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UNIVERSITY OF CALIFORNIA,
IRVINE

Investigating the Role of Snai2 (Slug) in Endothelial-to-Mesenchymal Transition and Vascular
Morphogenesis

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Molecular Biology and Biochemistry

by

Nan Wu Hultgren

Dissertation Committee:
Professor Christopher CW Hughes, Chair
Professor Xing Dai
Assistant Professor Matt Inlay

2018

DEDICATION

To my husband Eric, for his unconditional love and support through this challenging period of my life. I will forever remember all those “work dates” spent together in the lab on countless late nights and weekends. Thank you for always being there and for being an inspiration in life, and in science.

To my parents, for their love, trust and encouragement. Thank you for nurturing my curiosity for this world and for believing me to chase my dreams halfway across the globe.

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TABEL OF CONTENTS

	Page
LIST OF FIGURES	v
ACKNOWLEDGMENTS	vii
CURRICULUM VITAE	ix
ABSTRACT OF THE DISSERTATION	xiii
CHAPTER 1 Introduction	1
1.1 Vascular morphogenesis	1
1.2 Snai2 (Slug) is an EMT master regulator	9
1.3 3D models to study vascular morphogenesis	15
CHAPTER 2 Endothelial expression of Slug regulates sprouting angiogenesis <i>in vivo</i>	31
2.1 Slug germline knockout mice	31
2.2 Slug regulates developmental angiogenesis <i>in vivo</i>	32
2.3 Defective retinal angiogenesis is due to loss of Slug in endothelial cells	41
2.4 Loss of endothelial Slug expression decreases tumor growth by restricting tumor angiogenesis	46
2.5 Methods	53
CHAPTER 3 High levels of Slug in Endothelial Cells leads to disrupted vessel formation <i>in vitro</i>.	56
3.1 Slug Misexpression in EC Using Lentiviral Approach	56
3.2 Endothelial misexpression of Slug disrupts <i>in vitro</i> angiogenesis	60
3.3 Endothelial misexpression of Slug interrupts <i>in vitro</i> vasculogenesis	66
3.4 Methods	68
CHAPTER 4 Molecular mechanism of Slug-mediated angiogenesis	69
4.1 A role for partial endothelial-mesenchymal transitions in Angiogenesis?	69
4.2 SlugOE cells present an EndoMT-like gene expression profile	83
4.3 Slug regulates Notch-mediated VEGFR2 expression during sprouting	93
4.4 Inhibition of Notch signaling rescues impaired retinal angiogenesis in Slug knockout mice	101
4.5 Methods	106

CHAPTER 5	Upstream regulation of Slug in the endothelial cells	110
5.1	Slug is an SDF1 α downstream effector during sprouting angiogenesis	110
5.2	HGF induces transient Slug expression in EC	117
5.3	Methods	120
CHAPTER 6	Conclusions and future work	122
REFERENCES		140

LIST OF FIGURES

	Page
Figure 1.1 3D assays of vascular morphogenesis	18
Figure 2.1 Slug deficiency leads to impaired yolk sac vascular development	34
Figure 2.2 Slug deficiency leads to impaired neonatal retinal vascular development	38
Figure 2.3 Slug expression in different retinal cell types	43
Figure 2.4 Slug deficiency inhibits tumor growth by reducing tumor angiogenesis	48
Figure 2.5 Defective pathological angiogenesis in SlugKO mice is likely due to EC-intrinsic effect	51
Figure 3.1 Conditional mouse Slug overexpression lentiviral construct	59
Figure 3.2 Slug overexpression in EC disrupts sprouting angiogenesis	62
Figure 3.3 SlugOE chimera in 3D fibrin bead assay	65
Figure 3.4 Slug overexpression in EC disrupts vasculogenesis	67
Figure 4.1 Complete vs partial epithelial-to-mesenchymal transition (EMT)/endothelial-to-mesenchymal transition (EndoMT)	74
Figure 4.2 RNA-seq analysis reveals an EndoMT gene expression profile	85
Figure 4.3 SlugOE vascular network has reduced endothelial junctions and compromised barrier functions	90

Figure 4.4	Slug regulates EC sprouting via Notch-mediated VEGFR2 expression	95
Figure 4.5	Inhibition of VEGF and Notch signaling have opposing effects on angiogenic defects caused by Slug overexpression	99
Figure 4.6	Notch inhibition rescues delayed vascular expansion in SlugKO mouse retina	104
Figure 5.1	SDF1 α promotes sprouting angiogenesis by activating Slug transcription	112
Figure 5.2	SDF1 α induces Slug transcription sprouting by signaling through CXCR4 and ERK5	115
Figure 5.3	HGF induces transient Slug expression in EC	119
Figure 6.1	N-cadherin expression in EC is important for sprouting angiogenesis	131
Figure 6.2	Schematics of Slug-mediated pathological angiogenesis	135

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 - Study the effect of Slug deficiency on developmental and pathological angiogenesis using mouse retinal neonatal vascular development and Matrigel/syngeneic tumor angiogenesis as respective models.
 - Determine and verify the downstream targets of Slug during endothelial cell sprouting angiogenesis *in vitro* using RNA-seq, qPCR, western blot and immunofluorescent confocal microscopy techniques.
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- Gene expression profiling of various types of EC and their angiogenic/vasculogenic potentials
 - Culture HUVEC, EPC and iPS-EC in 2D and 3D culture systems and prepare samples for RNA-seq.
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ABSTRACT OF THE DISSERTATION

Investigating the Role of Snai2 (Slug) in Endothelial-to-Mesenchymal Transition and Vascular
Morphogenesis

By

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Doctor of Philosophy in Molecular Biology and Biochemistry

University of California, Irvine, 2018

Professor Christopher C.W. Hughes, Chair

The vascular network is arguably the most important organ in a human body. It connects all the other tissues and carries oxygen and nutrients to support their growth and maintenance. Vascular morphogenesis is a multi-step process that occurs in both embryogenesis and adults during pregnancy, wound healing and other pathological conditions. It is tightly regulated by a complex network of transcription factors and signaling pathways in endothelial cell (EC) intrinsic and extrinsic manners.

Slug (Snai2) is a member of the well-conserved Snail family of transcription factors and has roles in multiple developmental processes, including epithelial-to-mesenchymal transitions (EMT). Our lab has been previously demonstrated that Slug expression is up-regulated in angiogenic EC in vitro and is critical for sprouting, partly by controlling EC migration. Preliminary evidence suggests that Slug might play other roles in this process; however, a comprehensive investigation on the mechanisms of Slug-regulated vessel formation is lacking. In addition, although it has been suspected that Slug-mediated endothelial-to-mesenchymal

transition (EndoMT), a process similar to EMT, might have a role in angiogenesis, there is little direct experimental evidence supporting this hypothesis. Moreover, it is unclear whether the role of Slug in angiogenesis observed in our *in vitro* system also applies to *in vivo* models.

In this study, using various *in vitro* 3D culture models, as well as several *in vivo* models, we confirmed the critical role of Slug during both developmental and pathological vascular morphogenesis. Using RNA-sequencing analysis, we also demonstrated that Slug regulates an EMT-like suite of target genes. The level of Slug expression determines whether EC perform normal sprouting angiogenesis or excessive pathological angiogenesis by directly suppressing Dll4-activated Notch signaling to upregulate VEGFR2. *In vivo*, reduced Notch signaling completely rescues delayed retinal angiogenesis in SlugKO mice. Unlike Snail, Slug is absolutely required for pathological angiogenesis necessary for sustained tumor growth while it plays a transient role in developmental angiogenesis in mice. Slug, but not Snail, is activated in EC by the pro-angiogenic factor SDF-1 via its canonical receptor CXCR4 and subsequent ERK5 phosphorylation. These data are consistent with Slug regulating a partial endothelial-to-mesenchymal transition in angiogenic EC.

Chapter 1. Introduction

1.1 Vascular Morphogenesis

The vascular network runs through our entire body and connects all the tissues together in a circulatory system. It delivers oxygen and nutrients to support the growth and maintenance of our tissues, and carries away the waste products. The formation of the blood vessels largely occurs via two processes: vasculogenesis and angiogenesis¹⁻⁴. Vasculogenesis mainly takes place during embryonic development, when vessels form *de novo* from endothelial progenitor cells called angioblasts^{1,2}. During this process, angioblasts proliferate, migrate and arrange themselves into a primitive capillary plexus². A more complex vasculature then emerges out from this existing network via angiogenesis, and eventually organizes into a hierarchical structure that comprises arteries and veins that connect through capillary beds^{3,4}. Angiogenesis occurs in adults as well, during both physiological processes such as the menstrual cycle or wound healing, and pathological situations such as tumor growth^{4,5}. Too many, too few, or poorly-functioning vessels can lead to numerous pathologies⁶. Therefore, it is crucial to understand the regulatory mechanisms that govern each step of these complex processes.

Vasculogenesis

In mouse embryos around embryonic day 6.5 (E6.5), mesoderm-derived hemangioblasts give rise to the first blood islands, an aggregate of cells, some of which can proliferate and differentiate into endothelial progenitor cells called angioblasts². These angioblasts

then migrate and undergo adhesion to form cords that later lumenize into the primitive vascular plexus, first in the yolk sac by E7.5, and then in the embryo proper by E8.5⁷.

Many signaling molecules are involved in the regulation of endothelial lineage commitment and *de novo* vessel formation. The first signal committing the multipotent mesodermal cells to an endothelial lineage is thought to be Indian Hedgehog (IHH) derived from the adjacent endodermal cells⁸, which in turn activates BMP4⁹. Synergistic signaling from endoderm-derived IHH and bFGF then activate the expression of VEGFR2 in the mesodermal progenitor cells, which is critical for the induction and maturation of endothelial cells¹⁰. As angioblasts differentiate into mature EC, signals from bFGF and VEGF are critical to their survival, proliferation, and migration¹¹.

Adhesion molecules such as PECAM (CD31), CD34 and VE-cadherin (VE-cad) are expressed early in the angioblasts². Although the function of CD34 in developing embryonic vessels is still not clear, PECAM and VE-cad are primarily involved in homotypic adhesion at EC-EC junctions and are important for lumen formation, and vascular permeability¹². Furthermore, the interaction between endothelial integrin (ITG) proteins and various extracellular matrix (ECM) proteins secreted by both EC and other surrounding cell types also play an important role in vessel maturation and stability. Fibronectin (FN) is one of the most abundant ECM components in the blood islands and the primitive capillaries. Its expression and that of its receptor $\alpha 5\beta 1$ are critical for embryonic vascular development. In addition, laminin and vitronectin are also deposited by EC later during vasculogenesis and are characteristic of mature vessels¹².

In adult mammals, vasculogenesis has been observed in the endometrium during menstrual cycles and early placental vascularization⁵. The incorporation of bone marrow-derived circulating endothelial progenitor cells (EPC) has also been observed in ischemic tissues¹³. Similar to embryonic vasculogenesis, signaling pathways including VEGF, bFGF and Ang-Tie are also involved in the induction of adult vasculogenesis¹⁴. Interestingly, SDF1 α -mediated EPC recruitment and vasculogenesis has been described in cancer and plays a significant role in radio-resistance in solid tumors¹⁵.

Angiogenesis

Angiogenesis is the process where new blood vessels form from existing ones. There are two forms of angiogenesis. Sprouting angiogenesis forms new vessels by making new vascular sprouts that eventually anastomose with existing vessels³. Intussusceptive angiogenesis on the other hand forms new vessels by splitting an existing one into two¹⁶. This study focuses only on the first type of angiogenesis, and therefore the word “angiogenesis” is used interchangeably with “sprouting angiogenesis”.

Sprouting angiogenesis is a complex multi-step procedure requiring ECs to go through various morphological and behavioral changes, at the root of which are changes caused by alterations in gene transcription and protein expression. In a quiescent vessel, non-proliferative EC cells maintain junctions with one another. Together with perivascular cells and a basement membrane (BM), which is ECM deposited by both EC and pericytes, they form the vessel wall that provides selective barrier functions^{3,4}. EC defining the vascular lumen also possesses an apical-basal polarity that is marked by the

expression of Podocalyxin-like Protein-1 (PODXL) at the apical/lumen surface and the expression of Collagen IV (COL4A1-6) at the basal surface¹⁷.

In order to make a new sprout, EC from the existing vessel wall have to loosen their connections with the adjacent cells, alter their polarity from apical-basal to anterior-posterior, degrade the surrounding basement membrane, and move into the extracellular space³. The nascent sprout is led by specialized EC called “tip cells” that put forward actin-rich protrusions called “filopodia”¹⁸. These tip cells are highly migratory and can sense pro-angiogenic signals such as VEGF and move up a VEGF gradient^{19,20}. They also secrete a variety of matrix metalloproteinases (MMP) for ECM degradation, creating a path through the ECM for themselves and the cells trailing them^{21,22}. Following the tip cells are the stalk cells that proliferate to make more cells for the developing sprout. They also contribute to the deposition of the new BM²³. It was thought that the tip cell at the leading position was specified early in the process; however, recent advances in real-time imaging techniques have enabled the observation that tip cells and stalk cells can shuffle and change position multiple times during sprouting²⁴.

The developing sprout initially forms a cord-like structure with single cells following one another. Triggered by mechanical forces and interactions with surrounding ECM, stalk cells proliferate and contribute to lumen formation by either one or both of two mechanisms. The first mechanism is referred to as “cord-hollowing”, where a sheet of stalk cells adhere to the wall of a matrix space created by the tip cell when degrading the ECM²⁵⁻²⁷. Alternatively, “cell-hollowing” can take place when individual stalk cells form

small intracellular pinocytic vacuoles, which later fuse with those of the adjacent cells to form intercellular lumens^{28,29}. Both forms of lumen formation have been observed in various *in vitro* and *in vivo* models³⁰⁻³².

As the sprout matures, it will anastomose, or connect to other existing vessels and return to the quiescent state. EC up-regulate and secrete PDGFB, which recruits pericytes and smooth muscle cells through binding to PDGFR β ³³. These mural cells provide support to the newly-formed endothelium, contribute to the deposition of a new BM, and also generate signals that will promote and maintain vessel quiescence³⁴. Remodeling and pruning also occur via EC rearrangement and apoptosis to create a more differentiated, hierarchical vascular network³⁵.

Unsurprisingly, angiogenesis is tightly regulated by a variety of signaling pathways that can be roughly categorized as either pro-angiogenic or anti-angiogenic. The balance between these two opposing forces is referred to as an “angiogenic switch” that is critical to the formation and maintenance of a functional vasculature³⁶. VEGF signaling is the most important pro-angiogenic pathway³⁷. As mentioned above, together with bFGF, it affects all aspects of EC behaviors including survival, proliferation, migration and polarization³⁸. SDF1 α is another well-known pro-angiogenic factor³⁹. In EC, it primarily signals through its canonical receptor CXCR4 to activate either MEK/ERK or AKT/PI3K pathway to promote cell survival, proliferation and migration^{40,41}. As mentioned above, it is also a potent driver for the recruitment and incorporation of BM-derived EPC to the

sites of neovascularization³⁹. Moreover, it has also been shown to regulate vessel branching and maturation⁴².

Besides VEGF and SDF1 α , other signaling pathways such as HGF^{43,44}, TNF α ^{45,46}, and Wnt⁴⁷ also have been reported to promote angiogenesis. In fact, these have been referred to as the “escape mechanism” by which cancer cells overcome anti-VEGF therapies^{48,49}. The effect of TGF β signaling on angiogenesis is complicated and most likely context-dependent⁵⁰. Several studies reported that the addition of TGF β in various angiogenesis models has been shown to inhibit EC proliferation and tube formation while the others came to conflicting conclusion⁵⁰⁻⁵⁴.

Notch signaling also plays myriad roles in vascular network formation. The Notch receptor is a transmembrane protein. The activation of the receptor and the downstream signaling cascade requires the binding of Notch ligands (Delta, Jagged), which triggers the γ -secretase-mediated cleavage of the Notch intracellular domain (NICD) and its nuclear translocation. In the nucleus, the NICD associates with the CSL (CBF1/Su(H)/Lag-1) transcription complex and affects the expression of its target genes, including Myc, Hes and Hey families⁵⁵. During sprouting angiogenesis, Notch is involved in tip cell selection and branching. High levels of the Notch ligand Dll4 in the leading cells can activate Notch signaling in the adjacent cells, which in turn represses VEGFR2 expression and reduces their responsiveness to VEGF⁵⁶⁻⁵⁸. In the mouse retinal angiogenesis model, Jag1 acts as an antagonist of this process through the Fringe family glycosyltransferases⁵⁹, implying the importance of a delicate balance between different

Notch ligands. Meanwhile, activation of Notch downstream of VEGF signaling is important for arterial specification^{60,61}. The activation of Dll4-Notch1 signaling and the induction of Notch target genes lead to activation of the arterial marker EphrinB2 and suppression of the venous marker EphB4^{60,62}. In the absence of Notch signaling, vessels default into a venous fate with accompanying arterial-venous malformation (AVM), as seen in zebra fish embryonic development⁶⁰. Another Notch ligand, Dll1, has been shown to have a non-redundant role relative to Dll4 and Jag1 in the maintenance of arterial identity in the mouse embryo and postnatal arterialgenesis^{63,64}. In addition to regulating EC behavior, Notch pathway components are also expressed in perivascular cells and can regulate mural cell survival, EC-mural cell interaction and vascular stability^{65,66}.

Pathological Angiogenesis

Although angiogenesis is critical to embryonic development and wound healing in adults, it also plays a part in many diseases including inflammatory diseases, tissue ischemia, intraocular neovascular disorders, and cancer^{6,47}. Pathological angiogenesis shares many similar steps and regulatory pathways with those of physiological angiogenesis; however, the fundamental difference in pathological angiogenesis is that an imbalance exist between pro- and anti-angiogenic signals that results in either too many, too few, or non-functional vessels. In the case of age-related macular degeneration and cancer angiogenesis, the over-abundance of pro-angiogenic signals causes persistent EC activation and angiogenesis that never give rise to perfused mature vessels⁶⁷.

Ever since Dr. Judah Folkman proposed the idea of anti-angiogenic cancer-therapy in 1971, this has been a fruitful research area. Due to the unique characteristics of tumor vasculature compared to the normal vessels, and the relative genetic stability of the target cells—EC, anti-angiogenic therapy bears low risk and hence has become an attractive therapeutic strategy against cancer⁶⁸. Centering around the inhibition of VEGF signaling, the quintessential pro-angiogenic pathway, there have been multiple drugs approved by the FDA, including Bevacizumab (Avastin) and Pazopanib (Votrient), along with others targeting related pathways, including Sorafenib (Nexavar), and Sunitinib (Sutent). Additional drugs aimed at inhibiting angiogenesis in various human cancers are currently in Phase II and III clinical trials^{69,70}. However, this approach is not without its faults. Increasing evidence suggests that some cancers such as colorectal cancer (CRC) can quickly evolve to utilize alternative pathways to stimulate tumor angiogenesis, thus escaping VEGF inhibition, while continuing to hijack the surrounding vasculature for growth and metastasis⁷¹. This should not strike us as a surprise, however, since many pro-angiogenic pathways known to induce angiogenesis are activated in the tumor environment, including TGF β , Wnt, HGF, TNF α and SDF1 α ^{1,39,54,68}. They converge and cross-activate each other, so it is challenging to block all the pathways at once to halt tumor angiogenesis. Therefore, we need to better understand the “escape mechanism”, rethink the strategy of anti-angiogenic therapy, and identify new drug targets that are potentially further downstream and thus more likely to affect multiple angiogenic processes.

1.2 Snai2 (Slug) is an EMT master regulator

Snai2 (Slug) is a member of the well-conserved Snail family of transcription factors known to induce epithelial-to-mesenchymal transition (EMT), a critical process that occurs in both developmental and pathological conditions such as cancer metastasis and tissue fibrosis⁷². Since the expression of Slug alone is sufficient in driving a complete EMT program and alter the morphology and behavior of many cell types, it is considered a master regulator of EMT⁷³.

Slug in EMT & EndoMT

In developing *Xenopus* and chick embryos, Slug is expressed in the neural epithelial cells. Its expression specifies neural crest cell fate and induces EMT that is necessary for neural crest cell migration⁷⁴. Inhibition of Slug function leads to failure of neural tube closure in both *Xenopus* and chick embryos^{75,76}. Later on in development, Slug performs a similar function by inducing an endothelial-to-mesenchymal transition (EndoMT) in the heart⁷⁷. In this case, Slug expression is found in the arterioventricular canal (AVC) in the chick heart and is critical for endocardial cushion formation. In mice, Slug is also expressed in various embryonic tissues, including those of the cardiovascular system. However, Slug is not expressed in the migrating neural crest cells in the mouse embryo and therefore is not critical for inducing neural tube closure⁷⁸. Instead, Snail (Snail), another Snail family transcription factor, is expressed and functions to induce EMT⁷⁹. In the developing mouse heart, Slug expression is observed in the endocardial cells, however, Slug knockout (SlugKO) mice only display transient defects in EndoMT and

heart cushion formation, likely due to the compensatory effects of increased Snail expression⁸⁰. Although SlugKO mice survive, they do suffer from various defects, including growth deficiency, immune deficiency, sub-fertility and pigmentation deficiencies in the skin and fur, suggesting that Slug functions in melanocyte stem cells, hematopoietic stem cells and germs cells, all of which are neural-crest derived⁷⁸.

Although the importance of Slug expression in EMT/EndoMT during mammalian development is still being examined, its role in pathological EMT/EndoMT is well established. Slug expression is induced in many epithelial tumors including breast cancer, colon-rectal cancer, and ovarian cancer, and elevated Slug expression is often associated with poor prognosis and increased metastasis. In fact, the induction of Slug by TGF β and other factors is sufficient to drive an invasive phenotype in many types of tumor cells⁸¹⁻⁸³. Several studies have also demonstrated that EC expression of Slug can induce pathological EndoMT. For example, increased expression of Slug and several other EMT markers was observed in left ventricular myocardial tissues from patients with end-stage cardiac failure⁸⁴, suggesting a role of EndoMT in this process. One study, of patients suffering from coronary atherosclerosis found the extent of their disease to be closely associated with the activation of an EndoMT program and increased expression of Slug and other EMT markers⁸⁵. A recent study demonstrated that increased Slug expression and EndoMT contributes to arsenic-trioxide-induced cardiac fibrosis⁸⁶. These data suggest that a Slug-mediated EndoMT program can be reactivated in adults and contributes to diseases later in life⁸⁷.

Genes regulated by Slug

Slug is largely known as a transcription repressor. As a master regulator of EMT/EndoMT, it has been reported to down-regulate a variety of junction proteins including E-cad, VE-cad, and Occludin, likely through direct transcriptional repression⁸⁸⁻⁹¹. It is also involved in the disassembly of desmosomes in EC⁹². As a promoter of cell migration, it is known to indirectly up-regulate several MMP genes including MT1-MMP, MT4-MMP, MMP-2 and MMP-9^{82,93-96}. Moreover, Slug is also known to induce the expression of other EMT-related genes such as SPARC, PAI-1 and fibronectin⁹⁷. One interesting target gene of Slug is N-cad. During developmental EMT in chick embryos, Slug-mediated N-cad down-regulation in the neural epithelial cells is necessary for the initiation of the EMT program⁷⁵. However, during pathological EMT in cancer, upregulation of N-cad is often seen in the tumor cells and is associated with their metastatic phenotype^{73,98,99}. It is possible that other co-regulators are involved depending on the context. In progenitor cells and some cancer cells, Slug has been shown to regulate “stemness” and the proliferative potential in these cells^{100,101}. During pregnancy, Slug determines mammary gland stem cell fate by inducing robust expression of basal cell transcription factors including p63, ID4 Egr2, Mef2C and Twist2¹⁰². In BRCA1-/- breast cancer cells, increased Slug protein stability leads to impaired luminal cell lineage commitment and decreased luminal cell markers including CK8/18, EpCAM, and CD24¹⁰³. In epidermal progenitor cells, Slug expression inhibits the differentiation program and prevents expression of differentiated cell markers such as KRT1, FLG, TGM1, SPRR1A, GRHL3, and KLF4¹⁰⁴.

Upstream regulation of Slug

Slug, as a transcription factor that influences many essential cellular behaviors including survival, proliferation and migration, is regulated by a variety of signaling pathways at both transcriptional and post-translational levels. The TGF β /BMP pathway is the primary signal that activates Slug expression in developmental EMT^{77,81}. It is also one of the most commonly observed drivers of Slug expression in pathological EMT⁸¹. Many other growth factors that are known to induce cell migration, such as HGF, FGF, PDGF, and EGF, have also been shown to induce Slug transcription in different cell types in both development and disease^{92,105}. In particular, HGF has been shown to induce Slug-mediated partial EMT during tube formation by MDCK cells¹⁰⁶. Notch signaling has been shown to induce Slug transcription directly during heart cushion formation⁸⁰, or indirectly activate Slug expression and EMT in many types of cancers¹⁰⁷⁻¹⁰⁹. Moreover, Wnt, VEGF and HIF1 α have also been reported to induce Slug expression in pathological contexts¹¹⁰⁻¹¹².

Because so many different signaling pathways can activate Slug expression, and being an important transcription factor that regulates many target genes and downstream cellular processes, the level of Slug must be finely controlled. Indeed, Slug is known to bind its own promoter along with Sox9 and repress its own transcription¹⁰². It is also a highly labile protein with a half-life of only 20 minutes. In the cytoplasm, Slug is targeted by several ubiquitin ligases for proteosomal degradation. When phosphorylated by GSK3 β , it undergoes β -Trcp1-mediated ubiquitination and degradation^{110,113,114}. Slug degradation via a p53-P21-mediated, MDM-2-dependent mechanism has also been reported^{115,116}. In

addition, FXBL14 can also ubiquitinate Slug¹¹⁷. Interestingly, all the known degradation mechanisms require Slug to be in the cytoplasm; but unlike Snail, Slug does not have a nuclear export sequence¹¹⁸. Therefore, it would be interesting to know if any specific signal directs it to dissociate from the promoter of its target genes and translocate outside of the nucleus for degradation, or if this is a stochastic event.

Other EMT-associated transcription factors

Besides Slug, there are several other EMT-associated transcription factors. These include Snail, Twist, Zeb1/2, and Ovnl1/2, all of which are considered EMT inducers except for Ovnl1/2. Although these transcription factors all drive the EMT program, numerous studies have demonstrated that they have overlapping but non-redundant roles in this process. As mentioned above, Snail is the mammalian homolog of cSlug that is primarily responsible for inducing EMT and EndoMT during development^{78,80}. Similar to Slug, or maybe even to a greater extent, Snail is also known to drive EMT in cancer progression, mainly as a suppressor of the epithelial marker E-cad⁷³. Twist has also been reported to be important for neural tube closure^{119,120}. In cancer, Twist can be induced by many of the same factors as Slug, such as TGF β and Wnt; and its expression is associated with increased EMT and particularly with evading apoptosis and chemo-resistance¹²¹. Similarly, Zeb1/2 can be induced by TGF β to drive EMT in lung cancer and maintain tumor cell invasiveness^{122,123}. In skin, Zeb1 expression controls EMT and epithelial terminal differentiation¹²⁴. On the other hand, Ovnl1/2 have been called the “gatekeepers” of EMT. The induction of Ovnl1/2 has been associated with the repression of EMT inducers and the onset of an opposite process called mesenchymal-to-epithelial

transition (MET)^{125,126}.

Additional regulatory circuits among these EMT-associated factors have also been reported. For example, Snail can up-regulate Zeb1 and Zeb2 in oral squamous carcinoma and, at the same time, negatively regulate its own expression through direct promoter binding^{127,128}. Moreover, Slug indirectly up-regulates Snail through epithelial growth factor (EGF) and/or HGF signaling, thereby promoting mammary gland branching morphogenesis¹²⁹. Slug can also activate Zeb1 and its own expression through direct transcriptional regulation^{130,131}. Moreover, it is also speculated to be involved in a reciprocal regulatory loop with Twist¹³². In addition, many groups have shown that Twist1 can regulate the expression level of Snail and Slug by either directly influencing transcription^{133,134}, or through post-translational regulation via the NF- κ B/GSK-3 β axis¹³⁵. This body of evidence suggests that these EMT transcription factors most likely work in concert in a non-linear fashion and form a complicated regulatory network in regulation of the EMT process.

1.3 3D MODELS OF VASCULAR MORPHOGENESIS

Consensus guidelines for the use and interpretation of angiogenesis assays

4. 3D Models of Vascular Morphogenesis.

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Introduction

To generate blood vessels and vascular networks, endothelial cells (ECs) must undergo vascular morphogenesis, which can include either vasculogenic and/or angiogenic processes⁵⁰. In vasculogenesis, differentiating ECs migrate, proliferate, aggregate and rearrange to form cords that then undergo lumen formation to generate three-dimensional (3D), tubular blood vessels. In angiogenesis, stimulatory vascular endothelial growth factor (VEGF) promotes EC sprouting from existing vessel walls, the formation of new tubes, and anastomosis (joining) with other vessels to create an interconnected network of vessels. ECs recruit perivascular stromal cells (pericytes) to stabilize this newly-formed network and minimize leak upon blood perfusion. Importantly, not all sprouts become functional vessels. Pruning serves to selectively remove redundant or non-functional vessels to optimize fluid flow through the network³⁵.

In vitro assays have played a valuable role in our understanding of vascular morphogenesis. These assays provide a simpler platform than animal models for dissecting individual steps within the process while also incorporating 3D matrix to mimic native *in vivo* tissues. Here, we present several of the most reliable and informative assays developed to date and highlight the strengths and limitations of each (**Table 1**). While many types of ECs can be used in these assays, the most commonly

Table 1. Comparison of 3D Models of Vascular Morphogenesis.

ASSAY	PROCESS					
	Sprouting	Lumen Formation	Anastomosis	Pericyte Recruitment	Perfusion	Pruning
Fibrin Bead Assay	✓	✓	✓	✓		
Collagen Lumen Assay		✓		✓		
Retina Explant Assay	✓	✓	✓			
Vascularized Micro-Organ	✓	✓	✓	✓	✓	✓

used are human umbilical vein endothelial cells (HUVECs) and human endothelial colony-forming cell-derived EC (ECFC-ECs), which generally have a higher proliferative potential. Mouse ECs are not generally used in these assays as they are notoriously hard to maintain in culture. While we use “ECs” to reference both cell sources here, assays using a specific EC source are annotated accordingly.

Fibrin Bead Assay

Traditional Matrigel cord-forming or collagen I angiogenic invasion assays are insufficient to model the complexity of angiogenesis, as these assays are two-dimensional and ECs in these assays often form incomplete lumens. Moreover, lumen formation in Matrigel is not unique to ECs as several non-EC cell types (e.g. human prostate carcinoma and glioblastoma cells) also form lumens, complicating the interpretation of results from these assays ¹³⁶. In contrast, the fibrin bead assay provides a platform for

testing EC sprouting and lumen formation over an extended period (2-3 weeks), incorporates a 3D, extracellular matrix (ECM), and multiple cell types (i.e. stromal pericytes) to model native angiogenesis. ECs (HUVECs) are first allowed to adhere to collagen I-coated Cytodex beads to generate an EC monolayer that mimics the vessel wall of native vessels. These EC-coated beads are then embedded into a fibrin gel with human stromal cells either embedded within the gel or plated in monolayer on top. Tip

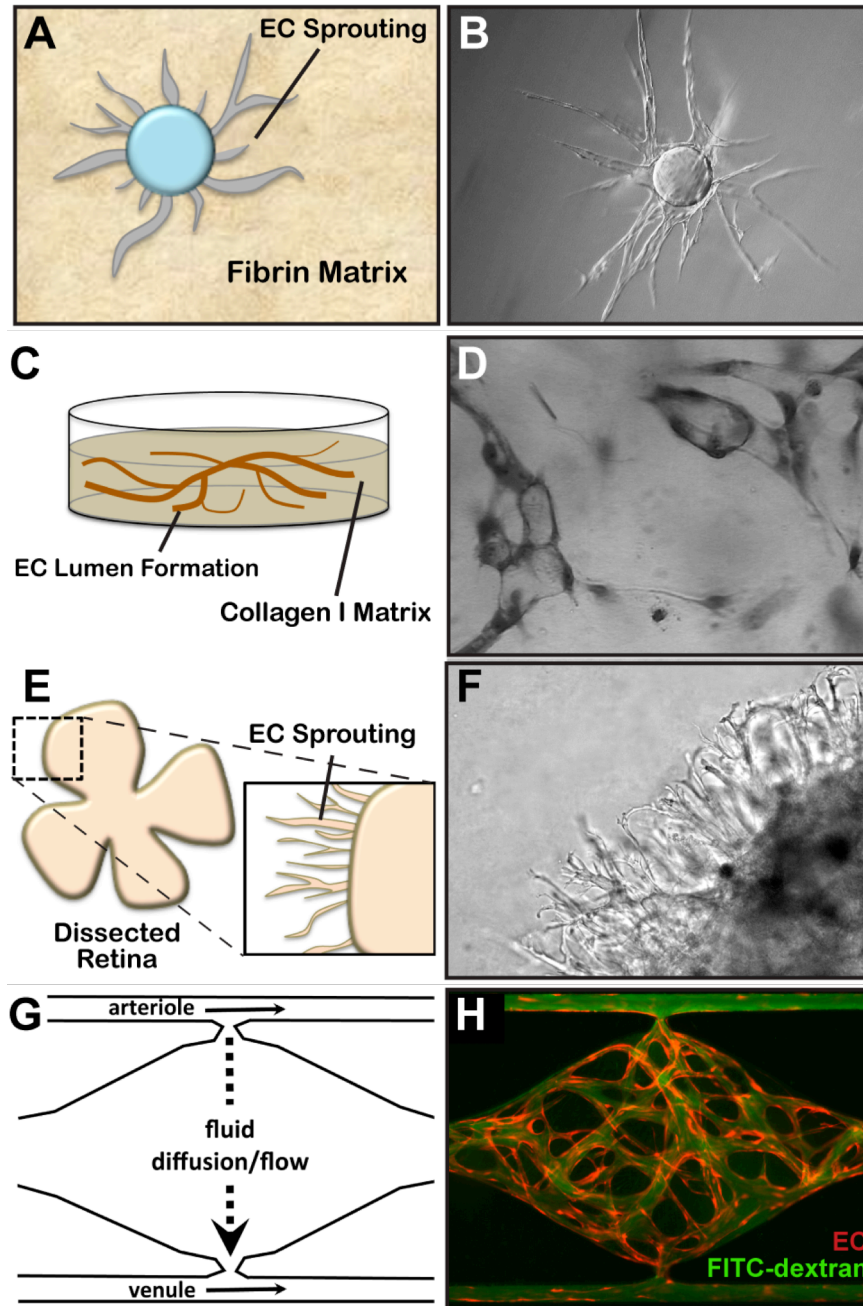


Figure 1.1 3D assays of vascular morphogenesis. (A) A fibrin bead assay uses collagen I- and EC-coated Cytodex beads embedded within a 3D fibrin gel matrix to measure EC sprouting and lumen formation. (B) These features are readily resolved using phase-contrast microscopy. (C) EC tube formation can be measured by embedding ECs within a collagen I matrix. (D) Once formed, these tubes can be visualized by toluidine blue staining and brightfield microscopy. (E) Whole-mount, dissected retinas from postnatal mice are mounted within collagen I-Matrigel matrix mix and cultured in pro-angiogenic medium to stimulate EC sprouting. (F) Sprout and lumen formation are resolved using phase-contrast microscopy. (G) The vascularized micro-organ (VMO) approach utilizes “arteriole” (high pressure) and “venule” (low pressure) microfluidic channels to drive medium diffusion and flow across a cell chamber where microvasculature forms. (H) The formed microvasculature (ECs, red) can be measured for leak by perfusion with 70kDa FITC-dextran (green).

cells are observed 2-3 days post-plating and elongating sprouts appear 2-4 days after this (**Fig. 1A, B**). When maintained in pro-angiogenic EGM-2 medium (Lonza), lumens form within a week and the assay remains viable up to three weeks, at which point anastomosis between sprouts is often apparent¹³⁷. A detailed video protocol of this assay is available¹³⁸.

Angiogenic sprouting from individual beads is evaluated by phase-contrast microscopy allowing for quantification of sprout number, length of sprouts, percentage of sprout lumenization, and the number of anastomoses. Genetic approaches (siRNA, lentiviral transduction) can modify gene expression in individual cell types to dissect cell-autonomous components of the angiogenesis process. Protein expression and localization is measured by fixing bead assays and using modified immunofluorescent staining techniques. More detailed gene expression analyses are made possible by harvesting individual cell types to track RNA expression changes over time through various stages of sprouting angiogenesis.

The use of stromal cells (i.e. lung fibroblasts) is critical to the success of this assay, as these cells secrete angiogenic factors necessary for EC sprouting and lumen formation, including growth factors (e.g. HGF, TGF- α , and Ang-1), as well as matrix molecules, matrix-modifying proteins and matricellular proteins (e.g. collagen I, procollagen C endopeptidase enhancer 1, secreted protein acidic and rich in cysteine [SPARC], transforming growth factor- β -induced protein ig-h3 [β IgH3], and insulin growth factor-binding protein 7 [IGFBP7]). These factors act to locally stiffen the matrix, which

supports sprouting and lumen formation⁴⁴. This assay represents a significant improvement over conventional, single cell-type angiogenic assays, as the inclusion of multiple cell types more closely mimics the physiological environment. Nevertheless, as this assay uses primary cultures of cells, rather than cell lines, it is important to remember that batch-to-batch variations in stromal cells (and HUVECs) can significantly affect assay results. Therefore, for this assay to be reproducible, it is crucial to identify stromal cell-HUVEC pairs that yield optimal angiogenic sprouts for this assay to be reproducible.

Collagen Lumen Assay

To understand EC lumen formation mechanics, early assays seeded ECs in monolayer on plastic dishes coated with ECM proteins (i.e. collagen I, collagen III, fibrin, or Matrigel). While these 2D assays are sufficient to induce EC cord formation, they cannot reproduce the necessary cues for true lumen formation found in native, 3D tissues. Collagen sandwich assays surround ECs within a 3D matrix by seeding the cells in monolayer on collagen I matrix, then covering them with a second layer of collagen¹³⁹. Nevertheless, tube formation fails to occur in a random, 3D growth pattern, forming only in the X-Y plane of the initial gel layer and not in the z-axis. As this does not adequately recapitulate normal vessel growth in a true 3D environment, George Davis and others further optimized these assays, opting instead to embed single ECs (HUVECs) randomly throughout a collagen I matrix. In the simplest version of these assays, HUVECs are seeded at low density (7×10^5 cells/mL) under serum-free growth conditions and with the addition of minimal growth factors (phorbol ester, VEGF, and fibroblast growth factor-2 [FGF-2]). After 48 hours, the embedded ECs form intact tubes throughout the gel, with

clearly demarcated lumens (**Fig. 1C, D**). Several variations on this assay have since enhanced and optimized lumen formation. First, the addition of several other growth factors, including stem cell factor (SCF), interleukin-3 (IL-3), stromal-derived factor-1 α (SDF-1 α), and FGF-2, further promote lumen formation while maintaining serum-free growth conditions. Second, when simultaneously seeded within the same matrix, stromal pericytes are recruited by ECs, recapitulating a key step in vascular morphogenesis. Lastly, to understand the process of EC sprouting and angiogenesis, ECs can be seeded on top of a 3D collagen gel containing the same growth factors and invasion of the underlying gel layer can be quantified. A detailed protocol of the collagen lumen assay and its variations is available for further reading¹⁴⁰.

Real-time imaging of tube formation can be achieved using fluorescent protein-transduced ECs. Alternatively, fixed vessels can be stained with 0.1% toluidine blue and imaged using brightfield microscopy (**Fig. 1D**). More in-depth analyses can be carried out on these fixed vessels using immunofluorescence staining of relevant protein markers or transmission electron microscopy to resolve structural details of formed lumen and remodeled ECM.

Regular users of collagen gels will note that the viscosity, pH, and contraction of these gels can hinder successful execution of assays in the hands of new users. As a result, special care should be taken when pipetting (such as when mixing cells and growth factors) and plating gels to ensure even gel coating of the bottom of the well plate. Perhaps most significantly, early gel contraction can limit the useful length of these

assays. Users will note that plating gels only in wells within the center of the 96 half-area wells and adding medium or water to the outer wells of the plate will minimize gel contraction, by maintaining local humidity levels. Additionally, seeding fewer ECs within the collagen (1.5×10^3 cells/mL) can minimize gel contraction and prolong the assay.

Retinal Explant Assay

Although *in vitro* assays are high-throughput and can mimic major steps in vascular morphogenesis, these assays do not fully recapitulate the *in vivo*, whole-organ environment¹³⁷. Several *in vivo* animal models, such as mouse retina or zebrafish fins, are valuable tools for studying vascular (re)establishment in a physiologically-relevant context^{136,141}. However, the added complexity of these systems makes it more difficult to ascertain the role of individual proteins and growth factors in the vascular morphogenesis process, relying on genetic manipulations or system-wide administration of pharmacologic inhibitors to dissect molecular pathways^{136,142}. As such, there is a need to increase assay complexity (and physiological relevance) while developing platforms amenable to *ex vivo* study in the laboratory. Retina explant assays are one such *ex vivo* platform, whereby dissected retinas are maintained and observed for vascular morphogenesis over several weeks in the laboratory. While multiple versions of this assay have been published, a protocol published by Sawamiphak, et al., is most widely used for the study of endothelial sprouting¹⁴³. Briefly, retina cups from embryonic, postnatal, or adult mice are harvested and cut radially to allow flat mounting of the retina interior surface onto a membrane insert. After recovery in media for 2-4 hours, the

explants can then be treated with stimulatory or inhibitory agents for up to 4 hours, followed by whole-mount microscopy analysis to evaluate the (anti-) angiogenic effect of these agents on vessel sprouting (**Fig. 1C**). When perfected, each pair of retinas can be harvested and dissected within minutes. Unfortunately, without the support of a 3D matrix, retinal cells cannot survive for long periods, thus making studies for later stages of angiogenesis impossible. To overcome this, Rezzola, et al., have improved the assay by embedding the retinas in different matrices after dissection¹⁴⁴. In this approach, retinas can be cross-cut into 4 equal pieces and left in serum-free media overnight. The retina fragments are then embedded in Matrigel, collagen I, or fibrin matrix and fed every 2 to 3 days. Depending on the age and the matrix used, sprouts can be observed between day 3 to 6 and anastomosis of neighboring sprouts, similar to what occurs *in vivo* can be observed in 10-14 days¹⁴⁵ (**Fig. 1D**). These explants can be maintained up to 3 weeks before the vessels eventually regress.

Vessel formation can be analyzed in real time using time-lapse imaging or the explants can be fixed and imaged by immunofluorescent microscopy at set experimental time points^{145,146}. Gene expression can be manipulated by genetic crossing of the donor mice or, more transiently, by treating retinas with lentivirus or siRNA. Moreover, embedded retinas can be treated with drugs over extended periods to dissect individual signaling pathways.

Several factors are critical to consistently achieve sprouting from dissected retina explants. First, the matrix proteins in which retinas are embedded can greatly influence

how vessels sprout. In our experience, Matrigel is far superior to single matrix proteins in inducing sprouting. However, the addition of 10-20% Matrigel in collagen I matrix is sufficient to stimulate sprouting compared to pure collagen I matrix. Second, the use of pro-angiogenic EGM-2 yields more sprouts as compared to basal medium alone. Lastly, as with any tissue explant, the age of the mouse can influence the degree of vessel sprouting. As such, special care should be taken to select mice appropriate for the experimental question at hand. There are many similarities between the mouse retinal explant assay and the traditional mouse/rat aortic ring angiogenesis assay or rat vena cava explant assay¹⁴⁷⁻¹⁴⁹. However, retinal explant models more closely model true capillary sprouting as the vasculature in these explants is actively developing and remodeling. This makes the retinal explant model uniquely suited to studying microvessel formation and its underlying mechanisms.

Vascularized Micro-Organ (VMO) Platform

To understand all the steps of vascular morphogenesis in a single platform, our group has optimized a vascularized micro-organ (VMO) approach to drive formation of a perfusable vascular network within a 3D hydrogel matrix environment. In contrast to the assays described above, VMO-embedded endothelial cells are exposed to and respond to shear stress, form lumenized vessels, and are perfused with a blood-substitute medium that delivers nutrients to tissues within the matrix, just as in the body. Specifically, this platform utilizes “arteriole” (high pressure) and “venule” (low pressure) microfluidic channels that are joined by a living microvascular network that forms by vasculogenesis in an intervening tissue chamber (**Fig. 1G**). A pressure differential between the two

channels is used to drive interstitial flow through the fibrin gel matrix during vessel development, induce vessel formation (through shear-sensing), and drive convective flow through the mature vasculature once formed and anastomosed to the outer channels. This pressure difference is induced by varying the level of medium within fluid reservoirs at either end of the microfluidic channels, thereby creating hydrostatic pressure heads that ensure continuous fluid convection across the cell chamber. To form vessels, human ECFC-ECs and human lung stromal cells are co-loaded within a fibrin matrix into the central cell chamber through an independent loading tunnel. When maintained in pro-angiogenic EGM-2 medium, vessels form within 4-6 days post-loading (**Fig. 1H**). When perfused with 70kDa rhodamine-dextran, a molecule similar in size to albumin, these vessels demonstrate minimal vessel leak – comparable to *in vivo* microvasculature. For readers interested in more information, a detailed protocol for loading and maintaining the VMO platform is available¹⁵⁰.

The VMO platform is fabricated from polydimethylsiloxane (PDMS), an optically clear, biologically inert polymer widely used in the microfluidics field. The use of this polymer and the dimensions of the platform ensure that live, GFP-transduced EPCs can be imaged and quantified throughout vessel formation. Specific parameters such as vessel network length, branching, and anastomosis can be measured in real-time as can vessel permeability by perfusion with fluorophore-tagged dextran molecules of various molecular weights. Additionally, immunofluorescent staining can be used to quantify expression of specific molecular markers or RNA can be collected to measure changes in

gene expression. Lastly, gene expression can be manipulated by treating individual cell types with lentivirus or siRNA prior to loading in the platform.

To ensure robust and reproducible vascular network formation, several steps are critical. First, the fibrin gel matrix must be consistently loaded within the VMO cell chamber. During normal loading, perfusion burst valves at the interface between the tissue chamber and the microfluidic channels ensures a gel/air interface (later a gel/fluid interface) is formed. To simplify loading and minimize specialized training for new users of the platform, current iterations of the VMO platform incorporate a pressure release valve at the loading tunnel that minimizes unintended gel bursting¹⁵¹. Second, robust vascular network formation requires that vessels within the chamber anastomose with the outer microfluidic channels. To facilitate the formation of these anastomoses, ECs can be either seeded directly within the microfluidic channels or induced to migrate from the gel by coating the external channels with extracellular matrix¹⁵². As with the fibrin bead assay, optimal stromal cell-EC pairs should be validated to ensure assay reproducibility.

Limitations & Other Considerations

With all EC assays, the source of ECs is critical to assay success. Although commercial versions of HUVECs and ECFC-ECs are available, cells from these sources show limited utility in many 3D assays, likely due to a larger than optimal number of cell doublings prior to shipment and use in the laboratory. As a result, the use of primary-isolated ECs will provide the most consistent results and is strongly encouraged. Readers will note that isolation protocols for both cell types are available^{153,154}. Additionally, patient-to-patient

variation between different EC isolations can lead to inconsistent assay results, an issue that may be avoided by pooling several EC lines prior to use.

Given the many differences between the assays described here, the useful length of these assays varies considerably. Even in well-trained hands, the contraction of gels in the collagen lumen assay effectively limits the useful time frame of assays to 72 hours or less. However, the other assays described here can persist for much longer periods of time, with the fibrin bead assay, retinal explant assay, and VMO platform all suitable for timepoints up to three weeks under appropriate conditions.

Lastly, the majority of these assays can be run in a relatively high-throughput manner, thereby accelerating the speed with which genetic, molecular, or pharmacologic screens can be conducted to understand vascular morphogenesis. This is especially true with the fibrin bead and collagen lumen assays, which utilize multiple beads or multi-well culture plates to increase assay throughput. Similarly, while initial versions of the VMO platform were cumbersome to load in high-throughput numbers, this platform is now used in an optimized configuration that incorporates up to 16 individual VMO devices within a standard 96-well plate ¹⁵⁵. This design simplifies translation to outside labs and interfacing with existing microscope and plate reader infrastructure. Of all the assays described in this section, retinal explants are most adversely affected by delays between initial dissection, mounting, and plating of tissue samples. This inherently limits the number of animals that can be dissected at once and, for now, limits the number of retinas that can be screened simultaneously.

Concluding Remarks

Vascular morphogenesis requires the interaction between several cell types and their surrounding, 3D microenvironment. The 3D vascular morphogenesis models described here provide unique *in vitro* culture systems that recapitulate this complex multi-step process. At the same time, the simplicity and consistency of these assays compared to *in vivo* models allows them to be conducted in a high-throughput fashion. Finally, the use of human cells in these assays improves the clinical relevance of such models, making them readily amenable to drug discovery applications.

Summary

Blood vessel formation, which includes the processes of vasculogenesis and angiogenesis, is a complicated multi-step process important for organismal development and wound healing^{1,156}. Out of context, however, these processes can both trigger and exacerbate an array of human diseases, including macular degeneration and cancer^{157,158}. While numerous advances have been made in our understanding of the molecular processes underlying blood vessel growth, most therapeutic approaches to limiting angiogenesis focus on the VEGF pathway^{70,157-159}. However, there is a growing awareness that multiple “escape” mechanisms exist that allow vessel growth even when VEGF signaling is limited^{68,160,161}. Growth factor/morphogen signaling pathways known to be involved in blood vessel growth include: VEGF, TGF β /BMP, Wnt, Hedgehog and Notch^{1,47,50,162,163}.

We and others have noted the similarities of angiogenic sprouting to epithelial-mesenchymal transitions (EMT)^{164,165}, where polarized epithelial cells lose apical-basal polarity, take on a more mesenchymal phenotype and migrate away from the parent tissue, either singly or in sheets. This process is driven by genes of the Snail family, including Snail (Snai1) and Slug (Snai2)^{72,73,166}. Endothelial “tip” cells that lead new sprouts can be thought of as having undergone a partial endothelial-mesenchymal transition (EndoMT) as they lose apical-basal polarity and lead the invading sprout. It should be noted that endothelial cells (EC) have also been reported to undergo a full EMT in some conditions, becoming fibroblastic^{87,164,167}. We followed up on this observation and found that both Slug and Snail are up-regulated in EC in an *in vitro* angiogenesis assay and that knockdown of either gene blocks sprouting⁹⁶. Interestingly, however, when Slug was over-expressed in EC and these cells were mixed with wild-type cells, they did not preferentially segregate to the tip position. We concluded that Slug is a marker of activated, angiogenic EC, but that its expression does not limit a cell to the tip position. We showed that Slug regulates MT1-MMP, but did not identify upstream activators nor other downstream targets.

As noted above, Slug is a member of the well-conserved Snail family of transcription factors that play important roles in the induction of EMT, a critical process in both development and pathological conditions such as cancer metastasis and tissue fibrosis^{73,74,83,88}. In mammals, Slug is expressed in numerous embryonic tissues, however, somewhat surprisingly, the global Slug knockout mouse is viable with few obvious

defects beyond mild runting at birth. Closer examination has revealed Slug functions in melanocyte stem cells, hematopoietic stem cells, and germs cells, but vascular defects have not been noted^{78,80,168,169}. Overexpression of Slug has been reported to result in cardiovascular system defects during development¹⁷⁰, suggesting that further study is warranted.

In this study we looked to reconcile our in vitro data with the mouse knockout studies by performing a more detailed analysis of the vasculature in knockout mice, in both physiologic and pathologic conditions. We find that retinal angiogenesis is delayed in the absence of Slug and that, remarkably, tumor growth in knockout animals is almost completely suppressed. We identify SDF1 (CXCL12) as an upstream regulator of Slug expression in EC and identify Dll4 as a critical downstream effector of Slug during angiogenesis.

Chapter 2. Endothelial expression of Slug regulates sprouting angiogenesis *in vivo*

2.1 Slug Germline Knockout Mice

Two slightly different Slug germline knockout (SlugKO) mice have been developed and are available through Jackson Laboratory⁷⁸. In the B6.129S1-Snai2^{tm1Grid}/J mice, only exon 2 and 3 of the Slug gene locus were deleted and these were replaced with a beta galactosidase fusion protein. Although the embryonic stem cells used were derived from the 129S1 mice, these mice were backcrossed with C57BL/6J mice for 10 generations and are maintained on a C57BL/6J background. Conversely, in the B6.129S1-Snai2^{tm2Grid}/J mice, the entire protein coding region of the Slug gene was deleted. These mice were also generated using 129S1 embryonic stem cells, injected in C57BL/6 blastocysts. However, the resulting animals were backcrossed with C57BL/6 mice only once, and therefore the colony is essentially maintained on a 129S1-C57BL/6 mixed background.

According to the Jackson Laboratory website, homozygous mutant mice (KO) in both lines are viable, though runted at birth with growth retardation throughout various developmental stages compared to their wild type (WT) littermates. They also suffer from varying levels of inflammation, despite having abnormal T cell differentiation and reduced leukocyte number. Males have been reported to be subfertile. In addition, they also show abnormal skin and fur pigmentation, and appear to have neural-behavioral defects. Interestingly, only the B6.129S1-Snai2^{tm1Grid}/J mice were reported to have

transient defects in endothelial-to-mesenchymal transition during cardiac cushion formation, which was later compensated by increased expression of Snail⁸⁰. To no one's surprise, all of the aforementioned defects can be attributed to defects in the neural crest derived cells, as developmentally Slug is an important neural crest specifier gene.

For the studies conducted in this thesis, the B6;129S1-Snai2^{tm2Grid}/J mice were obtained from the Jackson Laboratory (Stock # 010722). Upon receiving, the mice were crossed with either C57BL/6J, C57BL/N, or intercrossed. Therefore, three separate colonies were initially established. For the sake of time and efficiency, all of the results reported here were conducted with the intercrossed colony and their progenies crossed with other strains as mentioned below. The backcrossing with C57BL/6J and C57BL/N were continued and preliminary observation on body size, retinal vascular development, as well as tumor development did not show drastic difference between the two colonies. Therefore, after 10 generations of backcrossing, the C57BL/N line was cryopreserved and discontinued. Interestingly, after 10 generations, the C57BL/6J backcrossed mice appear to have decreased litter size (average of 5 to 6) compared to the intercrossed mice (average of 8 to 10). This might be due to having a pure background and therefore a reduced gene pool in the backcrossed colony.

2.2 Slug Regulates Developmental Angiogenesis *In Vivo*

Although SlugKO mice suffer from a variety of defects, a vascular phenotype has not been reported previously. This finding is surprising in light of our *in vitro* data showing

that Slug knockdown in HUVEC almost completely blocks sprouting⁹⁶. In the previous publications, the expression pattern of Slug in the yolk sac was examined at E7.5, and in the embryo at E8.5 to E11.5 (none investigated the postnatal retina)⁷⁸. Given that angiogenesis in murine yolk sac occurs at around E8.5, after a primitive capillary network has been established by vasculogenesis¹⁷¹, and in murine retina occurs postnatally, these time points were not ideal for observing Slug expression in vascular EC and its effects in developmental angiogenesis. Therefore, we decided to look more closely at angiogenesis during particular developmental stages in both the yolk sac and the retinas of the SlugKO mice.

We first examined the yolk sac. In the yolk sacs from the SlugKO mice at E9.5, the primitive vascular network appears more disorganized with less hierarchical structure (Fig. 2.1). We also saw enlarged vessels and increased vascular density in the SlugKO yolk sacs compared to the WT ones. These observations suggest there is indeed a vascular defect in the yolk sacs of the SlugKO mice.

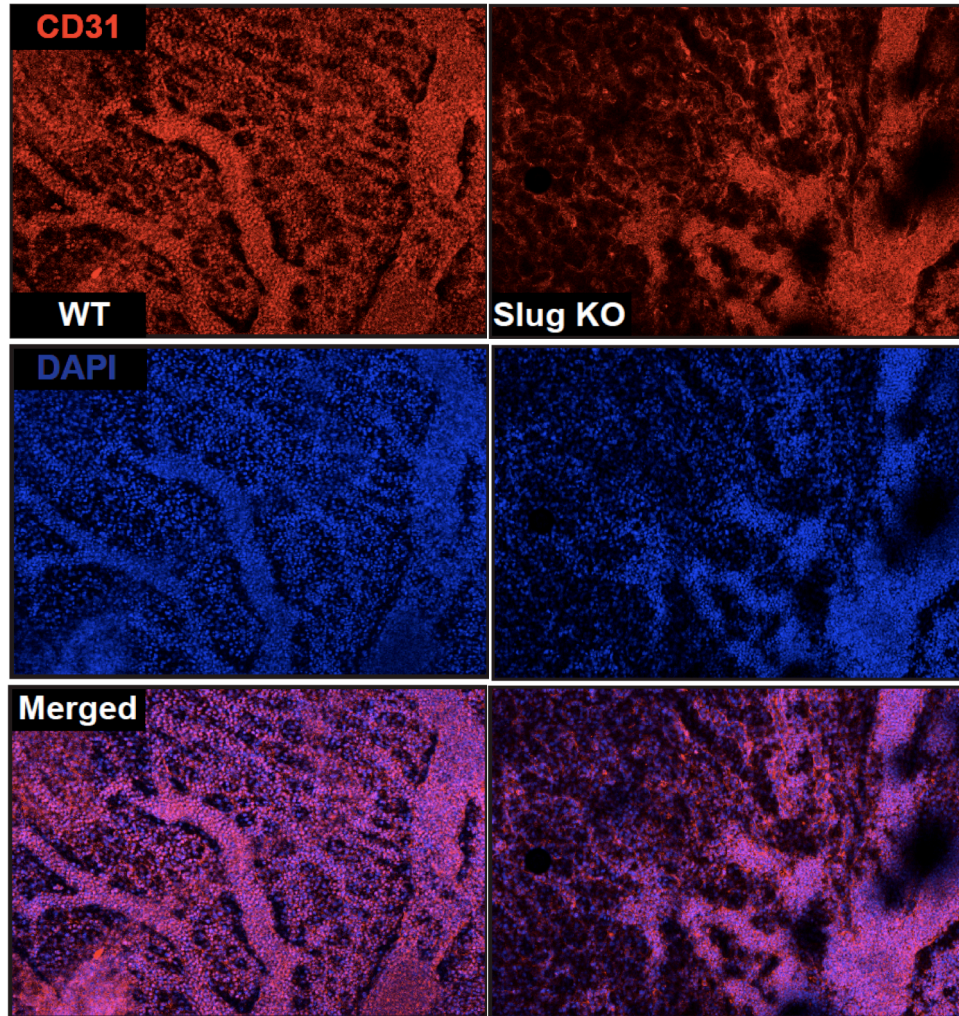


Figure 2.1: *Slug* deficiency leads to impaired yolk sac vascular development. CD31 and DAPI staining for E9.5 yolk sac showing enlarged and disorganized primitive vasculature in the *Slug*KO comparing to the WT animals.

Next, we evaluated the impact of Slug deficiency on neonatal retinal vascular development in the SlugKO mice. Mouse retina vascular development is a valuable model for angiogenesis. Mice are born with avascularized retinas that vascularize postnatally, mainly via angiogenesis, within two to three weeks of birth. There are three vascular network layers in the murine retina. The primary plexus (superficial plexus) that resides in the nerve fiber layer (NFL) develops during the first week after birth. During this time, the vascular EC enter the retina through the optic nerve disk located at the center of the retina and extend radially towards the periphery of the retina until they reach the edge at around postnatal day 8 (P8). Meanwhile, starting at around P7, the capillaries around the veins in the primary plexus start to sprout vertically into the retina to form the secondary plexus (deep plexus) between the outer nuclear layer (ONL) and the inner nuclear layer (INL) by P12. Finally, the tertiary plexus (intermediate plexus) forms between the ganglion cell layer (GCL) and the INL via angiogenic sprouting from both the primary and the secondary plexus. Only the primary plexus has arterial-venous specification, which occurs after P3, while the secondary and tertiary plexus are comprised of only capillary networks, although “diving veins” can be occasionally observed in these two plexus¹⁴¹.

The mouse retina vascular development model allows us to observe all the major vascular morphogenic events in an endogenous environment, including sprouting, anastomosis, pruning, arterial-venous specification as well as interactions that occur between EC themselves and with other cell types¹⁷². As previously described, we collected retinas

from time points specified below and performed immunofluorescent staining to visualize the vascular network¹⁷³.

We analyzed neonatal retinas at multiple developmental stages and first observed defects in the primary vascular plexus of SlugKO mice at postnatal day 5 (P5), shortly after the emergence of arteries and veins. Compared to the wild-type (WT) littermates, SlugKO mouse retinal vessels look underdeveloped with less regularity (Fig. 2.2A). In SlugKO retinas, the extension of the vascular network is delayed as indicated by a reduced distance from the optic disc to the leading edge of the vessels relative to WT littermates (Fig. 2.2A). Three measurements were taken from the center of the vasculature to the leading edge for each leaflet and the average was taken for each pup. The average of at least 6 pups for each genotype is plotted here (Fig. 2.2B). This is consistent with our *in vitro* data where loss of Slug impairs EC migration⁹⁶. Interestingly, further examination of the P5 retinas in SlugKO mice revealed an increase in both the number of tip cells and vessel density (Fig. 2.2D, E, F) at the leading edge of the vascular network relative to controls.

To determine if this morphological difference is due to changes in retinal EC proliferation, we performed EdU labeling *in vivo*. No significant difference was found in the number of EdU positive cells between these mice (Fig. 2.2G). This suggests that the increased density is a result of slower than normal migration of SlugKO EC resulting in a “pile-up” of EC at the leading edge of the primary vascular plexus. However, this delay

in vessel extension is transient and the difference between WT and SlugKO primary retina vasculature is almost overcome by postnatal day 9 (P9) (Fig. 2.2A, C).

After formation of the primary vascular plexus is complete, retinal EC begin to migrate vertically towards deeper retinal layers to form the secondary and tertiary plexus. Since the formation of these two plexus does not strictly follow the radial extension pattern, we assessed their level of vascularization by calculating vascular density using the grid method mentioned above for the whole retinal leaflets. As expected, vascular density at the secondary vascular plexus of these mice is reduced by 90% at P9 relative to the littermate controls (Fig. 2.2H, I). Again at P15, there is no significant difference in the vascular density in the secondary plexus of SlugKO versus littermate controls (Fig. 2.2J,K).

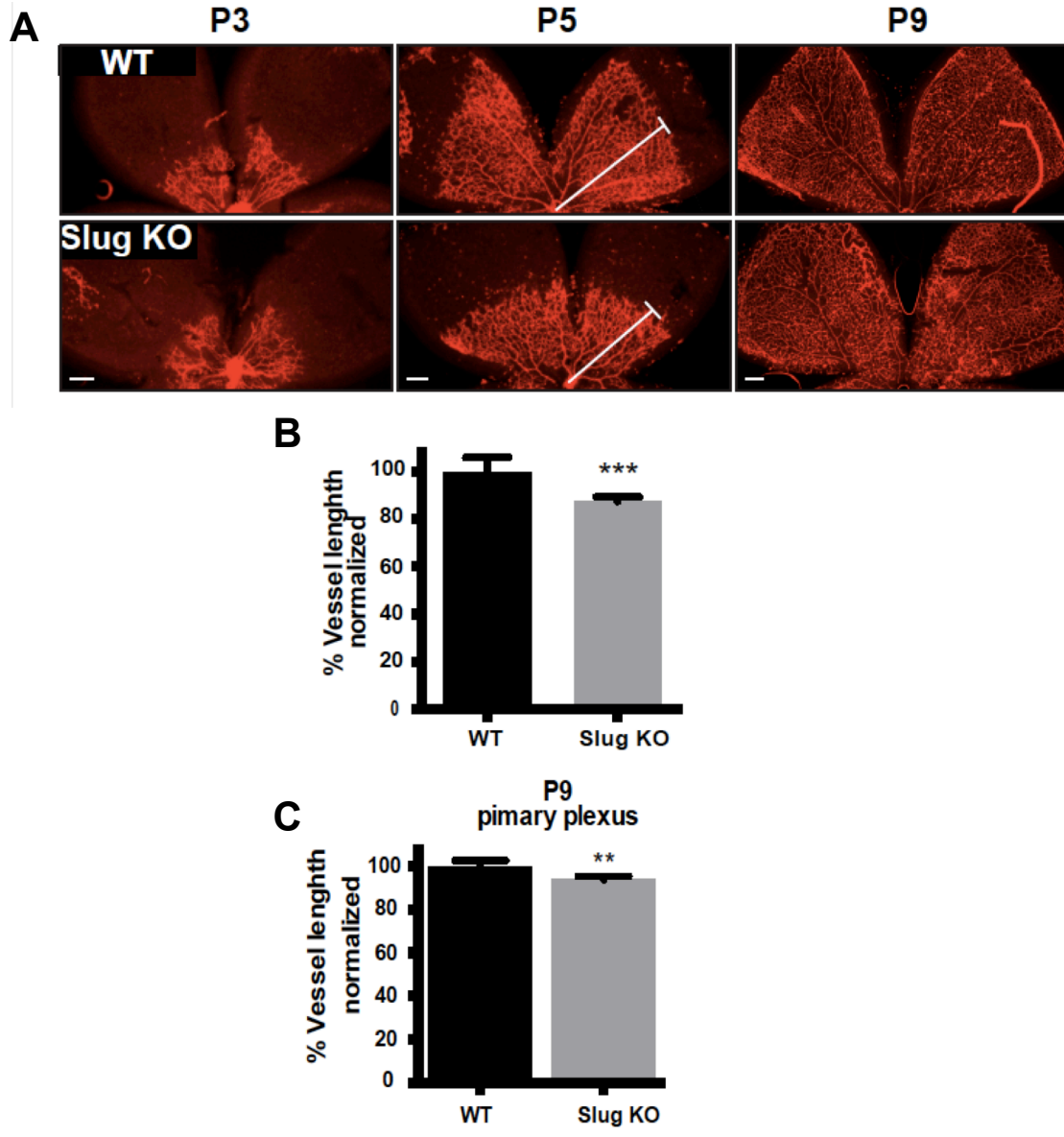
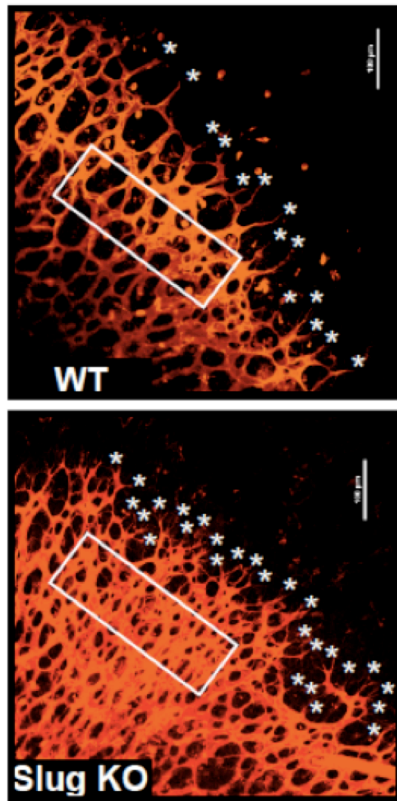
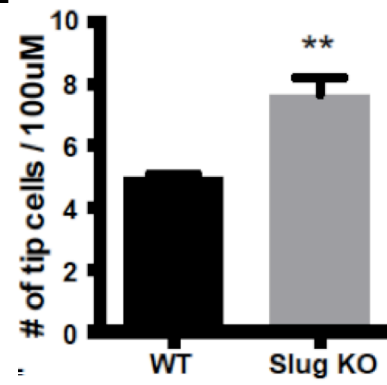


Figure 2.2: Slug deficiency leads to impaired neonatal retinal vascular development. A) IB4 staining of WT and SlugKO mouse retinal primary plexus at P3, P5 and P9. B) Average vessel length as a percentage of the retinal leaflet is quantified for P5 WT and SlugKO mice ($n > 10$). Vessel length is measured from the optical nerve center to the leading edge of the vessel growth, as indicated by white line in the middle panel in A. C) Average percentage vessel length of retinal leaflet length for secondary plexus in P9 WT and SlugKO mice ($n > 8$). D) IB4 staining of WT and SlugKO mouse retinal primary plexus vascular front. White rectangle specifies the area analyzed for leading edge vessel density. Each white star specifies an individual tip cells. E) Average number of tip cells per 100 μ m in WT and SlugKO mice ($n > 5$). F) Average percent vessel density at the leading edge of primary retinal vasculature in WT and SlugKO mice. G) Left: EdU staining of P5 WT and SlugKO retinas showing no difference in proliferation. Right: Average number of EdU⁺ nuclei per retinal leaflet ($n > 5$). H) IB4 staining of WT and SlugKO mouse retinal secondary plexus at P9. I) Average percent vessel density in the secondary plexus of the entire retina is quantified for P9 WT and SlugKO mice ($n > 8$). J) IB4 staining of secondary plexus in P15 WT and SlugKO mice. K) Average percentage vessel density of total retinal area for secondary plexus in P15 WT and SlugKO mice ($n > 5$). Scale bar: 100 μ m. Data represent mean $\pm$ s.e. two-tailed unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

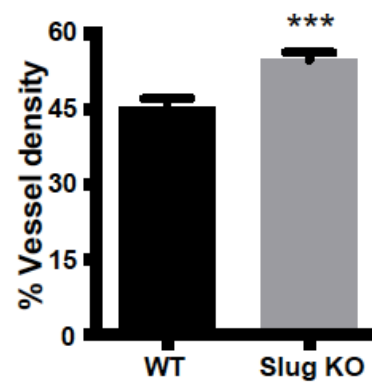
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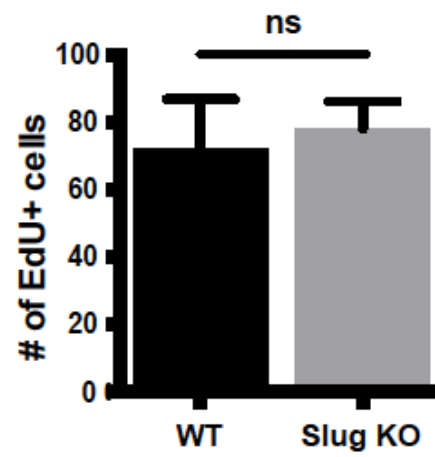
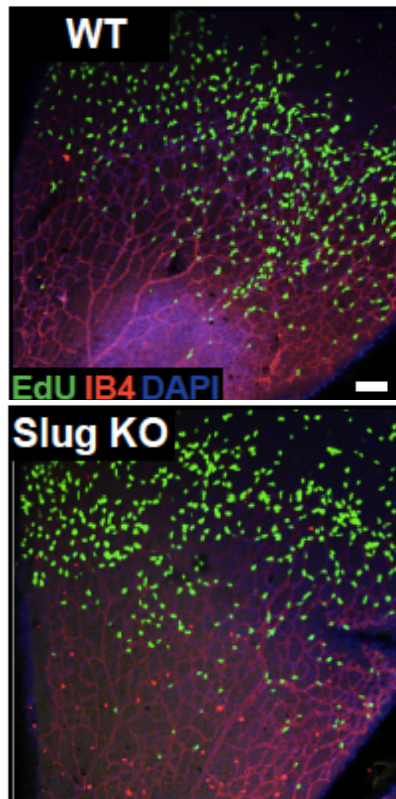
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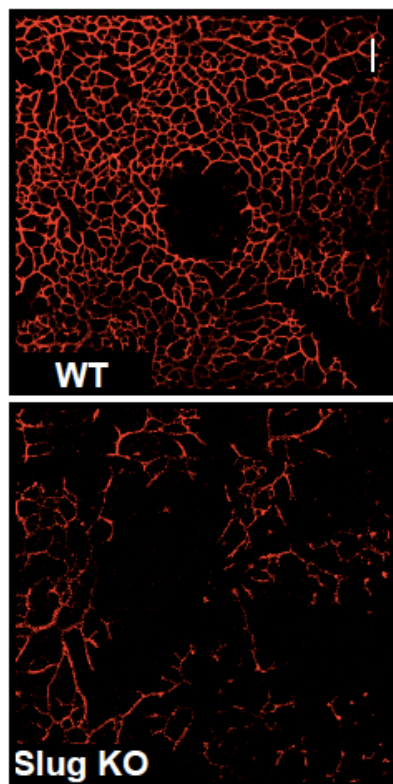
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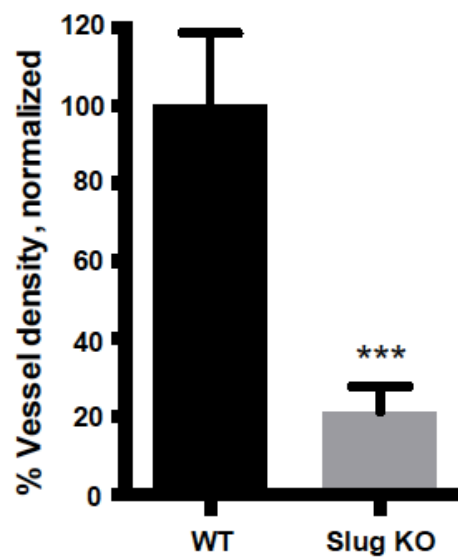
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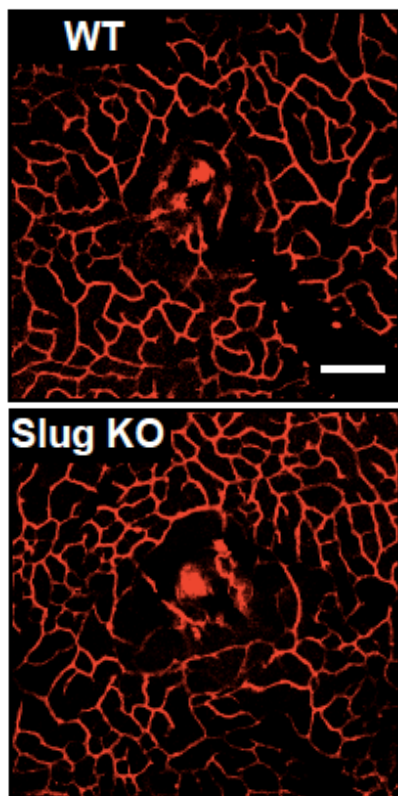
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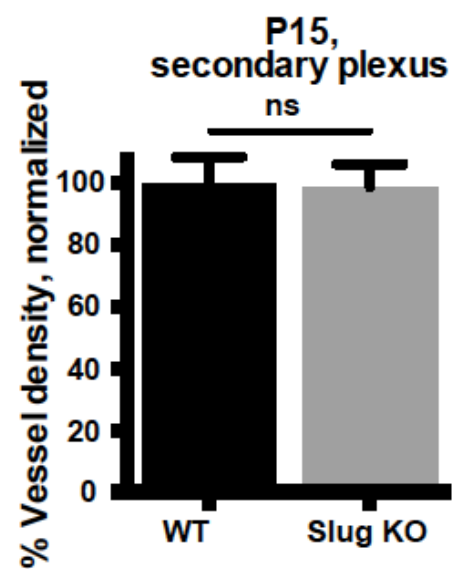
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2.3 Defective retinal angiogenesis is due to loss of Slug in endothelial cells

The development of mouse neonatal retinal vasculature is dependent on the cooperation of multiple cell types that reside within the retinal space. Upon entering the optic disc, EC migrate outwards and generate the vascular network by following a VEGF gradient laid down by the astrocytes¹⁴¹. This is followed by pericytes wrapping around the vessels to stabilize the network. In order to discern which cell type(s) is primarily responsible for the vascular defects we observe in Slug knockout mice, we first looked to see which cell types express Slug during retinal sprouting angiogenesis. Here, we used IsolectinB4 to label the vascular ECs, GFAP for the astrocytes and NG2 for the pericytes. DAPI was used as a general nuclear markers. As expected, we observed nuclear expression of Slug in the WT but not SlugKO retinal ECs (Fig. 2.3A), and in WT retina, Slug⁺ EC were distributed throughout the developing network (Fig. 2.3B). In contrast, we did not find any Slug⁺ astrocytes (Fig. 2.3B). Moreover, we did not see any changes in the distribution of GFAP⁺ astrocytes in SlugKO versus WT mice (Fig. 2.3C). This indicates that the vascular defects are not due to the loss of Slug in retinal astrocytes. On the other hand, we did find Slug expression in NG2⁺ pericytes, especially along the arteries (Fig. 2.3D). Therefore, we examined the distribution and coverage of the pericytes in the SlugKO mice compared to the WT controls. Again, we used a similar grid method as mentioned above with the modification that we percent pericyte coverage is calculated using the number of crosses intersecting both NG2⁺ pericytes and IsolectinB4⁺ vascular EC, divided by the total number of crosses intersecting EC (Fig. 2.3E). At least three pups per genotype were analyzed and no significant difference was found between

SlugKO and WT mice (Fig. 2.3F). These data indicate that loss of Slug in pericytes is unlikely to underlie the developmental angiogenic defects observed in SlugKO mouse retinas, and in combination with our *in vitro* studies, suggest that it is the loss of Slug in EC that accounts for the vascular phenotypes we observe.

In addition, we have observed possible defects in arterial-venous specification in the SlugKO mice, where the mutant animals appear to have fewer retinal veins than their WT littermates (data not shown). However, larger numbers of samples will need to be analyzed to confirm this observation. Together, these studies indicate that Slug has an important role in developmental angiogenesis, however the defects in Slug-deficient animals are transient, suggesting that other factors, possibly Snail, can compensate in this setting.

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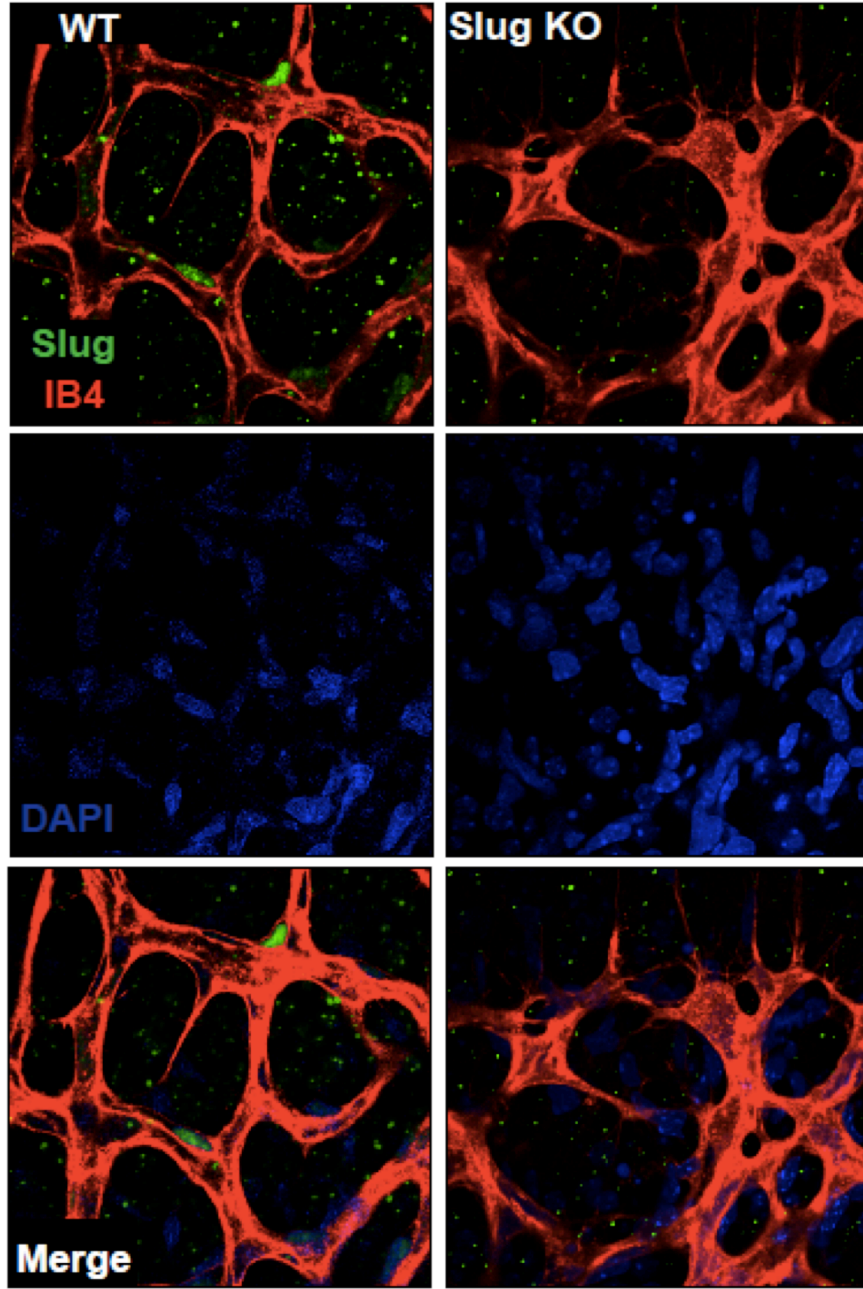
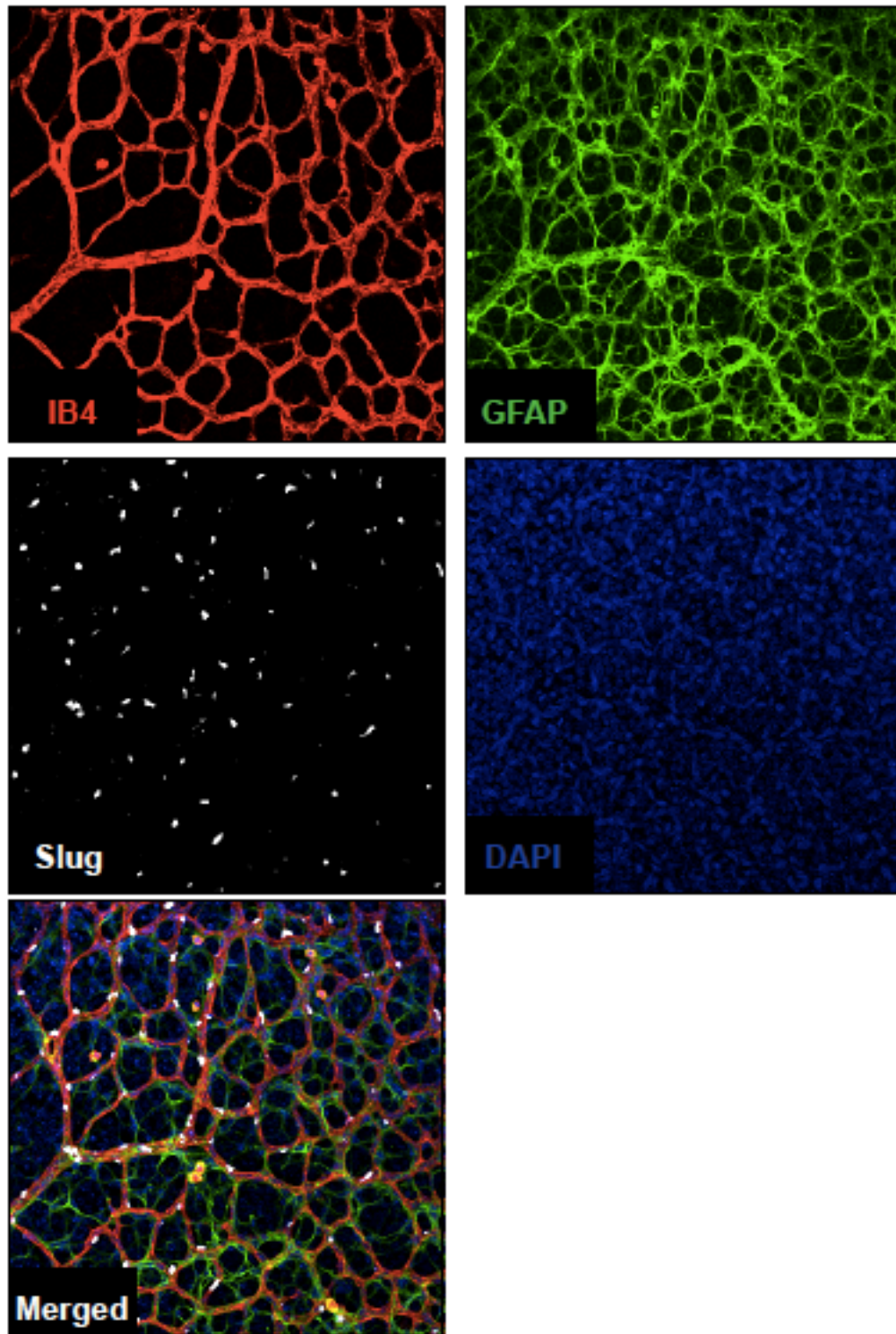
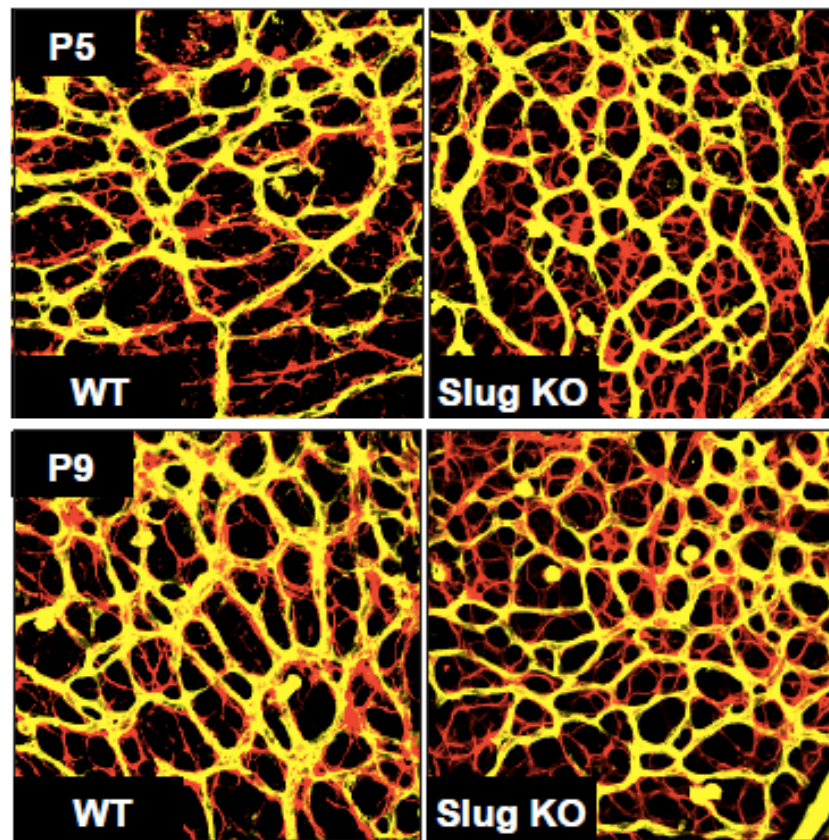


Figure 2.3: Slug expression in different retinal cell types. A) IB4, Slug and DAPI staining of WT and SlugKO mouse retinal primary plexus at P5 showing Slug is expressed in the nuclei of the retinal endothelial cells. B) IB4, Slug, GFAP and DAPI staining of WT mouse retinal primary plexus at P9 showing Slug is not expressed in the GFAP⁺ astrocytes. C) IB4 and GFAP staining of WT and SlugKO mouse retinal primary plexus at P5 and P9 shows no difference in astrocyte distribution. D) Immunofluorescent staining of WT P5 retina showing Slug expression in both retinal EC and NG2⁺ pericytes. White arrow: Slug⁺ pericytes. E) Immunofluorescent staining images with overlaid grid showing method of pericyte coverage quantification. F: Average percentage pericyte coverage in P5 WT and SlugKO mouse retinas showing no difference (n>5). Scale bar: 100μm. Data represent mean ± s.e. two-tailed unpaired t-test. *p<0.05, **p<0.01, ***p<0.005.

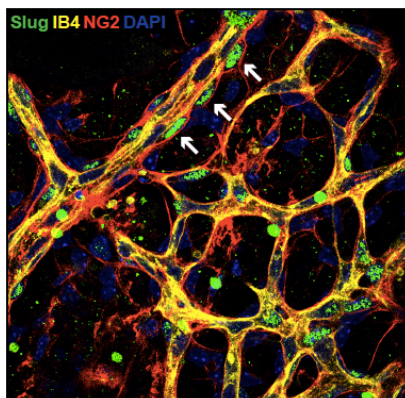
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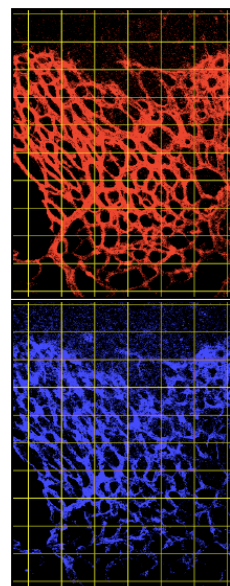
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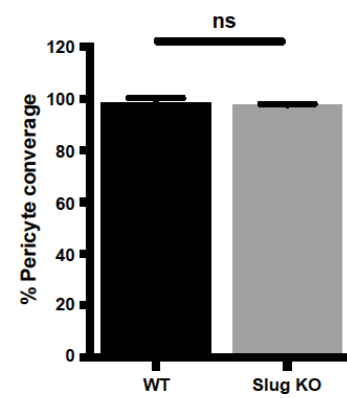
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2.4 Loss of endothelial Slug expression decreases tumor growth by restricting tumor angiogenesis.

Although Slug was originally discovered as a developmental gene, recent studies have been focused on its significant role in regulating EMT in pathological conditions such as tissue fibrosis and tumor progression^{81,82}. In order to determine if Slug expression in non-cancer cells, particularly EC, plays a role in tumor angiogenesis, we used a syngeneic tumor transplantation model. Mouse colorectal cancer cell lines MC-38 and CMT-93 were used.

While the tumors grew well in the WT mice, we observed a significant decrease in tumor growth in the SlugKO animals at all time points measured for both CMT93 and MC38 tumors (Fig. 2.4A, B). Of note, in many Slug knockout animals, tumor development was completely halted after a brief initial growth period (Fig. 2.4C, D, data not shown). Similar results were also observed with syngeneic B16 melanoma tumor cells (data not shown). Since all the cancer cell lines used in this study have “normal” Slug expression, this strongly suggests that lacking Slug expression in the tumor stroma, most likely the EC, has a substantial effect on the growth and progression of wild type tumor cells.

To directly assess tumor angiogenesis, we also harvested the CMT-93 tumors from the WT mice and from the few SlugKO mice that had tumors large enough to harvest (Fig. 2.4C). In those tumors we observed significantly less vasculature, particularly in the center of the tumors, compared to tumors from WT mice (Fig. 2.4E, F). Consistent with

the reduced vasculature, an increase in necrosis was also noted in the tumors from SlugKO mice compared to those from WT mice (Fig. 2.4G). Together, these data suggest that restricted tumor angiogenesis in SlugKO mice attenuated tumor growth.

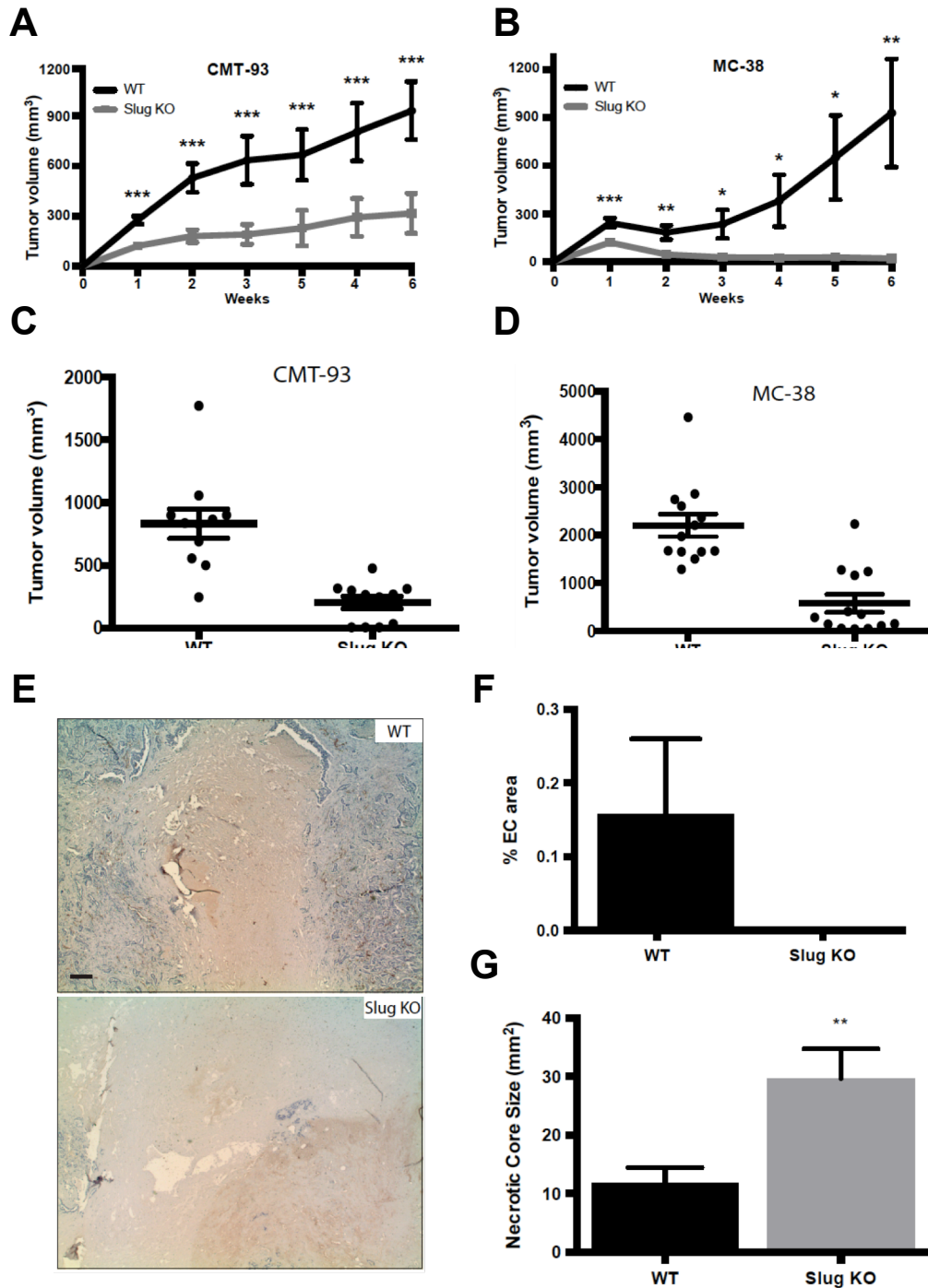


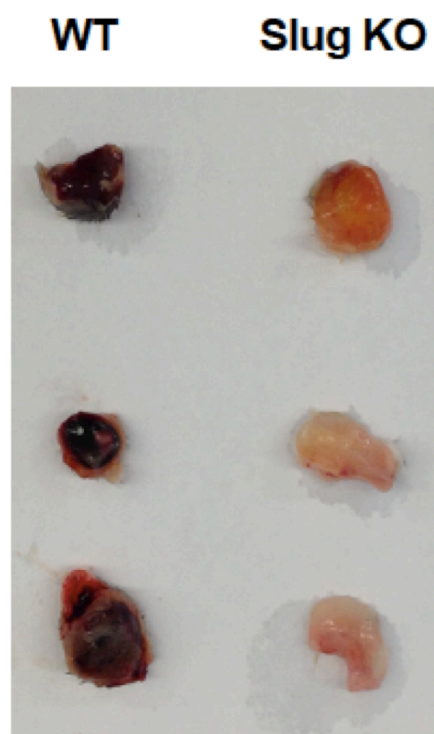
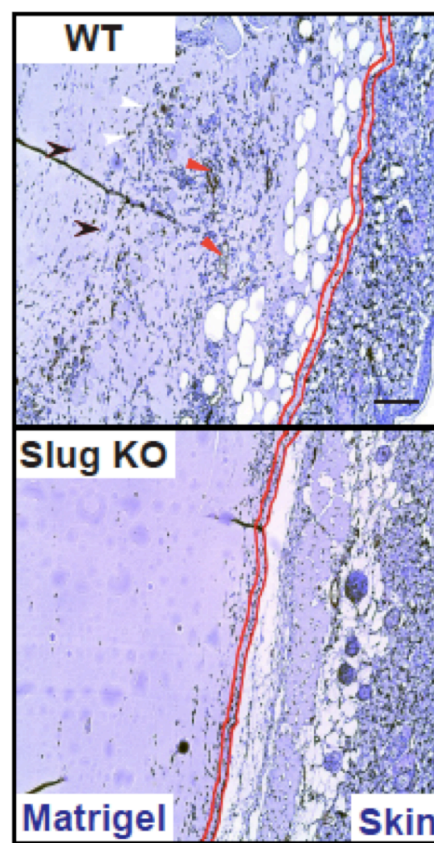
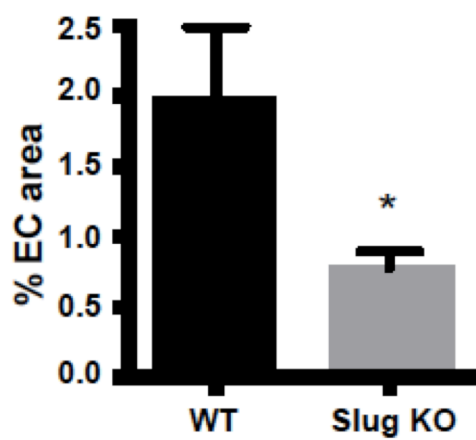
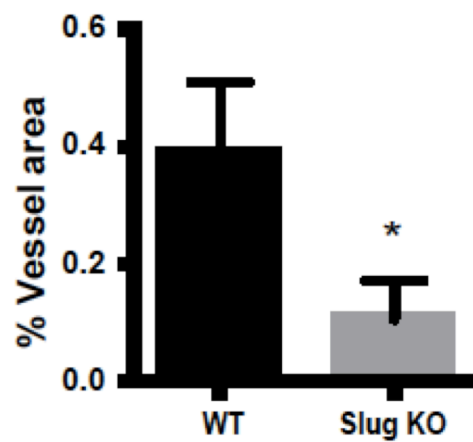
Figure 2.4: Slug deficiency inhibits tumor growth by reducing tumor angiogenesis. A) Growth curve of CMT-93 colorectal tumor in WT and SlugKO mice (n> 10). B) Growth curve of MC-38 colon tumor in WT and SlugKO mice (n>10). C) Endpoint CMT-93 tumor size in each WT and SlugKO mice. D) Endpoint MC-38 tumor size in each WT and SlugKO mice. E) CD31 staining in the core regions of CMT-93 tumors from WT and SlugKO mice. Nuclei were counterstained with hematoxylin. Scale bar: 200µm. F) Average percentage of CD31+ areas in CMT-93 tumors from WT and SlugKO mice. G) Average size of necrotic core areas in CMT-93 tumors from WT and SlugKO mice. Data represent mean $\pm$ s.e. two-tailed unpaired t-test. *p<0.05, **p<0.01, ***p<0.005.

To gain a clearer picture of the role of Slug expression in EC during tumor angiogenesis, we employed the Matrigel plug *in vivo* angiogenesis assay as a simplified model. Matrigel is a type of solubilized basement membrane matrix extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma. It is mainly composed of laminin (60%) and collagen IV (30%), along with 8% entactin, which serves as a bridging molecule that stabilizes the matrix structure. Regular Matrigel, as used in the tumor study described above, also contains various growth factors including EGF, TGF β , PDGF and IGF-1, all of which are naturally occurring in the EHS tumor environment. However, for the Matrigel plug *in vivo* angiogenesis assay, growth factor reduced (GFR) Matrigel was used.

The assay was performed as previously described with minor modifications¹⁷⁴. Strikingly, while the WT plugs were filled with blood, the SlugKO plugs were relatively pale (Fig. 2.5A). Consistent with this, IHC-staining revealed a significant degree of CD31⁺ EC infiltration in the WT but not the SlugKO plugs (Fig. 2.5B), which was quantified as the percent CD31⁺ area relative to total area in the plugs (Fig. 2.5C). Moreover, an even greater reduction was seen in the percentage of lumenized vessel area in the Slug KO plugs compared to the controls (Fig. 2.5D), suggesting that, unlike WT EC, the SlugKO EC that infiltrated the plugs failed to form functional vessels. Together, these data indicate that impaired pathological angiogenesis due to reduced EC recruitment likely explains the decreased tumor growth in SlugKO mice.

Interestingly, we also observed reduced infiltration of CD31⁺ stromal cells in the SlugKO mice (Fig. 2.5B). We and others have previously published that stromal cells such as fibroblasts play a critical role during angiogenic sprouting by synthesizing and remodeling extracellular matrix^{44,175}. In order to rule out the possibility that the lack of EC recruitment in the SlugKO plugs was a secondary effect of reduced stromal cell infiltration, we conducted a rescue experiment using mouse stromal fibroblasts. Matrigel was prepared with (200,000 cells per plug) or without WT mouse fibroblasts (mF) and each SlugKO mouse in the study received one Matrigel implant of each kind on either side of the ventral mid-line. We also implanted Matrigel without mF in WT mice as a control. After 7 days the plugs were harvested and stained for CD31. Again, there was very little ingrowth of vasculature into Matrigel plugs without mF in the SlugKO mice, whereas there was robust growth into plugs in WT mice (Fig. 2.5E). Consistent with our previous data, the addition of WT mF to the Matrigel plugs failed to induce vessel ingrowth in SlugKO mice (Fig. 2.5E, F). Taken together, these data confirm that the poor angiogenic response in a pathological environment in the SlugKO mice is due to an EC-intrinsic defect.

In summary, our study has shown that SlugKO mice have an impaired angiogenic program in both developmental and pathological settings, likely due to EC-intrinsic defects in the absence of Slug. These data support our previous *in vitro* finding that Slug expression in EC is important for sprouting angiogenesis. Furthermore, this is the first time angiogenic defects were reported in SlugKO mice.

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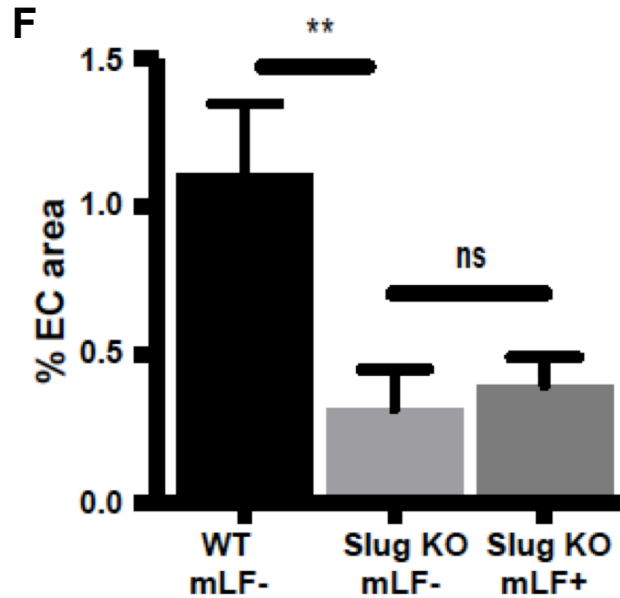
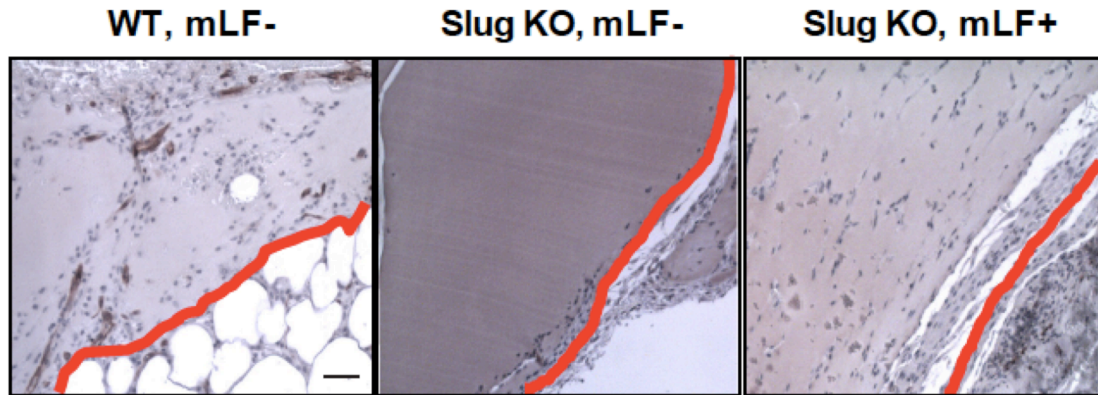


Figure 2.5: Defective pathological angiogenesis in SlugKO mice is likely due to EC-intrinsic effect. A) Representative images of Matrigel plugs harvested from WT and SlugKO mice. Scale bar: 1cm. B) CD31 staining of Matrigel sections reveals reduced EC recruitment, signified by CD31⁺ area (white arrow heads), reduced vessel formation, signified by vessel area (red arrow heads), and reduced stromal cells, signified by CD31⁻ hemotoxylin counter stained cells (black arrow heads) in SlugKO mice. Double red lines separate native mouse skin tissue from the Matrigel area. E) Average percent CD31⁺ area in Matrigel sections from WT and SlugKO mice (n>15). F) Average percent vessel area in Matrigel sections from WT and SlugKO mice area (n>15). G) CD31 staining of Matrigel sections suggests the addition of WT mLF cannot rescue the impaired EC recruitment in SlugKO mice. Red line separates the native mouse skin tissue from the Matrigel area. H) Average percent CD31⁺ area in Matrigel sections from WT and SlugKO mice (n>10). Scale bar: 100μm. Data represent mean ± s.e. two-tailed unpaired t-test. *p<0.05, **p<0.01, ***p<0.005.

2.5 Methods

Vascular Development in Yolk Sac

8 to 12 week-old females were mated during estrus and sacrificed to obtain embryos at E9.5. The embryos were collected and their yolk sacs were dissected in cold PBS. The yolk sacs were then fixed in 4% PFA at room temperature for 20 minutes. For immunofluorescent staining, the yolk sacs were permeablized in 0.1% Triton-100X for 20 minutes and blocked with 5% BSA for 1 hour at room temperature. The yolk sacs were incubated with primary antibodies against mouse VE-cad (Abcam, 1:100) and mouse CD31 (BD, 1:50) overnight at 4°C and washed thoroughly. Secondary antibodies were added and incubated for 1 hour at room temperature. Yolk sacs were then washed thoroughly and mounted in ProlongGold. Confocal imaging was performed at 100x magnification with tiling. Images were processed and cropped with Adobe Photoshop.

Vascular Development in neonatal mouse retina

Neonatal pups at specified age were euthanized and their eyes were enucleated and immediately fixed in 4% PFA on ice for up to 4 hours. The eyes were then washed thoroughly with PBS and dissected. The obtained retinas were then permeablized with 0.3% Triton-X 100 in PBS (permeablization buffer) and blocked with 5% BSA in permeablization buffer overnight at 4°C. The retinas were incubated with primary antibodies for 2 to 4 days at 4°C and washed extensively in PBS before secondary antibodies (and DAPI nuclear staining when used) were incubated for 2 days. Retinas were then washed thoroughly, post fixed with 4% PFA and mounted in Prolong Gold. Number of tip cells was counted for each leaflet and the average was taken for each pup.

For vessel density, an area 100 μm x 300 μm of size at the front of the vasculature was overlaid with a randomly-generated grid (grid size: 80 μm^2) and the number of crosses intersects with the vessels and the total number of crosses within was counted. The vessel density was determined by taking the ratio of the two numbers. Average of at least three pups per genotype are plotted.

EdU labeling in vivo

WT and SlugKO P5 pups were injected intraperitoneally with 20 μL of EdU (50mM in PBS) and euthanized 2 hours later for analysis. The development and the detection of EdU were done using Click-iT EdU AF488 Imaging Kit. The number of EdU⁺ cells was counted for each leaflet and averaged for each pup. The average of at least three pups was plotted.

Mouse syngeneic tumor transplantation model

Mouse syngeneic colorectal cancer cell lines MC-38 (NCI) and CMT-93 (ATCC) were expanded *in vitro* prior to implantation. Cancer cells were mixed with Matrigel and injected subcutaneously on the right flank at 5 million or 10 million cells per implant (100 μL) respectively. WT and SlugKO mice at 20-24 weeks of age were used. Tumors were allowed to develop for 6 weeks while measurements were taken weekly or until they reached the maximum size allowed by the IACUC protocol. The tumors were then harvested and fixed in 10% formalin overnight at room temperature before paraffin embedding and sectioning for IHC analysis. Each experiment was repeated at least three times with at least 10 mice per genotype each time.

Matrigel plug *in vivo* angiogenesis assay

Briefly, growth factor reduced Matrigel was mixed with 20U/mL Heparin, 400ng/mL recombinant mouse bFGF and 400ng/mL recombinant mouse VEGF165. WT and SlugKO mice at 20-24 weeks of age were used to keep consistent with the subcutaneous tumor model. Matrigel mix (400 μ L) was injected subcutaneously on the ventral lower abdominal area, close to the mid-line. For the rescue experiment, WT mouse lung fibroblasts or PBS control of the same volume was added to the Matrigel prior to implanting. The plugs are left in mice for 7 days, then harvested and fixed in 10% formalin overnight at room temperature. The plugs were then paraffin embedded and sectioned. IHC staining of CD31 was performed to visualize vascular EC and the nuclei were counter stained with hemotoxylin.

Chapter 3. High levels of Slug in Endothelial Cells leads to disrupted vessel formation *in vitro*.

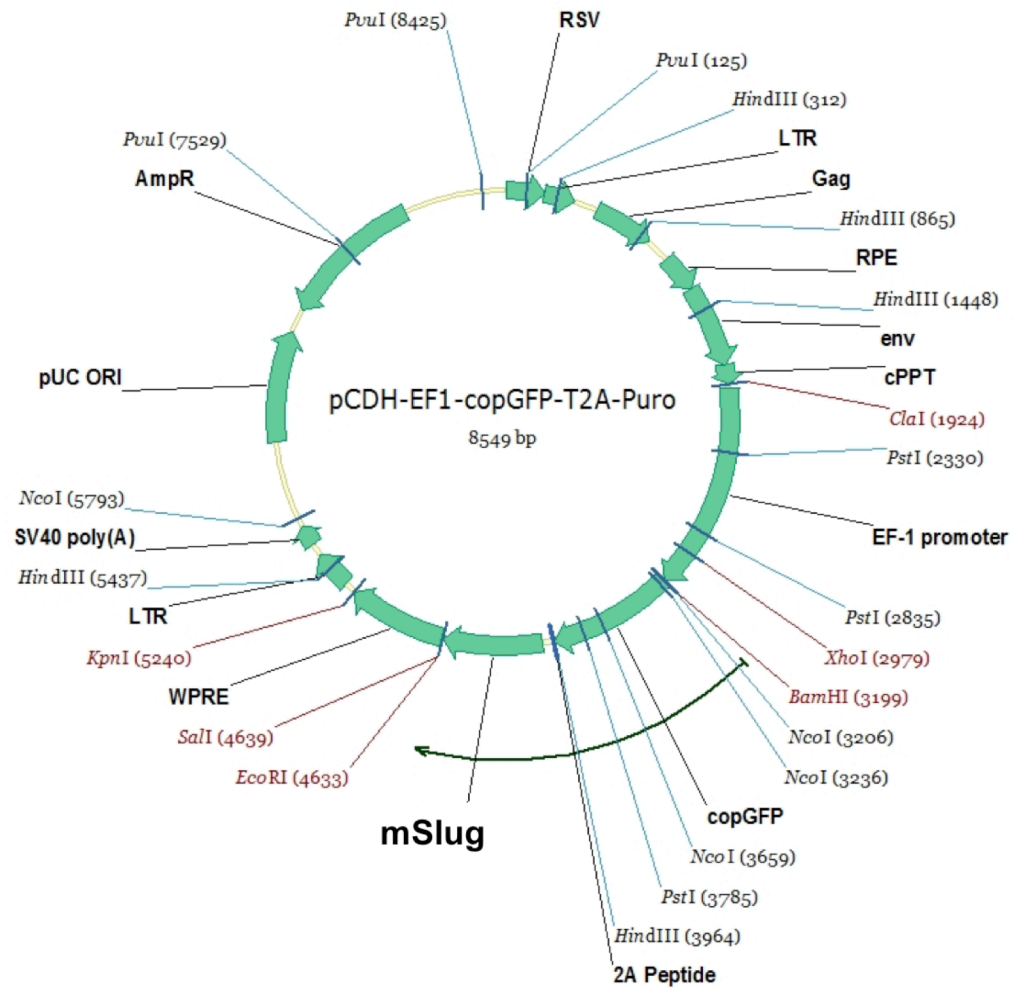
3.1 Slug Misexpression in EC Using Lentiviral Approach

To further analyze the role of Slug in EC during angiogenesis we decided to misexpress the gene through lentiviral transduction. Lentiviruses place very little load on transduced cells and do not need to be used with cells undergoing proliferation, in contrast to retroviruses. Several lentiviral constructs containing either human SLUG or mouse Slug gene cDNA sequences were generated and used in this study. The human SLUG overexpressing construct has been generated previously⁹⁶ (Fig. 3.1A). Here, the expression of Slug as well as a copGFP fluorescent tag are constitutively activated and driven by a human EF1 α promoter. The construct is designed so that the translated copGFP and Slug polypeptides are linked by a T2A peptide, which auto-cleaves once translation is complete. This design allows both transcripts to be driven by the same promoter and made at a 1:1 ratio so that the level of GFP fluorescence corresponds to the level of SLUG expression. This construct was used to achieve Slug misexpression in HUVEC in most of the *in vitro* experiments we conducted.

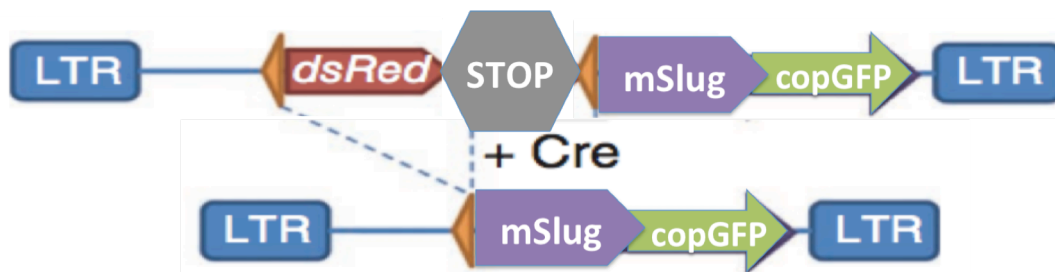
We also designed mouse Slug (mSlug) overexpressing constructs for conditional overexpression upon Cre-mediated recombination (cSlugOE). In these constructs, the T2A-linked copGFP-mSlug expression cassette follows a dsRed-STOP expression cassette that is flanked by two loxP sites (Fig. 3.1B). When integrated, only cells that express Cre recombinase will express mSlug and the GFP tag, whereas other virally-

transduced cells will express the dsRed tag. To account for the difference in promoter efficiency and the need for differential expression level, this design was cloned into two backbones with different promoters: one driven by a human EF1 α promoter and the other one by an MSCV promoter. To test these, we generated HUVEC expressing Cre recombinase at a low MOI so that only about 50% of cells would express Cre-recombinase. We then transduced the HUVEC with either the cSlugOE virus, or the control virus that does not contain the mSlug cDNA. Four days after transduction, we saw that about 50% of the cells expressed the GFP tag while the rest expressed the dsRed tag in either control or cSlugOE transduced cells (Fig. 3.1C, D). We also performed western blot for Slug expression in these cells to confirm that we achieved an increase in Slug expression in cSlugOE HUVEC compared to the control HUVEC (Fig. 3.1E, the antibody cross-reacts with both human and mouse Slug proteins).

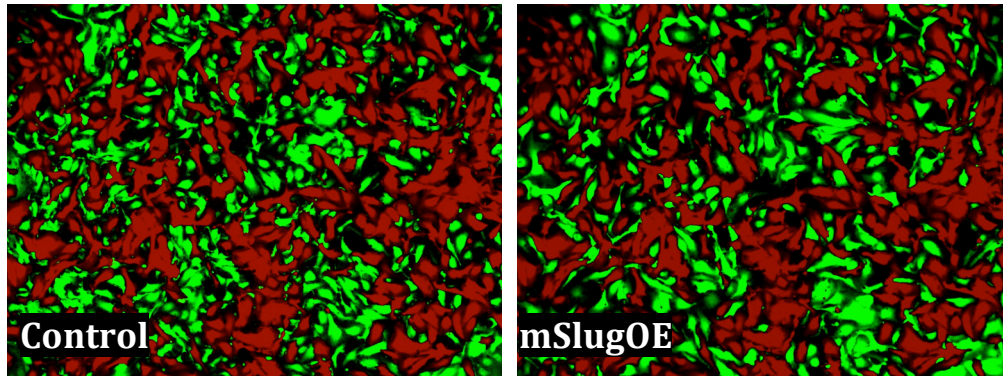
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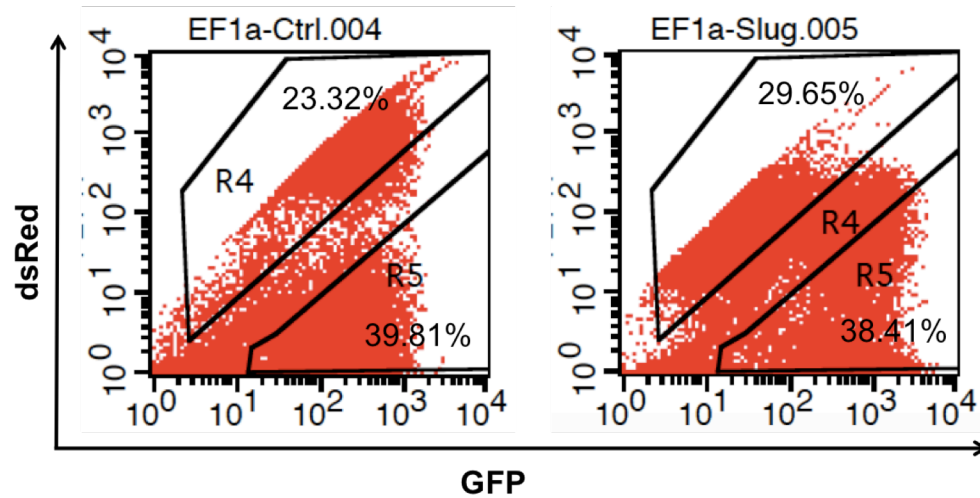
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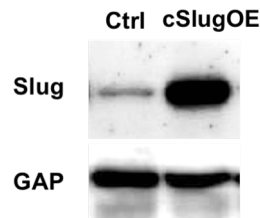


Figure 3.1: Conditional mouse Slug overexpression lentiviral construct. A) Human SLUG cDNA is cloned into the pCDH lentiviral backbone (CD521A-1, System Biosciences). B) Using the same backbone, loxP flanking dsRed expression cassette with a stop codon was cloned upstream of copGFP and mouse Slug cDNA sequence was cloned downstream of copGFP. Both transcripts are driven by the EF1 α promoter. When expressed in cells that do not express Cre-recombinase, the EF1 α promoter drives dsRed expression but not GFP-Slug expression. When transduced into cells expressing Cre-recombinase, the dsRed expression cassette is excised out and the EF1 α promoter now drives the expression of GFP and Slug at 1:1 ratio. C) HUVEC with a mixed population of Cre⁺ and Cre⁻ cells transduced with either the control or the cSlugOE construct. D) FACS plot showing percentage dsRed and GFP-tagged cells. E) Western blot of HUVEC (mixed population) expressing either the control or the cSlugOE construct.

3.2 Endothelial misexpression of Slug disrupts *in vitro* angiogenesis

Here, we misexpressed human SLUG in HUVEC at two different levels by transducing them with the lentivirus with different titer. Based on the qPCR analysis results, we were able to achieve expression of SLUG at either 3-fold (SlugOE^{low}), or 25-fold of the normal induction level (SlugOE^{high}, Fig. 3.2A). We then performed the 3D fibrin bead assay as previously described¹³⁸ and observed angiogenic behaviors at different stages of the sprouting process. Briefly, HUVEC were coated onto Cytodex 3 beads and embedded in a fibrin gel with human lung fibroblasts seeded on top. The assay was maintained in EGM2 for the number of days specified.

On day 2 of the assay, tip cells started to appear in the GFP control cells. However, more tip cells formed per bead in both SlugOE^{low} and SlugOE^{high} cells. In addition, increased filopodia could be observed on tip cells of SlugOE cells. In SlugOE^{high} cells, pre-lumenized sprout is also apparent at this time, almost 48 hours earlier than the control cells (Fig. 3.2B). These observations suggest that high levels of Slug expression in HUVEC led to not only increased numbers of tip cells, with higher environment-sensing and migratory potential due to more filopodial protrusion, but also early sprouting, likely due to increased migration.

By day 6 or 7 of the fibrin assay, sprouts in the control condition started to lumenize and mature (Fig. 3.2C). However, sprouts in the SlugOE conditions showed similar but distinct types of defects depending on the level of Slug expression. In the SlugOE^{low}

condition, hypersprouting was observed. In addition, these sprouts show increased lumen diameter (Fig. 3.2C). This could be due to either heightened proliferation or increased matrix degradation^{26,176}. EdU staining confirmed that SlugOE cells do proliferate faster (Fig. 3.2D). However, almost all the sprouts maintained their connections. On the other hand, we observed not only hypersprouting in the SlugOE^{high} condition, but also increased fragmentation of sprouts, a phenomenon previously observed⁹⁶. There appear to be cells that had completely lost connection from each other (Fig. 3.2C), while some cells appeared to have lost polarity and looked rounded (Fig. 3.2C). It is intriguing to speculate whether these cells started as part of a sprout but lost their junctions during the process and became disconnected, or were migrating as single cells from the beginning (as seen in a full EndoMT), instead of migrating as a group, characteristic of endothelial sprouting.

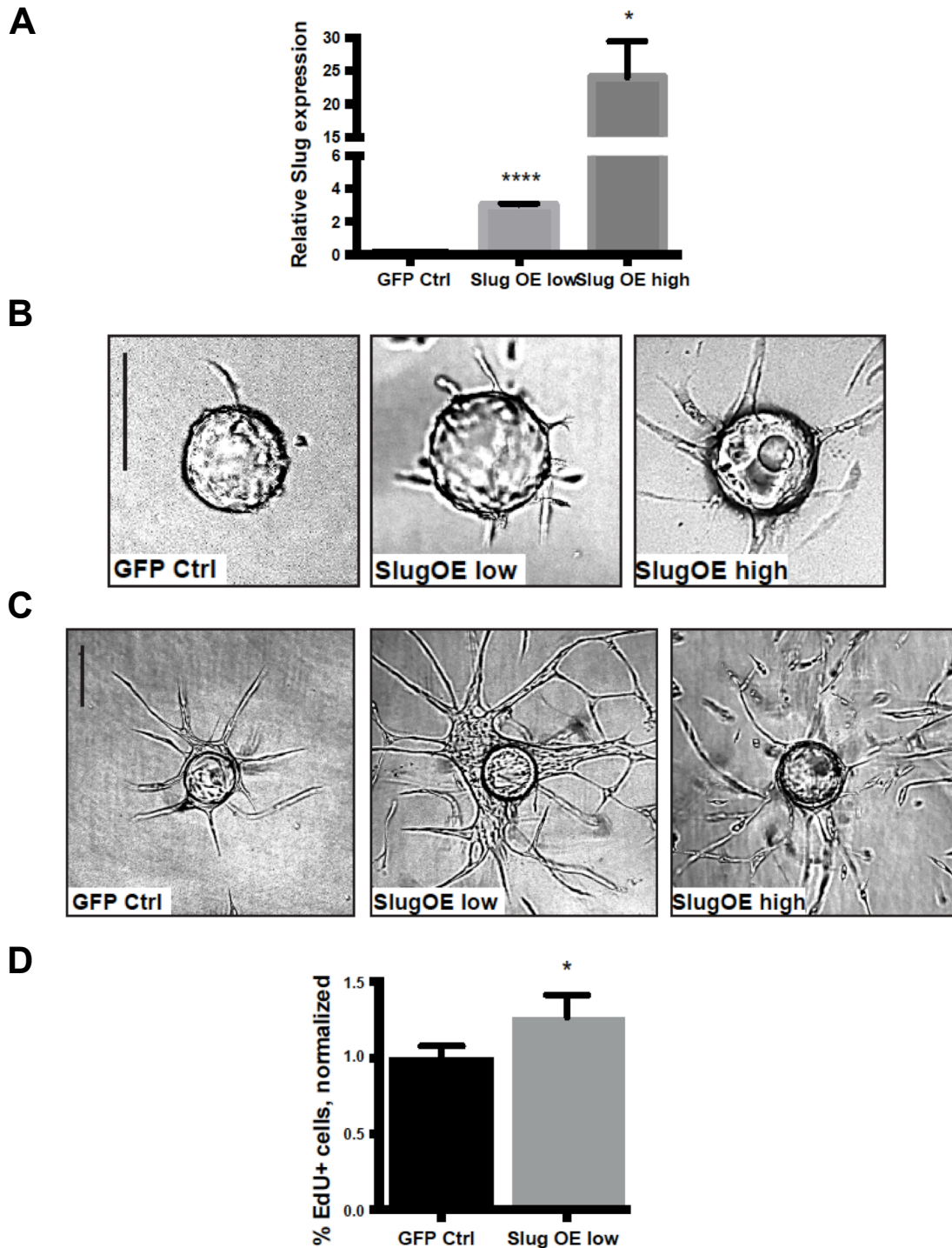


Figure 3.2: Slug overexpression in EC disrupts sprouting angiogenesis. A) Two levels of Slug overexpression at either 3-fold (Low) or 25-fold (High) of normal induction level. B) 24 hours (day 1) after embedding of the 3D fibrin bead sprouting assay. SlugOE in EC induces increased tip cell formation (middle) and accelerates sprouting (right). C) Day 7 of the 3D fibrin bead sprouting assay. SlugOE in EC leads to dilated lumen (middle) and fragmentation of the sprouts (right). D) Average percentage EdU⁺ nuclei per view in GFP control and SlugOE low cells. Scale bar: 100 μ m. Data represent mean $\pm$ s.e. two-tailed unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Another puzzling observation previously made was that when SlugOE cells (GFP⁺) were mixed with control cells (non-transduced), even at a low ratio (25% of the total cells), almost all the sprouts (>90%) contained SlugOE cells and, remarkably, these sprouts were composed almost completely of SlugOE cells⁹⁶. Our conclusion was that the SlugOE cells could either be actively suppressing the control cells from becoming integrated into the developing sprouts, or they may have simply out-competed them.

To test this, a similar chimera experiment was conducted, where SlugOE-GFP or GFP⁺ cells at various ratios (0, 25%, and 100%) were mixed with control cells expressing a different fluorescent tag (mCherry or BFP) so that both populations can be visualized. We observed both control (BFP) and SlugOE-GFP cells incorporated into and evenly distributed through the sprouts (Fig. 3.3A), and we observed a moderate increase in the ratio of SlugOE-GFP cells to control cells (Fig. 3.3A). This increase can likely be explained by increased proliferation in the SlugOE cells as described above. Moreover, mCherry/BFP cells were distributed throughout the sprouts along with the SlugOE-GFP cells, suggesting that the SlugOE cells were unlikely to be actively suppressing and excluding the control cells from becoming part of the developing sprout.

Human SLUG and mouse Slug proteins share 96% overall sequence homology, and 100% homology in both SNAG and zinc-finger binding domains¹¹⁸. Therefore, we speculated that mouse Slug might retain some functions in HUVEC. When we performed a bead assay with the Cre-HUVEC that were transduced with either the control or the cSlugOE virus, we saw similar results as in the chimera experiment described above with

the SlugOE-GFP cells. The cSlugOE condition had more and longer sprouts than the control condition, with both dsRed-tagged (cSlugOE negative) and GFP-tagged (cSlugOE positive) cells integrated into the sprouts at comparable ratios (Fig. 3.3B). Again, this result supports the notion that SlugOE cells do not actively suppress or exclude Slug-low cells from sprouting. Surprisingly, dsRed-tagged cells in the cSlugOE chimera bead assay also appear to be forming more sprouts or are part of long sprouts that can only be observed in SlugOE conditions. This seems to suggest that Slug overexpression is inducing more and longer sprouts at least partly in a paracrine manner. Additional quantification experiments with confocal microscopy and FACS analysis are needed to confirm these conclusions.

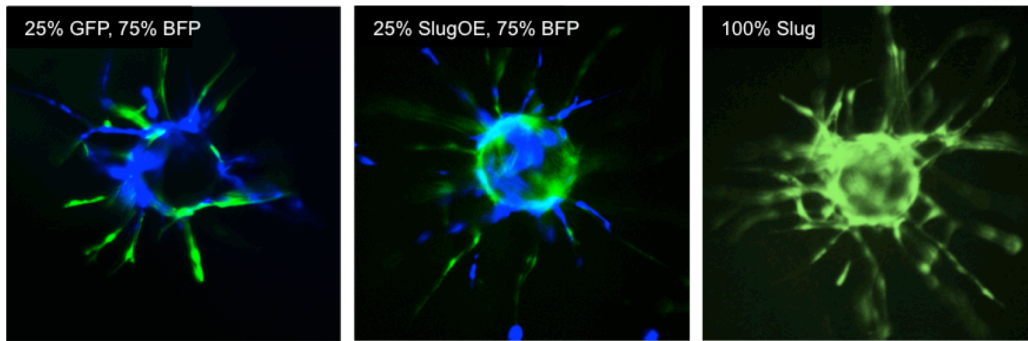
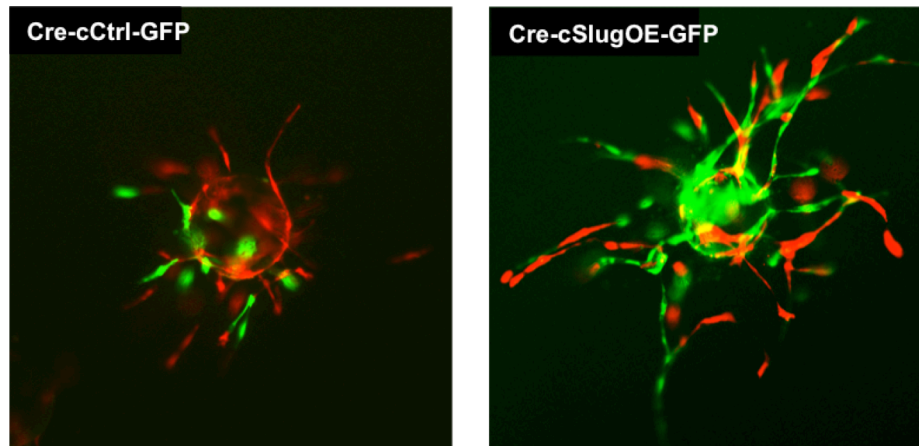
A**B**

Figure 3.3: SlugOE chimera in 3D fibrin bead assay. A) 25% SlugOE-GFP or GFP cells are mixed with 75% BFP cells. Bead assay was performed and images are taken at day 5. B) Mixed Cre^{+/+}-HUVEC are transduced with either control-GFP or cSlugOE-GFP construct as shown in Fig. 6C,D. Cells were used to perform 3D fibrin bead assay and images are taken at day 5.

3.3 Endothelial misexpression of Slug interrupts *in vitro* vasculogenesis

To determine whether SlugOE alters the ability of EC to form a vascular network, as opposed to single sprouts, we used an *in vitro* vascularized micro-organ (VMO) platform¹⁵⁵ in which EC form a capillary bed that anastomoses to microfluidic “arteries” and “veins” allowing flow of a blood substitute through the network. Compared to control EC the SlugOE^{low} cells formed distorted vessels with larger diameters and fewer branch points (Fig. 3.4A-E), while the SlugOE^{high} cells were mostly incapable of forming networks (Fig. 3.4A).

In aggregate these data demonstrate that Slug is a critical transcription factor for vascular morphogenesis and precise control of its expression level is required. Too much or too little Slug expression each disrupts proper vessel formation. It is interesting to note that elevated Slug expression in EC can lead to enlarged and irregular vessel formation, similar to what is seen in the tumor-associated vasculature. Together with the observation described in Chapter one that lack of Slug limits tumor angiogenesis and halts tumor growth, one can conclude that Slug might be a necessary and sufficient factor in tumor angiogenesis, and therefore a potential target for new anti-angiogenic therapies.

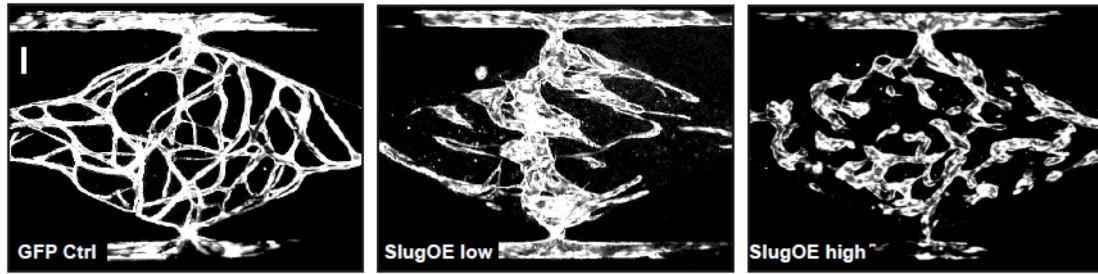
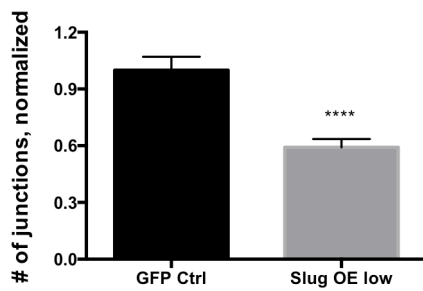
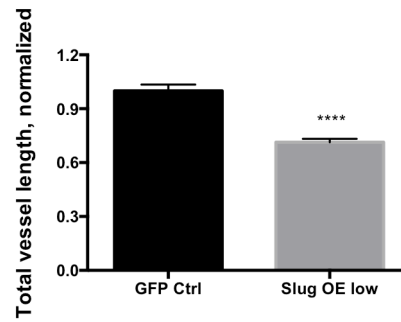
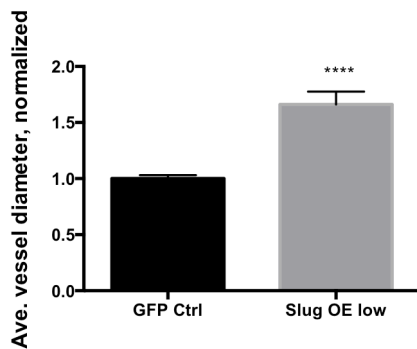
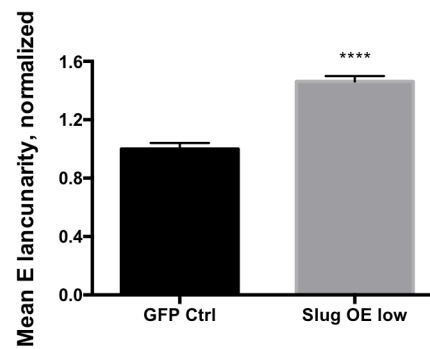
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Figure 3.4: Slug overexpression in EC disrupts vasculogenesis. A) SlugOE in EC leads to dilated lumen (middle) and fragmentation of the network (right) in *in vitro* vasculogenesis assay. This is quantified as B) reduced number of junctions, C) reduced total vessel length, D) increased average vessel diameter, and E) increased average lacunarity. Scale bar: 100 μ m. Data represent mean $\pm$ s.e. two-tailed unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

3.4 Methods

Vascularized micro-organ and perfusion

Transduced HUVEC (control-GFP or SlugOE-GFP) and NHLF were harvested and loaded into the tissue chambers on the microfluidic platform. Hydrostatic pressure was established at the inlet and outlet medium reservoirs to generate laminar flow along the microfluidic channels and interstitial flow across the tissue chambers. Cell culture medium was replaced every other day. After 5 days of culture, control-GFP and SlugOE-GFP VMOs were subjected to a vascular perfusion test. Briefly, 70 kDa Rhodamine B dextran (25 μ g/mL) was introduced to the medium reservoir with the highest pressure-head and allowed to perfuse through the vascular networks for 30 minutes. AngioTool was used to quantify and assess various characteristics of the networks. Each experiment was repeated with at least three different HUVEC lines.

Chapter 4. Molecular mechanism of Slug-mediated angiogenesis

4.1 A role for partial endothelial-mesenchymal transitions in Angiogenesis?

A role for partial endothelial-mesenchymal transitions in Angiogenesis?

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Abstract

The contribution of epithelial-to-mesenchymal transitions (EMT) in both developmental and pathological conditions has been widely recognized and studied. In a parallel process, governed by a similar set of signaling and transcription factors, endothelial-to-mesenchymal transitions (EndoMT) contribute to heart valve formation and the generation of cancer-associated fibroblasts. During angiogenic sprouting, endothelial cells express many of the same genes and break down basement membrane; however, they retain intercellular junctions and migrate as a connected train of cells rather than as individual cells. This has been termed a partial endothelial-to-mesenchymal transition. A key regulatory checkpoint determines whether cells undergo a full or a partial epithelial-to-mesenchymal transitions/endothelial-to-mesenchymal transition; however, very little is known about how this switch is controlled. Here we discuss these

developmental/pathological pathways, with a particular focus on their role in vascular biology.

Introduction

Morphological changes in tissues are invariably associated with phenotypic changes in the cells that comprise them. Often these are limited to temporary changes in protein expression patterns, but more dramatic changes can also occur, during which cells undergo changes in transcriptional programs that lead to significant changes in morphology and function. One class of such changes is called the epithelial-to-mesenchymal transition (EMT), and variants of traditional EMT include endothelial-to-mesenchymal transition (EndoMT) and partial EMT/EndoMT. Our focus will be to highlight the distinctions among the subsets, with an emphasis on angiogenesis as a unique example of a partial EndoMT.

Endothelial-to-Mesenchymal Transition

Endothelial cells (EC) have many epithelial characteristics, including strong apical–basal polarity, the ability to form tubes, and the potential to undergo a transition to a mesenchymal-like cell (EndoMT). There are many reasons, therefore, to think that this process is related to EMT and may thus share some of the same pathways and effectors, including the key transcription factors Snail, Slug, Twist, Zeb1, and Zeb2, which we describe in detail further. There have been several excellent reviews published on EMT^{72,73,166,177}, and so we focus on EndoMT, with reference to EMT where clear overlaps exist. During embryogenesis, subsets of EC in the developing heart undergo EndoMT,

acquire mesenchymal markers, invade the surrounding tissue, and form the valves and septa of the adult heart¹⁷⁸, a process that involves transforming growth factor- β (TGF β), bone morphogenetic protein, and Notch signaling pathways^{179,180}. These pathways converge on a complex network of transcription factors that includes HES, HEY1/2, Twist, and SOX9^{181,182}. Pathologically, EndoMT can be reactivated in the adult heart and has been shown to contribute to cardiac fibrosis, a characteristic common to most forms of heart failure. Using lineage-tracing techniques, Kalluri's group demonstrated that 27% to 35% of fibroblasts present in fibrotic heart tissue were of EC origin, strongly suggesting a role for EndoMT in this process. Importantly, EndoMT was TGF β 1-dependent, whereas bone morphogenetic protein-7 preserved the EC phenotype and consequently reduced fibrosis¹⁸³. Interestingly, however, a more recent study suggests that the accumulation of cardiac fibroblasts is not because of an EndoMT, but rather, the cells derive from a previously unrecognized fibroblast population, itself derived from ECs during development¹⁸⁴. EndoMT has also been implicated as a source of fibroblasts in hypertrophic cardiomyopathy¹⁸⁵, diabetes mellitus-induced cardiac fibrosis¹⁸⁶, and chronic pulmonary hypertension^{187,188}, although these studies lacked definitive lineage-tracing analyses.

There is also evidence supporting a role for EndoMT during both acute and chronic kidney injury¹⁸⁹. In three distinct mouse models of chronic kidney disease, ~ 30% to 50% of fibroblasts co-expressed the EC marker CD31 along with markers of myofibroblasts and fibroblasts, including fibroblast-specific protein-1 and α -smooth muscle actin. Lineage tracing experiments confirmed the EC origin of these cells¹⁸⁹. More recent work has suggested that only $\approx$ 10% of the myofibroblasts present in kidney fibrosis derive

from an EndoMT, whereas the remainder come from proliferation of local fibroblasts and differentiation from bone marrow cells¹⁶⁷. Other studies, however, have suggested that these fibroblasts may be derived from pericytes¹⁹⁰. It should be borne in mind, however, that these are all mouse studies and strain-specific differences are always a possibility. Other fibrotic diseases where EndoMT has been implicated as a source of fibroblasts/stromal cells include intestinal fibrosis¹⁹¹ and scleroderma^{192,193}.

In aggregate, these studies provide evidence that EndoMT likely provides a source of fibroblasts in both damaged heart and kidney (although the extent is unclear) and may function to facilitate tissue remodeling and fibrosis.

Finally, EndoMT also has a significant role to play in cancer. For example, Zeisberg et al¹⁹⁴, using 2 different mouse models of cancer, demonstrated that EndoMT accounts for $\leq 40\%$ of cancer-associated fibroblasts. A distinct population of fibroblasts co-expressed the EC marker CD31 along with either fibroblast-specific protein-1 or α -smooth muscle actin. Use of transgenic mice with irreversibly tagged EC revealed strikingly similar results—unique populations of fibroblasts co-expressing endothelial and mesenchymal markers. These data suggest that EndoMT is a significant source of cancer-associated fibroblasts in tumors. Remarkably, it has also been demonstrated that Twist overexpression in head and neck cancer cells can drive them into an EC phenotype¹⁹⁵.

Angiogenesis: A Partial EndoMT

When epithelial cells commit to a mesenchymal phenotype, the event is designated as a complete EMT. Partial EMT is also possible, and this occurs when ≥ 1 of the key

characteristics of complete EMT are not exhibited, such as loss of cell–cell contact. For example, during re-epithelialization of cutaneous wounds, keratinocytes undergo a series of changes reminiscent of EMT, including loss of polarity, rearrangement of the actin cytoskeleton, alterations in cell–cell contacts, and breakdown of basement membrane; however, these cells retain some intercellular junctions and migrate as a cohesive cell sheet¹⁹⁶. Similarly, during Madin–Darby canine kidney (MDCK) cell tubulogenesis, chains of epithelial cells migrate although again retaining intercellular junctions—a partial EMT driven by Slug activity¹⁰⁶. Angiogenesis, the formation of new blood vessels from the preexisting vasculature, is essential during development and many normal physiological processes, but is also important in numerous pathological processes, including tumor growth. Interestingly, comparison of angiogenesis and EMT reveals several similarities. Among these, the tip cells that lead emerging sprouts lack apical–basal polarity, degrade both basement membrane and extracellular matrix and, by definition, are migratory. However, angiogenic EC do not usually separate from their neighbors, suggesting that angiogenesis may involve a partial EndoMT^{96,164} (Fig. 4.1).

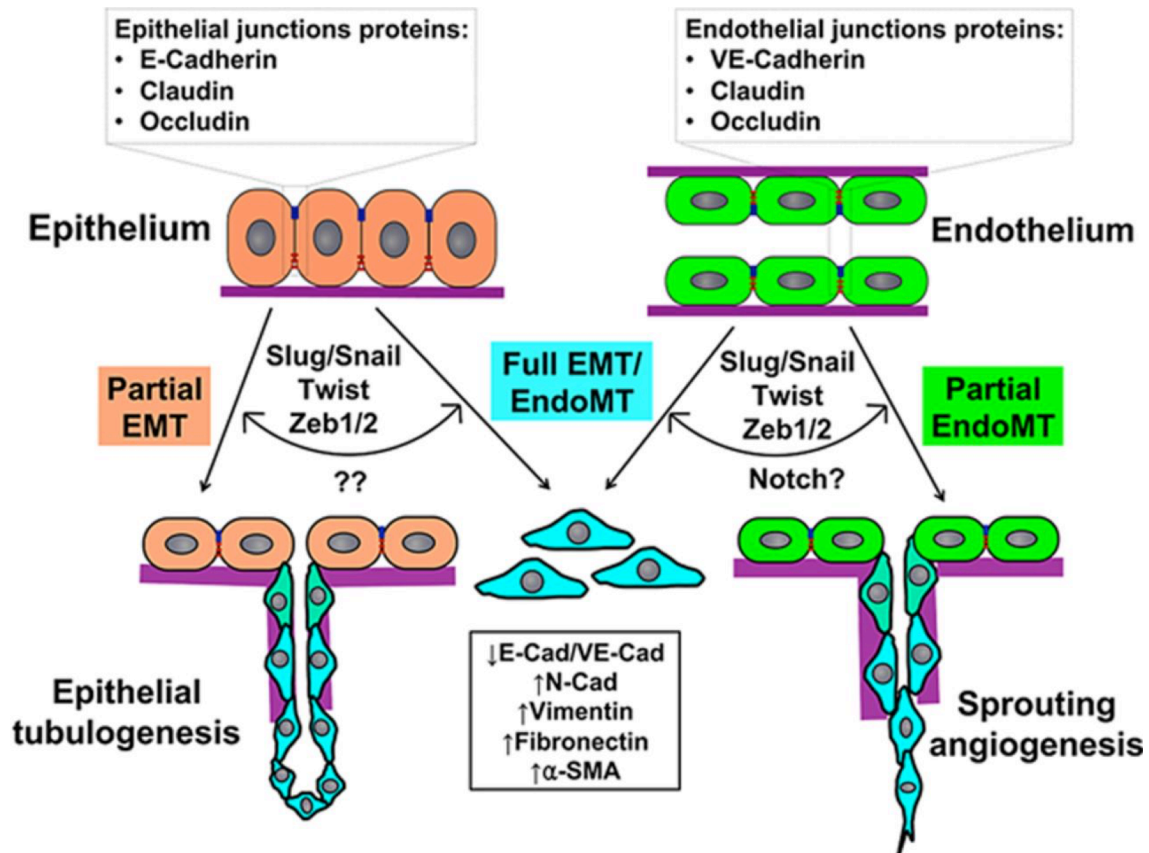


Figure 4.1. Complete vs partial epithelial-to-mesenchymal transition (EMT)/endothelial-to-mesenchymal transition (EndoMT). Epithelial and endothelial cells comprise the quiescent epithelium and endothelium, respectively, and use junctional proteins to maintain connections. Once transcriptional reprogramming is initiated, an event led by the EMT/EndoMT transcription factors Slug, Snail, Twist and Zeb1/2, the epithelial/endothelial cells lose apical-basal polarity, severe intercellular junctions, and become motile cells. However, the regulatory signal(s) that determines whether these cells undergo a complete EMT/EndoMT or partial EMT/EndoMT remains unclear. In the case of sprouting angiogenesis, the contact-dependent Notch signaling pathway may have a major role to play in this process. E-cadherin/E-cad indicates epithelial cadherin; N-cad, neural cadherin; α-SMA, α-smooth muscle actin; and VE-cadherin/VE-cad, vascular endothelial-cadherin.

Although much is known about the growth factors, receptors, and signaling pathways that govern angiogenesis, there is still much to learn about transcriptional changes that regulate each phase of angiogenesis, including sprouting. Our laboratory has recently published preliminary evidence demonstrating that the transcription factors Snail (*SNAIL* in human, *Snai1* in mouse) and Slug (*SNAIL2/Snai2*) are indeed expressed and regulated by angiogenic EC during in vitro angiogenesis⁹⁶. We demonstrated that inhibition of Snail or Slug expression results in a reduced ability of angiogenic EC to invade and migrate through multiple extracellular matrix environments. Importantly, lentiviral-mediated re-expression of membrane type-1 matrix metalloproteinase (MT1-MMP) rescued the inability of EC lacking Slug to migrate. This finding, therefore, suggests that membrane type-1-MMP is a critical downstream target of Slug during angiogenesis. Importantly, we and others have observed increased expression of Snail and Slug in the vasculature of colon, breast¹⁹⁷, and ovarian carcinoma¹⁹⁸. It is interesting to speculate that the same factors that drive epithelial cells toward a mesenchymal, prometastatic phenotype may also drive EC toward a proangiogenic phenotype, which is also associated with metastasis. Key factors here may include vascular endothelial growth factor (VEGF), TGF β , bone morphogenetic proteins, hepatocyte growth factor, and Wnts. The permanently activated phenotype of tumor vasculature may well reflect the chronic activation of the EndoMT process, driven by persistently elevated VEGF and a hypoxic environment, leading to excessive sprouting and a failure to settle back into the mature, stable phenotype associated with nontumor EC. It is possible therefore that drugs that target EMT/EndoMT, potentially through these pathways, may be doubly effective in slowing metastatic spread of epithelial tumors. Finally, we have preliminary data

suggesting that Slug deficiency in mice leads both to impaired developmental and to pathological angiogenesis (Welch-Reardon KM, Wu N, Hughes CCW, unpublished data, 2014). In aggregate, these data clearly point to a role for the Snail family of transcription factors during angiogenesis. Furthermore, the findings that cell–cell contact is retained and expression of vascular endothelial-cadherin is not reduced are reminiscent of the partial EMT seen during keratinocyte migration in wound closure and during mammary gland or kidney epithelial cell tubule formation¹⁹⁹. We therefore think that angiogenic sprouting may represent a partial EndoMT.

Signaling pathway Governing EndoMT

EndoMT and EMT share many of the same regulators, with members of the TGF β superfamily being arguably the most prominent players. TGF β signaling through Smad-dependent and independent pathways leads to direct transcriptional regulation of multiple genes, including several EMT/EndoMT-inducing transcription factors⁸¹. Expression of these transcription factors subsequently drives loss of cell–cell adhesion by repression of epithelial/endothelial genes encoding junction proteins, regulation of cytoskeletal rearrangement, and increased expression and activity of both MT-MMPs and secreted MMPs²⁰⁰. Moreover, during EndoMT, up-regulation of EC Slug by TGF β and other growth factors results in increased migration and invasion into extracellular matrices of diverse composition, and this is due in part to the indirect activation of membrane type-1-MMP, MMP-2, and MMP-9⁹⁶. Interestingly, nuclear Smads form multiprotein complexes with EMT/EndoMT-transcription factors, including Snail, Zeb1, and Zeb2, resulting in suppression or activation of promoters of epithelial (E-cadherin, Occludin, ZO-1) or

mesenchymal (Vimentin, N-cadherin) genes, respectively⁷³. TGF β can also activate Smad-independent pathways, such as MAPK/ERK/JNK (mitogen-activated protein kinases/extracellular signal-regulated kinases/c-Jun N-terminal kinases), all of which are implicated in EndoMT^{81,201,202}. Finally, a recent study has shown a requirement for PKC δ (protein kinase C) and c-Abl in mediating TGF β -induced EndoMT in mouse pulmonary EC²⁰³.

Aside from TGF β , several other signaling pathways associated with EMT have also been reported to regulate EndoMT. The relationship between canonical Wnt signaling and the onset of EMT and metastasis is well established in many cancer models. In human prostate cancer, the expression and nuclear activity of β -catenin correlates with the level of hypoxia-induced factor 1 α and hypoxia-induced factor 1 α -induced EMT²⁰⁴. The degree of hypoxia-induced EMT can also be enhanced by Wnt3a-induced activation of β -catenin in hepatic carcinoma²⁰⁵. Furthermore, it has been demonstrated that canonical Wnt signaling stabilizes Slug expression through regulating glycogen synthase kinase 3- β phosphorylation and β Tcrp-1-mediated ubiquitination, thereby inducing EMT in triple-negative breast cancer¹¹⁰. In contrast, the understanding of canonical Wnt signaling in EndoMT was mostly limited to developmental processes until recently. In an experimentally induced myocardial infarction model, Aisagbonhi et al, using lineage-tracing experiments, demonstrated that the canonical Wnt pathway is transiently activated in ECs, and this in turn leads to EndoMT²⁰⁶. More recently, the effect of Wnt7a and its antagonist Dkk-1 on EndoMT during arteriosclerosis was explored. In contrast to its effect on myofibroblasts, the activation of Wnt signaling through Wnt7b expression preserves the phenotype of ECs, whereas the expression of Dkk-1 promotes EndoMT²⁰⁷.

Fibroblast growth factor (FGF) has been proposed as a gatekeeper of partial EndoMTs through its regulation of the *let-7* miRNA, which normally acts to suppress TGF β -induced EndoMT²⁰⁸. When FGF signaling is reduced in a murine model of transplant arteriopathy, in this case by inflammatory signals that down-regulate the FGF receptor, *let-7*-mediated suppression of TGF β signaling is relieved and EC undergo an EndoMT, leading to intimal fibrosis. Given that the sprouty genes, which regulate FGF receptor signaling, have previously been implicated in the regulation of EC sprouting²⁰⁹, it is tempting to speculate that they may be acting as a rheostat to fine-tune FGF signaling²¹⁰ and thereby control whether EC undergo a partial or full EndoMT in response to TGF β signaling.

Notch activation is a well-known regulator of angiogenesis^{56,57,211,212} and is linked to both EMT and EndoMT events. The cleavage and nuclear translocation of the Notch intracellular domain can induce transcriptional alterations and hence a series of morphological and functional changes related to a mesenchymal transition²¹³. Notch can suppress (or activate) gene expression directly or through up-regulation of Snail and Slug in both epithelial cells and EC and, thus, initiate EMT and EndoMT in both developmental and pathological conditions^{80,214,215}. Notch ligands can also be induced by TGF β signaling to activate Notch receptors⁵⁰ and enhance EMT synergistically²¹⁶. Blockage of either Jagged-1, or its downstream signaling target Hey-1, can attenuate TGF β -induced EMT in mammary gland, kidney tubule, and epidermal epithelial cells^{215,217}. Notch and VEGF are both induced in the hypoxic tumor environment, and they work together to drive metastasis. On the one hand, interaction of Notch and hypoxia-induced factor pathways leads to increased stemness of cancer cells, self-

renewal ability, and a complete EMT^{215,218}. On the other hand, hypoxia-dependent induction of VEGF expression augments tumor angiogenesis, which provides increased opportunities for tumor cell intravasation. Finally, the crosstalk between Notch and VEGF pathways in the context of hypoxic tumors also promotes partial EndoMT in angiogenic tumor EC, leading to the formation of unstable, leaky vessels⁵⁰. Altered vessel integrity and permeability correlates with enhanced tumor cell dissemination to distant sites²¹⁹.

Notch-mediated EMT/EndoMT is unusual, and somewhat paradoxical, as it is contact-dependent. Importantly, the ability of cells to retain intercellular adhesion complexes although migrating as a group is crucial to tubulogenesis. As described earlier, processes involving tubulogenesis, such as angiogenesis and kidney tubule formation, both require a partial EMT/EndoMT, during which the participating cells temporarily lose apical-basal polarity and gain migratory capacity, but never fully acquire all mesenchymal phenotypes, nor completely lose cellular adhesion. Although other signaling pathways, such as TGF β , hepatocyte growth factor, and FGF, are capable of promoting this process, it is intriguing to speculate that Notch activation, perhaps in conjunction with sprouty, is a crucial determinant of a partial versus full EMT/EndoMT.

Aside from the major signaling pathways discussed earlier, miRNA, epigenetic regulation, and histone modification have also recently emerged as regulators of EMT^{199,200} and may also have a role in EndoMT²⁰⁸. These alterations control the expression level of the Snail/Slug, ZEB, and Twist families of transcription factors, and these in turn feed back to affect either the expression of the miRNA or histone-modifying

enzymes, or their activity^{199,200}. Clearly, the relationship(s) between the master regulators governing EMT/EndoMT are extremely complex^{73,81}.

Transcription Factor Interactions Governing EndoMT

Snail (*SNAIL* in human, *Snail* in mouse), Slug (*SNAIL2/Snai2*), Zeb1 (*ZEB1/Zeb1*), Zeb2 (*ZEB2/Zeb2*), and Twist (*TWIST1/Twist1*) have been identified as the key transcriptional regulators of EMTs and EndoMTs. A shared function of these proteins is their ability to repress the transcription of epithelial-cadherin (E-cadherin); however, numerous studies have demonstrated that they have overlapping but nonredundant roles in EMT and tumor progression. In human carcinomas, it is generally accepted that Snail plays a major role in inducing EMT, whereas Zeb1/2 and Twist are mainly involved in maintaining the invasive mesenchymal phenotype²⁰⁰. However, our recent study on EndoMT suggests that at least in the case of sprouting angiogenesis, Slug is the primary initiator of this process, whereas the induction of Snail occurs at a much later time⁹⁶. It is therefore unclear if each of these transcription factors has a distinct and specific role during EMT/EndoMT or if they rather act in symphony to promote a mesenchymal phenotype. Accumulating evidence from studies observing their expression patterns and their ability to regulate each other has begun to reveal a nonlinear map that suggests that these transcription factors mostly act in concert. For example, Snail can up-regulate Zeb1 and Zeb2 in oral squamous carcinoma and, at the same time, negatively regulate its own expression through direct promoter binding^{127,128}. Moreover, Slug indirectly up-regulates Snail through epithelial growth factor or hepatocyte growth factor signaling, thereby promoting mammary gland branching morphogenesis¹²⁹. Slug can also activate Zeb1 and

its own expression through direct transcriptional regulation^{130,131}. In addition, many have shown that Twist1 can regulate the expression level of Snail and Slug by either directly influencing transcription^{133,134} or through post-translational regulation via the NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells)/glycogen synthase kinase 3- β axis¹³⁵.

Dynamic Functions of EndoMT Transcriptional Regulators

The master regulators of EMT mediate repression of E-cadherin expression, and this is often described as the hallmark of EMT. However, several recent studies show that in both in vitro and in vivo models, EMT master regulators can induce EMT/EndoMT-like phenotypes in cells without complete loss of membrane E-cadherin—a partial EMT. Similarly, we observed that overexpression of Slug in EC promotes EC sprouting, a process suggestive of a partial EndoMT, without altering the mRNA levels or surface expression of vascular endothelial-cadherin, the EC equivalent of E-cadherin⁹⁶. Interestingly, Leroy et al others have shown that Slug up-regulation prevents apoptosis and promotes cell proliferation through p53¹⁰⁶, two processes associated with angiogenic sprouting. The induction of Twist alone, perhaps surprisingly, is sufficient to induce single cell dissemination/local invasion without the loss of epithelial identity, and moreover, E-cadherin expression is required for this process²²⁰. Conversely, the deletion of E-cadherin alone is not sufficient to induce EMT. Indeed, in the absence of E-cadherin, and despite a reduction in multiple classes of junction proteins, epithelial cells are still able to invade an extracellular matrix as a chain rather than single cells²²⁰. Collectively, these data suggest that master regulators of EMT and EndoMT serve more functions than simply acting as repressors of the epithelial/endothelial phenotype.

Remaining Questions and Perspectives

Our knowledge of the mechanisms underlying EMT and EndoMT is rapidly advancing; however, there are still several critical questions that have to be answered, including the following:

- Are Snail, Slug, Twist, Zeb1, and Zeb2 all required for an EndoMT?
- Do these genes work sequentially, in parallel or in feedback loops?
- What regulates expression of EndoMT-promoting transcription factors?
- What are the target genes for EndoMT transcription factors?
- What controls whether cells undergo a full or partial EndoMT?
- How is vascular endothelial-cadherin protected from downregulation in partial EndoMT?
- Are there fundamental differences between EMT and EndoMT or are the basic mechanisms identical?
- Do the same factors that promote EMT in cancer promote EndoMT in angiogenic EC?
- How does EndoMT contribute to progression of diseases, such as cancer, arteriosclerosis, and fibrosis?
- Are there fundamental differences between pathological EndoMT and developmental EndoMT?
- Can EndoMT be targeted therapeutically in cancer and other diseases involving pathological angiogenesis?

Answers to these questions have the potential to fundamentally affect how we target pathological angiogenesis.

4.2 SlugOE cells present an EndoMT-like gene expression profile

As described previously⁹⁶ and above, Slug misexpressing EC develop angiogenic tip cells sooner upon receiving pro-angiogenic stimuli and show an increase in sprouting. In an attempt to explore the underlying mechanism by which EC Slug regulates vascular formation, and to identify its downstream target genes during angiogenesis, we performed RNA-sequencing analysis on EC harvested from a fibrin 3D bead angiogenesis assay.

We compared gene expression profiles between GFP control and GFP-SlugOE EC at day two (D2) and six (D6) of the assay, which represents the initiation of sprouting and the establishment of lumenized vessels, respectively. EC from three different isolations were used for this experiment (Fig. 4.2A) to minimize donor variability, and each was processed separately. We analyzed gene expression and identified 1992 genes whose expression showed significant changes between the GFP control and GFP-SlugOE over the time-course (Fig. 4.2B). We performed K-means clustering on the 1992 differentially expressed genes and identified 10 clusters that showed dynamic expression profiles (Fig. 4.2A). Gene ontology (GO) analysis performed on genes from each cluster identified cluster-specific enrichment for various angiogenesis-related terms, including terms related to cell proliferation, migration, junction assembly and matrix protein regulation (Fig. 4.2C).

Several clusters are of particular interest. Cluster 8 (271 genes) includes genes whose expression level was drastically increased by Slug misexpression during the initiation of

sprouting (D2) but not after the sprouts were established (D6). The most enriched GO terms in this cluster are cell proliferation and cell cycle regulation (Fig. 4.2C), suggesting that during the early phase of sprouting angiogenesis Slug expression in EC primarily designates a proliferation program in these cells. Cluster 2 (427 genes) includes the largest number of genes whose expression decreases as the level of Slug increases across the four groups. As expected, the top-ranked GO terms for this cluster are Angiogenesis and epithelial-to-mesenchymal transition. Finally, cluster 6 (167 genes) includes genes whose expression level primarily depends on the stage of angiogenesis. Again, EMT is among the top-ranked GO terms in this cluster, supporting our hypothesis that an EndoMT-like process is required during angiogenesis.

A

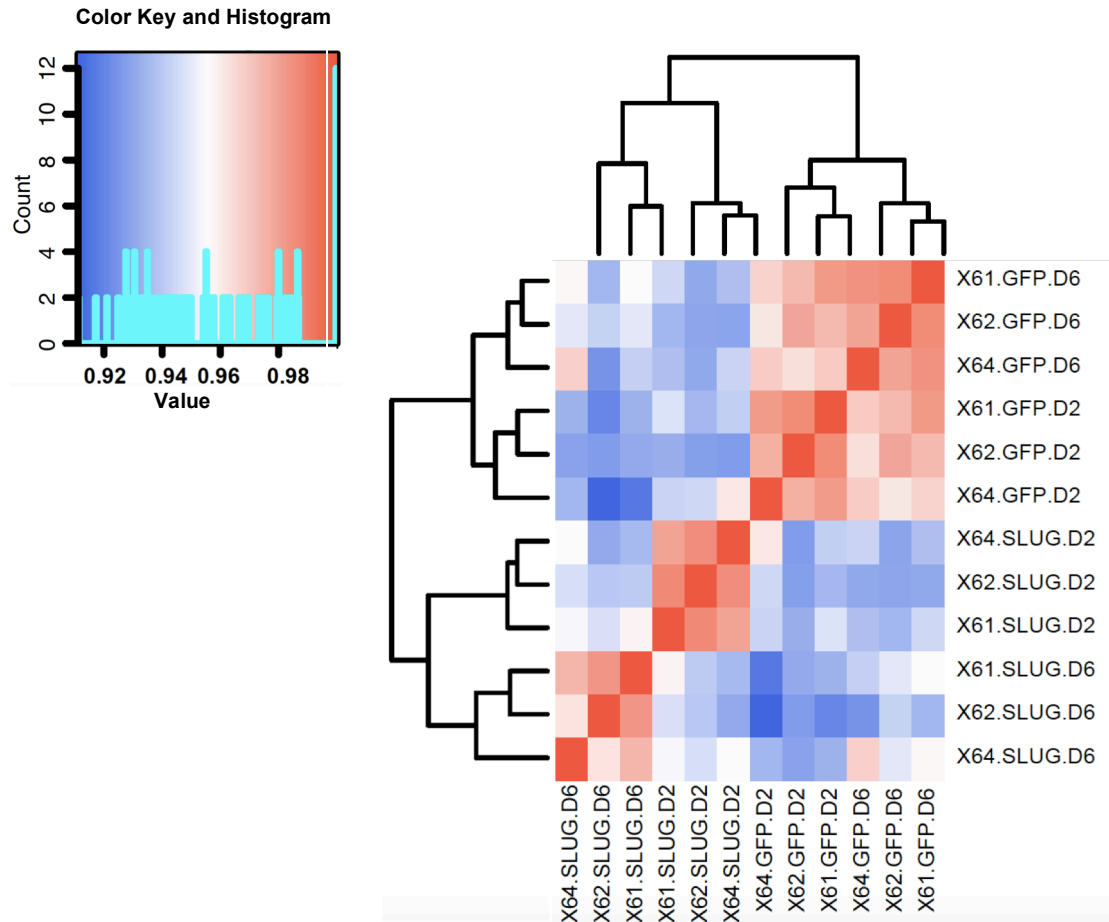
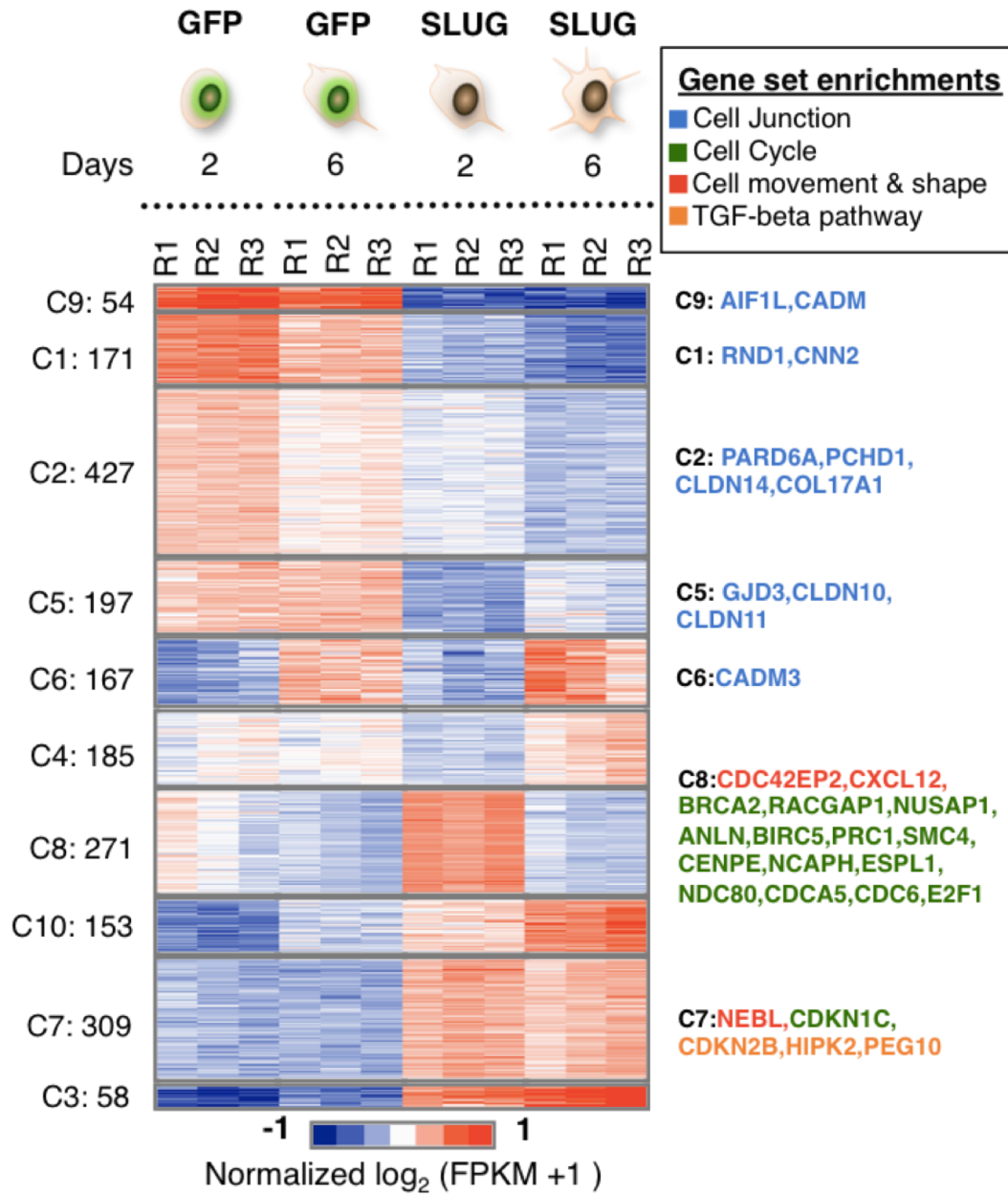
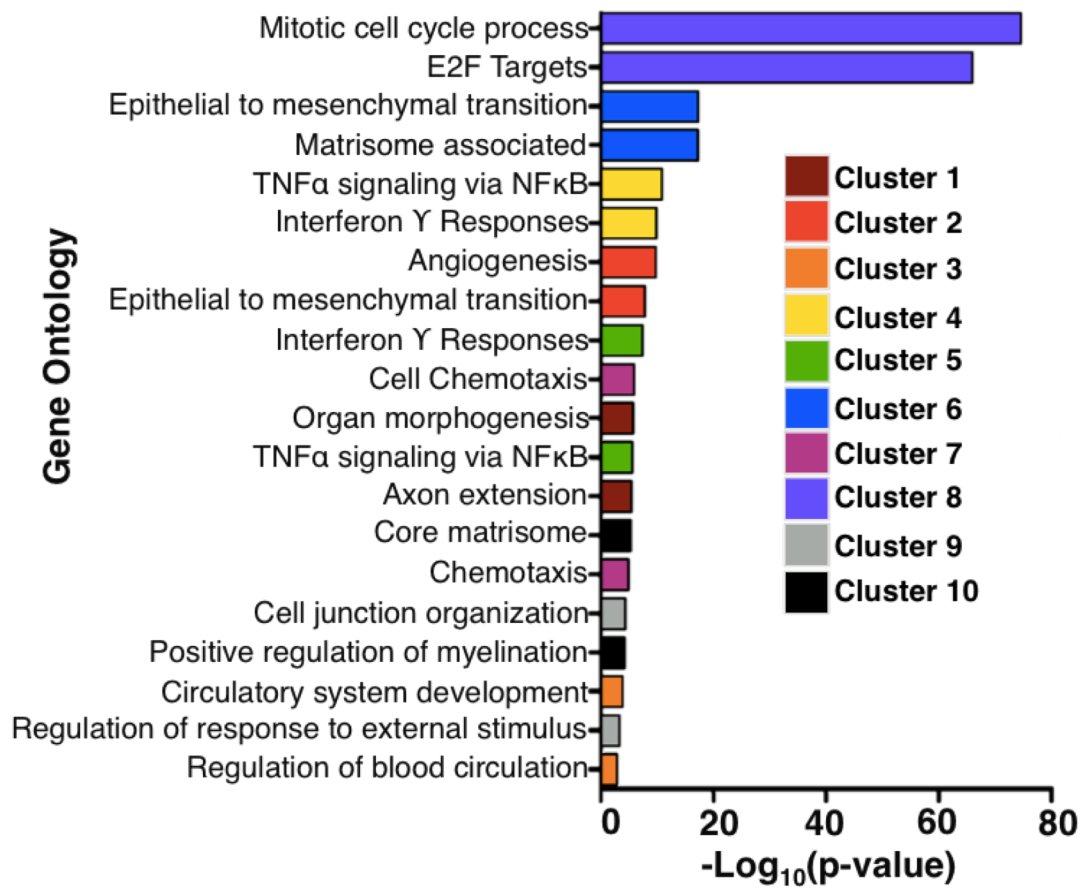


Figure 4.2: RNA-seq analysis reveals an EndoMT gene expression profile. A) RNA-seq analysis of gene expression in GFP control and SlugOE EC from day 2 and day 6 of the fibrin bead assay (n=3) showing high concordance among biological replicates. B) Left: Heat map for differentially expressed genes in GFP control and SlugOE EC on day 2 and day 6 of the fibrin 3D bead assay, grouped into 10 unique K-mean clusters. Right: Representative genes from each of the four main GSEA terms as shown in their respective K-mean clusters. C) Top two ranked Gene Ontology terms enriched in genes from each K-mean group.

B



C



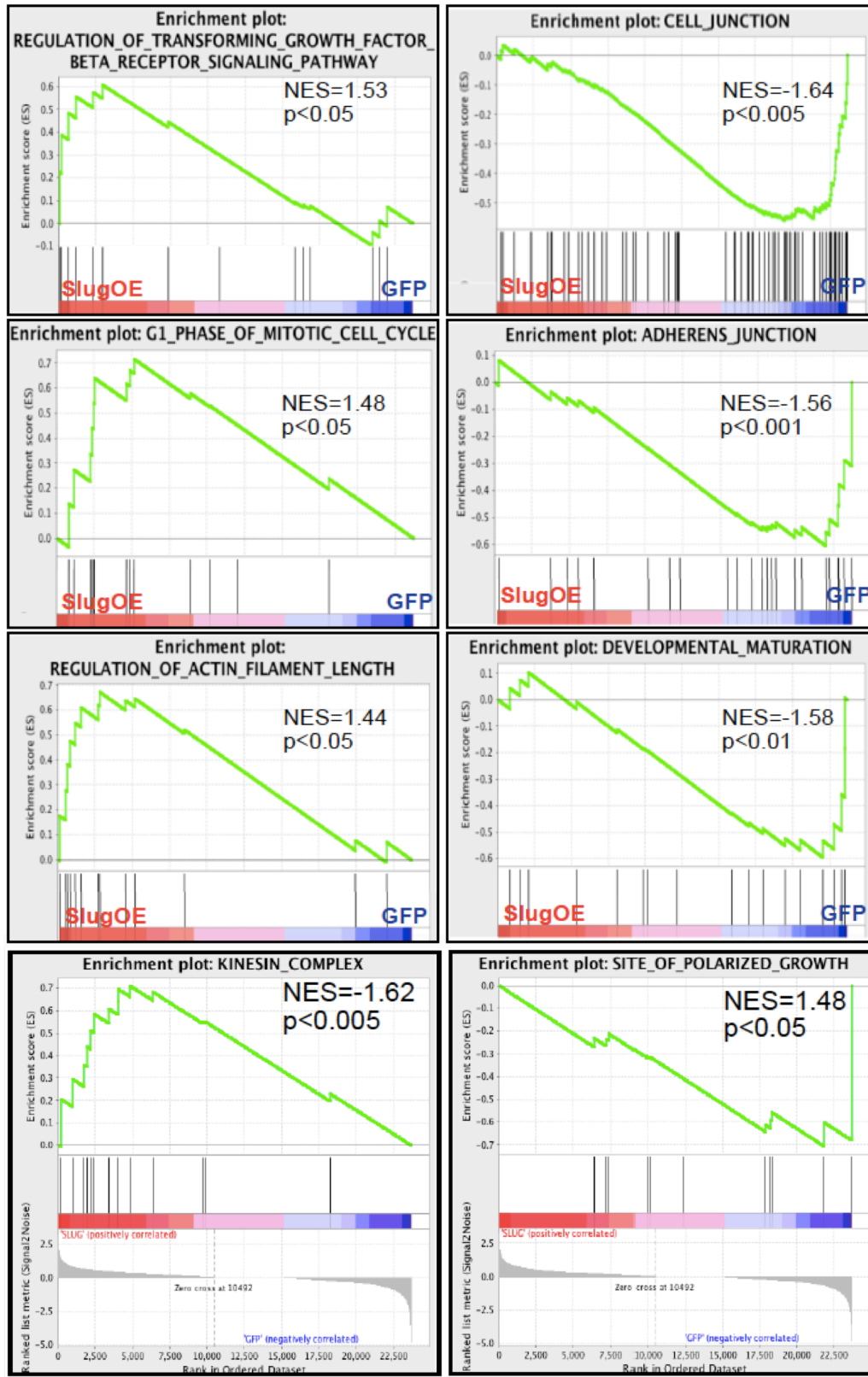
We also performed Gene Set Enrichment Analysis (GSEA) independently using all the genes expressed in our experiment. Consistent with the aforementioned analysis, SlugOE leads to up-regulation of genes that are enriched in the TGF β receptor pathway, regulation of cell cycle, shape and cell movement, whereas genes that are related to junction proteins and cell/developmental maturation and polarized growth were down-regulated (Fig. 4.3A). More importantly, several genes including SPARC, ACTC2, and FSCN1 that are known to be associated with EMT/EndoMT were up-regulated in SlugOE cells (Fig. 4.3B).

Since the loss of endothelial junctions is considered a hallmark of an EndoMT, we focused on this finding. To verify that the changes in gene transcription are also reflected at the protein level, we performed immunofluorescence staining and found a reduction in the surface expression of the endothelial junction proteins Claudin-5, VE-cadherin and Occludin (Fig. 4.3C). Interestingly, not all junction proteins were down-regulated at the transcriptional level (Fig. 4.3B). In order to test if the global loss of junction proteins has any impact on EC functions, we utilized our VMO platform to analyze vascular leak. We ran 70 kDa FITC Dextran through the networks formed by either GFP control or GFP-SlugOE EC for 30 minutes and analyzed extravascular immunofluorescence levels. While control vessels retained the marker we observed severe leakage throughout the networks formed by SlugOE EC (Fig. 4.3D), consistent with the loss of key junctional proteins.

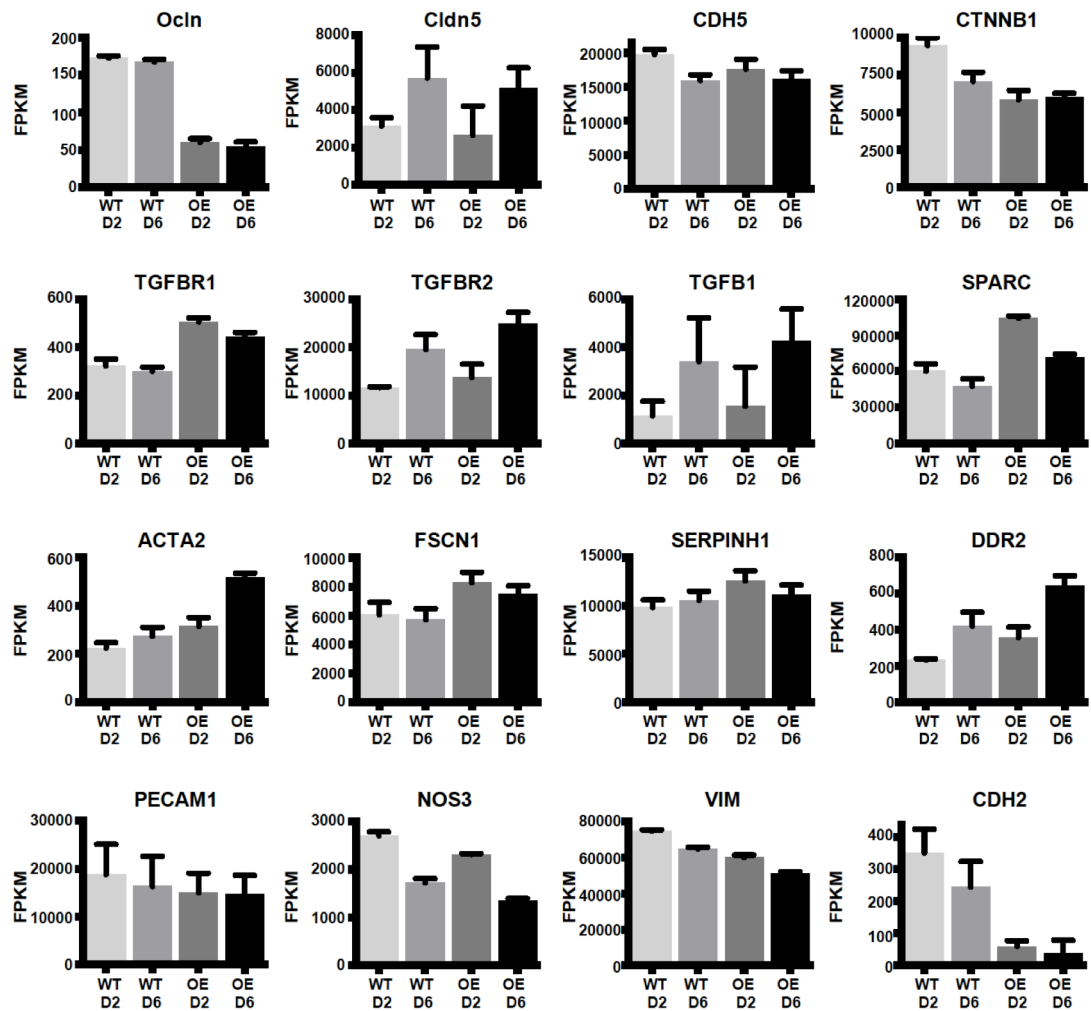
Interestingly, N-cadherin (N-cad) and Vimentin (Vim), two cell adhesion molecules that often increase in expression during EMT are down-regulated in the SlugOE cells. This might seem in contradiction to our other observations, however, unlike in epithelial cells, these two genes are both expressed and have important functions in normal EC^{232,233}. Therefore, their reduced expression in SlugOE further confirms the trend that SlugOE EC are losing their resting EC identity and normal EC function and undergoing an EndoMT.

Taken together, these data support a role for Slug in EC in regulating genes associated with an EndoMT, including those that control cell migration, proliferation and the formation of cell-cell junctions.

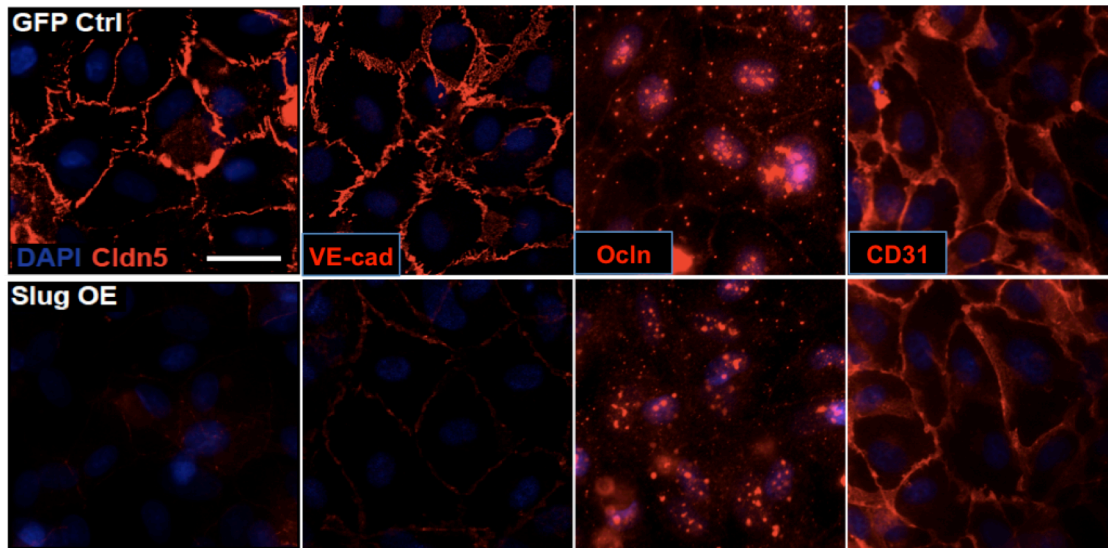
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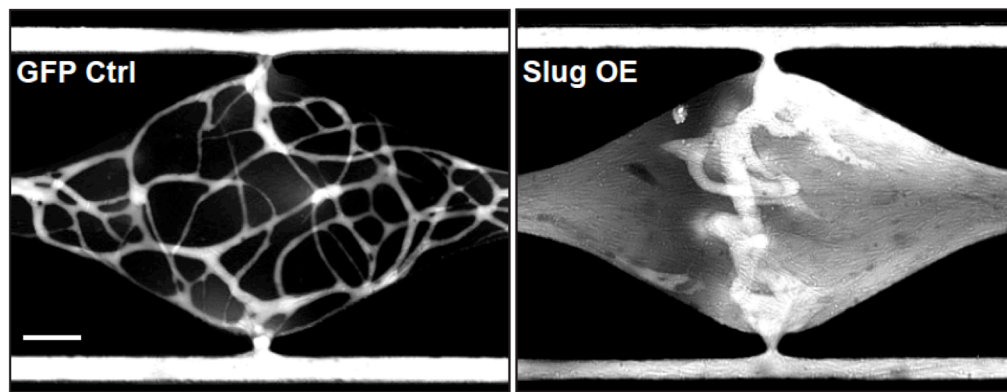


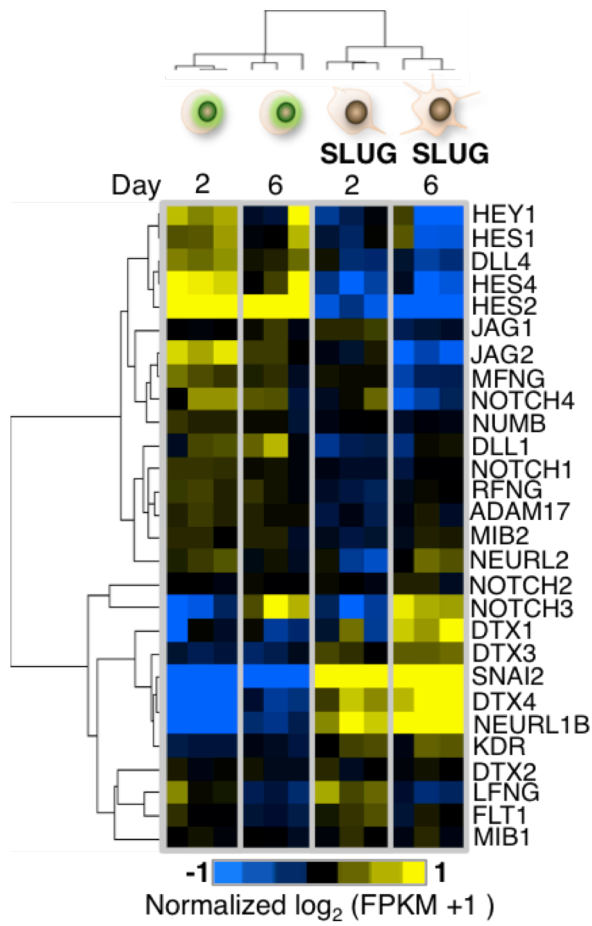
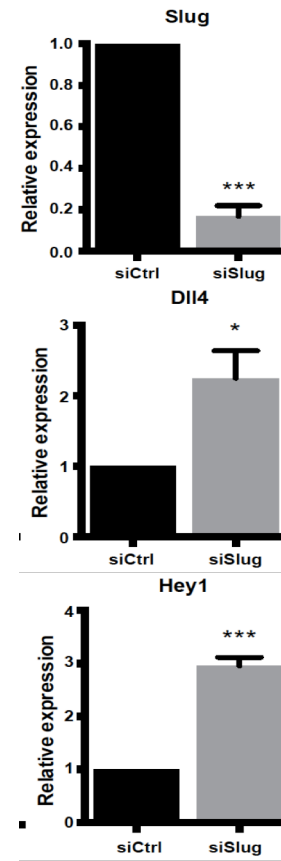
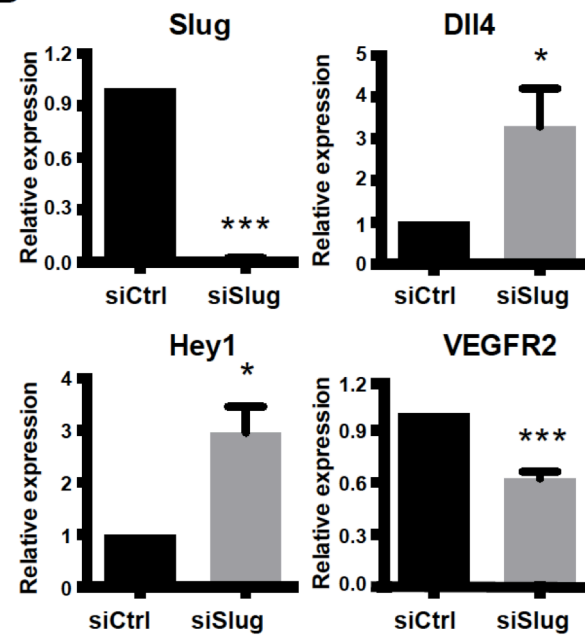
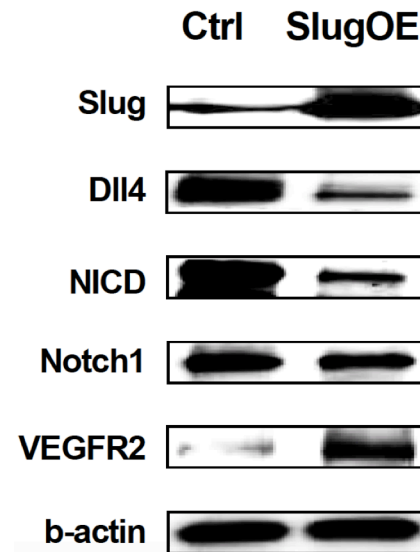
Figure 4.3: SlugOE vascular network has reduced endothelial junctions and compromised barrier functions. A) GSEA analysis showing enrichment in EMT-related terms up-regulated in SlugOE EC and down-regulated in GFP control EC. B) Average FPKM values of genes involved in cell-cell junctions, TGF β signaling, EMT and normal EC functions. C) Immunofluorescence staining showing reduced surface expression of Cldn5 and VE-cad, but not CD31 in SlugOE EC. Scale bar: 50 μ m D) 70KD Rhodamine-dextran perfusion of the vascularized-micro-organ model showing SlugOE network is leaky. Scale bar: 200 μ m.

4.3 Slug regulates Notch-mediated VEGFR2 expression during sprouting

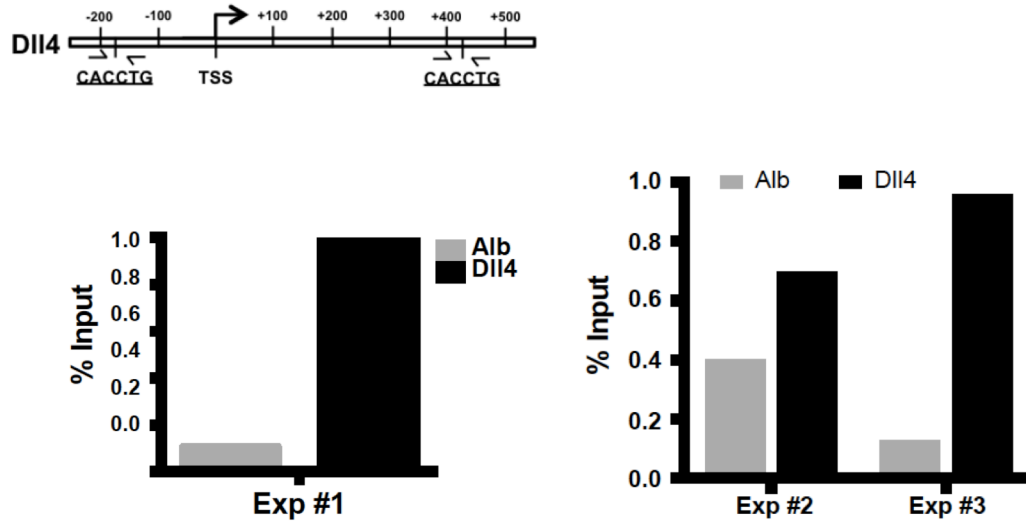
Notch signaling is among the most important pathways involved in vascular development. Our RNA-seq study identified changes in gene expression for several components of the Notch pathway, including reduced expression of the Notch ligand Dll4, and the EC-specific Notch target Hey1 (Fig. 4.4A). We validated our RNA-seq results by siRNA knockdown in SlugOE EC and observed Slug-dependent regulation of these genes, with strong rescue of both Dll4 and Hey1 expression (Fig. 4.4B). We also performed siRNA knockdown of the endogenous Slug in EC and observed similar results (Fig. 4.4C). In addition, to see if the changes in gene expression are reflected at the protein level, we performed western blot analysis on either GFP-control or SlugOE EC coated on the cytodex beads. The same number of cells from each condition were lysed directly into 2x SDS sample loading buffer and the proteins were immediately denatured by boiling at 95°C for 10 minutes. As expected, SlugOE EC expressed less Dll4 and Hey1 protein but had much higher VEGFR2 levels than the control cells (Fig. 4.4D). Importantly, we also detected reduced levels of the Notch intracellular domain (NICD) in SlugOE EC while total Notch1 protein level was not affected (Fig. 4.4D), confirming that Slug is indeed decreasing Notch signaling in EC.

Slug is known to modulate transcription by binding to the E-box sequences in the promoter region of its target genes, and there are two E-box sequences (CACCTG) within +/- 500 bp of the Dll4 transcription start site (TSS) (Fig. 4.4E). In order to test if Slug directly suppresses Dll4 transcription, we performed ChIP-qPCR with primers flanking the first E-box sequence 180bp upstream of the Dll4 TSS (Fig. 4.4E). Compared to the

control gene Albumin (ALB), we observed a significant enrichment for the Dll4 E-box binding site in the SlugOE EC (Fig. 4.4E). Slug also regulates a second Notch ligand Jag2 (Fig. 4.4A) and we found that the promoter of this gene was also directly bound by Slug (Fig. 4.4F). Therefore, we conclude that Slug regulates Notch signaling by direct suppression of its ligands.

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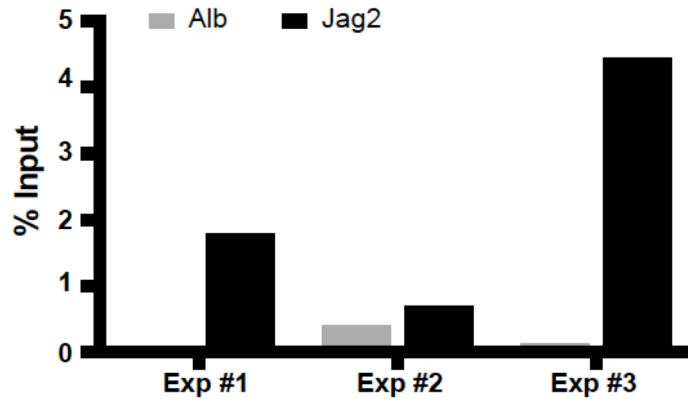


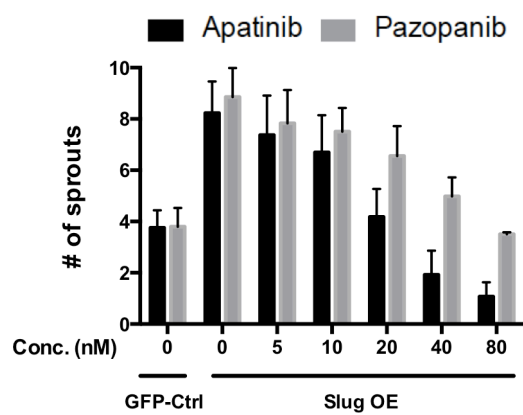
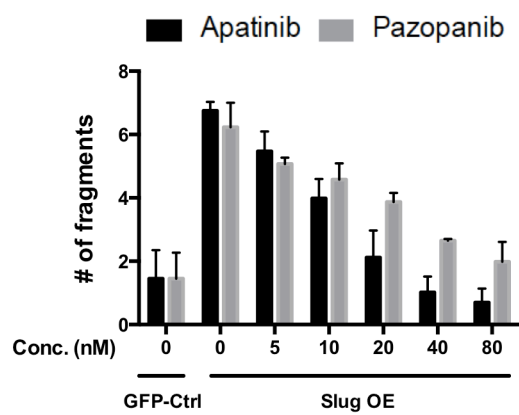
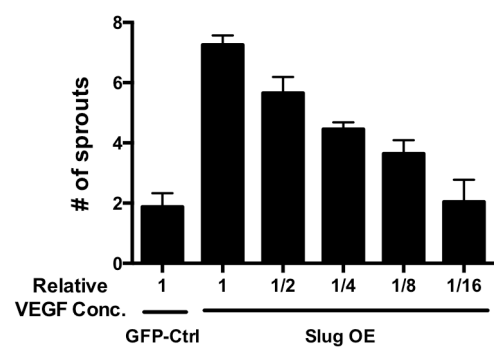
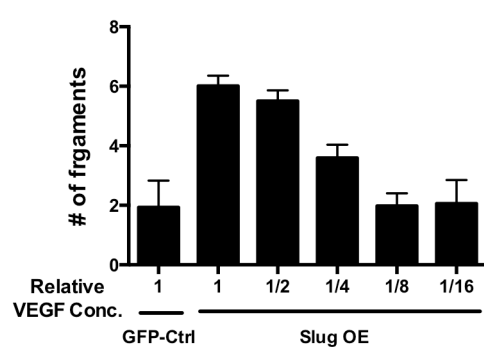
Figure 4.4: Slug regulates EC sprouting via Notch-mediated VEGFR2 expression. A) Heat map for differentially expressed Notch pathway components in GFP control and SlugOE EC on day 2 and day 6 of the fibrin 3D bead assay. B) qPCR analysis of Slug and its Notch related target genes in SlugOE cells when treated with siControl or siSlug. C) qPCR analysis of Slug and its Notch related target genes in control HUVEC when treated with siControl or siSlug. D) Western blot analysis showing Slug and its Notch related target genes. E) ChIP qPCR analysis showing enrichment of Slug binding to Dll4 promoter region in SlugOE EC in 3 separate experiments. F) ChIP qPCR analysis showing enrichment of Slug binding to Jag2 promoter region in SlugOE EC in 3 separate experiments. Data represent mean $\pm$ s.e. two-tailed unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

We and others have previously reported that increased Hey1 expression upon Notch activation represses the transcription of VEGFR2^{56,58,234}, thereby dampening the EC response to VEGF and decreasing sprouting^{56,211}. We would expect, therefore, that knockdown of Slug should, through up-regulation of Notch signaling, lead to suppression of VEGFR expression, and this was indeed the case (Fig. 4.4B). We further hypothesized that the hyper-sprouting phenotype exhibited by SlugOE cells might be due to overexpression of VEGFR2 and that this phenotype might be attenuated by moderate inhibition of receptor activity. To test this we used the fibrin bead assay and titrated-in the VEGFR2 inhibitors Apatinib and Pazopanib. As shown in Fig 4.5A, both VEGFR2 inhibitors were able to curb the Slug-driven hyper-sprouting phenotype, and the effect was dosage-dependent. Interestingly, the number of disconnected fragments also decreased as the VEGFR2 inhibitor concentration increased (Fig. 4.5B), suggesting that reduced VEGF signaling also attenuated the EndoMT-like phenotype in SlugOE EC. In order to rule out off-target effects of the drugs, we also titrated down the VEGF concentration in the media and again, completely corrected the hypersprouting and EndoMT phenotype in the SlugOE EC (Fig. 4.5C, D).

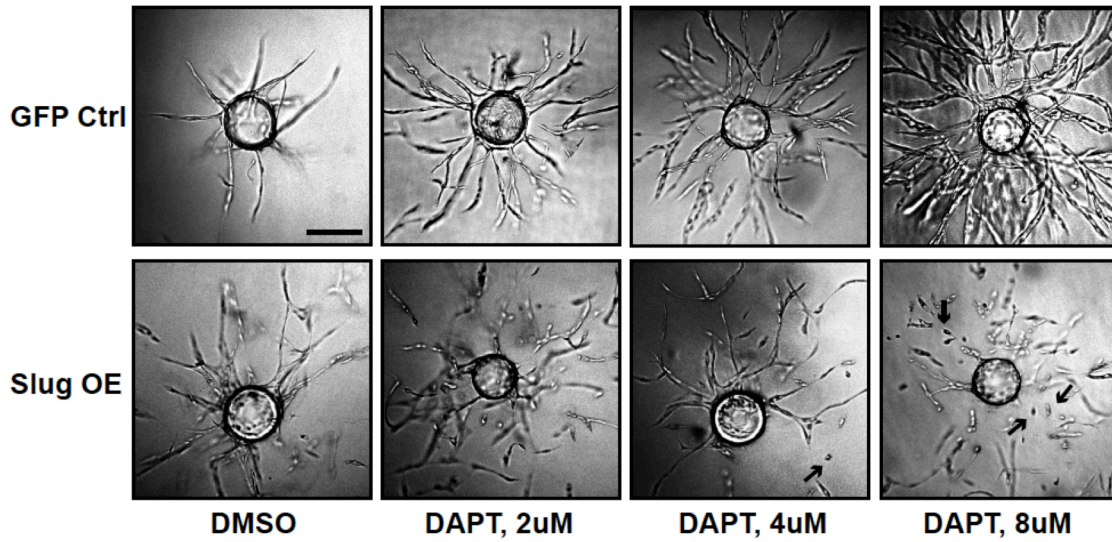
In order to test whether direct Notch inhibition would synergize with SlugOE we treated either control or SlugOE EC with DAPT, which is a γ -secretase inhibitor that blocks NICD cleavage and therefore the intracellular transduction of Notch signaling, and counted the number of disconnected fragments as an indicator of complete EndoMT. While increased DAPT concentrations induced hyper-sprouting of control cells, as previously reported^{58,234}, treatment of SlugOE cells resulted in the complete disassembly

of the sprouts into single cells (Fig. 4.5E). Quantification confirmed that the number of disconnected fragments increased in with DAPT treatment in a dose-dependent manner (Fig. 4.5F).

Together, these data confirm that Slug expression in EC promotes sprouting angiogenesis by modulating Notch signaling through transcriptional repression of Dll4, and increased expression of VEGFR2. Moreover, these data suggest that the loss of Notch signaling and subsequently the increased VEGF sensitivity contribute to the enhanced EndoMT phenotype we observe in SlugOE EC.

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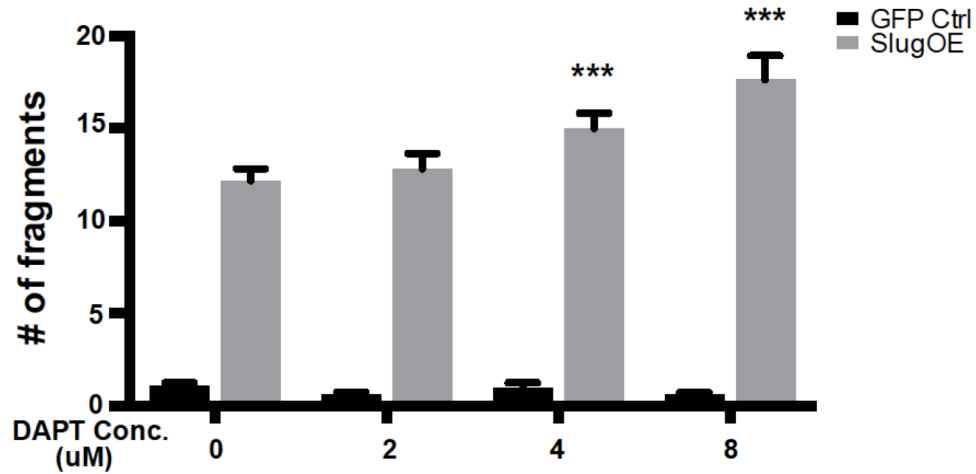


Figure 4.5: Inhibition of VEGF and Notch signaling have opposing effects on angiogenic defects caused by Slug overexpression. A). Number of sprouts per bead was quantified for GFP-control EC and SlugOE EC treated with either Apatinib or Pazopanib at different concentrations. B) Number of disconnected fragments around each bead was quantified for GFP-control EC and SlugOE EC treated with either Apatinib or Pazopanib at different concentrations. C) Number of sprouts per bead was quantified for GFP-control EC and SlugOE EC fed with media containing different levels of VEGF. D) Number of disconnected fragments around each bead was quantified for GFP-control EC and SlugOE EC fed with media containing different levels of VEGF. E) DAPT treatment in the fibrin 3D bead assay leads to increased fragmentation and higher level of EndoMT in SlugOE EC. Black arrow: individual EC that have lost polarity completely and appear round. Scale bar: 100µm F) Number of disconnected fragment in SlugOE EC increases in a does-dependent manner following DAPT treatment. Data represent mean $\pm$ s.e. two-tailed unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

4.4 Inhibition of Notch signaling rescues impaired retinal angiogenesis in Slug knockout mice

Given our *in vitro* findings, we wanted to test whether manipulating the Notch pathway *in vivo* could improve the Slug knockout phenotype in the mouse retina vasculature. Firstly, we examined whether the expression pattern of the key pathway components are altered in our knockout animals using immunofluorescent staining and confocal microscopy. Again, we harvested and dissected the retinas at P5 and stained for Dll4 and VEGFR2 expression. Consistent with previous studies, the WT controls showed Dll4 expression primarily in EC at the leading edge of the developing retina (Fig. 4.6A). However, in the SlugKO animals, we observed mis-expression of Dll4, with signal not only at the front, but also in the middle of the developing network (Fig. 4.6A). Moreover, we also observed a significant reduction in VEGFR2 expression in areas expressing Dll4, particularly in the EC of SlugKO retinas (Fig. 4.6B), suggesting that lack of VEGF signaling, specifically in the retinal EC, is responsible for the delayed retinal angiogenesis in SlugKO mice.

Next, to confirm that the down-regulation of VEGFR2, and therefore the impaired sprouting in the SlugKO mice, is due to overactive Notch signaling, we sought out ways to inhibit Notch signaling *in vivo*.

We first took a pharmacological approach by injecting these mice with DAPT, a Notch inhibitor as mentioned above. The experiment was performed as previously described⁵⁸.

Analysis of P5 retinas showed that DAPT treatment significantly increased network advancement in the SlugKO retinas to levels comparable to WT retinas (Fig. 4.6C). Therefore, inhibition of Notch signaling in the SlugKO animal was able to completely rescue the delayed retinal angiogenesis in the primary plexus. However, little rescue effect was observed in the secondary plexus of the P9 retinas (data not shown), suggesting that a different regulatory mechanism might be involved with the formation of the secondary plexus²³⁵.

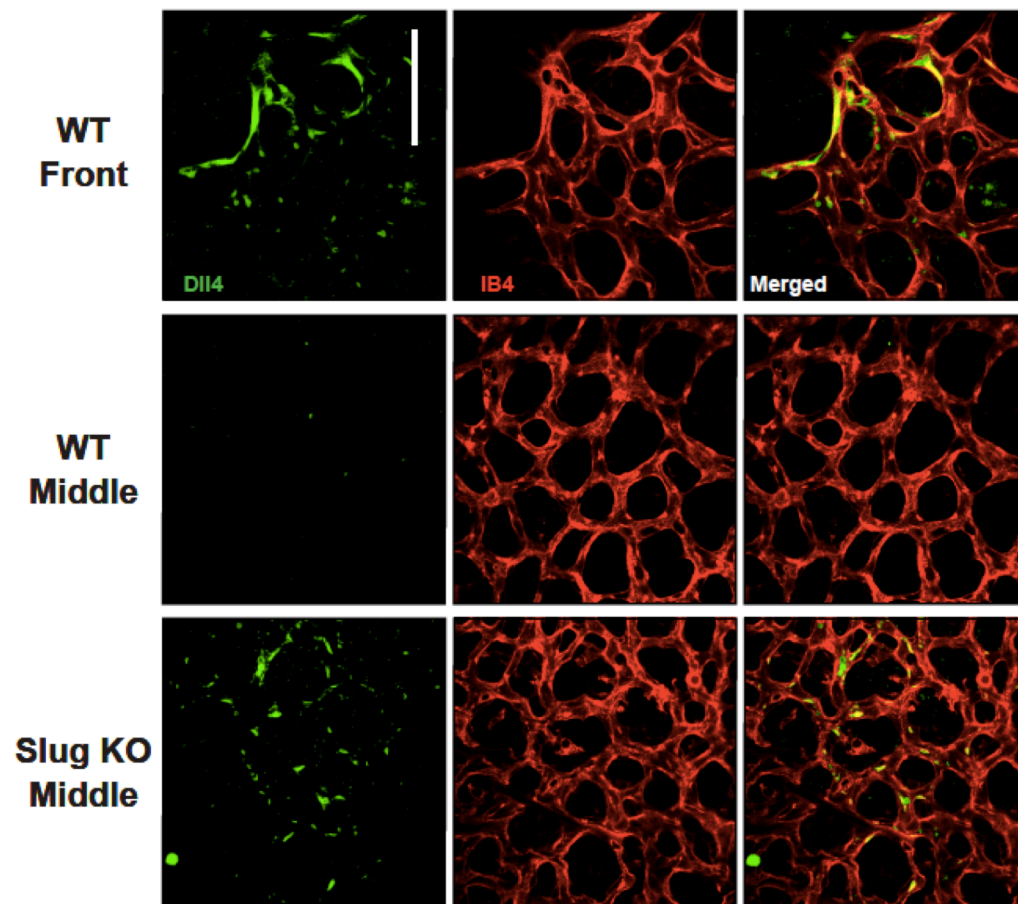
We wanted to confirm that Slug mediates Notch signaling during retinal angiogenesis by specifically regulating Dll4 expression, and also to rule out potential off-target effects by DAPT. Thus, we next took a genetic approach to reduce Dll4 expression in SlugKO mice using a transgenic mouse model.

Dll4 homozygous knockout is embryonic lethal in all inbred strains of mice tested, and Dll4 heterozygous knockout is also lethal in many inbred strains²³⁶. However, Dll4 heterozygous (Dll4Het) animals on an outbred background are viable with a strong vascular overgrowth phenotype in the retina. Consistent with our findings using DAPT, the partial loss of Dll4 on a SlugKO background (SlugKO/Dll4Het) almost completely restored the delayed vascular expansion (Fig. 4.6D). In addition, over-proliferation of EC and increased vascular density at the leading edge of the network was also noted in these animals (Fig. 4.6D).

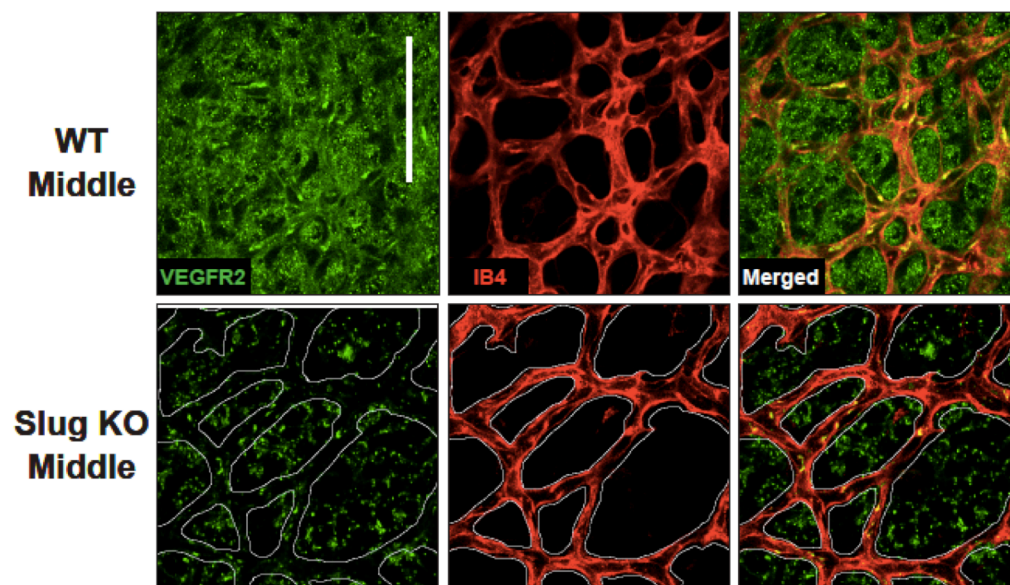
Taken together, these data support the notion that Slug regulates Notch signaling *in vivo* specifically through Dll4 suppression, and Notch signaling *in vivo* can rescue the impaired retinal angiogenesis seen in the SlugKO mice.

Figure 4.6: Notch inhibition rescues delayed vascular expansion in SlugKO mouse retina. A) Immunofluorescent staining showing localization of Dll4 expression at the angiogenic front and the middle of the network in WT and SlugKO mice. B) Immunofluorescent staining showing reduced VEGFR2 expression in SlugKO retinas comparing to the WT. C) IB4 staining of P5 retinas from WT and SlugKO retinas treated with DMSO control or DAPT. See quantitation on top right corner (n>5). D) IB4 staining of P5 retinas from WT, SlugKO and SlugKO/Dll4Het. See quantitation on top right corner (n>5). Scale bar: 100µm. Data represent mean ± s.e. two-tailed unpaired t-test. *p<0.05, **p<0.01, ***p<0.005.

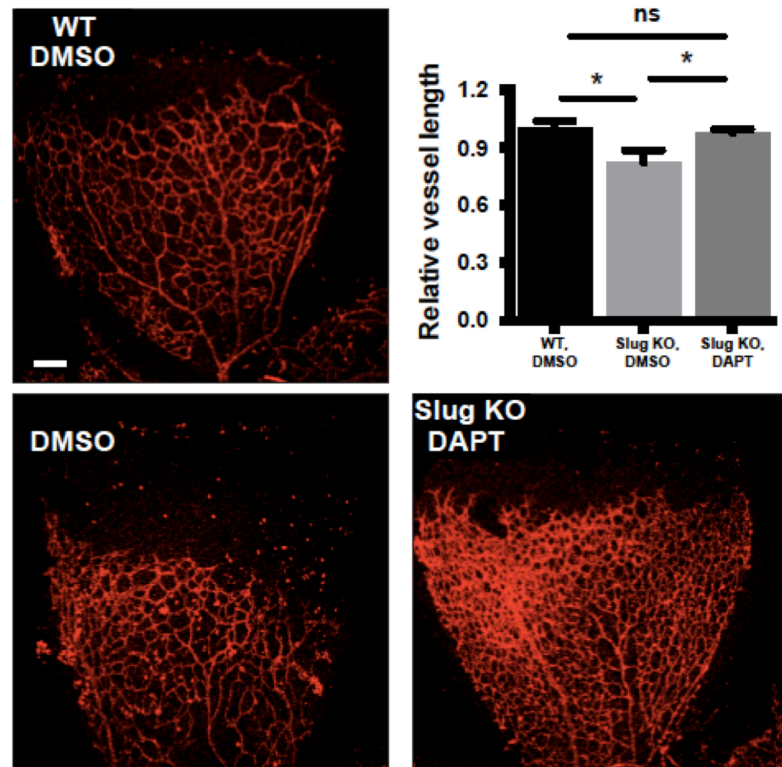
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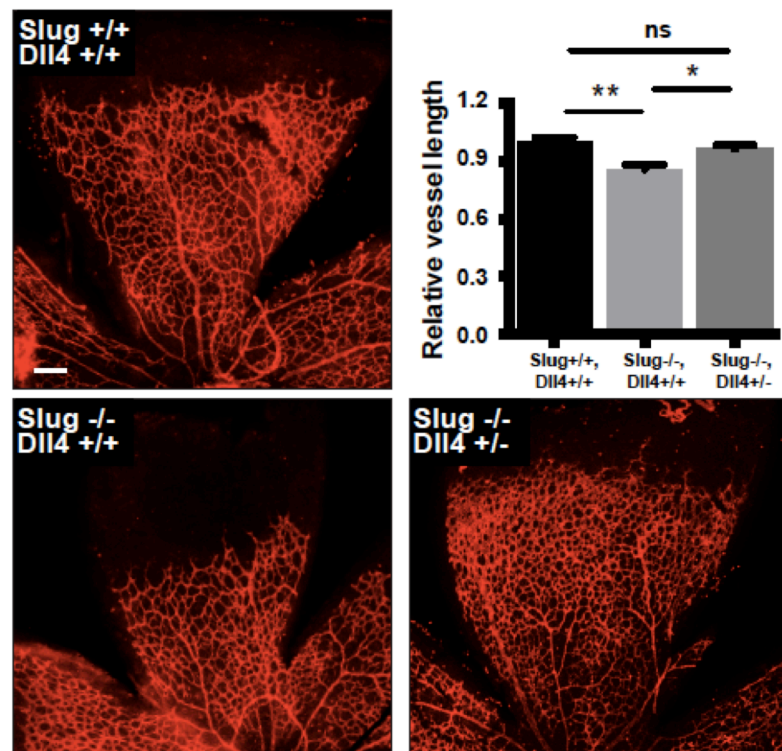
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B



4.5 Methods

RNA-sequencing and data analysis

The 3D Fibrin bead assay was performed using either Slug overexpressing (SlugOE) or GFP control HUVEC. The HUVEC were harvested at day 2 and day 6 post-embedding as described above. The HUVEC-coated Cytodex beads were further treated with collagenase IV to release the cells from the beads and filtered through a 75µm filter top tube to remove the beads. The cells were then lysed in RNA lysis buffer and incubated at 55°C with Protease K for 15 minutes. The RNA was then isolated following manufacturers protocol using a Qiagen RNA Mini Kit.

RNA integrity (RIN) was measured for all samples using the Bioanalyzer Agilent 2100 series. All sequencing libraries analyzed were generated from RNA samples measuring a RIN score ≥ 9 . The Illumina TruSeq mRNA stranded protocol was used to obtain poly-A mRNA from all samples. A total of 200ng of isolated mRNA was used to construct RNA-seq libraries respectively. RNA-seq libraries were quantified and normalized using the Library Quantification Kit from Kapa Biosystems and sequenced as paired-end 100 bp reads on the Illumina HiSeq 2500 platform.

Quality control was performed on data using FASTQC (v. 0.11.2)²²¹, and reads were trimmed using Trimmomatic (v.0.32)²²² with Illumina TruSeq adapter sequences using a PHRED quality score 15 and minimum length 20 bases. Trimmed reads were then aligned to the human hg19 reference genome using the Ensembl GRCh37 annotations

and post processed using Tophat2 (v.2.0.12)²²³, Bowtie2 (v.2.2.3)²²⁴ and Samtools (v.0.1.19)²²⁵. Expression level was quantified both with FPKM (Fragment per kilobase per million mapped reads) using Cufflinks (v. 2.1.1)²²⁶ and with raw counts using HTSeq (v.0.6.1p1.)²²⁷. Differential analysis was done using DESeq2²²⁸ and significant genes were considered (p-value < 0.05, 2 fold change). K-means clusterin was performed to extract dynamic gene clusters based on differential expression between GFP and SLUG knockdown time-points. Gene set enrichment analysis (GSEA)²²⁹ was performed on expressed genes to identify significant gene sets across sample comparisons and reported. Additional gene ontology analysis was performed for each cluster and reported using Metascape²³⁰. Clustering was performed in R and heatmaps were generated using JavaTree²³¹.

Chromatin Immunoprecipitation (ChIP)

The ChIP experiment was conducted following the protocol provided at <http://www.genomecenter.ucdavis.edu/farnham>. Briefly, HUVEC expressing either GFP or Slug-GFP were grown to confluency and cross-linked with formaldehyde in cold PBS for 10 minutes at room temperature. Cross-linking was stopped with glycine and cells were collected on ice, pelleted and snap froze in liquid nitrogen. Before sonication, pellets were thawed on ice, lysed in 5mM PIPES buffer with 85mM KCl, 0.5% NP-40 and Protease Inhibitor Cocktail (Roche) and passed through a 20-gauge needle repeatedly to break open cells. After centrifuging, supernatant was removed and RIPA buffer was added to lyse the nuclei. Chromatins were then sonicated on ice to obtain chromatin fragments that are around 300bp. To attach antibody to the magnetic beads, the sheep

anti-rabbit beads (Invitrogen 11204D) were first blocked with 5mg/mL BSA, then incubated with polyclonal rabbit anti-Slug antibody (Santa Cruz Biotch) on a rotator overnight at 4°C. Save 100uL chromatin as input control and use the rest to perform ChIP by adding the chromatin to the antibody-bound beads and incubating on a rotator overnight at 4°C. To reverse crosslinking, beads were washed thoroughly and incubated at 65°C for 5 hour with 1 minute of vortexing every 15 minutes. After centrifuging, supernatant was transferred to a new tube and incubated at 65°C overnight to complete reverse corss-linking. Both input control and ChIP'ed chromatins were purified with Qiagen PCR purification kit (Qiagen 28104) and concentration was determined using a Qubit Fluorometer.

VEGFR2 inhibition in bead assay

Either GFP-control or SlugOE EC were coated onto cytodex beads and embedded.

Apatinib and Pazopanib were titrated to specified concentrations. The inhibitor treatments started on day 2 after embedding, shortly after the initiation of sprouting, and medium with new inhibitor was changed every 2 days.

In vivo DAPT treatment

The DAPT treatment was performed as previously described⁸. Briefly, DAPT was prepared in DMSO and further diluted in corn oil for injection (100mg/kg per mouse, up to 20 ul per injection). For retinas that were harvested and analyzed at P5, subcutaneous injection was performed at P3 and P4, once daily. For retinas that were harvested and analyzed at P9, injection was performed at P3, P4, P7 and P8, once daily. Control wild

type and Slug knockout mice received DMSO/corn oil at the same ratio as the treatment group. The experiment was repeated three times with at least two mice per genotype per litter.

Dll4 heterozygous mouse breeding scheme

Since all of our mice are inbred, to minimize embryonic lethality when crossing with the Dll4 mutant mice, we first crossed the SlugKO animals with WT outbred stocks to create Slug heterozygous mice with a 50% outbred, 50% inbred background. We then intercrossed the F1 progenies to create the SlugKO-outbred (SlugKO-O) breeders. SlugKO-O mice were then crossed with Dll4Het and their Slug-heterozygous/Dll4-heterozygous (SlugHet/Dll4Het) F1 progenies were intercrossed to examine the rescue effect from the loss of Dll4 in Slug knockout mice. WT littermates of the respective backgrounds were used as controls in each experiment.

Chapter 5. Upstream regulation of Slug in the endothelial cells

5.1 Slug is an SDF1 α downstream effector during sprouting angiogenesis

SDF1 α (CXCL12) is known to regulate sprouting angiogenesis and vessel maturation as well as arterial specification³⁹. Moreover, elevated levels of SDF1 α have been reported in various tumors and are associated with augmented tumor angiogenesis⁴⁰. In our 3D fibrin bead angiogenesis assay, where the formation of lumenized sprouts strongly depends on fibroblast-secreted factors, SDF1 α was a soluble factor only present in the conditioned medium from highly angiogenic fibroblasts, but not from those that were less angiogenic. This suggests that SDF1 α is an important factor in our *in vitro* assay system and that we can use this assay to study SDF1 α 's mode of action. Despite being a well-known pro-angiogenic factor, the mechanism by which SDF1 α regulates vessel formation is not completely understood. We recently identified mTORC2 (but not mTORC1) as one of the critical downstream targets of CXCR4 signaling in angiogenesis²³⁷. We have also shown that SDF1 α can induce Slug expression in EC⁹⁶. Therefore, we wanted to see if SDF1 α -mediated Slug activation in EC is important for sprouting angiogenesis.

When we treated EC coated on the cytodex beads and embedded in the fibrin gel with SDF1 α (100ng/mL) for three days, we saw an early and increased formation of tip cells (Fig. 5.1A). Concurrently, we observed an increase in Slug, but not Snail transcription in these cells (Fig. 5.1B). Interestingly, this activation of Slug transcription cannot be readily observed in quiescent HUVEC grown in monolayer on gelatin (data not shown).

To determine whether Slug activation plays a role in its induction of sprouting angiogenesis by SDF1 α , we utilized a mouse retinal explant model. The explants were prepared as previously described with some modifications¹⁴³. We found that while SDF1 α treatment led to increased sprouting in the WT explants, this effect was abolished in the SlugKO explants (Fig. 5.1C, D). These data suggest that SDF1 α induces angiogenesis by regulating Slug expression.

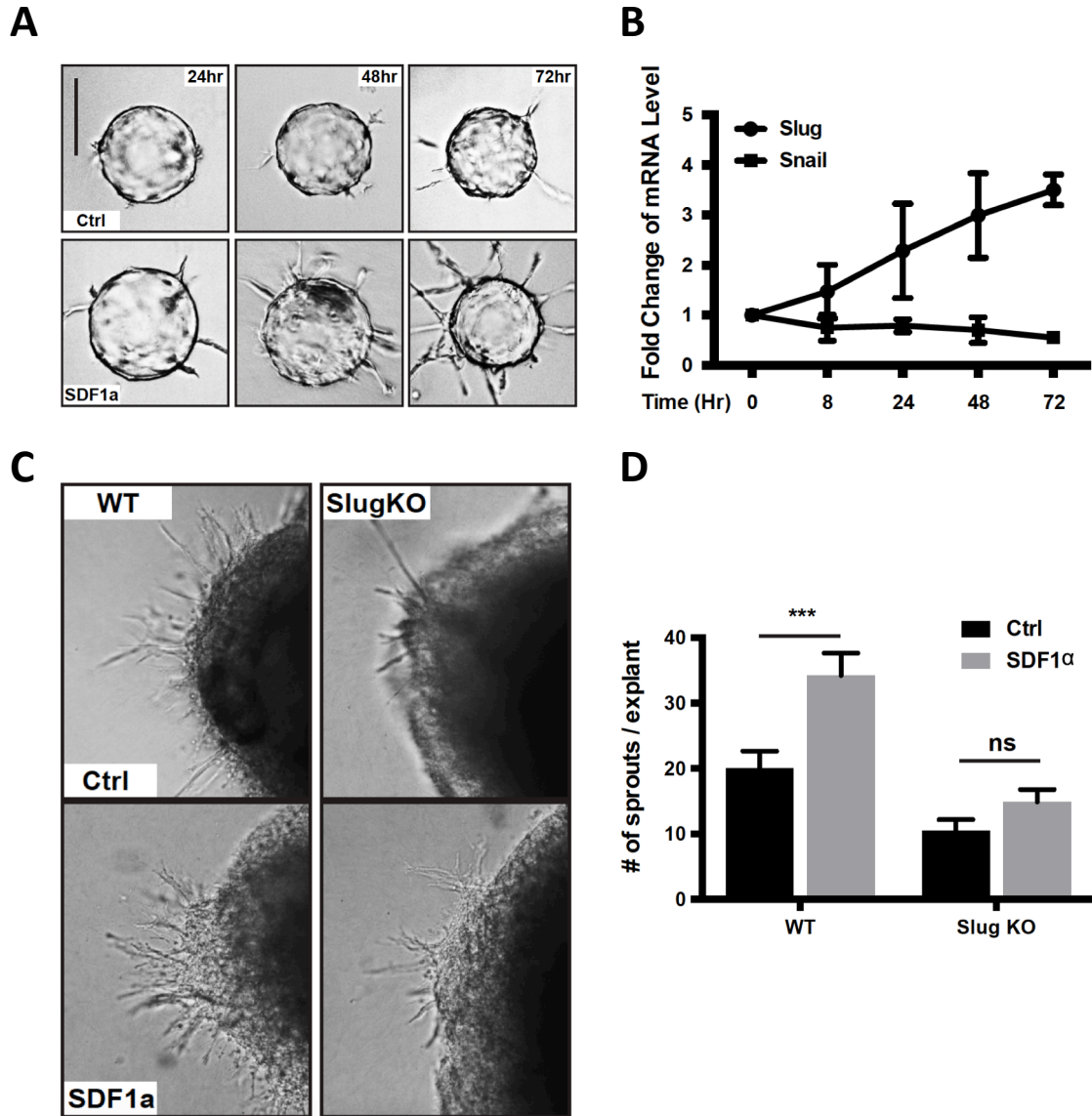


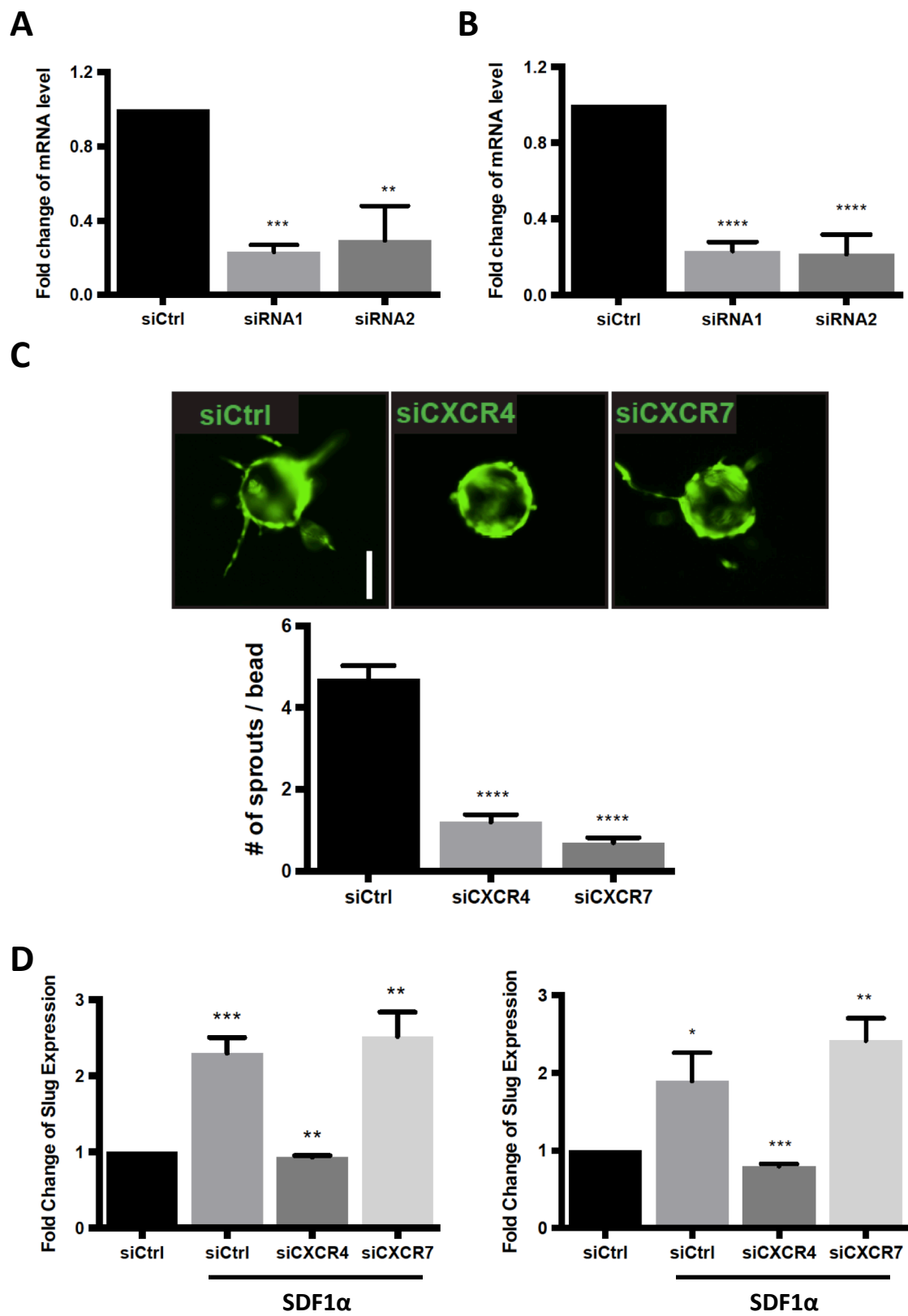
Figure 5.1: SDF1 α promotes sprouting angiogenesis by activating Slug transcription. A) SDF1 α treatment leads to early and increased tip cell formation and sprouting. B) SDF1 α treatment leads to activation of Slug but not Snail transcription. C) SDF1 α induces increased sprouting in WT but not SlugKO mouse retina explants. D) Number of sprouting per retina explant is quantified. Scale bar: 100 μ m. Data represent mean $\pm$ s.e. two-tailed unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

SDF1 α has two known receptors in EC, CXCR4 and CXCR7⁴¹. Although CXCR4 is thought to be the canonical receptor, some recent studies have suggested a role for CXCR7 in tumor angiogenesis^{41,238}. In order to determine which receptor is used by SDF1 α to induce Slug expression, we knocked down each of the receptors in EC using respective siRNA and then checked for Slug induction upon SDF1 α treatment. We used 2 individual siRNAs against each of the receptors and observed over 70% reduction for all of them on day 4 post-transfection (Fig. 5.2A, B). Consistent with previous reports^{39,239}, we saw inhibition of sprouting when either of the receptors was knocked down (Fig. 5.2C). However, only when we knocked down CXCR4 did we observe complete blockage of Slug induction, while the knockdown of CXCR7 had little effect (Fig. 5.2D), implying a different mechanism of SDF1 α -CXCR7-mediated sprouting angiogenesis. Furthermore, when the bead assay was treated with AMD3100, a small molecule inhibitor for CXCR4, starting on day 2, we observed a similar reduction in sprouting (Fig. 5.2E). Finally, when we conducted the same experiment using SlugOE EC, we saw a significant level of rescue of the CXCR4-inhibited cells and normalization of sprouting (Fig. 5.2E, F). Taken together, these data suggest that SDF1 α signals through CXCR4 to induce Slug transcription in EC and promotes sprouting angiogenesis.

Next, we wanted to see how the SDF1 α -CXCR4 signal is transduced into the nucleus to activate Slug transcription. It is known that SDF1 α -CXCR4 binding can activate ERK phosphorylation³⁹. Indeed, we observed increased phosphorylation for both ERK1/2 and ERK5, which can be blocked by the CXCR4 inhibitor AMD3100 (Fig. 5.2G). However, only when ERK5 phosphorylation was inhibited by XMD8-92, a specific ERK5 inhibitor

(Fig. 5.2G), did we lose Slug induction by SDF1 α (Fig. 5.2H). This was not seen with ERK1/2 inhibition (data not shown). Furthermore, knockdown of CXCR4, but not CXCR7 largely blocked ERK5 phosphorylation (Fig. 17I).

Together, these data indicate that SDF1 α -CXCR4 binding leads to the phosphorylation of ERK5, which in turn activates Slug transcription and its downstream angiogenic programs.



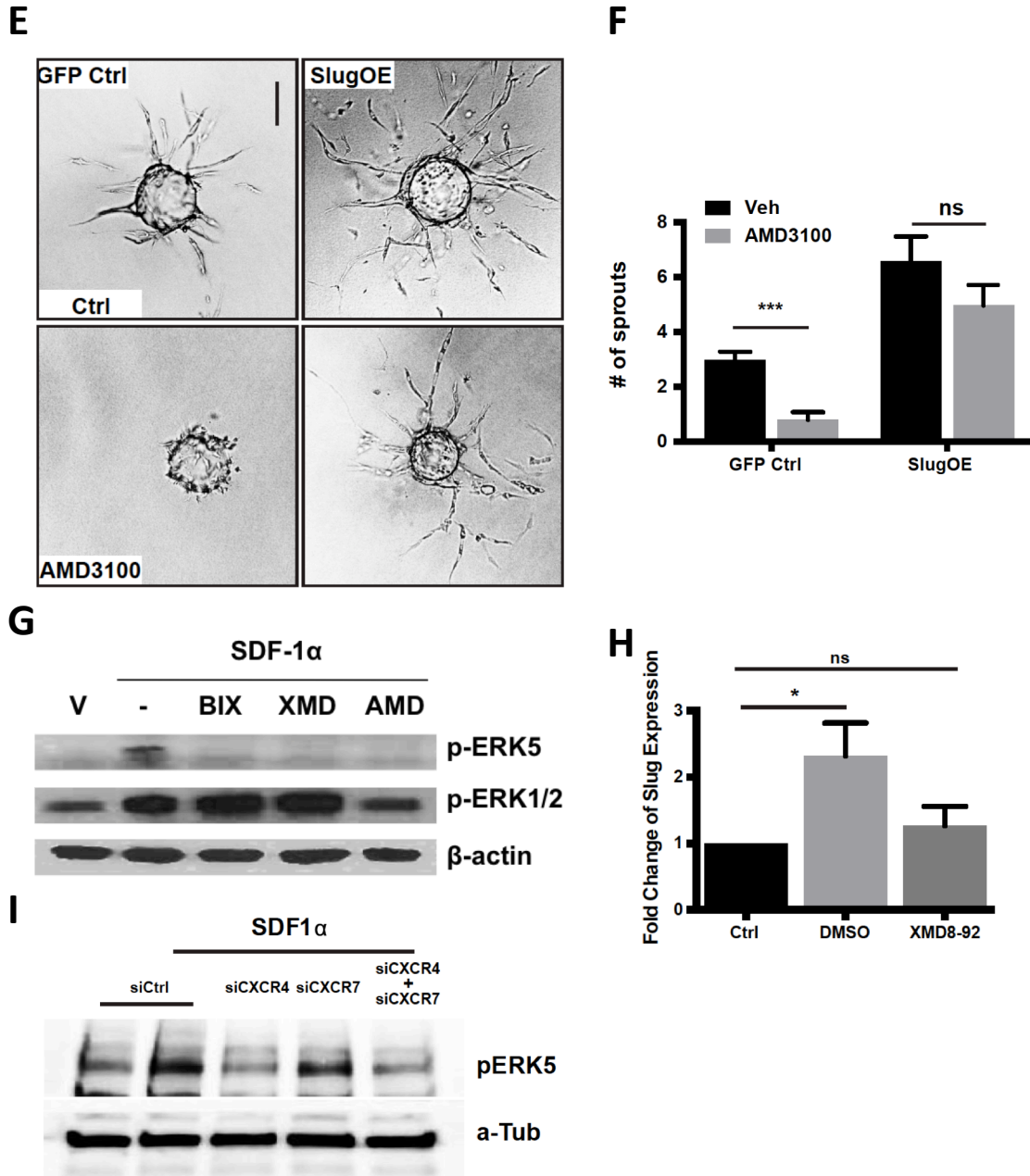


Figure 5.2: SDF1 α induces Slug transcription sprouting by signaling through CXCR4 and ERK5. A) qPCR analysis showing CXCR4 knockdown using two individual siRNAs. B) qPCR analysis showing CXCR7 knockdown using two individual siRNAs. C) Top: knockdown of either CXCR4 or CXCR7 leads to reduced sprouting in the fibrin 3D bead assay. Image taken on day 5 of the assay. Bottom: Average number of sprouts per bead with siControl, siCXCR4 and siCXCR7 EC. D) qPCR analysis of Slug induction by SDF1 α in EC treated with two separate sets of siRNA for CXCR4 or CXCR7. E) AMD3100 inhibition of sprouting can be rescued by Slug overexpression in EC. F) Number of sprout per bead is quantified. G) SDF1 α activation of ERK5 phosphorylation and it can be blocked by MEK5, ERK5 or CXCR4 inhibition. BIX: MEK5 inhibitor, XMD: ERK5 inhibitor, AMD: CXCR4 inhibitor. H) SDF1 α induction of Slug can be blocked by ERK5 inhibitor XMD8-92. I) SDF1 α activation of ERK5 phosphorylation is abolished by CXCR4 knockdown. Scale bar: 100 μ m. Data represent mean $\pm$ s.e. two-tailed unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

5.2 HGF induces transient Slug expression in EC

We have previously shown that HGF is secreted by angiogenic fibroblasts and is essential for sprouting *in vitro*⁴⁴. Moreover, HGF-induced Slug expression in epithelial cells is important for the partial EMT phase of Madin-Darby canine kidney (MDCK) cell tubulogenesis¹⁰⁶. We therefore speculated that HGF might be a key factor for Slug induction in angiogenic ECs.

We grew EC in monolayer, treated them with HGF and harvested RNA and protein at various time points. We observed peak Slug transcription activation 1 hour after the HGF treatment (Fig. 5.3A), which corresponds to 2.5 hours at the protein level (Fig. 5.3B). However, this induction was only transient and by 24 hours, the amount of Slug RNA and protein had dropped back to the basal level. This is consistent with previous results showing that HGF induction of Slug at 24 hours is minimal⁹⁶. Since the EC in bead assays are exposed to numerous angiogenic stimuli, these data suggest co-stimulation with other factors might be necessary for stable induction of Slug.

Next, the effect of HGF treatment on HUVEC migration was assessed with a 2D scratch assay. Interestingly, we only observed increased cell migration (accelerated wound closure) upon HGF treatment with cells growing on collagen-coated plates, but not gelatin or fibronectin (Fig. 5.3C). This result was confirmed in a 3D collagen invasion assay. HGF treatment led to an increased number of cells invading into the Col I gel (Fig. 5.3D). These data imply that collagen-specific integrin or MMPs might be activated by

HGF that are involved in EC migration.

Interestingly, the RNA-seq data indicated that SlugOE EC expressed much higher level of HGF compared to the control cells both at the initiation (day 2) and maturation of sprouts (day 6). Meanwhile, the expression level of MET, the receptor for HGF, does not seem to be controlled by Slug, but is rather associated with the early stage of angiogenesis (Fig. 5.3E). These data suggest a potential feedback loop between HGF signaling and Slug expression during sprouting.

In summary, these data suggest that Slug can be induced in EC by various growth factors and cytokines. However, unlike in epithelial cells, stable Slug induction in EC appears to be dependent on a combination of signals including co-stimulation of multiple signaling pathways and the presence of specific extracellular matrixes such as Collagen I, all of which can be found in a pathological environment such as a fast developing tumor. This further supports a role for Slug specifically in regulating pathological angiogenesis.

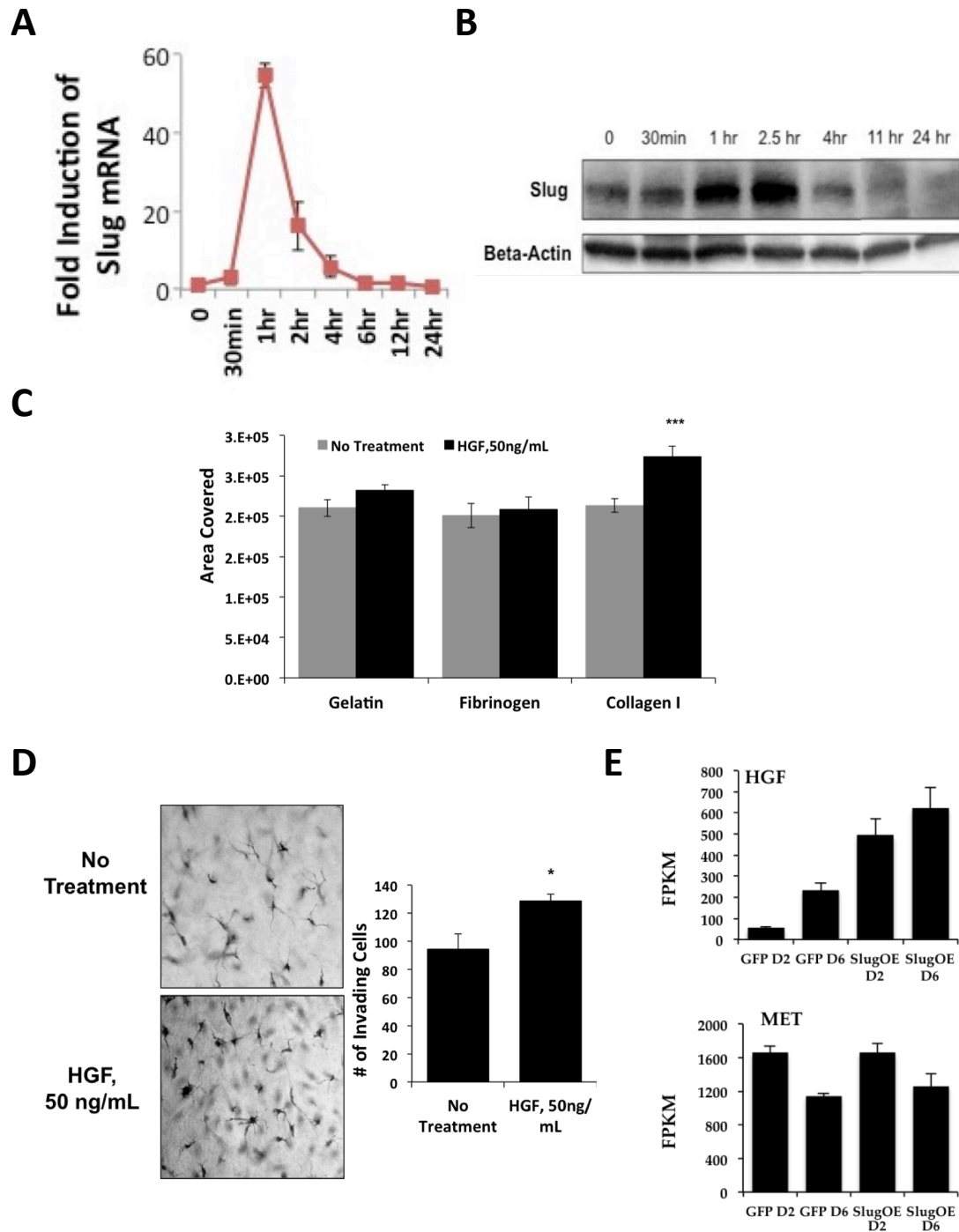


Figure 5.3: HGF induces transient Slug expression in EC. A) qPCR analysis showing changes of Slug transcription upon HGF treatment (50ng/mL) over time. B) Western blot showing changes in Slug protein level upon HGF treatment (50ng/mL) over time. C) Average areas covered after 24 hours by EC migration upon HGF treatment (50ng/mL) on different ECM coatings in 2D scratch assay. D) Left: Invading HUVEC after 48 hours in 3D Col I invasion assay $\pm$ HGF. Right: Average number of invading cells per gel $\pm$ HGF. E) FPKM values from RNA-seq data showing HGF and MET in GFP control and SlugOE EC at day 2 and day 6 3D fibrin sprouting assay. Data represent mean $\pm$ s.e. two-tailed unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

5.3 Methods

Mouse retina explant model

P4 pups were euthanized and sterilized with ethanol. The eyes were immediately enucleated and the retinas were dissected out in cold EBM2 in sterile conditions and cross-cut into four pieces. The retina pieces were serum starved overnight and embedded in a mixture of Col I and Matrigel (70% + 30%) the next day. The retinas were fed with 3% EBM2 with or without SDF1 α every other day for 6 days. The experiment was repeated three times with retina pieces from at least two mice per genotype.

siRNA treatment

Fluorescently tagged EC were transfected with siRNA against either CXCR4 (50nM) or CXCR7 (25nM) individually or together using Lipofectamine 2000. The siRNA transfection concentration used here were the lowest concentration required to achieve the maximum knockdown of the respective target genes. The next day, cells were coated onto the cytodex beads. A portion of the beads were embedded with fibroblasts (40,000 cells/well) mixed in the fibrin gel to promote sprouting angiogenesis. The assay was fed every other day and fixed on day 5 (6 days post-transfection) for imaging and quantification. The remaining beads were embedded in the fibrin gel without the fibroblasts, treated with vehicle control or SDF1 α and harvested on day 3 (4-days post-transfection) for RNA and qPCR analysis to determine the knockdown efficiency.

2D scratch assay

Plates were first coated with gelatin, fibronectin or Collagen I. HUVEC were plated and allowed to grow to confluency. Two hours prior to scratching, cells were treated with 10 $\mu\text{g/mL}$ of mitomycinC to stop proliferation. After a vertical scratch was made in the center of the plate using a pipette tip, cells were rinsed with HBSS buffer three times and fresh medium without mitomycinC was added. The cells were then imaged at various time points and the width of the scratch wound was measured with ImageJ.

Chapter 6. Conclusions and future work

The role of Slug as a master regulator of EMT in normal epithelial cell and various types of cancer cell has been extensively studied. However, until recently, the role of Slug in mammalian EC appeared to be dispensable, potentially due to the compensatory effect of Snail⁸⁰. Previously, our lab demonstrated, using our *in vitro* 3D fibrin bead angiogenesis assay, that Slug expression in the EC regulates angiogenic sprouting, and this is partly due to up-regulated EC migration as a result of induced MMT1-MP⁹⁶. Nonetheless, further investigation is required to uncover the effects of Slug expression at a global level during EC spouting. Furthermore, before our work the understanding of EC-specific effects of Slug on vascular formation *in vivo* was still lacking, with initial reports of the Slug germline knockout mice not reporting any vascular defects.

Here, we demonstrated for the first time that endothelial expression of Slug controls both developmental and pathological angiogenesis *in vivo*. We first observed abnormalities in the primitive vascular network in SlugKO mouse yolk sac at E9.5. Later in the postnatal animals, using retina vascular development as a model, we detected transient but significant delay in the growth of both primary and secondary vascular plexus in the SlugKO animals. Consistent with our previous *in vitro* finding, this is partly due to impaired retinal EC migration. Using a mouse subcutaneous tumor model and Matrigel plugs as an *in vivo* angiogenesis assay, we showed that Slug deficiency led to reduced tumor angiogenesis and in many cases, this was enough to completely halt tumor growth.

These studies reconciled the discrepancy between our previous *in vitro* findings and the initial analysis of the Slug global knockout mouse.

While Slug is important for developmental angiogenesis, the vascular defects we observed in SlugKO mouse retinas were only transient. This could be due to the compensatory effect from other factors such as Snail. Indeed, defects in EndoMT and cardiac cushion formation were observed in SlugKO embryos at E9.5. However, this was overcome by E10.5 by increased Snail expression. To see if this was the case in SlugKO retinal vasculature, EC can be FACS sorted from freshly dissected retinas and qPCR analysis can be performed to compare Snail expression level in WT versus SlugKO cells. We did not see consistent increases in Snail expression when Slug was knocked down in EC using siRNA (data not shown). However, it is possible that the adaptive response of increased Snail expression can only be observed with chronic loss of Slug.

Another caveat in the retinal developmental model is the investigation of cell type specific effects of Slug deficiency. Multiple cell types are involved in the retinal vascular network formation, and there are differences in the regulatory mechanism for each of the three vascular plexus¹⁴¹. For example, during the formation of the primary plexus, the presence of astrocytes is critical, as they lay down the VEGF gradient to attract EC to migrate and sprout towards the periphery. However, other cell types such as neurons also play a role in this process by expressing VEGFR2 and therefore limiting the availability of VEGF in specific areas²⁴⁰. More recently, it has been shown that retinal pericytes promote sprouting and regulate vessel branching via expression of VEGFR1²⁴¹. In WT

mice, we observed Slug expression in EC, but not astrocytes. Moreover, we did not see changes in astrocyte abundance or distribution in SlugKO retinas. Therefore, it is unlikely that astrocytes are responsible for the angiogenic defects. However, we did observe Slug expression in retinal pericytes. Although we did not see changes in pericyte coverage in the SlugKO animals, it is possible that the loss of Slug in pericytes could alter its function and interaction with EC and the microenvironment. In addition, we observed a very moderate increase in VEGFR1 expression in SlugOE EC, suggesting the possibility that Slug might regulate VEGFR1 expression. Thus, it would be interesting to determine if VEGFR1 expression in the SlugKO retinal pericytes was reduced.

The most definitive way to prove a cell-autonomous effect of Slug deficiency would be to use cell-type specific Slug knockout mice. However, to date, no conditional Slug knockout mouse has been successfully generated. As a second approach, we are developing a Cre-loxP mediated lentiviral rescue system to specifically restore Slug expression only in the retinal EC. The details of the viral construct were described in Chapter 2. We plan to either deliver the virus in live pups through intravitreal injection, and harvest the eyes 4 days later for whole mount fluorescence analysis; or, we will mix the virus in the matrix gel where retinal explants are embedded and observe the changes in real time *ex vivo*. If EC-specific restoration of Slug can rescue the angiogenic defect in SlugKO retina, we would be able to prove a cell (EC)-autonomous role for Slug. It is worth noting that the current designs of the viral constructs rely on strong ubiquitous promoters (EF1 α or MSCV) that would most

likely provide non-physiologically high levels of Slug expression. As we have demonstrated in Chapter three, abnormally high levels of Slug would also disrupt vascular formation. Therefore, the usage of endogenous promoters might be necessary.

Contrary to what happens in developmental angiogenesis, Slug appears to have a much more profound role in pathological angiogenesis as its loss leads to sustained suppression of tumor angiogenesis and tumor growth in SlugKO mice. Since the addition of WT stromal cells was not able to rescue the lack of EC recruitment and vessel formation in SlugKO Matrigel plugs, the phenotype is most likely caused by EC-intrinsic defects.

Another possible player here is the immune cell. Different immune cells are known to play differential roles in tumorigenesis. The production and recruitment of cytotoxic T cells is important for inducing apoptosis in immunogenic tumors²⁴², whereas the infiltration of neutrophils into the tumor has been associated with activation of the “angiogenic switch” via their production of cytokines and growth factors^{243,244}. In SlugKO mice, the T cell development is impaired, resulting in reduced production of CD4⁺ and CD8⁺ T cells²⁴⁵. Therefore, the failure to establish tumors in these mice is unlikely due to increased cytotoxic T cell functions. On the other hand, abnormal neutrophil infiltration has also been observed in these mice. Jiang et al, the authors of the first paper describing the SlugKO mice, reported chronically inflamed eyelids/conjunctivitis caused by increased neutrophil infiltration in the SlugKO mouse eyes⁷⁸. However, Newkirk et al. observed reduced neutrophil infiltration into the skin of SlugKO mice following UV-induced DNA damage compared to the WT animals. Due to

these conflicting observations, it is hard to predict if neutrophils are involved in the restricted tumor angiogenesis and the reduced tumor growth we observed in the SlugKO mice. More investigation is warranted to determine if neutrophil infiltration is impaired in SlugKO animals upon implantation of the tumor, and if there is any difference in the production of inflammatory cytokines that leads to reduced pathological angiogenesis.

Nevertheless, these findings suggest that inhibition of Slug expression in not only cancer cells, but also cancer-associated fibroblasts, EC and maybe even neutrophils could target cancer progression from multiple angles simultaneously. Since Slug is only elevated in angiogenic endothelium and tumor (-associated) cells, it might be a uniquely suitable new target for tumor-specific therapies. Furthermore, the recent development of nanoparticle-based *in vivo* delivery for endothelial-specific siRNA has now opened a new door for targeting transcription factors and other intracellular proteins as therapeutics²⁴⁶.

Although Slug expression is induced in angiogenic EC, and is necessary for sprouting angiogenesis, abnormally high Slug levels in EC can also disrupt normal vessel formation. In our 3D fibrin bead *in vitro* angiogenesis assay, depending on the level of misexpression, SlugOE sprouting defects ranged from overgrowth to increased fragmentation. Similar defects were observed in our vascularized-micro-organ (VMO) model, which in the early stages relies on vasculogenesis to form the networks. Moreover, we observed increased lancunarity—inhomogeneity or gappiness in the vascular network, suggesting SlugOE vessels are more contorted and irregular in shape, much like what is seen in the tumor vasculature.

In order to understand how elevated Slug expression augments pathological angiogenesis, we again took advantage of the 3D fibrin bead angiogenesis assay, which enables us to isolate EC from various stages of angiogenesis for RNA-seq analysis. We were able to compare the expression profiles of SlugOE with WT EC collected from both the initiation (day2) and establishment (day6) of the nascent angiogenic sprouts. This experimental design allowed us to discern not only the influence of SlugOE on the angiogenic process, but also the differential role of Slug at different stages of angiogenesis.

Consistent among several independent analyses, our data indicate that Slug has many actions, including: induction of TGF β signaling, promoting changes in cell shape and movements, altering cell junction and matrix adhesions, and particularly, during the early phase of sprouting, driving the proliferation program in EC. These findings are in agreement with previously reported roles for Slug in epithelial cells, smooth muscle cells and many types of solid tumor cells⁸³.

In both the RNA-seq data and by immunostaining, we saw a dramatic loss of cell junctions. Occludin gene expression was strongly decreased and both VE-cad and Cldn5 showed reduced surface expression in SlugOE EC, but little change in gene transcript levels. The decrease in VE-cad at the surface could be a consequence of decreased CTNNB1 (β -catenin) in SlugOE EC, leading to a loss of VE-cad anchoring at the cell surface. The loss of N-cad might also contribute to decreased VE-cad²⁴⁷. Since the

maturation of adherens junctions has been shown to promote the assembly of tight junctions, the loss of VE-cad surface expression might explain the loss of Cldn5 surface expression. The loss of junctions is an important feature of EMT and might explain the disconnected fragments/individual EC we observe when we test Slug overexpressing EC in the fibrin gel assay.

Interestingly, the gene expression changes we see support our previous suggestion that EC undergo a partial EndoMT during angiogenic sprouting. Several diagnostic genes were up-regulated, including SPARC, α SMA, FSCN1 and DDR2, which showed increased expression both during normal sprouting (WT D2 vs D6), and in response to overexpressed Slug. We also confirmed our previous finding of increased MT1-MMP, which is critical for EC migration during angiogenesis⁹⁶. Interestingly, N-cad, which is a key gene up-regulated during EMT, showed reduced expression in SlugOE EC. This may be a context-specific difference in that epithelial cells do not normally express N-cad, but up-regulate it during EMT. Conversely, N-cad is normally expressed in EC to a level comparable to VE-cad where it plays numerous roles²³². More importantly, the suppression of N-cad (and another adhesion molecule NCAM1), potentially by Slug, is required for the induction of EMT and proper neural crest migration in the chick embryo⁷⁵. Therefore, the down-regulation of N-cad, together with reduced expression of other EC-specific genes such as eNos and Vimentin, may be a similar indicator of an epithelial-type cell transition into a more mesenchymal-like/activated cell. Interestingly, SlugOE EC showed increased levels of T-cad, the function of which is unrelated to cell-cell junctions, but has been associated with tumor angiogenesis²⁴⁸. In summary, we

believe a “cadherin switch”²⁴⁹, rather than the simple loss or gain of N-cad, is a better marker for EMT/EndoMT.

In EC, N-cad has been shown to function as a “nascent” junction protein – it only localizes to cell-cell junctions when they first establish. As junctions mature, N-cad gives way to VE-cad, as they share the same membrane anchor proteins p120 and α , β -catenin, and becomes more diffusely expressed throughout the membrane. It has been shown to be involved in EC-pericyte and EC-tumor interactions, as well as in regulation of vascular permeability²⁵⁰. In MDCK cells with HGF-induced N-cad expression, it is shown to be involved in directional group migration in a 3D matrix²⁵¹. We therefore speculate that the loss of N-cad might account for many phenotypes observed in the SlugOE EC. More specifically, the loss of N-cad might be causing the increased fragmentation and increased permeability in SlugOE vessels. Consistent with this hypothesis, our preliminary results indicate that N-cad knockdown using siRNA (Fig. 6.1A) in EC leads to hypersprouting and increased branching in the 3D fibrin bead angiogenesis assay, much like what was seen in the SlugOE EC (Fig. 6.1B). N-cad overexpression (N-cadOE) in EC (Fig. 6.1C) seems to have little effect on sprouting (Fig. 6.1D), although there might be an increase in rounded non-polar cells. Notably, N-cad has been shown to play a role in neural cell polarities²⁵². Since vascular EC establish polarity in response to directional flow and shear stress, it would be interesting to use N-cadOE EC or EC treated with siNcad in the vascularized-micro-organ (VMO) platform and see if changes in the level of N-cad would alter EC behavior under different flow conditions. Since N-cad expression is reduced by more than 50% in the SlugOE EC, it would also be

interesting to see if N-cadOE would partially rescue the SlugOE phenotype. Although lower MOI or an endogenous promoter might be needed to achieve more physiological re-expression of N-cad using a lentiviral approach, since abnormally high level of N-cad have been associated with EMT/EndoMT as well.

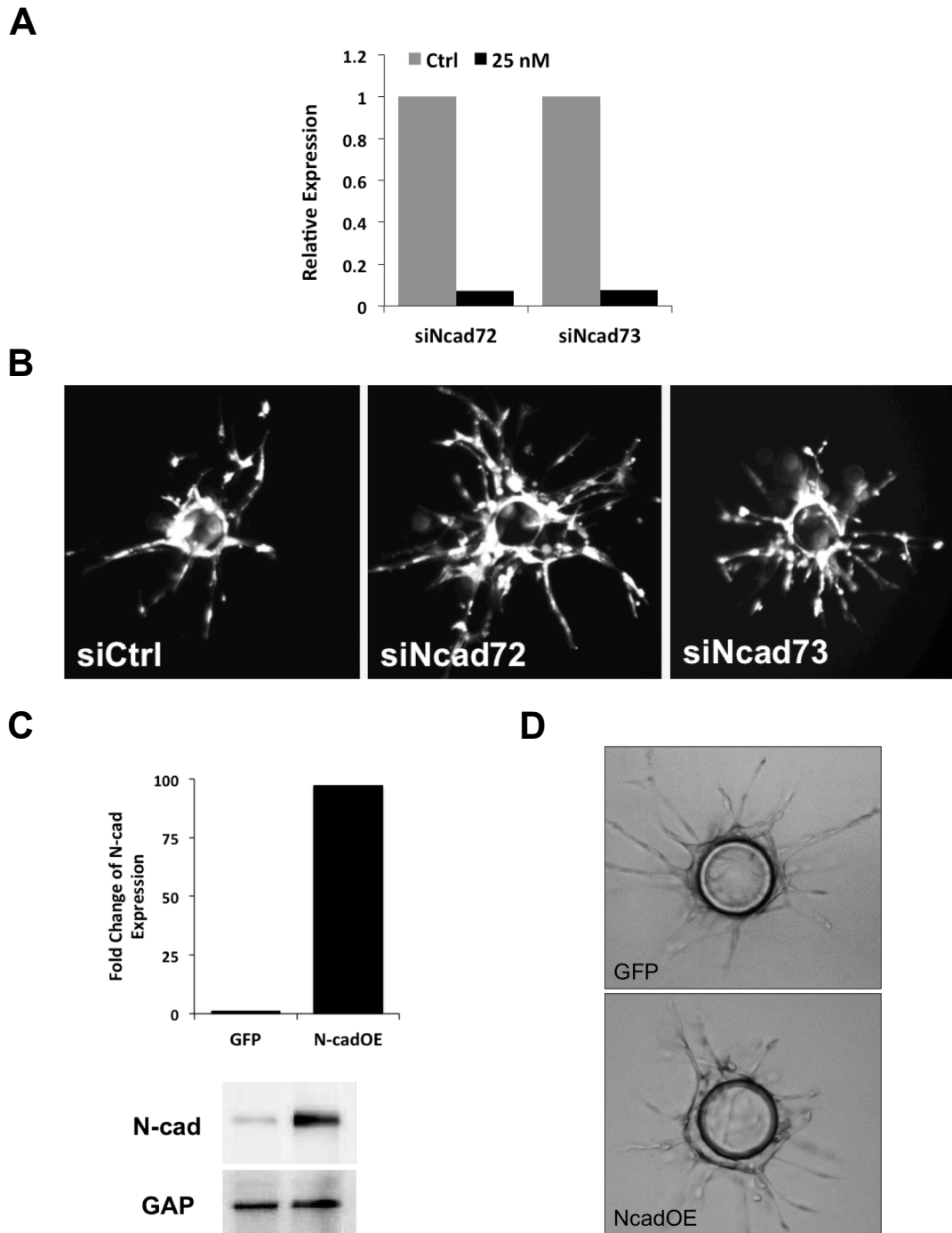


Figure 6.1: N-cad expression in EC is important for sprouting angiogenesis. A) qPCR analysis showing N-cad knockdown using two individual siRNA. B) N-cad knockdown in EC leads in increased sprouting in 3D fibrin bead assay by day 5. C) qPCR and western blot analysis showing misexpression of N-cad using a lentiviral approach. D) N-cadOE in EC does not lead to an obvious phenotype in a 3D fibrin bead assay.

In addition to the gene expression changes noted above, we also found a strong downregulation of Notch pathway components, including Dll4, Jag2 and Neurl1B in SlugOE EC. A recent study has shown that Snail, the canonical Snail family protein, inhibits Notch signaling by directly suppressing Dll4 transcription, which results in vascular defects and prenatal lethality. Since Slug and Snail recognize similar E-box sequences²⁵³, it is possible that Slug can bind to the same site. Indeed, another study has shown that Slug binds to the Dll4 promoter in human epidermal progenitor cells¹⁰⁴. Using ChIP-qPCR, we showed here that Slug can directly repress the Notch ligands Dll4 and Jag2. The down-regulation of Dll4 in SlugOE EC leads to reduced Notch signaling as indicated by decreased NICD, as well as the reduction in expression of an EC-specific Notch target Hey1. We and others have previously published that Hey1 negatively regulates VEGFR2 expression and hence controls the angiogenic potential of EC²³⁴. As expected, we observed increased VEGFR2 expression in SlugOE EC. In addition, inhibition of VEGF signaling not only corrected the hypersprouting phenotype in SlugOE EC, but also reduced the level of EndoMT in these cells, as indicated by a reduced number of disconnected fragments. Finally, Notch inhibition in the neonatal mouse retina was able to rescue the vascular defects caused by Slug deficiency.

Slug has previously been considered only as a downstream effector of Notch signaling, particularly during EndoMT. To our knowledge, this is the first study to show that Slug is an upstream regulator of Notch signaling through Dll4 inhibition. We have previously shown that Slug expression in EC does not force a tip location on the cell, rather it allows EC to outcompete EC expressing lower levels of Slug for position in a developing

sprout⁹⁶. Indeed, low Dll4 expression, as a result of high Slug levels, would not necessarily be consistent with a tip cell position, as these cells, in mouse at least, are thought of as expressing high levels of Dll4. However, close examination of retinas stained for Dll4 suggest that it is more a marker of EC close to the developing front than an absolute tip marker. In addition, recent publications have suggested that Dll4 expression is not required for sprouting EC to acquire or retain tip-cell identities, but rather it controls the arterial specification among the leading cells during later stages of network formation^{254,255}. We therefore speculate that during normal angiogenesis, stochastic elevation of Slug expression in individual EC potentiates them towards a tip-cell fate, yet expression of Slug in angiogenic EC generally marks them as activated cells.

Numerous factors present in the tumor microenvironment including TGF β , Wnt, HGF and TNF α , can induce Slug expression in epithelial cells⁸³. However, little is known about the upstream regulation of Slug in EC. SDF1 α has been shown to regulate sprouting and network maturation^{39,256}, and elevated SDF1 α -CXCR4 signaling has been associated with increased tumor angiogenesis. Interestingly, we and others have shown that SDF1 α activates Slug transcription in both EC and various cancer cells, and is an inducer of EMT^{81,96}. In addition, we have identified SDF-1 α as a key mediator of EC sprouting induced by angiogenic fibroblasts⁴⁴. These data strongly suggest SDF1 α as a potential upstream regulator of Slug during sprouting angiogenesis. In this study, we tested this hypothesis directly using several *in vitro* and *ex vivo* models and found that SDF1 α indeed activates Slug transcription mainly by signaling through its canonical

receptor CXCR4. Although its ancillary receptor CXCR7 has been recently shown to promote tumor angiogenesis⁴¹, it does not seem to work through Slug-mediated angiogenic program. In fact, knockdown of CXCR7 seems to lead to slight increase in Slug, possibly due to increased ligand availability for CXCR4. Finally, SDF1 α -CXCR4 has been previously shown to signal through ERK1/2 to promote cell motility and survival³⁹. However, here we report that SDF1 α -CXCR4 induced ERK5 phosphorylation, but not ERK1/2, to activate Slug transcription in angiogenic EC, which is consistent with previous reports²⁵⁷.

In aggregate, our data suggest Slug as a critical regulator of vessel formation, particularly in the pathological setting. Upon activation by various inducing factors (e.g. SDF1 α , TGF β , VEGF, HGF, TNF α , etc.), Slug down-regulates Dll4, which reduces Notch activity and increases VEGFR2 expression in EC. This allows them to be more responsive to angiogenic stimuli. Meanwhile, Slug expression also affects various EC behavior by initiating a partial EndoMT program, which puts them in an activated state to be more proliferative and migratory (Fig. 6.2).

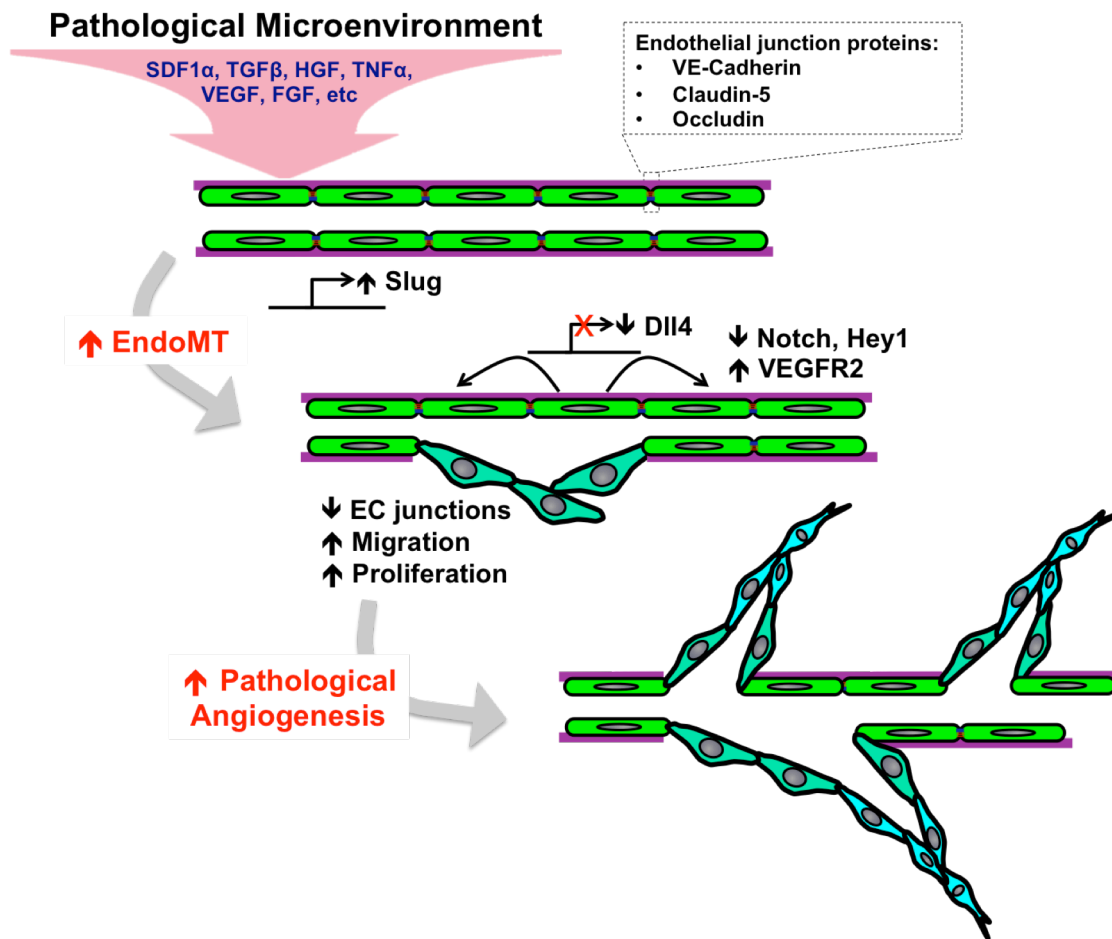
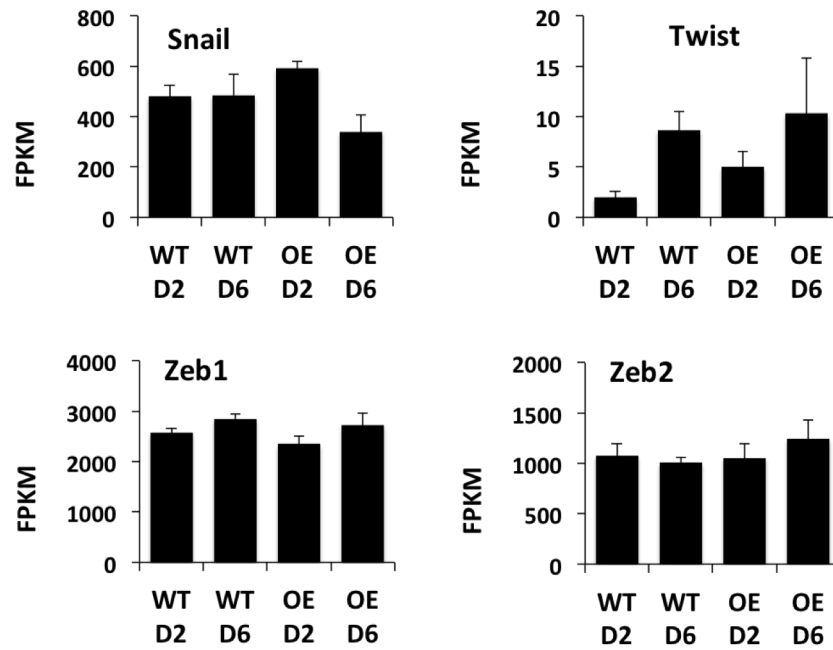
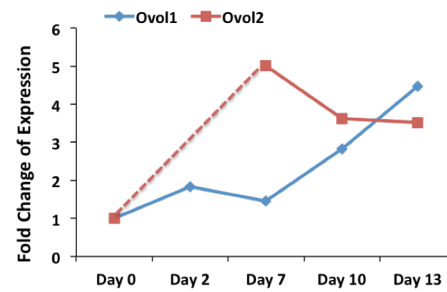
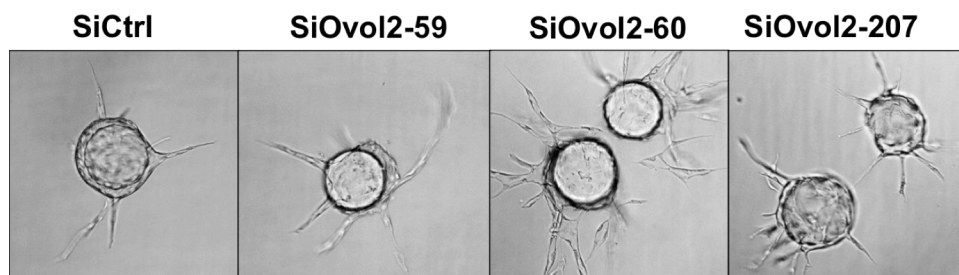
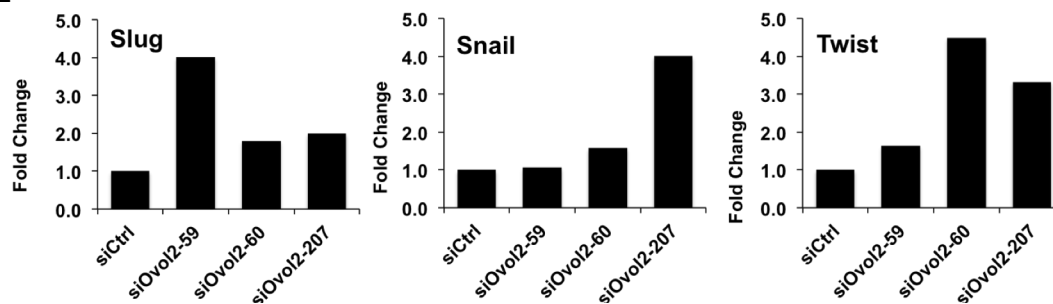


Figure 6.2: Schematics of Slug-mediated pathological angiogenesis. The combination of cytokines, growth factors, and extracellular matrix in a pathological environment such as a developing tumor can activate Slug transcription in the vascular endothelial cells. Elevated Slug expression in turn activates EC in quiescent blood vessels and initiate an EndoMT-like phenotype where they lose cell-cell junctions, start to proliferate and become more migratory. Meanwhile, Increased Slug also suppresses Dll4 expression, lower Notch signaling in EC and therefore leads to increased branching and sprout formation. This ultimately leads to a rise in pathological angiogenesis and the formation of abnormal blood vessels.

If Slug (and Snail) play such significant roles in vascular formation, one might reasonably wonder if that is the case for all of the EMT transcription factors, such as Twist, Zeb, and Ovol⁷³. Indeed, based on our RNA-seq data, Snail, Twist, Zeb1 and Zeb2, but not Ovol1/2, are all expressed in angiogenic EC (Fig. 21A). While the expression levels of Zeb1 and Zeb2 do not appear to be associated with a specific angiogenic stage, Twist1 expression seems to increase after the sprouts have become more established (Fig. 21A). This is consistent with our previous qPCR results showing that Slug, Snail and Twist expression are activated sequentially in angiogenic EC (Fig. 21B) while the expression of Ovol is not detectable until late stage when sprouts have lumenized and anastomosed (Fig. 21C). Our preliminary data also suggest that knockdown of Snail or Ovol2 does affect sprouting angiogenesis *in vitro*⁹⁶ (Fig. 21D). Consistent with the consensus that Ovol2 is the “gatekeeper” of EMT, we also observed an increase in the RNA level of pro-EMT transcription factors when Ovol2 was knocked down in HUVEC (Fig. 21E). All of these preliminary studies suggest differential roles of the various EMT transcription factors in EC functions. It would be interesting to perform immunofluorescence staining of all these factors at various stages of angiogenesis and vasculogenesis to see when and where each of the factors is activated and functioning (Fig. 21F). Ultimately, FISH-seq could be applied to the 3D fibrin bead sprouting assay or the VMO model so we could preserve structural integrity while performing quantitative gene expression analysis.

This group of transcription factors is known to have overlapping but differential roles during EMT, and regulatory interactions among them have been reported¹⁶⁵. Our

preliminary data suggest that a similar situation might occur in vascular EC. Ultimately, the suggestion we put forward for future work will be to elucidate a transcriptional regulatory network involving all of these EMT factors, identifying how each governs multiple aspects of EC behavior; and how each factor is spatially and temporally regulated to orchestrate this complex yet beautiful symphony of vascular morphogenesis.

A**B****C****D****E**

F

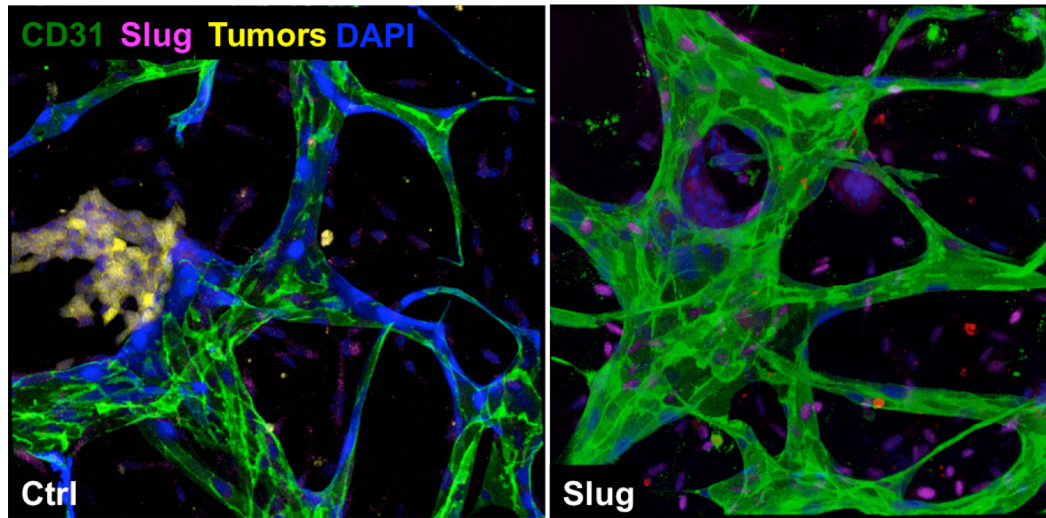


Figure 21: EMT transcription factors are expressed and function in EC. A) Other EMT transcription factors are also expressed in angiogenic EC. B, C) Various EMT transcription factors are expressed at different stages during angiogenesis. D) Ovol2 knockdown leads to increased sprouting in 3D fibrin sprouting assay. E) Ovol2 knockdown leads to increase in pro-EMT transcription factors. F) Immunofluorescent staining of nuclear Slug expression in EC (CD31⁺) in the VMO model. Note Slug expression can also be observed in perivascular cells (CD31⁻).

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