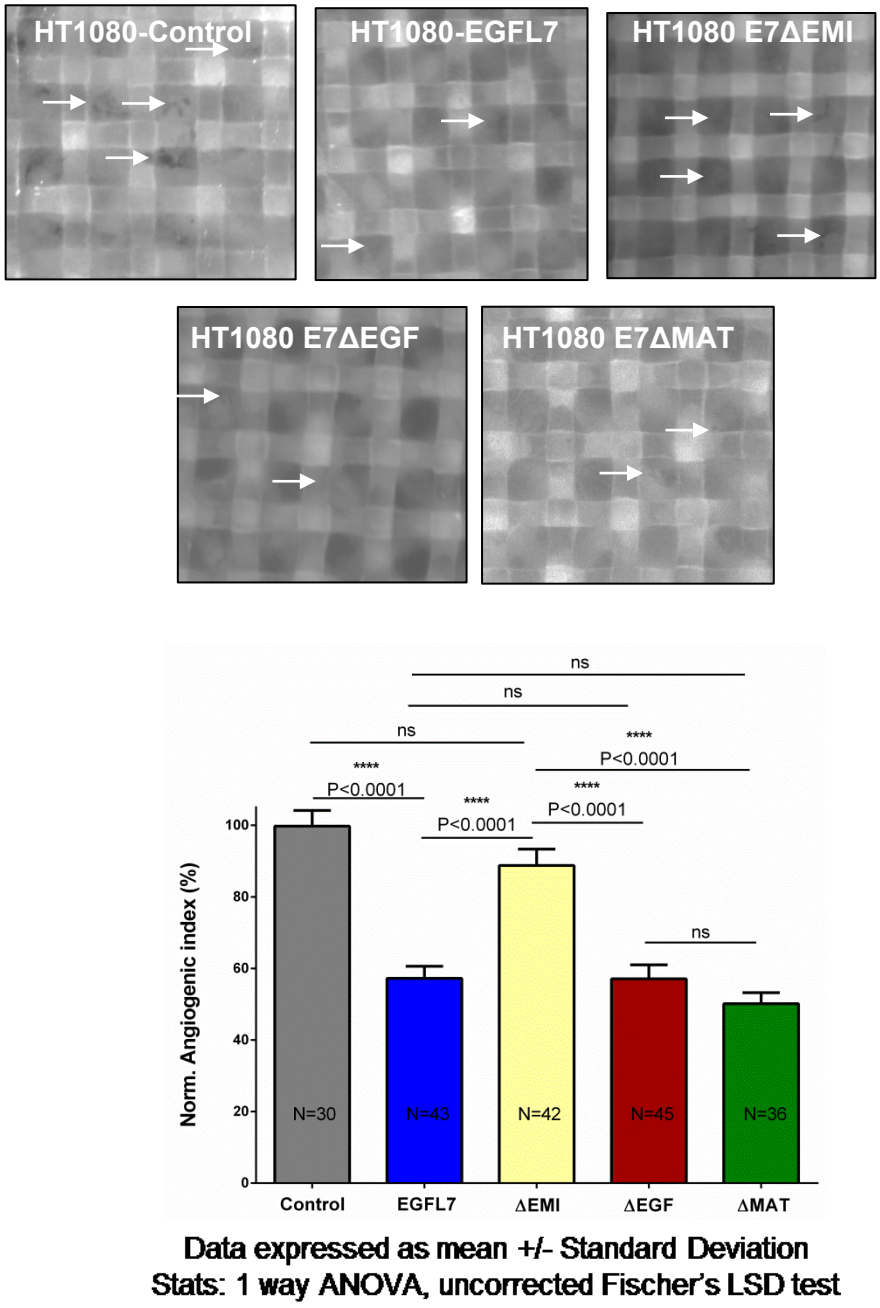


Figure 3.5: EGFL7 inhibits tumor angiogenesis *in vivo* via its Emilin-like domain. HT1080 control, EGFL7-expressing and deletion variants were embedded into collagen onplants and placed on the chorioallontoic membrane of chicken embryos. The EGFL7 onplants had significantly fewer angiogenic vessels compared to control. But deleting the Emilin-like domain negated EGFL7's anti-angiogenic phenotype, suggesting that the Emilin-like domain is required for angiogenic inhibition. ($N \geq 30$, $p<0.0001$).

This suggested that the Emilin-like domain within EGFL7 is for its anti-angiogenic phenotype *in vitro*. To confirm this phenotype, control or EGFL7-expressing (full-length and deletions) HT1080 variants were embedded into collagen that was sandwiched between two nylon meshes to make a collagen 'onplant'. These onplants were placed onto the CAM of day 10 chicken embryos and angiogenic vessels were allowed to invade the tumor cell-containing collagen. The onplants were monitored every day and images were captured on day 3 of the experiment (embryonic day 13). The angiogenic index of the onplant was determined as the percentage of grids that contained newly formed blood vessels out of the total number of grids in the upper mesh. Similar to the *in vitro* beads assay, the onplants with EGFL7-expressing HT1080 cells had significantly fewer blood vessels penetrating the collagen medium compared to control HT1080 cells. But interestingly, the EGFL7-ΔEMI expressing HT1080 cells did not lead to reduced blood vessel penetration, suggesting that the Emilin-like domain is required to inhibit angiogenesis. The EGFL7-ΔEGF and EGFL7-ΔMAT collagen onplants had similar blood vessel penetration as the full-length EGFL7 expressing cells, as the Emilin-like domains in both these cell lines were intact (Fig 3.5). Both the *in vitro* and *in vivo* experiments showed that deleting the Emilin-like domain negates the anti-angiogenic phenotype of EGFL7. In addition to the results of the tumor growth assay *in vivo,* we confirmed that the Emilin-like domain is the functional domain within EGFL7 that is required for its anti-angiogenic activity.

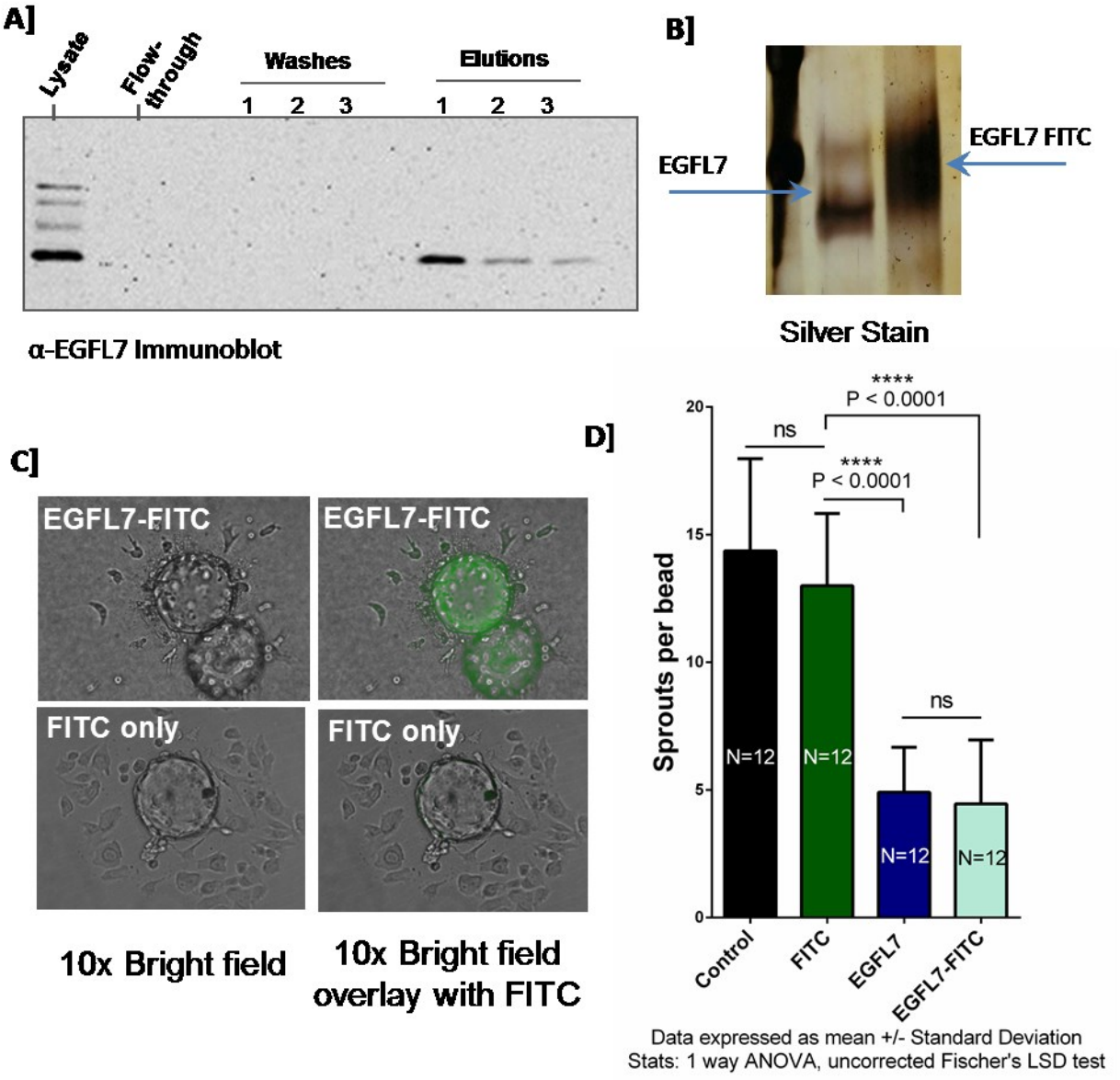


Figure 3.5.1: EGFL7 protein co-localizes with endothelial cells. A] Recombinant EGFL7-His tag protein was purified using cobalt resin. The approximate concentration of the eluted protein was 40 ng/ml. B] FITC was conjugated to the purified EGFL7-his tag protein. C] Endothelial cell-coated beads were embedded into a fibrin clot and purified EGFL7 protein was added on top of the clot in low-serum media. Images captured on day 3 show that EGFL7 co-localized with the endothelial cells (N = 24). There was no signal in FITC-only treated beads. D] Purified EGFL7 and EGFL7-FITC protein significantly reduced endothelial cell sprouting (N = 12, $p<0.0001$).

3.3.1 EGFL7-FITC associates with endothelial cell surface *in vitro*

We purified recombinant EGFL7 protein via the baculovirus expression system and conjugated it with FITC (fluorescein isothiocyanate) to validate EGFL7's permeation through the fibrin clot used in the *in vitro* angiogenesis assay in the previous section (Fig 3.4). We hypothesized that EGFL7 associated directly with the endothelial cell surface in the *in vitro* bead angiogenesis assay as a strong anti-angiogenic phenotype was observed.

Cytodex microcarrier beads were coated with endothelial cells and embedded into fibrin clots as previously described. FITC-EGFL7 was added on top of the fibrin clot in low-serum medium at a final concentration of 2 nM. In control wells, only FITC was added on top of the fibrin clot in low-serum medium at a final concentration of either 2 nM or 10 nM for comparison.

Bright-field and FITC overlay Images at 10x were captured on day 3 after incubation and it was observed that FITC-EGFL7 co-localized with the endothelial cells in the fibrin clot. There was no FITC signal on the endothelial cells that were incubated with only FITC proving that the association was EGFL7-mediated (Fig 3.5.1).

This assay confirmed that EGFL7 not only easily penetrated the fibrin clot, but also localized specifically on the endothelial cell surface.

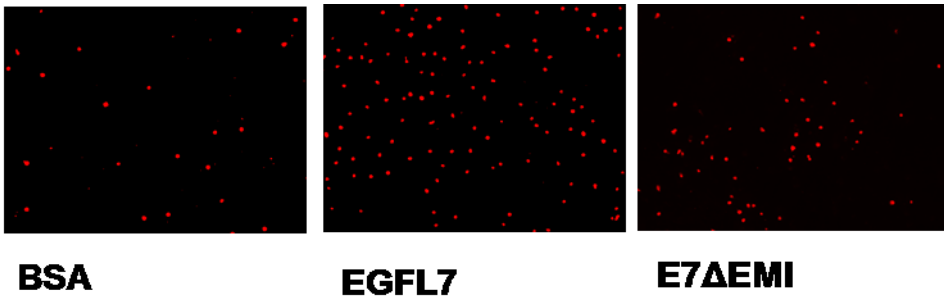
3.4 EGFL7 increases endothelial cell adhesion

EGFL7 was first identified as a protein expressed in the endothelium during embryogenesis[39]. In adults, EGFL7 expression is mostly restricted to proliferating endothelial cells (eg. wounds)[41,181]. We hypothesized that EGFL7 reduced tumor angiogenesis by regulating endothelial cell behaviour like adhesion.

Endothelial cell adhesion to the extracellular matrix is essential to the process of vascular development and angiogenesis[182]. The ECM-endothelial cell adhesion is usually mediated through Integrin αβ dimers on the cell surface that interact with specific ECM constituents. EGFL7 is also known to co-localize with a variety of ECM constituents like fibronectin, collagen IV and vitronectin[41]. EGFL7 potentially acts as a mediator between the ECM and endothelial cells by regulating their interaction.

From the previous experiments, we know that the Emilin-like domain regulates angiogenesis *in vitro* and *in vivo*. We purified EGFL7 and EGFL7-ΔEMI protein to test if the Emilin-like domain is required to regulate endothelial cell adhesion as well.

Cell culture plates (12-well) were coated with 40 ng/ml of either BSA, EGFL7 or EGFL7-ΔEMI protein overnight and EA.hy926 endothelial cells were seeded onto each well. The endothelial cells were allowed to adhere for 3 hours followed by 5x washes with PBS. The adherent cells were fixed, stained and counted. EGFL7-coated plates had a significantly higher number of endothelial cells still adhered to the plate surface compared to the BSA control. Interestingly, deleting the Emilin-like domain did not have an effect on the number of adhered cells, suggesting that the Emilin domain is not involved in regulating cell adhesion (Fig 3.6).



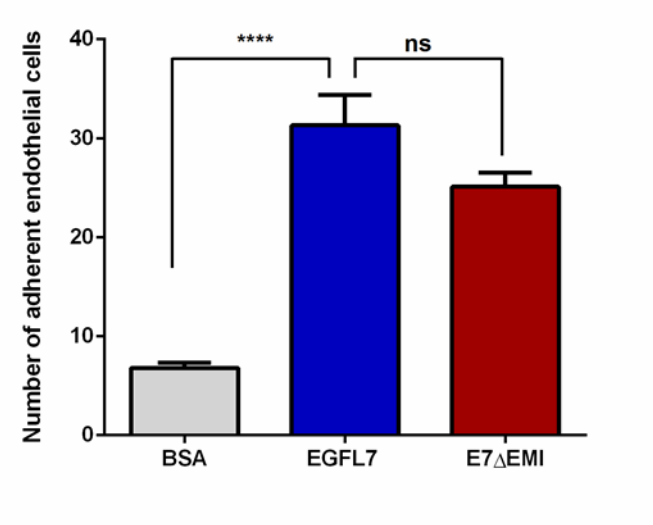


Figure 3.6: EGFL7 increases endothelial cell adhesion, but not via its Emilin-like domain. Recombinant EGFL7 and EGFL7-ΔEMI protein was purified and coated onto cell culture plates overnight. EA.hy926 endothelial cells were allowed to adhere onto these plates for 3 hours followed by 5x PBS washes. EGFL7 increased endothelial cell adhesion as expected. But this adhesion was not mediated via its Emilin-like domain as there was no significant reduction in adhesion when the Emilin-like domain is deleted.

3.5 EGFL7 interacts with Integrin β_1 and Thrombospondin-1

Although EGFL7 is known to be involved in the process of angiogenesis, very little is known about its mechanism of action. Since EGFL7 is known to associate with the extracellular matrix (ECM) and regulate endothelial cell behaviour, we hypothesized that EGFL7 may interact with other key ECM-associated proteins that are also involved in endothelial cell communication, adhesion or migration[41,94,95]

To identify potential EGFL7 binding partners, we employed the technique of Immunoprecipitation (IP). HT1080 control and EGFL7-expresssing cell lysates were incubated with anti-V5 tag antibody followed by pull-down with protein A-coated magnetic IP beads. The samples were resolved via 10% SDS polyacrylamide gel electrophoresis gels and transferred onto a nitrocellulose membrane. The membrane was blotted with anti-EGFL7 antibody to confirm adequate precipitation of the EGFL7 protein. Integrin β_3 was used as a positive control as it is a known interactor of EGFL7[181].

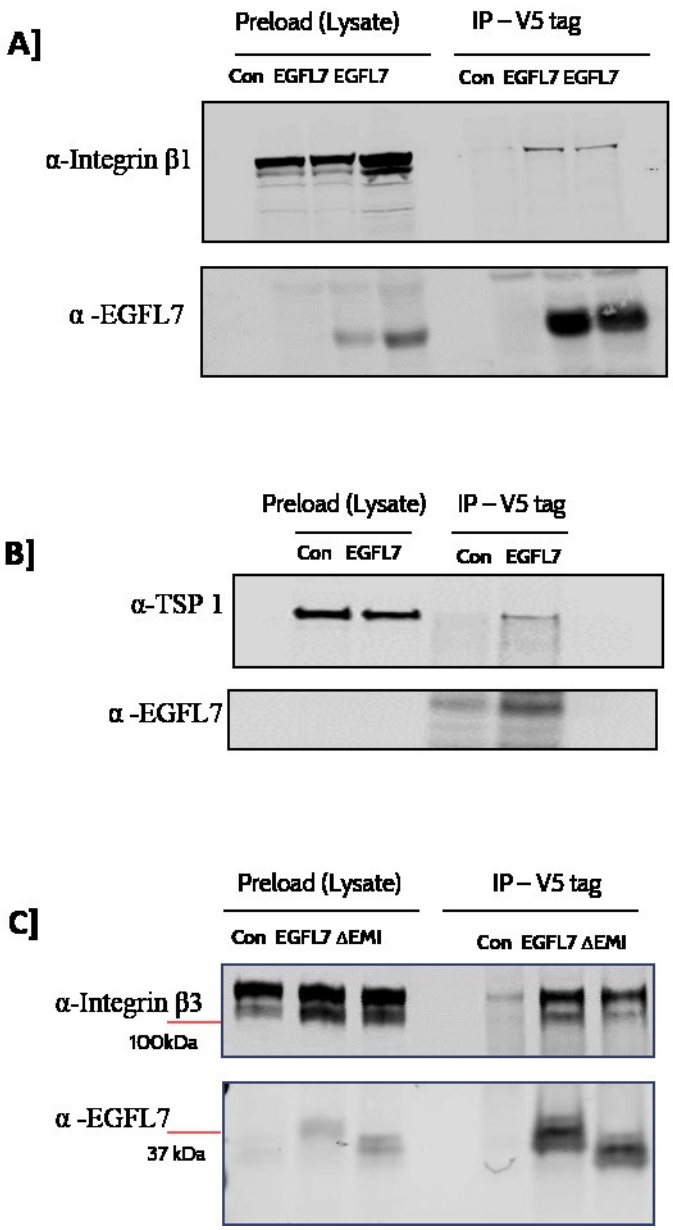


Figure 3.7: EGFL7 interacts with Integrin β_1 and Thrombospondin-1. Anti-V5 tag antibody was used to immunoprecipitate EGFL7 from HT1080 cell lysates. EGFL7 was found to interact with A] Integrin β_1 and B] Thrombospondin 1. C] EGFL7 is known to interact with Integrin β_3. Deleting the Emilin-like domain from EGFL7 did not cancel this interaction, suggesting that another domain within EGFL7 is responsible for this interaction.

The nitrocellulose membrane was blotted with antibodies against various proteins involved in endothelial cell communication with other cells and the ECM. Through this process, we discovered that EGFL7 also interacted with Integrin β_1 and Thrombospondin-1 (Fig 3.7).

3.5.1 The anti-angiogenic phenotype of EGFL7 is not mediated through its interaction with Integrin β_3

EGFL7 is known to interact with Integrin β_3[181], as also verified by the previous immunoprecipitation experiments (Fig 3.7C). Integrin β_3 plays a crucial role in angiogenesis as it interacts with many ECM proteins like fibronectin and vitronectin[183,184]. Integrin β_3 is expressed on the surface of endothelial cells and helps the cells adhere to the ECM[181]. Since EGFL7 is one of the ECM-associated proteins, we hypothesized that EGFL7 mediates its anti-angiogenic phenotype via its interaction with Integrin β_3.

We knocked down (KD) the expression of Integrin β_3 in EA.hy926 endothelial cells using Integrin β_3 siRNA. We coated the Integrin β_3-KD endothelial cells onto cytodex microcarrier beads and embedded them in fibrin clots. HT1080-control or HT1080-EGFL7 cells were plated on top of the fibrin clots. Beads were imaged and analysed on day 3 after treatment. KD of Integrin β_3 had no effect on EGFL7's ability to inhibit angiogenesis, as there was no significant difference in the number of angiogenic sprouts per bead in the control and KD wells (Fig 3.8).

Interestingly, deleting the Emilin-like domain had no effect on EGFL7`s interaction with Integrin β_3 (Fig 3.7C).

3.6 EGFL7 is expressed at the cell surface of EGFL7-expressing HT1080 cells

EGFL7 is a secreted angiogenic factor that is deposited on the ECM[41]. EGFL7 is also known to be expressed in cancer cells and regulate the adhesion and migration of hepatocellular carcinoma cells[108]. However, it is unknown whether EGFL7 is expressed on the cell surface of cancer cells.

To test this, we immobilized control HT1080 and EGFL7-expressing HT1080 cells with non-permeabilizing paraformaldehyde fixative on glass cover slips. Both cell lines were stained with anti-EGFL7 antibody, AF-647 phalloidin stain for actin and Hoechst 33342 marker for cell nuclei. Z-stacked images were captured via confocal microscopy and EGFL7 staining was observed along the cell surface of EGFL7-expressing cells only (Fig 3.9 B).

We also performed another experiment to show that EGFL7 is expressed on the tumor cell surface. We biotinylated HT1080-control and HT1080-EGFL7 cells using cell-impermeable Sulfo-NHS-Biotin. Immunoprecipitation using Sulfo-NHS-Biotin is a well-established protocol to selectively precipitate proteins that are only expressed at the cell surface[185].

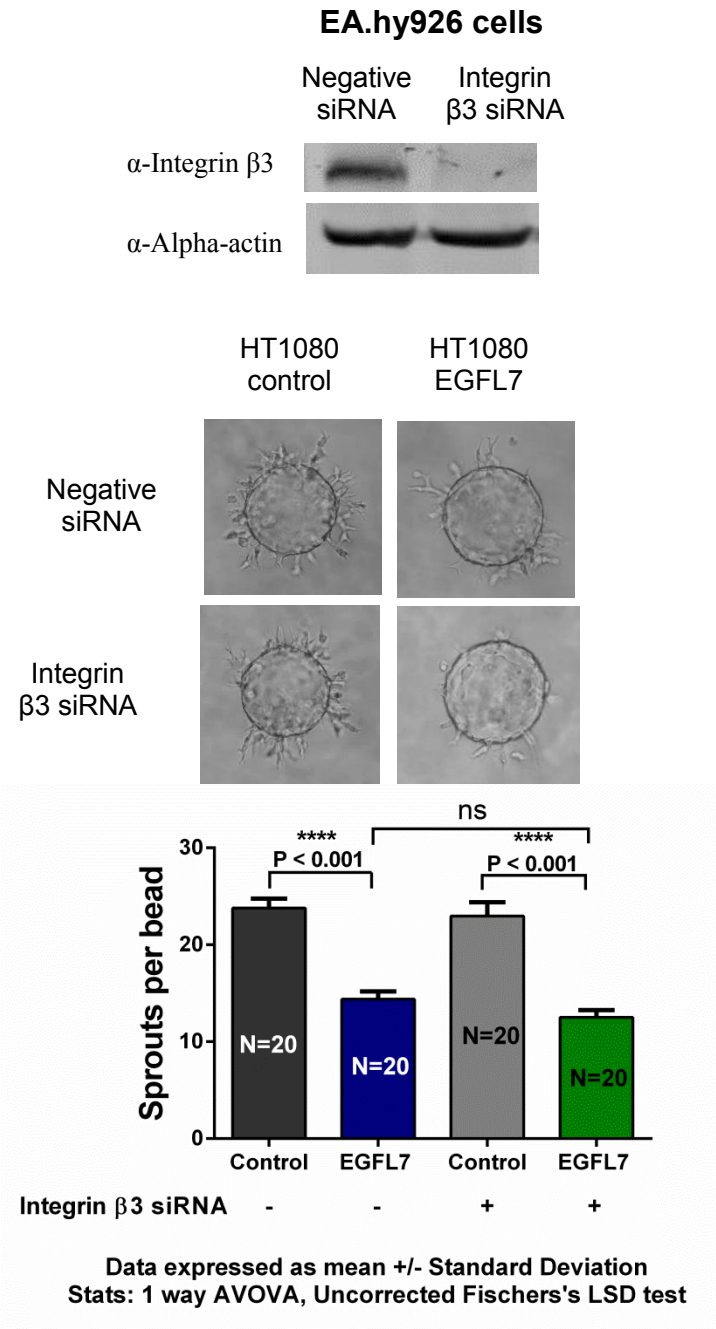


Figure 3.8: The anti-angiogenic function of EGFL7 is not mediated through its interaction with Integrin β_3. A] Integrin β_3 was knocked down (KD) in endothelial cells using Integrin β_3 siRNA. B] An *in vitro* angiogenesis assay revealed no significant difference in endothelial cell sprouting in Integrin β_3 KD endothelial cells. (N=20, $p<0.001$).

We immunoprecipitated the biotinylated proteins using protein-A magnetic beads coated with anti-biotin antibody and resolved the samples on 10% SDS PAGE gels. Next, we immuno-blotted the Western blot membrane with anti-EGFL7 antibody and anti- β-Tubulin antibody. β-Tubulin was used as a negative control as it is not expressed at the cells surface[142]. A 37 kDa band corresponding to EGFL7 was observed only in the immunoprecipitation samples from EGFL7-expressing cancer cells (Fig 3.9 A). While similar amounts of β-Tubulin were observed in the cell lysates of both control and EGFL7-overexpressing HT1080 cells; no detectable β -Tubulin bands were observed in immunoprecipitation samples of either cell variants, as expected[186].

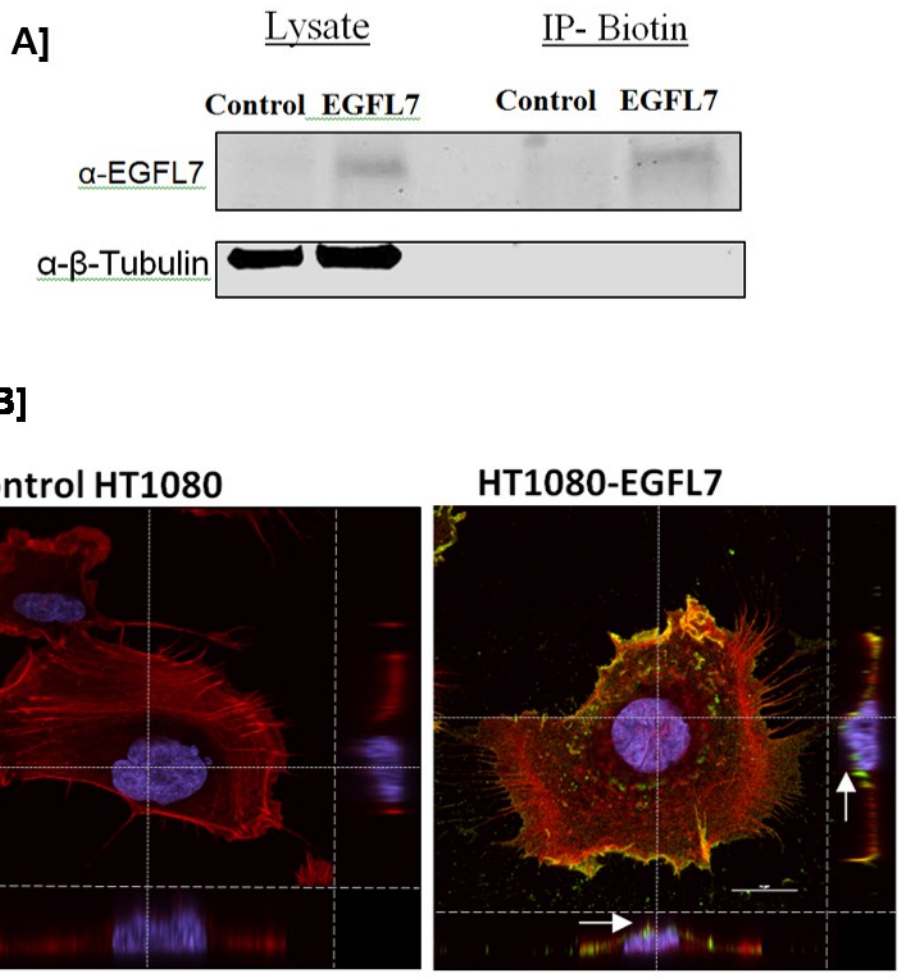


Figure 3.9: EGFL7 is expressed on the tumor cell surface. A] Western blot analysis of biotinylated EGFL7 immunoprecipitation using anti-Biotin antibody. Proteins only at the cell surface can be biotinylated, proving that EGFL7 is expressed at the tumor cell surface. B] Confocal images of non-permeabilized Control-HT1080 and EGFL7-expressing HT1080 fibrosarcoma cells captured at 63x magnification. Phalloidin-AF 647 (red), white arrows indicating cell surface EGFL7 (green) and nuclei staining (blue). Scale bar, 20 microns.

Also published in Cho *et al*, 2017.

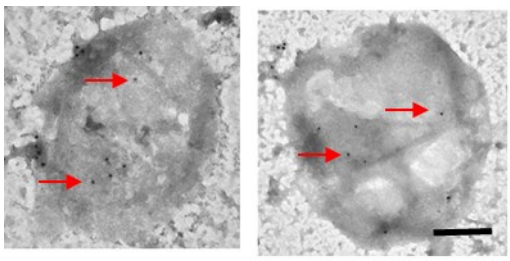
3.7 EGFL7 is secreted from cancer cells via extracellular vesicles

In addition to being expressed on the tumor cell surface, EGFL7 is known to be deposited onto the ECM[41]. EGFL7 is composed of domains that are commonly found in other ECM constituents as well (eg. the EGF-like domain is found in laminins)[187]. Although EGFL7 is known to be targeted for secretion, little is known about how it is released from the cell membrane[95].

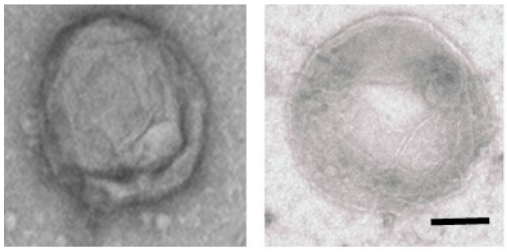
We hypothesized that EGFL7 is secreted from the tumor cell surface via extracellular vesicles (EV) as cancer cells have been implicated in modulating the cellular components of their microenvironment by transferring proteins, mRNAs and miRNAs via extracellular vesicles[188,189].

To test this hypothesis, we incubated HT1080 control and EGFL7-expressing cells in no-serum DMEM medium for 12 hours. The conditioned medium was then centrifuged at 500x g to remove live cells, 1000xg to remove cell debris and then ultracentrifuged at 100,000xg to precipitate the extracellular vesicles. The EV pellet was resuspended in 100 µl of PBS and analysed using Western blotting and TEM imaging.

Control and EGFL7-expressing tumor cell derived EVs were fixed and stained with anti-EGFL7 antibody followed by immunogold staining for visualization via transmission electron microscopy (TEM). The images revealed that HT1080-EGFL7 derived EVs expressed EGFL7, while no EGFL7 staining was observed on control HT1080 derived EVs (Fig 3.10).



HT1080 - EGFL7
Extracellular vesicles



HT1080 - control
Extracellular vesicles

Figure 3.10: EGFL7 is secreted via extracellular vesicles. A] Extracellular vesicles (EV) were isolated from control and EGFL7-expressing fibrosarcoma (HT1080) cells via ultracentrifugation and stained with anti-EGFL7 antibody followed by streptavidin-gold. TEM imaging revealed that tumor-cell derived EVs were positive for EGFL7 expression. Scale bar = 200 nm.

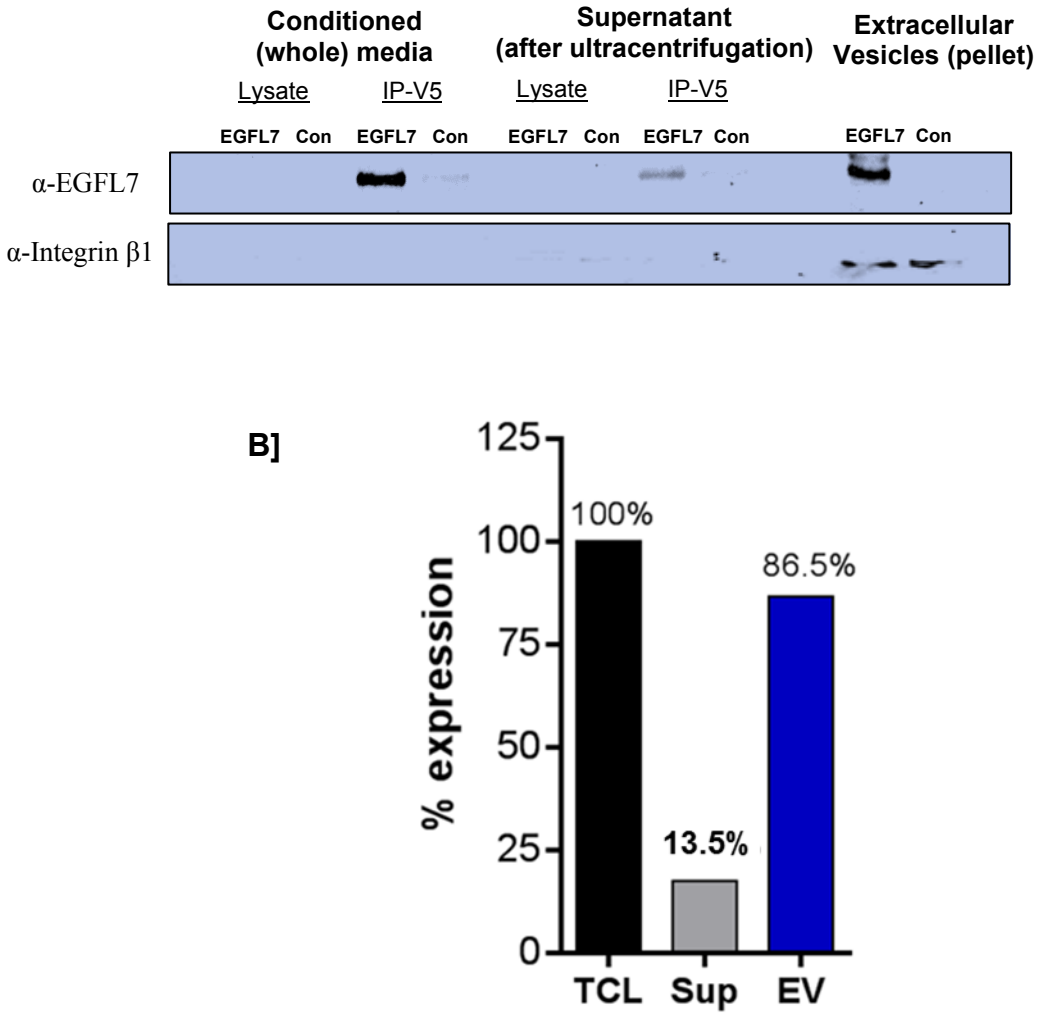


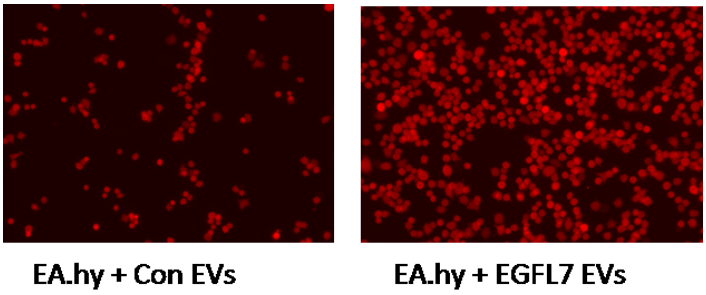
Figure 3.11: Majority of EGFL7 is secreted from tumor cells via extracellular vesicles. A] HT1080 cells (control and EGFL7-expressing) were incubated in no-serum DMEM media for 12 hours. Half of this enriched media was ultracentrifuged to pellet EV. The other half was incubated with V5-tag antibody to immunoprecipitate EGFL7 using protein-A beads. Additionally, any remaining EGFL7 from the post-ultracentrifugation enriched media was also immunoprecipitated for comparison. B] Western blot densitometry analysis shows that majority of EGFL7 protein is secreted via extracellular vesicles

An additional experiment was carried out where EGFL7 from both conditioned medium and post-ultracentrifugation supernatant was immunoprecipitated using Protein A magnetic beads coated with anti-V5 tag antibody. Immunoprecipitation allows for an approximate quantification of the total amount of a selected protein in lysates[190]. Pelleted EVs were also collected for comparison of EGFL7 expression. We re-suspended all samples in Laemmli gel loading buffer and resolved them using a 10% SDS polyacrylamide gel. The Western blot analysis indicated that only a small fraction of the secreted EGFL7 protein is not associated with EVs (13.5%) and is freely dispersed in the conditioned medium (Fig 3.11). Nearly 84.5% of EGFL7 was pelleted, suggesting it was EV-associated.

To verify that EGFL7 was indeed expressed in tumor cell-derived EVs, we also immunoblotted the membrane with anti-TSP-1 antibody as TSP-1 is a known EV-associated protein[191,192]. There was no TSP-1 staining in the enriched media; TSP-1 bands were clearly visible in both control and EGFL7-derived EVs.

3.7.1 EGFL7-EVs increase endothelial cell adhesion *in vitro*

From previous experiments, we know that purified recombinant EGFL7 protein increases endothelial cell adhesion (Fig 3.6). However, it is unknown if tumor extracellular vesicle-associated EGFL7 also shares this phenotype. We carried out an EA.hy926 endothelial cell adhesion assay to verify the



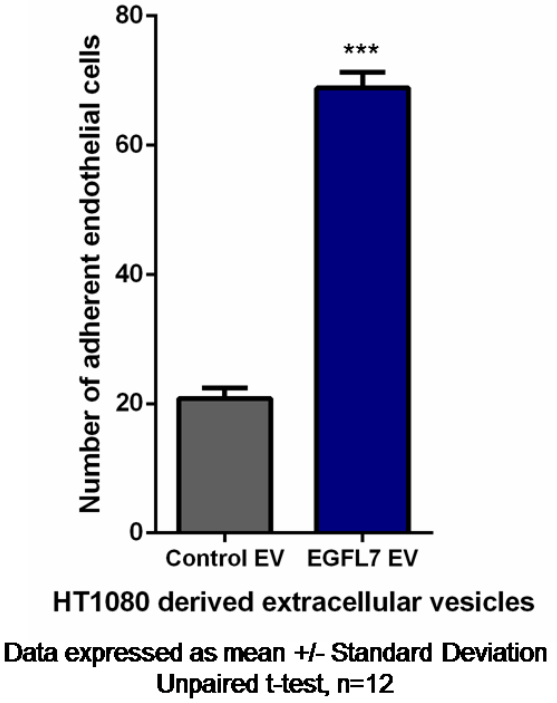


Figure 3.12: EGFL7 extracellular vesicles increase endothelial cell adhesion. Extracellular vesicles (EVs) were isolated from control and EGFL7-expressing HT1080 fibrosarcoma cells via ultracentrifugation. EA.hy926 endothelial cells were treated with the EVs for 3 hours before 5x PBS washes. EGFL7-EVs significantly increased endothelial cell adhesion. (N=12, $p<0.001$).

functionality of tumor-derived EVs and its effect on endothelial cell adhesion. Control and EGFL7 EVs were isolated from HT1080 conditioned medium and resuspended in DMEM medium with 10% FBS. The EV-rich medium was mixed with trypsin-detached EA.hy926 cells and added to 6-well cell culture plates. The endothelial cells were allowed to adhere for 3 hours followed by 5x PBS washes. The remaining adherent cells were fixed, imaged and counted. Similar to the EGFL7 protein, EGFL7-EVs also led to a significant increase in endothelial cell adhesion, compared to control-EVs (Fig 3.12).

3.7.2 The EGF-like domain repeats are required for EV-mediated secretion of EGFL7

In ECM associated proteins, Emilin-like and EGF-like domains are usually found towards the N-terminal signal peptide and play a role in targeting and secretion[97]. To identify which domain within EGFL7 facilitates its EV-mediated secretion, we isolated EVs from the conditioned medium of all deletion mutant cell lines. All EVs were stained with anti-EGFL7 antibody followed by the AF-647 secondary antibody.

We analysed the number of EGFL7 antibody-positive EVs in all samples using the Apogee micro flow cytometer and found that deleting the EGF-like domain significantly reduces EV production in HT1080 cells (Fig 3.13). As expected, EGFL7 secreted significantly more EVs compared to control HT1080 cells; deleting the Emilin-like domain did not affect EV production and secretion. Another interesting observation from this experiment was that deleting the

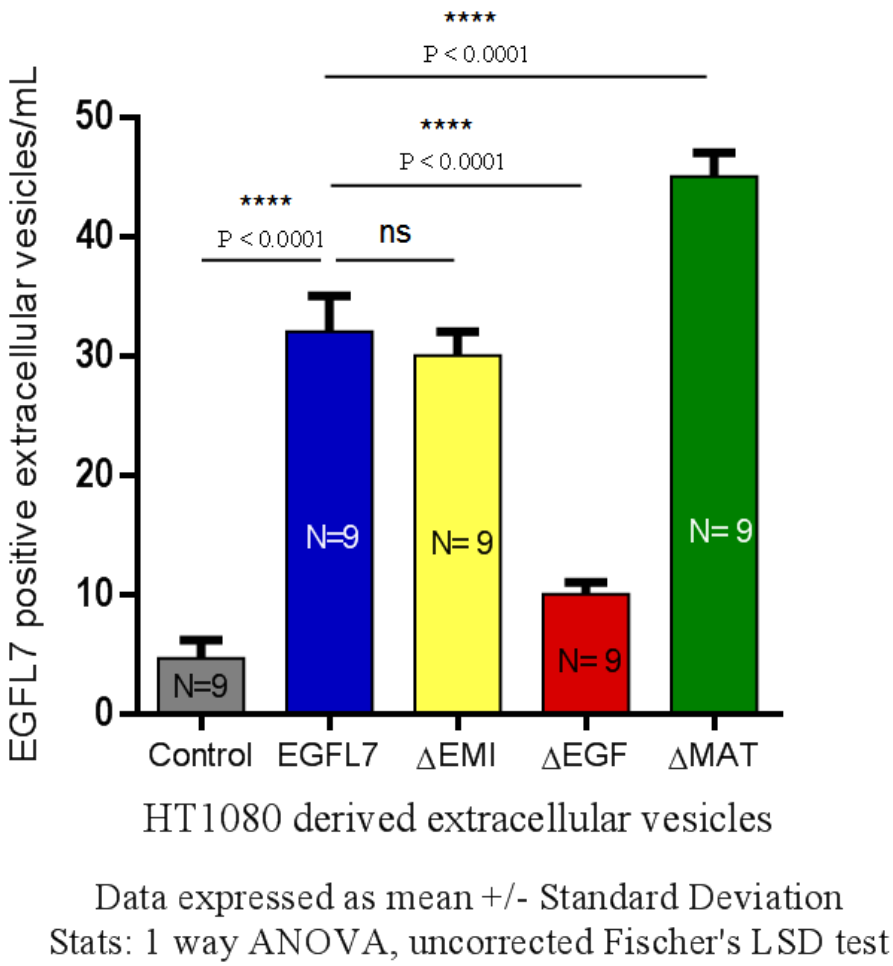


Figure 3.13: The EGF-like domain is required for EGFL7-EV secretion. Particle count of AF-647 and anti-EGFL7 antibody tagged HT1080 derived extracellular vesicles (EVs) was performed using a micro flow cytometer. EGFL7-expressing cells produced more EVs as expected. Deleting the EGF-like domain significantly reduced EV production. Conversely, deleting the Matrilin-like domain significantly increased EV production. (N=9, p<0.0001).

Matrilin-like domain significantly increases EV secretion compared to all other domains.

We performed an additional experiment to confirm the requirement of the EGF-like domain for EV secretion. Glass cover slips were coated with fibronectin, an ECM constituent, and HT1080 cell variants were coated on top for 48 hours. We fixed and stained the cells with anti-EGFL7 antibody along with AF-647 Phalloidin (actin) and Hoechst 33342 stain for nuclei. Secreted EGFL7 protein was easily visualized around HT1080-EGFL7, HT1080 EGFL7-ΔEMI and HT1080-EGFL7ΔMat cells. However, only a sparse amount of secreted EGFL7 was visible around the HT1080 EGFL7-ΔEGF and control HT1080 cells (Fig 3.14).

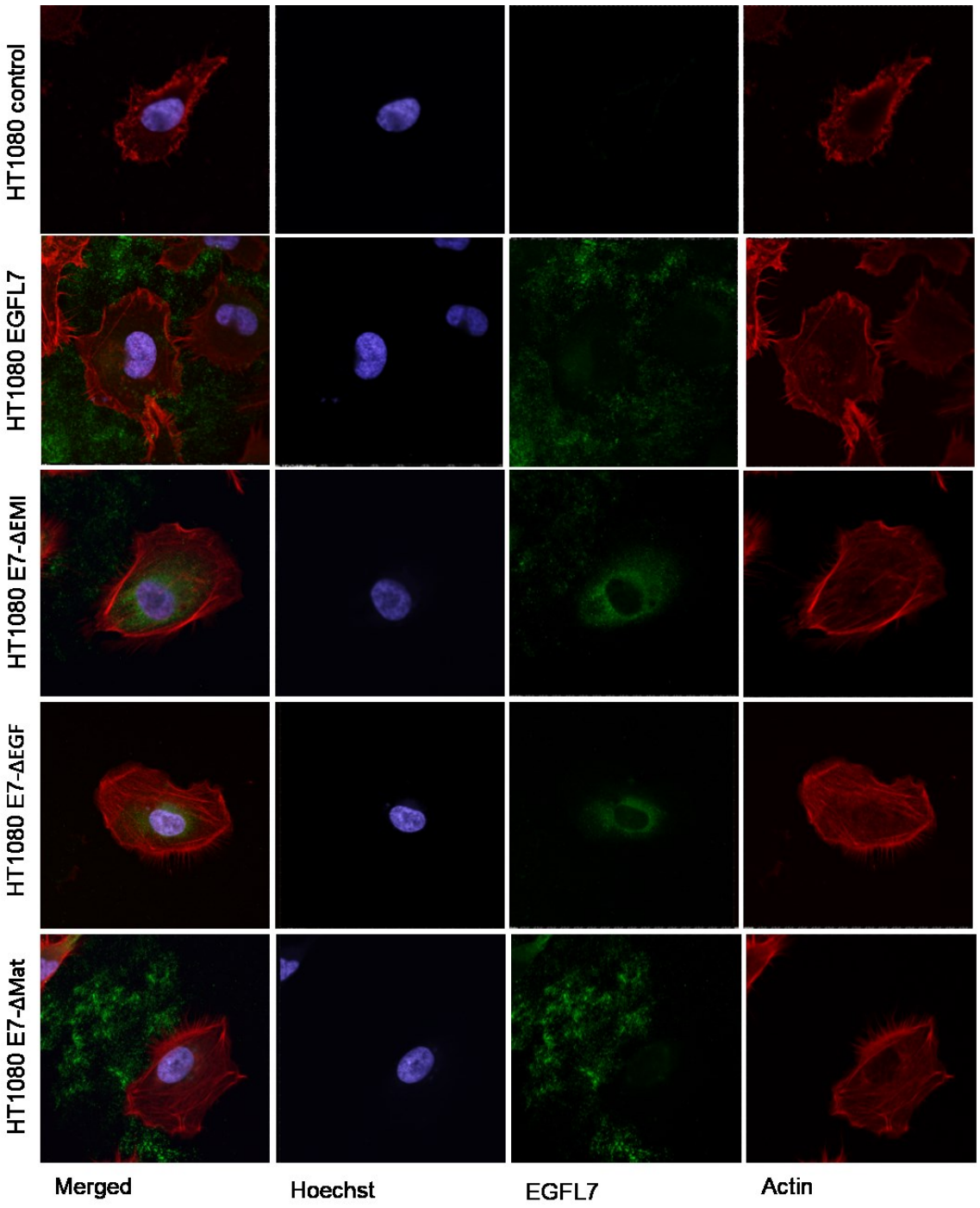


Figure 3.14: EGFL7 is secreted from tumor cells via the EGF-like domain. Confocal images of HT1080 control, HT1080-EGFL7 and its deletion variants plated onto fibronectin. Secretion of EGFL7 is significantly reduced when the EGF-like domain is deleted. (n=6)

Chapter 4: Discussion

4.1 Discussion

EGFL7 is expressed both in cancer cells and in endothelial cells[41,108]. In this study, we focused on the effect of tumor-derived EGFL7 on the endothelial cells in the tumor microenvironment. We also aimed to decipher how EGFL7 is secreted from the cancer cells and deposited onto the ECM.

We utilized an *in vivo* tumor growth, both *in vivo* and *in vitro* angiogenesis and an *in vitro* endothelial cell adhesion assays to elucidate EGFL7's influence on the endothelial cells in the tumor microenvironment. In addition, extracellular vesicle secretion and function assays were performed to decipher EGFL7's mechanism of action. Finally, immunoprecipitation assays were completed to identify potential binding partners for EGFL7 that may play a role in facilitating its anti-angiogenic phenotype.

4.1.1 EGFL7 inhibits tumor growth and angiogenesis

From the published literature so far, EGFL7 has been implicated as a marker for poor prognosis in many human cancers, especially in glioblastomas[112,121]. But to our surprise, EGFL7-expressing HT1080 tumors were significantly smaller than control HT1080 tumors in the CAM *in vivo* model (Fig 3.3).

The rate of proliferation of EGFL7-expressing cells was similar to the control HT1080 cells, indicating that the smaller tumor size was not a result of decreased proliferation (Fig 3.2). Next, we tested the effect of EGFL7

expression on angiogenesis and found that tumor-derived EGFL7 inhibited angiogenic sprouting in both *in vivo* and *in vitro* models (Fig 3.4 and 3.5). The smaller tumor size of EGFL7-expressing tumors is likely due to the suppression of angiogenesis in the EGFL7-expressing tumors. Additional assays showed that extrinsic EGFL7 co-localized with endothelial cells and promoted their adhesive properties (Fig 3.4.1 and 3.6).

EGFL7 has been shown to promote endothelial cell adhesion, migration, sprouting, and proliferation during embryonic angiogenesis to form healthy blood vessels from existing vasculature[40]. In adults, EGFL7 expression is downregulated in normal tissues and restricted to the proliferating endothelium in wounds, sites of atherosclerosis and the uterus[39,40]. In the tumor microenvironment, EGFL7 is overexpressed compared to normal physiology as cancer cells secrete EGFL7 in an attempt to attract angiogenic sprouts[39,95]. And although EGFL7 causes an increase in endothelial cell adhesion and migration in the tumor microenvironment, the blood vessels formed as a result of excess EGFL7 expression are tortuous and leaky, with a thicker endothelium[100,193].

Since the discovery of EGFL7 in the tumor microenvironment, a few studies have attempted to therapeutically target EGFL7 to disrupt tumor angiogenesis *in vitro*[111,121–125]. However, a phase II clinical trial adding anti-EGFL7 antibody (Parsatuzumab) to a combination of mFOLFOX6 and anti-VEGF-A antibody (Bevacizumab) therapy to a cohort of 127 patients showed no improvement compared to the placebo results[127]. The overall response rate was 59% in the parsatuzumab (anti-EGFL7) arm and 64% in the placebo arm[127].

The results from our study show that the role of tumor-EGFL7 in the tumor microenvironment is more complex than previously understood and antibody-targeted inactivation of EGFL7 may not be the ideal method for anti-cancer therapy.

4.1.2 The Emilin-like domain in EGFL7 is required for its anti-angiogenic effect

The Emilin-like (EMI) domain in EGFL7 is also commonly found in the EMILIN/Multimerin family proteins, like Emilin1 and Multimerin2[97], that are present in the ECM. The EMILIN/Multimerin family proteins are involved in multimerization and adhesion of ECM-associated proteins[194]. The EMI domain in these proteins is a cysteine-rich domain that is suggested to function as a protein-protein interaction molecule[99,195].

In our study, the tumor size of EGFL7-expressing cells was significantly smaller than non-EGFL7 expressing cells (Fig 3.3). Deleting the EMI domain in EGFL7 led to a loss of the tumor inhibitory phenotype of the protein. Next, we tested the effect of the EMI domain deletion on EGFL7's regulation of endothelial cell adhesion and sprouting. We utilized both *in vitro* and *in vivo* assays to verify the result and discovered that although the EMI domain was required to inhibit EGFL7-mediated angiogenesis, it was not the domain responsible for promoting endothelial cell adhesion (Fig 3.4, 3.5 and 3.6).

ΔEMI led to a complete loss of EGFL7's anti-angiogenic phenotype *in vitro* and *in vivo,* signifying the requirement of the EMI domain for EGFL7-mediated angiogenic inhibition. Although the role of EGFL7's EMI domain has never been documented in published literature, in other proteins the presence of Emilin-like domains suggest that the proteins are involved in the deposition of elastic fibres in the ECM[99,196,197]. Interestingly, EGFL7 has been shown to inhibit the deposition of mature elastic fibers by repressing lysyl oxidase (LOX)– mediated conversion of tropoelastin into elastin[103]. It is probable that the EMI domain is responsible for the (LOX)-mediated regulation of elastin maturation by EGFL7.

Deletion of the EMI domain did not have a significant effect on the adhesion of endothelial cells compared to full-length EGFL7, but the trend suggested a slightly lowered capacity for adhesion (Fig 3.6). The EGFL7 protein in endothelial cells has been shown to bind to the ECM via its interaction with Integrin $\alpha_v\beta_3$ in an RGD motif-dependant mechanism[181]. The RGD motif is expressed in the EGF-like domain in EGFL7, suggesting that endothelial cell adhesion may be regulated via the EGF-like domain. Unfortunately, we were unable to purify recombinant EGFL7ΔEGF and EGFL7ΔMAT protein to confirm the domain that regulates endothelial cell adhesion.

4.1.3 EGFL7 interacts with Integrin β_1 and TSP-1

EGFL7 is known to interact with Lysyl Oxidase (LoxL2) and $\alpha_V\beta_3$ integrin in the extracellular matrix[103,181]. EGFL7 is also suggested to antagonistically bind

to Notch receptors as it contains a Delta/Serrate/LAG-2 domain in its first EGF-like domain repeat[95,100].

We used anti-V5 tag antibody coated magnetic beads to immunoprecipitate EGFL7 to identify potential binding partners that may co-precipitate with it. Integrin β_3 precipitation was used as a positive control. We discovered that Integrin β_1 and Thrombospondin-1 readily precipitated with EGFL7 (Fig 3.14). Both Integrin β_1 and Thrombospondin-1 are key players in cancer progression and endothelial cell regulation[198,199]. Depending on their levels of expression, Integrin β_1 and TSP-1 are found to be pro- or anti-tumorigenic[199–201].

Integrin β_1 plays an important role in vascular differentiation and development; these integrins also facilitate the adhesion between the ECM and surrounding cells[184,198]. ECM constituents (like EGFL7) are known to interact with Integrin β_1 to regulate the adhesion and migration of endothelial cells for the formation of angiogenic blood vessels[49,184].

Conversely, TSP-1 is a potent inhibitor of angiogenesis[202,203]. Both TSP-1 and TSP-2 directly antagonize the activity of VEGF, leading to reduced migration, adhesion and proliferation of endothelial cells[203]. Moreover, TSP-1 has been shown to collaborate with Integrin β_1 to produce this anti-angiogenic effect[204].

Overall, the binding partners of EGFL7 build strong supporting evidence for EGFL7's involvement in the regulation of tumor angiogenesis. Like Integrin β_1

and TSP-1, our data suggests that the expression of EGFL7 may also be dependent on its concentration in a given cellular microenvironment.

4.1.4 Functional EGFL7-expressing extracellular vesicles are secreted by fibrosarcoma cells

EGFL7 is a secreted protein that is expressed in both endothelial and cancer cells[108]. Extracellular vesicles (EVs) isolated from the conditioned medium of HT1080-EGFL7 expressing cells were positive for EGFL7 expression, as confirmed via TEM imaging and Western blotting (Fig 3.9 and 3.10).

In recent years, tumor-derived extracellular vesicles have been found to facilitate intercellular communication within the tumor microenvironment[205]. EVs are capable of transferring proteins, and nucleic acids like mRNA and microRNAs to nearby or distal recipient cells[188,205]. In a particularly interesting study, Zomer *et al* discovered that EVs secreted by malignant cancer cells carry mRNAs that enhance the migratory behaviour and metastatic capacity of less malignant cancer cells nearby[206]. Tumor cell-derived EVs also have an effect on the endothelial cells, stromal cells, and the ECM in the tumor microenvironment[189,205].

We tested the effect of control HT1080 and EGFL7-expressing HT1080 EVs on endothelial cell adhesion and observed that they increased endothelial cell adhesion *in vitro* similar to purified EGFL7 protein (Fig 3.11). This shows

that functional EGFL7 is secreted via cancer cells into the tumor microenvironment.

In addition to being secreted via extracellular vesicles, we observed that soluble EGFL7 is also freely secreted into media. According to our study, 86.5% of EGFL7 is EV-bound, while a small fraction (13.5%) of EGFL7 was not pelleted via ultracentifugation (Fig 3.10). A reason for the biased EV-bound secretion may be that EVs are able to travel to distant sites via the blood circulation as EVs are often able to evade and even suppress the immune system[207–210].

We performed an additional experiment that studied the effect of domain deletions on the secretion of EVs using micro flow cytometry. We stained all EVs with anti-EGFL7 antibody and calculated the number of EGFL7-expressing (full-length and deletion variants) EVs per uL. EGFL7-expressing cells secreted significantly more EVs than the control HT1080 cells as expected; the HT1080-EGFL7ΔEMI cells secreted a similar amount to HT1080-EGFL7 (no significant difference). Remarkably, deletion of the EGF-like domain significantly reduced the number of EVs secreted by HT1080 cells and the deletion of the Matrilin-like domain seemed to have an opposite effect as EV secretion increased dramatically (Fig 3.12).

The EGF-like domains are usually found towards the N-terminal signal peptide and play a role in targeting and secretion of ECM-associated proteins[97,99]. This may also be the case with EGFL7 as deleting the EGF-like

domain severely reduced EV secretion. Additionally, there is an increase in EV secretion upon deletion of the Matrilin-like domain which suggests a potential role for the Matrilin-like domain in retaining EGFL7 in the cytoplasm. Although Matrilin-like domains have not been documented in other proteins yet, they are similar to the C-terminal coiled-coil region of the Matrilin family proteins[211,212]. Long coiled-coil proteins have been suggested to play a role in tethering vesicles to intracellular organelles[213,214]; it is possible that the Matrilin-domain plays a similar anchoring role in EGFL7-EVs.

We confirmed this EGF-like domain dependant secretion of EGFL7 by imaging intercellular and secreted (extracellular) tumor-EGFL7 on fibronectin-coated glass slides. From the confocal microscopy, large deposits of secreted EGFL7 protein were observed all around the HT1080-EGFL7 cancer cells; similar patterns were observed around the HT1080-EGFL7ΔEMI cells. There was significant intracellular staining for EGFL7 observed in the cytoplasm of EGFL7ΔEMI and EGFL7ΔEGF cancer cells, but no detectable secreted protein for the EGF-like deletion. Large deposits were again visible for the HT1080-EGFL7ΔMat cells confirming the increased EV-mediated secretion (Fig 3.12).

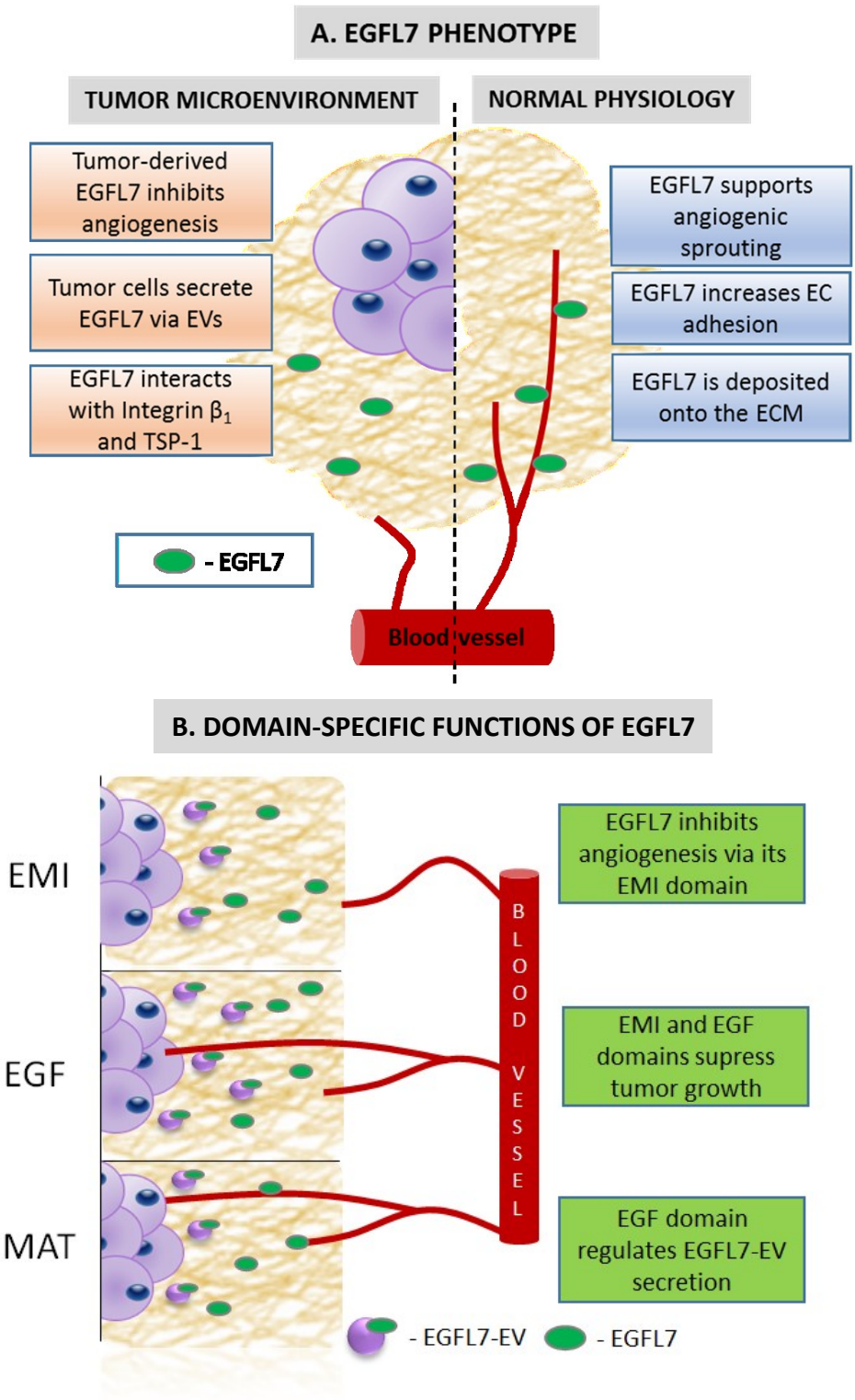


Figure 4.1: Conclusion. EGFL7 inhibits tumor angiogenesis via its EMI domain. EGFL7-EV secretion is regulated by the EGF domains.

4.2 Significance

Tumor angiogenesis is one the hallmarks of cancer progression that can potentially lead to metastasis[53]. In the past few decades, targeting and inactivating pro-angiogenic molecules has been a successful addition to chemotherapy[96]. Understanding the complexities of tumor angiogenesis regulation will further add to the development of better anti-cancer therapies and diagnostic tools.

EGFL7 plays a significant part in endothelial cell adhesion, migration, and proliferation in normal tissues as well as the tumor microenvironment, as seen from published literature and this study. We were able to clarify that tumor-derived EGFL7 functions as an anti-angiogenic molecule in the tumor microenvironment. We also implicated the Emilin-like domain within EGFL7 as the functional anti-angiogenic domain. This information can be used to potentially develop an EMI domain-based anti-angiogenic therapy.

Moreover, we identified two novel interactors of EGFL7: Integrin β1 and Thrombospondin-1. These interactions can be further studied to regulate angiogenesis.

4.3 Limitations

Although *in vitro* 2D cell cultures cannot completely replicate the *in vivo* conditions, results from 2D *in vitro* culture experiments are a good indicator of

cellular responses to stimulations from biochemical cues. Using 3D cultures to perform in vitro assays will provide a more robust analysis[216].

There are limitations to our chosen approach of immunoprecipitation as we are only testing autocrine interactions. A more thorough method to identify binding partners for EGFL7 would be to study the interaction of tumor-derived EGFL7 protein with cell surface endothelial cell proteins.

4.4 Future Directions

The EMI domain in EGFL7 was identified by us to be responsible for its anti-angiogenic phenotype. But the EMI domain is 78 amino acids in length and it would be beneficial to identify the exact anti-angiogenic sequence. There are six cysteine disulphide bonds in the EMI domain that may be responsible for this phenotype. It would also be beneficial to recognize the domains that are responsible for EGFL7's interaction with Integrin β_1, Integrin β_3 and TSP-1.

It is possible that EGFL7's anti-angiogenic phenotype is concentration-dependant. Purified EGFL7 or tumor cell lines expressing varying amounts of EGFL7 can be used to identify EGFL7`s mechanism for anti-angiogenesis.

4.5 Conclusion

We identified EGFL7 as a potent angiogenic inhibitor via its Emilin-like domain in both *in vitro* and *in vivo* models. Our experiments revealed that

EGFL7 co-localized with endothelial cells and enhanced their adhesive properties. The Emilin-like domain did not participate in the adhesion of endothelial cells.

Next, we recognized two key angiogenic players, Integrin β_1 and Thrombopondin-1, as binding partners for EGFL7. The integrin β_3-EGFL7 interaction was proven insignificant for EGFL7's anti-angiogenic phenotype.

Additionally, through confocal imaging, we showed that EGFL7 is expressed on the cellular surface of cancer cells as well as secreted onto the ECM via extracellular vesicles. We identified the requirement of the EGF-like domains for the targeting and secretion of EGFL7-expressing extracellular vesicles.

In summary, this study provides several new insights into EGFL7's functions and secretory mechanisms. The data from this study can be used to further develop EGFL7 as a therapeutic agent against solid tumor angiogenesis.

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