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#### **Publication Date**

2014

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UNIVERSITY OF CALIFORNIA,  
IRVINE

Defining a role for Snail family transcription factors during angiogenesis

DISSERTATION

Submitted in partial satisfaction of the requirements  
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Katrina Marie Welch-Reardon

Dissertation Committee:  
Professor Christopher C.W. Hughes, Chair  
Professor David A. Fruman  
Professor Steven C. George

2014

Chapter 2 © Arteriosclerosis, Thrombosis, and Vascular Biology  
Chapter 3 © 2014 Journal of Cell Science  
All other materials © 2014 Katrina Marie Welch-Reardon

## **DEDICATION**

To my husband Matt, I am forever grateful for your endless support and continual patience. Your constant encouragement enabled me to conquer this chapter of my life, thank you for always loving me and believing in me.

To my parents and siblings, you have been my cheerleaders. During points of near collapse you provided me with renewed faith and confidence in my abilities. Mom and Dad, you gave me the foundation and confidence to achieve my dreams, I could not have accomplished my goals without your help.

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## **ACKNOWLEDGMENTS**

My sincere gratitude to my mentor, Dr. Christopher Hughes for the support and guidance he provided throughout my Ph.D. career. I would also like to extend my appreciation to my committee members Dr. David Fruman, Dr. Steven George, Dr. Melissa Lodoen and Dr. John Lowengrub. A heartfelt thanks to previous and current members of the Hughes Lab, I feel so blessed to have been part of such a unique and inspiring group of scientists. A special thanks to Andrew Newman for understanding my struggles and reminding me to “settle” when times got tough, to Ashley Fong for keeping us all informed on UCI gossip, to Monica Romero for being my desk partner and providing me with constant entertainment, to Stephanie Popson for encouraging me to always “keep my chin up”, to Mary Ziegler for being an inspirational woman, incredible scientist and providing continual guidance, and to Nan Wu for continuing the work I started and motivating me during my last year.

Sources of funding included:

- American Heart Associated Pre-doctoral Fellowship awarded to Katrina Welch-Reardon
- US National Institutes of Health grant RO1HL60067 to Dr. Christopher C.W. Hughes

# CURRICULUM VITAE

## **Katrina Marie Welch-Reardon**

### **EDUCATION AND CERTIFICATIONS**

---

- University of California, Irvine** - Irvine, CA 2008 – 2014  
**Ph.D.**, Biological Sciences - Vascular Biology  
Department: Molecular Biology and Biochemistry
- Keck Graduate Institute of Applied Life Sciences** - Claremont, CA 2013  
Bioscience Management Bootcamp
- BioCollaborative Life Science Immersion Certificate Program** 2013  
**Certification** - Awarded 125 Professional Development Units  
San Diego State University
- Santa Clara University** - Santa Clara, CA 2002 – 2006  
**Bachelor of Science**  
**Major:** Cell and Molecular Biology **Minor:** Biotechnology
- CIEE Study Abroad** - Costa Rica 2004  
Studies Abroad Program, Santa Clara University

### **PROFESSIONAL EXPERIENCE**

---

- University of California, Irvine** 2008 – present  
*Graduate Student Researcher* | Irvine, CA
- Completed research in the laboratory of Dr. Christopher Hughes focusing on vascular biology with particular emphasis on the vasculature of tumors.
  - Managed and completed multiple research projects by formulating hypotheses, collecting and effectively analyzing data, identifying multiple avenues to solve complex problems, and publishing results in reputable peer-review journals.
  - Identified novel genes and the processes they regulate during angiogenesis – thesis work focuses on identifying a role for transcription factor Slug (Snai2) during this process.
  - Expert in the manipulation and design of multiple in vitro angiogenesis assays.
  - Collaborated with multidisciplinary research groups resulting in co-author publications in peer-review journals.
  - Successfully managed and trained undergraduate researchers in molecular biology laboratory techniques.
- University of California, Irvine** 2014 – present  
*Office of Technology Alliances Intern* | Irvine, CA
- Authored non-confidential description (NCD) documents published on the OTA website encouraging and facilitating licensing agreements thereby promoting UC, Irvine intellectual property.
  - Performed prior art searches to ensure novelty of inventions.
  - Completed market analysis resulting in identification of potential licensing partners for licensors.

**Oxbridge Biotech Roundtable, Los Angeles Chapter** 2013 – present  
*Ambassador* | Los Angeles, CA

- Served as an advocate in the LA region by fostering communication between academia and industry.
- Assisted in planning and marketing OBR events.

**University of California, Irvine** 2010 – 2012  
*Teaching Assistant* | Irvine, CA

- Teaching Assistant for the following undergraduate courses in the school of Biological Sciences: Immunology Lecture, Experiment Microbiology Laboratory and Molecular Biology Lecture.
- Responsibilities included, preparing lecture plans and weekly quizzes, leading and teaching microbiology laboratory techniques, holding and leading weekly discussion sections, and grading all assigned course material.

**Stanford University** 2006 – 2008  
*Life Science Research Assistant* | Palo Alto, CA

- Involved in developing a procedure for massive parallel resequencing of multiple human genes by combining a highly multiplexed and target-specific amplification process with a high-throughput parallel sequencing technology; Illumina Solexa Genome Analyzer Sequencing Technology. Research completed in the laboratories of Dr. Hanlee Ji, Dr. James Ford, and Dr. Ronald Davis.
- Collaborated with Affymetrix and trained in using Affymetrix Molecular Inversion Probes.
- Research and collaborations resulted in four co-author publications in leading peer-review journals and completion of one book chapter.

**UW Medicine- Harborview Medical Center** 2005  
*Research Intern* | Seattle, WA

- Researched Human Papilloma Virus by culturing, cloning, identifying, and sequencing unique HPV strains. Research completed in the laboratory of Dr. Nancy Kiviat.

**Santa Clara University** 2005  
*Lab Assistant and Student Advisor* | Santa Clara, CA

- Lab Assistant and Student Advisor for Investigations in Evolution and Ecology. Assisted the professor and students in the laboratory and advised on topics related to evolution and ecology.

**Fred Hutchinson Cancer Research Center** 2004  
*Research Intern* | Seattle, WA

- Studied the expression levels of rRNA in E6, a Myc target gene, in Human Papilloma Virus cell lines. Perfected techniques including ChIP and qRT-PCR. Research completed in the laboratory of Dr. Denise Galloway.

**Xcyte Therapies** 2003  
*Research Intern* | Seattle, WA

- Processed incoming human tissue samples, phenotyped tissue by flow cytometry, and maintained laboratory records.

## LEADERSHIP AND MEMBERSHIP

---

- Associated Graduate Students – University of California, Irvine 2012 – present
  - *Council Member* | 2012 - present
    - Elected represented from the school of Biological Sciences.
    - Represented students on AGS and UCI committees by communicating important issues and events to constituents.
  - *Funding Committee* | 2013 – present
    - Responsible for allocating \$70,00 to highly qualified graduate student applicants seeking travel money for conferences within their field.
  - *Outreach Committee* | 2013 – present
    - Helped to organize and host a research symposium event at UCI in which one hundred student research talks, from all disciplines, are presented to a non-technical audience.
    - Identified and applied for outside funding from governmental bodies and businesses to fund the symposium.
    - Personally responsible for recruiting Dr. Art Riggs as the keynote speaker for the symposium.
  - *Business and Finance Committee* | 2012 – 2013
    - Fundraising and entrepreneurship hub responsible for overseeing efforts to explore and enact new business ventures.
    - Involved in the development of AntTutors, a program in which graduate students tutor undergraduates in science related courses.
  
- Kappa Alpha Theta – Sorority at Santa Clara University 2003 – 2006
  - Founding member of the Eta Lamda chapter.
  - Recruitment Day Chair, Fall 2003.

## FELLOWSHIPS AND AWARDS

---

- Journal of Cell Science 2014
  - “Angiogenic sprouting is regulated by endothelial cell expression of Slug (Snai2)” was selected for highlighting in the ‘In This Issue’ section of JCS.
  - An image from a figure in this manuscript was selected by the Executive Editor for use as the cover image for the cover of the journal.
- F1000Prime article recognition 2014
  - “Angiogenic sprouting is regulated by endothelial cell expression of Slug (Snai2)”, published in the Journal of Cell Science, was selected for F1000Prime, it was recommended as being of special significance in its field by faculty member Dr. Michael Simons.
- American Heart Association Pre-doctoral Fellowship 2012 – 2014
  - Received a priority score of 1.73 and a percentile rank of 15.83%
    - The most meritorious ranked application corresponded to priority scores close to 1.0 and a low percentile rank. Applicants that received a ranking of 22% or lower were awarded \$22,000/year.
- Outstanding Poster Award 2013
  - Highest scored poster out of 200+ poster presentations at the North American Vascular Biology Organization (NAVBO) Conference held in Hyannis, MA.

## PUBLICATIONS

---

### Publications

1. Ehsan SM, **Welch-Reardon KM**, Waterman ML, Hughes CCW, George SC. A three-dimensional *in vitro* model of tumor angiogenesis and intravasation. *Integrative Biology*, 2014 April (Epub ahead of print).
2. **Welch-Reardon KM**, Ehsan SM, Wang K, Wu N, Newman AC, Romero-Lopez M, Fong AH, George SC, Edwards RA, Hughes CCWH. Angiogenic sprouting is regulated by endothelial cell expression of Slug (Snai2). *Journal of Cell Science*, 2014 May; 127(9): 2017-2028.
3. Popson SA, Ziegler M, Chen X, Holderfield M, Shaaban C, Fong A, **Welch-Reardon K**, Papkoff J, Hughes CCW. Interferon-induced transmembrane protein 1 regulates endothelial lumen formation during angiogenesis. *Arteriosclerosis, Thrombosis and Vascular Biology*, 2014 May; 34(5): 1011-9.
4. Newman AC, Chou W, **Welch-Reardon KM**, Fong AH, Popson SA, Phan DT, Sandoval DR, Nguyen DP, Gershon PD, Hughes CC. Analysis of Stromal Cell Secretomes Reveals a Critical Role for Stromal Cell-Derived Hepatocyte Growth Factor and Fibronectin in Angiogenesis. *Arteriosclerosis, Thrombosis and Vascular Biology*, 2013 March; 33(3): 513-522.
5. Ji HP, Morales S, **Welch K**, Yuen C, Farnam K, Ford JM. Identification of a novel deletion mutant strain in *Saccharomyces cerevisiae* that results in microsatellite instability phenotype. *Biodiscovery*, 2012 Jul; (1) 4.
6. Schiffman JD, Wang Y, McPherson LA, **Welch K**, Zhang N, Davis R, Lacayo NJ, Dahl GV, Faham M, Ford JM, Ji HP. Molecular inversion probes reveal patterns of 9p21 deletion and copy number aberrations in childhood leukemia. *Cancer Genetics Cytogenetics*, 2009 Aug; 193(1): 9-18.
7. Fredriksson S, Baner J, Dahl F, Chu A, Ji H, **Welch K** and Davis RW. Multiplex amplification of all coding sequence within 10 cancer genes by Gene-Collector. *Nucleic Acids Research*, 2007 April; 35(7): e47.
8. Dahl F, Stenberg J, Fredriksson S, **Welch K**, Zhang M, Nilsson M, Bicknel DL, Bodmer WF, Davis RW, and Ji H. Multigene amplification and massively parallel sequencing for cancer mutation discovery. *Proceedings of the National Academy of Sciences*, 2007; 104(22): 9387-92.

### Book Chapter

1. Hanlee Ji and **Katrina Welch**. Chapter 6: Molecular inversion probe assay for allelic quantitation. In *Microarray Analysis of the Physical Genome*. Series: *Methods in Molecular Biology*, 2009 May; 556: 67-87.

## VOLUNTEER WORK

---

### Santa Clara University Alumni

*Volunteer Tutor* | Downey, CA

2012 – present

- Assisted 8<sup>th</sup> graders from low income families prepare for the Catholic High School entrance exam at St. Matthias High School.

### Habitat for Humanity

*Volunteer* | Long Beach, CA

2011

- Assisted in the building of homes for families in need.

## **ADDITIONAL SKILLS**

---

- Biology:** Most molecular and biochemistry laboratory skills including cell culture and comfortable with mouse work.
- Computer:** Proficient in all Microsoft Office Programs, Adobe Illustrator and familiar with Adobe Photoshop.
- Language:** Semi-fluent in spoken Spanish.
- Soft skills:** Leader, problem solver, extremely organized, excellent communicator and public presenter, team player, excellent at project and time management, outstanding multitasking skills, ability to successfully complete research grant writing, excels at written communication, superb teaching skills, capable of generating novel ideas and possesses strong analytical skills.
- Interests:** I have always been fascinated by other cultures and to quench my thirst I have made traveling around the globe a top priority. I also love being outdoors and growing up in the Pacific Northwest fostered my love for hiking, camping, water sports and snow skiing.

# **ABSTRACT OF THE DISSERTATION**

Defining a role for members of the Snail family of zinc-finger transcription factors during angiogenesis

By

Katrina Marie Welch-Reardon

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2014

Professor Christopher C.W. Hughes, Chair

Angiogenesis is a tightly regulated multi-step process in which new blood vessels form from the preexisting vasculature. Due to extensive research in the field of vascular biology, a plethora of knowledge exists about the growth factors, receptors, and signaling pathways that drive angiogenesis. However, our understanding of the transcription factors that bridge the gap between these signals and new gene expression remains incomplete.

Members of the Snail superfamily of zinc-finger transcription factors are expressed and required during both embryonic development and progression of malignant epithelial tumors. Specifically, this protein family drives developmental and pathological events by inducing epithelial to mesenchymal transitions (EMT). Snail (Snai1) and Slug (Snai2) are transcriptional repressors that belong to the Snail family of zinc-finger proteins. Importantly, expression of Snail or Slug in tumor cells is known to contribute to invasion and metastasis, and these genes have also been observed in angiogenic endothelial cells

(EC) *in vivo*. Based on these observations, we asked whether angiogenic sprouting might share common attributes with EMT.

Using our *in vitro* angiogenesis model, we show that sprouting EC express and regulate Slug and Snail. To demonstrate a functional role for these genes, we utilized small interfering RNA (siRNA) to inhibit Slug or Snail expression and examine phenotypic alterations in EC undergoing angiogenesis. Interestingly, both Slug and Snail appear to be required for angiogenic sprouting as siRNA-mediated knockdown of either gene inhibits EC sprouting. In addition, we demonstrate that Slug regulates expression of membrane-type 1 matrix metalloproteinase (MT1-MMP; itself a regulator of vascular morphogenic events), but not vascular endothelial-cadherin (VE-Cadherin). Furthermore, lentiviral-mediated re-expression of MT1-MMP rescues the loss of sprouting induced by Slug knockdown, confirming that MT1-MMP is a crucial downstream target of Slug during sprouting angiogenesis. Importantly, we also observe enhanced Slug and Snail expression in tumor-associated blood vessels in multiple cancers. Taken together, these data identify a critical role for Slug expression in regulating pathological angiogenesis.



# **CHAPTER 1**

## **Introduction**

### **VASCULAR DEVELOPMENT**

The adult vasculature is initially formed in the embryo by vasculogenesis, a process in which vessels are formed de novo from endothelial cell precursors termed angioblasts.<sup>1-3</sup> During vasculogenesis, angioblasts proliferate and coalesce into a nascent network of vessels referred to as the primary capillary plexus. This primitive arrangement of endothelial cells (EC) then serves as a scaffold for angiogenesis, a process in which new blood vessels form from the pre-existing vasculature.<sup>1</sup> Most angiogenesis takes place in the embryo ensuring that each developing organ receives an adequate supply of nutrients and oxygen, and although very little vascular turnover occurs in the adult, angiogenesis does take place during the ovarian cycle and during physiological repair processes such as wound healing.

Angiogenesis is a tightly regulated process governed by the balance between pro- and anti-angiogenic signals – disruption of this equilibrium has been termed the “angiogenic switch.”<sup>4</sup> The angiogenic switch has been depicted as a balance with pro-angiogenic factors on one side and anti-angiogenic factors on the other. Extensive studies reveal a multitude of growth factors responsible for tipping the balance in either direction thereby promoting or inhibiting angiogenesis. Angiogenic stimuli include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), placental growth factor (PIGF), and hepatocyte growth factor (HGF) to name a few.<sup>5-7</sup> Occupying the other side of the scale are angiogenic inhibitors including thrombospondin-1 (TSP-1), soluble Flt1 (sFlt1), angiostatin, and endostatin.<sup>8</sup> During development and normal physiological conditions, the balance between these signals is strictly

controlled. However, aberrant expression of these signals leads to the absence or overabundance of blood vessels contributing to the pathologies of many disorders such as heart and brain ischemia, cancer, arthritis, and diabetes-related blindness. It is therefore crucial to understand the cellular and molecular signals that regulate each step of angiogenesis to ameliorate these disease states.

### **ANGIOGENESIS: A MULTI-STEP PROCESS**

During angiogenesis a subset of quiescent EC respond to angiogenic growth factors, the most well studied being VEGF. Once these angiogenic cytokines are bound to their cognate EC receptors, a cascade of tightly regulated molecular and cellular events ensues. The initial stages of angiogenesis require EC to lose their apical-basal polarity and degrade the adjacent basement membrane (BM) and extracellular matrix (ECM). Matrix breakdown thereby permits nascent sprouts to migrate towards angiogenic stimuli. Developing vessels then proliferate, form intricate branching patterns, form lumens, and anastomose with neighboring vessels. Finally, as the newly formed vessel matures, EC recruit mural cells to their abluminal surface, synthesize new BM and return to a quiescent phenotype. Together, these highly coordinated steps of angiogenesis result in functional vascular networks that support transportation of oxygen and nutrients throughout the body.

#### **Selection of EC for sprouting**

During initiation of sprouting angiogenesis, the transition of EC into two distinct cell types emerges. These discrete EC types are referred to as tip cells and trunk cells and each subpopulation has a distinct function, phenotype, and molecular profile.<sup>9</sup> Tip cells assume the

lead and guide the nascent sprout along a gradient of angiogenic stimuli.<sup>10</sup> Consequently, tip cells are highly polarized and extend both fillopodia and lamellipodia permitting exploration of the surrounding environment. Trailing behind the tip cells is a thread of trunk cells. Unlike tip cells, trunk cells are proliferative, form the vessel lumen, and contribute to the synthesis of new BM.<sup>11</sup> Aside from these phenotypic differences, gene expression profiles reveal unique molecular variances between tip and trunk cells. Tip cells have elevated expression levels of delta-like ligand-4 (Dll-4), Jagged-1 (Jag-1), platelet derived growth factor-B (PDGF-B), unc-5 homolog B (UNC5B), VEGF receptor-2 (VEGFR-2), membrane type-1 MMP (MT1-MMP), neuropilin receptor-2 (NRP-2), C-X-C chemokine receptor-4 (CXCR-4), and have low levels of Notch signaling activity.<sup>10, 12-21</sup> Importantly, the Notch pathway is a significant driver of tip and trunk cell selection through spatial differentiation and lateral inhibition.<sup>22</sup>

The necessity of Notch signaling in the endothelium is well established as loss of a single copy of Dll-4 or deletion of Notch-1 results in vascular defects and embryonic lethality.<sup>23, 24</sup> Angiogenic EC express various Notch receptors (Notch-1, 3, 4) and ligands (Dll-1, Dll-4, Jagged-1, Jagged-2), and their differential expression results in tip or trunk cell identity.<sup>25</sup> Specifically, when a nascent vessel is formed, VEGF binds VEGFR-2 causing upregulation of Notch ligand, Dll-4 in tip cells.<sup>10, 11, 17, 26</sup> Dll-4 subsequently activates Notch on adjacent EC resulting in downregulation of VEGFR-2 thereby creating a feedback loop that maintains active VEGF signaling and a migratory phenotype in tip cells while suppressing this phenotype in trunk cells.<sup>15, 16, 27</sup> Furthermore, Notch receptor signaling drives greater expression of VEGFR-1 in trunk cells serving as a competitive inhibitor of VEGF signaling.<sup>26</sup> Interestingly, more recent studies have challenged this concept of tip and trunk cell exclusivity arguing that tip-trunk cell

specification is rather dynamic and transient, dependent on continually competition between these cell types.<sup>17</sup> Regardless of these controversial data, it is clear that proper sprout formation requires coexistence of both tip and trunk cells in a nascent vessel.

### **EC interactions with the surrounding matrix**

Once tip and trunk cells are defined during the angiogenic cascade, they must collectively migrate through the surrounding matrix. This action requires detachment of mural cells from the preexisting vasculature followed by proteolytic degradation of the interstitial matrix. Secretion of angiopoietin-2 (Ang-2) facilitates mural cell release placing tip cells in contact with the BM, a matrix rich in type IV collagen and laminin.<sup>9, 28, 29</sup> Past the BM, a sprout encounters an ECM comprised of collagen type I under normal physiological conditions or, during pathological angiogenesis, a matrix abundant in fibrin, fibronectin, and vitronectin.<sup>30-32</sup> Degradation of these BM and ECM components requires EC to utilize proteolytic enzymes including plasminogen activators, the related ADAM (a disintegrin and metalloproteinase) protein family, and matrix metalloproteinases (MMPs).

Angiogenesis is an invasive process and EC require MMPs to move through diverse matrices. MMPs are either secreted or tethered to the cell membrane, and those bound to the cell surface are classified as membrane-type MMPs (MT-MMPs). MMPs are maintained in an inactive form and upon cleavage of a propeptide domain enzymatic activation is achieved. In the field of vascular biology, particular emphasis has been placed on MT1-MMP, MMP-2, and MMP-9 as critical regulators of angiogenesis. Interestingly, gene knockout studies reveal these three proteases to be required during both normal and pathological angiogenesis, but dispensable

during embryonic vascular development.<sup>33-36</sup> MT1-MMP mutant mice lack adequate vascular invasion at secondary ossification centers and MMP-9 mutant mice present defects in angiogenesis at the growth plate of bones.<sup>33, 34</sup> MMP-2 mutant animals show strong reduction in retinal neovascularization after injury, an observation that is amplified in MMP-2/MMP-9 double knockouts.<sup>35</sup> Finally, MMP-2/MT1-MMP double mutants have vascular defects including capillaries with extremely small lumens.<sup>36</sup>

*In vivo* studies and extensive *in vitro* work have unraveled cellular and molecular mechanisms behind the actions of MT1-MMP, MMP-2, and MMP-9 during angiogenesis. MT1-MMP is indispensable in several steps of the angiogenic process including degradation of collagen and fibrin, EC invasion and migration, and formation of capillary tubes.<sup>37-39</sup> In addition, ample evidence indicates that MT1-MMP is localized to the invading tip cell, while its expression is downregulated in trunk cells.<sup>9, 20, 21, 28</sup> MT1-MMP is also required for the activation of MMP-2. The propeptide of MMP-2 is cleaved by a cell-surface complex that consists of a homodimer of MT1-MMP and a single molecule of tissue inhibitor of metalloproteinase-2 (TIMP-2).<sup>40</sup> Once activated by this complex, MMP-2 is critical during pathological angiogenesis. For example, MMP-2 exposes hidden  $\alpha_v\beta_3$  binding sites on collagen IV facilitating EC adhesion and migration.<sup>41-43</sup> Finally, degradation of ECM by EC expression of MMPs also liberates growth factors including VEGF and basic fibroblast growth factor (bFGF) which otherwise remain sequestered in the matrix.<sup>2</sup> For instance, MMP-9 releases matrix-bound VEGF to stimulate vascular patterning and also cleaves VEGF creating a 16 kDa fragment that promotes decreased vascular density and larger vessel diameter.<sup>44, 45</sup>

While migrating and degrading matrix, EC process signals from the ECM through EC-surface expression of integrins – heterodimeric membrane glycoproteins composed of an alpha and beta subunit, each with a unique ECM ligand-binding profile. Importantly, these integrin-ECM interactions promote EC behavioral alterations including cell attachment and migration, making these proteins key contributors during angiogenesis.<sup>46-49</sup> *In vivo* and *in vitro* data have implicated several integrins as regulators of angiogenesis including  $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$ ,  $\alpha 4\beta 1$ ,  $\alpha 5\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha 6\beta 4$ ,  $\alpha 9\beta 1$ ,  $\alpha v\beta 3$ , and  $\alpha v\beta 5$ . Of particular importance is the  $\alpha v\beta 3$  integrin. This integrin complex has high affinity for ECM proteins including vitronectin, fibronectin, fibrinogen, and osteopontin, all of which are key components of the ECM, particular during pathological angiogenesis.<sup>50, 51</sup> It is therefore of no surprise that this integrin is widely expressed on tumor blood vessels but is absent on the vessels of normal endothelium.<sup>51</sup> Finally,  $\alpha v\beta 3$  binds directly with active MMP-2 generating a single cell-surface receptor capable of regulating both matrix degradation and motility, thereby facilitating directed cellular invasion.<sup>52</sup>

### **Mechanisms of EC lumen formation**

The formation of the vascular lumen is a pivotal step during the process of angiogenesis as the luminal space of a blood vessel is required to transport blood, nutrients, and oxygen to tissues. It is generally accepted that trunk cells are the subset of EC responsible for forming tubes during the growth of a vascular sprout, although some controversy remains regarding the exact mechanism(s) of this process. In one mechanism, referred to as cord hollowing, trunk cells flatten onto the wall of a matrix space created by the tip cell, initiating a luminal area and leading to a tube that emerges from the trailing chain of trunk cells. This mechanism is observed during *in vitro* sprouting angiogenesis, in the retina and aorta of developing mice, and during

zebra fish major axial and ISV formation *in vivo*.<sup>53-57</sup> Alternatively, a cell hollowing mechanism has been described in which individual trunk cells generate small intracellular pinocytic vacuoles that, through exocytic events, fuse with adjacent trunk cells to form intercellular lumens. Cell hollowing has been observed during lumen formation in 3D collagen gels *in vitro* and during zebrafish ISV formation and mouse arteriolar lumenogenesis *in vivo*.<sup>58-61</sup> It is clear that lumenogenesis is a highly dynamic process that may occur by different cellular mechanisms dependent on the organism, stage of development, or size and site of the developing vessel.

### **Vessel Maturation**

During the final stages of angiogenesis the newly formed vascular network must return to a quiescent state. This is achieved through wrapping of mural cells around the newly formed vessels, followed by vascular remodeling. First, EC recruit vascular support cells to their abluminal surface. The type of mural cell and the area of coverage are dependent on the caliber of the vessel – smooth muscle cells (SMC) to arteries, and pericytes (PC) to venules and capillaries.<sup>1</sup> Recruitment of these mural cells is mediated by secretion of platelet-derived growth factor B (PDGFB) by tip cells, which signals through PDGF receptor- $\beta$  (PDGFR- $\beta$ ) located on mural cells.<sup>62</sup> Stabilization between EC and mural cells is then mediated by binding of Ang-1, secreted by smooth muscle cells or PC, to its receptor Tie-2 on EC.<sup>63, 64</sup> These support cells then synthesize new basement membrane (BM) thereby sending signals to the newly formed vessels to assume a quiescent phenotype. Finally, EC remodeling takes place to meet the requirements of the surrounding tissue. During this process some EC apoptose and a subset of vessels regress creating a more differentiated vascular network. These final stages of angiogenesis result in a

functional and mature vascular network that acts as a conduit for blood, oxygen, nutrients, and waste.

## **PATHOLOGICAL ANGIOGENESIS**

Angiogenesis is a process that is critical during physiological conditions such as embryonic development, wound healing, and reproduction. It also plays an important role in many disease states including ischemic and inflammatory diseases, diabetes, rheumatoid arthritis, and cancer.<sup>65-69</sup> In cancer, sprouting angiogenesis is required to meet the metabolic demands of a rapidly dividing tumor. In fact, a tumor will fail to grow larger than ~2 mm without blood vessel recruitment due to the diffusion limit of oxygen and nutrients. Angiogenesis is therefore required for a primary tumor's survival and is also involved in metastasis formation and further outgrowth of metastases.<sup>70</sup> However, due to an imbalance between pro- and anti-angiogenic signals, tumors constantly produce pro-angiogenic signals in excess of angiogenic inhibitors, the tumor vasculature is architecturally abnormal. Vessels in tumors are highly disorganized, tortuous and dilated, with uneven diameter, excessive branching and shunts. As a result, blood flow in these vessels is chaotic and variable leading to hypoxic and acidic regions in the tumor.<sup>71, 72</sup> Moreover, the tumor vasculature is highly permeable due to a number of fenestrae, widened junctions, discontinuous or absent BM, and improper or decreased vessel association with pericytes.<sup>73, 74</sup>

In 1971, Judah Folkman proposed that tumor growth was dependent on angiogenesis and inhibiting this dependency could be a strategy to arrest tumor growth.<sup>75</sup> Progressive research has since proven Folkman's theory, setting in motion the pursuit of anti-angiogenic molecules and strategies for cancer treatment. Included in these blocking therapies are Bevacizumab (Avastin),



Sorafenib (Nexavar), Sunitinib (Sutent), Pazopanib (Votrient), and Everolimus (Afinitor). Bevacizumab, for example, is a monoclonal antibody that binds VEGF preventing VEGF from activating VEGFR2.<sup>76</sup> This drug is used as a monotherapy to treat glioblastoma and in combination with other cancer drugs to treat metastatic colorectal cancer, some non small-cell lung cancers, and metastatic renal cell cancer. Many of these therapies provide improvement in progression-free or overall survival; however, they are nonetheless met with eventual drug resistance and side effects due to the complexity and heterogeneity of the tumor microenvironment. Furthermore, both animal and preliminary human clinical trials reveal that different tumors respond differently to anti-angiogenic therapy complicating treatment regimens. Continual efforts to understand the molecular mechanisms that regulate tumor angiogenesis will certainly lead to the development of novel targets for anti-angiogenic therapies.

## **METHODS FOR STUDYING ANGIOGENESIS *IN VITRO***

Mechanistic insights into physiological and pathological processes *in vivo* have often come from *in vitro* culture systems. Conventionally, single cell populations are grown on two-dimensional (2D) substrates such as tissue culture polystyrene and experiments in this format have provided the basis for our understanding of intricate biological processes. However, these traditional culturing methods were challenged by Bissel and colleagues in the early 90's in a study demonstrating that human breast epithelial cells developed like tumor cells when cultured in 2D, but reverted to normal growth behavior when cultured in three-dimensional (3D) analogs of their native microenvironment.<sup>77</sup> Subsequently, numerous studies have shown that cells grown *in vitro* are dramatically perturbed by their new microenvironments resulting in gene expression alterations.<sup>78, 79</sup> These findings collectively reveal the insufficiency of monolayer cultures and, as

a result, scientists have developed numerous 3D scaffolds that recapitulate aspects of native cellular microenvironments for *in vitro* cell culture. These 3D models more adequately represent the complexity of an endogenous microenvironment and are therefore becoming the method of choice for more physiologically relevant modeling of cell behavior *ex vivo*.

### **The fibrin-gel angiogenesis assay**

During angiogenesis, EC undergo complex morphological changes that a 2D environment insufficiently supports. To overcome this, our lab has developed a 3D assay termed the fibrin-gel angiogenesis assay which beautifully recapitulates angiogenesis as observed *in vivo*.<sup>80</sup> In this assay EC (Figure 1.1 Ai) are coated onto Cytodex<sup>TM</sup> microbeads and embedded into a 3D fibrin matrix, an environment that is physiologically relevant to pathologic wound healing (Figure 1.1 B). To further mimic an *in vivo* microenvironment, mural cells (Figure 1.1 Aii) are seeded on top of the fibrin-gel. These support-cells secrete pro-angiogenic growth factors that, along with elements in endothelial growth media (EGM), induce angiogenesis. Over the course of approximately ten days, each step of *in vivo* angiogenesis is elegantly recapitulated including sprouting, migration, lumen formation, anastomosis and vessel network maturation (Figure 1.1 C). This assay therefore serves as a powerful tool to study molecular and cellular events during angiogenesis. For example, EC treated with inhibitors such as small interfering RNA (siRNA) or expression plasmids provide insight into the roles particular genes play during this process. In addition, EC can be isolated from the assay at specific time points to acquire mechanistic knowledge behind observed phenotypic alterations after EC manipulation.

### **The collagen I invasion and lumen assays**

Pioneers in the field of vascular biology have developed additional 3D assays to investigate particular steps of angiogenesis. Dr. George Davis and colleagues established 3D *in vitro* assays designed to scrutinize initial stages of sprouting as well as lumen formation during angiogenesis.<sup>58, 59</sup> First, the collagen I invasion assay provides an ideal platform to study preliminary steps of angiogenesis (Figure 1.2 B). In this 3D model system, EC (Figure 1.2 A) are seeded on top of collagen I gels containing pro-angiogenic cytokines thereby stimulating EC to invade into the collagen gel, a process reminiscent of tip-cell selection and migration (Figure 1.2 C). Alternatively, EC (Figure 1.3 A) are embedded into a collagen I gel spiked with cytokines to promote EC lumen formation through the development of intracellular vacuoles that coalesce to form capillary lumens and tubes (Figure 1.3 B, C). These two assays enable investigation of discrete morphologic steps and molecular events controlling EC sprouting and lumen formation. Similar to the fibrin-gel angiogenesis assay EC can be manipulated and harvested from these assays to complete gene expression analysis leading to identification of novel genes required during these processes.

### **The 3D vascularized tumor assay**

Although the assays described above sophisticatedly mimic crucial stages of *in vivo* angiogenesis, they certainly lack the complexity of a tumor microenvironment. Collaborators in the lab of Dr. Steven George have therefore developed a multicellular model that more accurately captures angiogenesis as it ensues during tumor progression. In this assay, EC and tumor cells (Figure 1.4 A) are formulated into spheroids which are then embedded in a fibrin matrix containing mural cells (Figure 1.4 A, B). Over the course of approximately seven days,

robust sprouting angiogenesis into the matrix, as well as contiguous vascularization within the spheroid is observed (Figure 1.4 C). This multicellular model facilitates tumor-directed EC behavior in a more physiologically relevant model. Importantly, this model reflects the cellular heterogeneity of native tumors in which EC and mural cells communicate directly with cancer cells to influence the progression of cancer.

## **THE SNAIL FAMILY OF ZINC-FINGER TRANSCRIPTION FACTORS**

### **Epithelial- and Endothelial-to-Mesenchymal Transitions**

The epithelial-to-mesenchymal transition (EMT) is a highly conserved cellular event that permits polarized, immotile epithelial cells to transform into motile, mesenchymal cells. During this transition epithelial cells lose apical-basal polarity, sever intercellular junctions, degrade basement membrane components and become migratory, mesenchymal-like cells. These events, however, do not necessarily occur in one particular order and not all components of EMT are present in any given example. EMT was initially observed during embryonic development in a variety of tissue remodeling events including mesoderm formation and neural crest development.<sup>81</sup> More recently, this cellular program has been implicated in promoting invasion and metastasis of many carcinomas.

A specialized form of EMT is endothelial-to-mesenchymal transition (EndMT). This particular transition has been well characterized during heart formation where a subset of endothelial cells in the developing heart acquire mesenchymal markers, invade the surrounding tissue and form the valves and septa of the adult heart.<sup>82</sup> EndMT also occurs in pathological events. In cancer, it was demonstrated that a subset of cancer-associated fibroblasts (CAFs) arose from an EndMT

mechanism.<sup>83</sup> In the case of cardiac fibrosis, fibroblasts, the cells responsible for excessive deposition of ECM and progression of the pathology, were found to originate from EC.<sup>84</sup> Additional evidence suggests that EndMT may occur in other disease settings such as chronic pulmonary hypertension, atherosclerosis, wound healing, and in both acute and chronic kidney injury.<sup>85-88</sup>

During both EMT and EndMT, genes involved in cell adhesion, migration, and invasion are transcriptionally altered. The Snail family of zinc-finger transcription factors are evolutionarily conserved proteins responsible for many of these transcription alterations. Importantly, in 2000, the zinc-finger transcription factor Snail (Snai1) was found to directly bind the E-box element of the E-Cadherin promoter leading to downregulation of E-Cadherin expression and dissolution of cell-cell adhesion, a step required during EMT.<sup>89, 90</sup> Other transcription factors have since been identified as key regulators of vertebrate EMT programs including Slug (Snai2), Zeb-1, Zeb-2 and Twist. However, Snail and Slug will be the transcription factors predominately discussed throughout this thesis.

### **Snail genes in developmental processes and cancer progression.**

Without EMT, multicellular organisms would be incapable of getting past the blastula stage of embryonic development. Pointedly, EMT in vertebrates is required for the formation of the heart, the musculoskeletal system, most craniofacial structures, and the peripheral nervous system. Two transcription factors, Snail and Slug, are well recognized as significant regulators of EMT events and are often required for these cellular programs to progress. For example, Snail knockout mice are embryonic lethal due to severe defects in gastrulation and mesoderm formation.<sup>91</sup>

Furthermore, conditional deletion of Snail mid-gestation results in embryonic lethality in part due to severe cardiovascular defects.<sup>92, 93</sup> In other vertebrates such as zebrafish and *Xenopus*, Snail is expressed in the neural crest and plays a key role in specification and migration of these cells.<sup>94, 95</sup> Finally, in chicken, Slug performs many of the functions that Snail carries out in other vertebrates.

Since the discovery of the Slug gene in chicken, homologues in several vertebrates including *Xenopus*, zebrafish, mouse, and human have been identified. In several vertebrate species, expression of Slug is implicated in regulating the formation and delamination of the mesoderm and neural crest. In chicken, Slug is expressed in cells undergoing EMT during gastrulation, neural crest formation, and limb development.<sup>96-98</sup> Slug is also required for EndMT in the heart of chicken and for EMT during neural crest emergence in both *Xenopus* and chicken.<sup>96, 99, 100</sup> However, dissimilar to many vertebrate species, the Slug gene is not essential for mesoderm or neural crest development in mice.<sup>101</sup> In fact, Slug homozygous null mutant mice are viable although they do exhibit postnatal growth deficiency among other defects.<sup>101</sup> These results indicate that neither the expression pattern nor the biological function of Slug is conserved among all vertebrates. Other defects of Slug mutant mice include a diluted coat color and areas of depigmentation, development of eye infections, and male subfertility and reduced testes size due to reduced seminiferous tubules. In humans, loss of Slug has been observed in a very rare subset of patients with Waardenburg-Shah syndrome; a disease characterized by varying degrees of deafness, minor defects in structures arising from the neural crest, and pigmentation anomalies.<sup>102</sup>

It is clear that the Snail family of zinc-finger transcription factors is essential during embryonic development. As previously described, the predominant function of Snail and Slug is to regulate EMT, which is required during developmental processes including gastrulation and neural crest formation. Interestingly, many of the cellular alterations that occur during tumor progression are reminiscent of developmental EMT. For this reason, Snail and Slug expression have been implicated in tumor progression. For example, Snail expression in breast carcinomas is associated with metastasis, tumor recurrence, and poor prognosis.<sup>103-105</sup> Similarly, Slug has been associated with poor clinical outcome in breast and ovarian tumors.<sup>104</sup> Studies in colorectal cancer show Snail overexpression likely contributes to distant metastases.<sup>106</sup> In addition, Slug overexpression in colon cancer is recognized as an independent marker for poor prognosis.<sup>107</sup> Snail and Slug overexpression have also been observed in a variety of other carcinomas including ovarian carcinoma, squamous cell carcinoma, hepatocarcinoma, and lung adenocarcinoma.

### **Induction and maintenance of Snail genes**

Snail and Slug are well recognized as being essential drivers of both developmental and pathological EMT and EndMT events. As a result, many signaling pathways that regulate Snail and Slug expression have been described. During development, Snail family members are induced by receptor tyrosine kinases (RTK), which are activated by signals such as FGF, PDGF, and epidermal growth factor (EGF). Transforming growth factor- $\beta$  (TGF- $\beta$ ), the bone morphogenetic protein (BMP) pathway, as well as Wnt and Notch signaling cascades have also been cited as inducers of Snail and Slug.<sup>108-111</sup> Furthermore, *in vitro* and *in vivo* studies confirm that similar pathways stimulate expression of these two transcription factors during EMT

associated with cancer progression.<sup>108, 110, 112, 113</sup> Finally VEGF, HGF, hypoxia, and laminin 5 have also emerged as regulating expression of Snail and Slug.<sup>114-116</sup> Given the diversity of these signals, it is easy to speculate that Snail and Slug expression depends on the process and cell type being scrutinized.

Snail and Slug are labile proteins with extremely short half-lives, approximately 25 minutes. Glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) binds to and phosphorylates Snail at two consensus motifs; phosphorylation of the first motif regulates its  $\beta$ -Trop-mediated ubiquitination, whereas phosphorylation of the second controls its nuclear export.<sup>117</sup> Hence, inactivation of GSK-3 $\beta$  via the Wnt, PI3K/Akt, or MAPK signaling cascades may promote Snail stability and nuclear import. Phosphorylation of Snail not only leads to its degradation, but can also result in its stabilization. In breast cancer cells, p21-activated kinase-1 (Pak-1) phosphorylation of Snail results in protein accumulation in the nucleus thus facilitating its repressor functions.<sup>118</sup> Unfortunately, stabilization of Slug is less studied and to date very little is known regarding phosphorylation sites, subcellular localization or degradation. However, one study found p53 suppressed cancer cell invasion through negative regulation of Slug.<sup>119</sup> MDM2, an E3 ubiquitin ligase, was identified as being induced by p53 resulting in MDM2-mediated Slug degradation. Therefore, in cancers with p53 mutations, low levels of MDM2 prevent Slug degradation leading to increased cancer invasion as a result.

### **Genes regulated by Snail family members**

Snail proteins act as molecular triggers of EMT/EndMT programs by repressing a subset of common genes that encode cadherins, claudins, cytokeratins, integrins, mucins, occludin and ZO



proteins. More specifically, Snail downregulates or alters cellular localization patterns of epithelial markers such as E-Cadherin, desmoplakin, occludin, claudin-3, ZO-1, MUC-1, and cytokeratin-17 and -18.<sup>90, 111, 120</sup> Similarly, Slug downregulates occludin, integrin  $\alpha$ -3, and E-Cadherin although to a lesser extent than Snail.<sup>121-123</sup> In addition, Slug expression causes disassembly of desmosomes.<sup>121</sup> Once these epithelial genes are repressed, mesenchymal markers including vimentin, fibronectin, and N-Cadherin are consequently upregulated.<sup>90</sup>

Although Snail and Slug were initially characterized as repressors, the upregulation of numerous genes following their expression suggests that they might also act as gene inducers. Of particular interest is the observed induction of several MMP genes subsequent to expression of Snail or Slug, although this observation is unlikely due to direct promoter activation. In many carcinomas, Snail expression increases or accelerates expression of MT1-MMP, MMP-1, MMP-2, MMP-7 and MMP-9, contributing to the invasive capabilities of epithelial cancers.<sup>112, 124-127</sup> Slug has a similar story; MT1-MMP, MT4-MMP, MMP-2, and MMP-9 expression increase as a result of Slug expression in numerous carcinomas.<sup>128-132</sup> Indeed, increased expression of these proteolytic enzymes aids in degradation of the ECM thereby contributing to a cell's enhanced ability to invade and migrate. Moreover, in Madin-Darby canine kidney (MDCK) cells, Snail and Slug expression results in upregulation of genes that encode several collagens and ECM-related proteins, such as SPARC, plasminogen activator inhibitor-1 (PAI-1), and TIMP-1.<sup>133</sup> Combined, these data strongly suggest that Snail and Slug not only function as repressors, but also as inducers of genes critical for EMT/EndMT events.

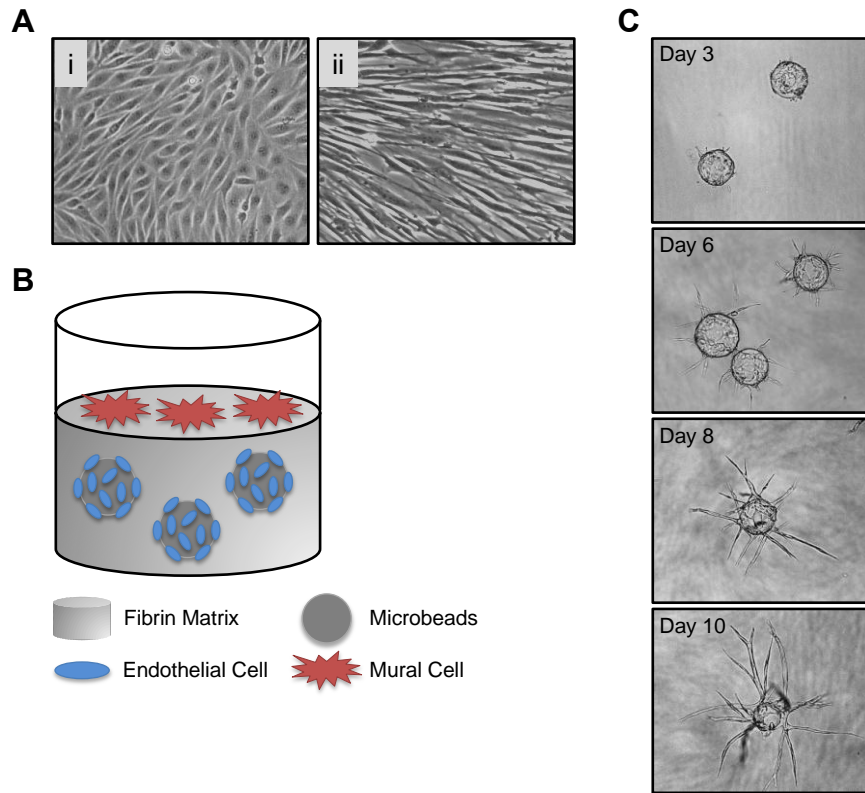
Several lines of evidence also point to a role for Snail superfamily members in regulating cell death or survival. In humans, a translocation event converts the repressor hepatic leukemic factor (HLF) into an activator that, as a result, induces Slug and leads to aberrant cell survival and to the development of leukemia.<sup>134</sup> Furthermore, haematopoietic progenitors in Slug null-mutant mice demonstrate sensitivity to death induced by gamma-irradiation.<sup>135</sup> Snail too acts as a survival factor. Examples include Snail-expressing cells surviving despite being deprived of survival factors, demonstrating resistance to direct apoptotic stimuli that signal through the death receptor, and exhibiting resistance to DNA damage.<sup>123, 136, 137</sup>

## **SUMMARY**

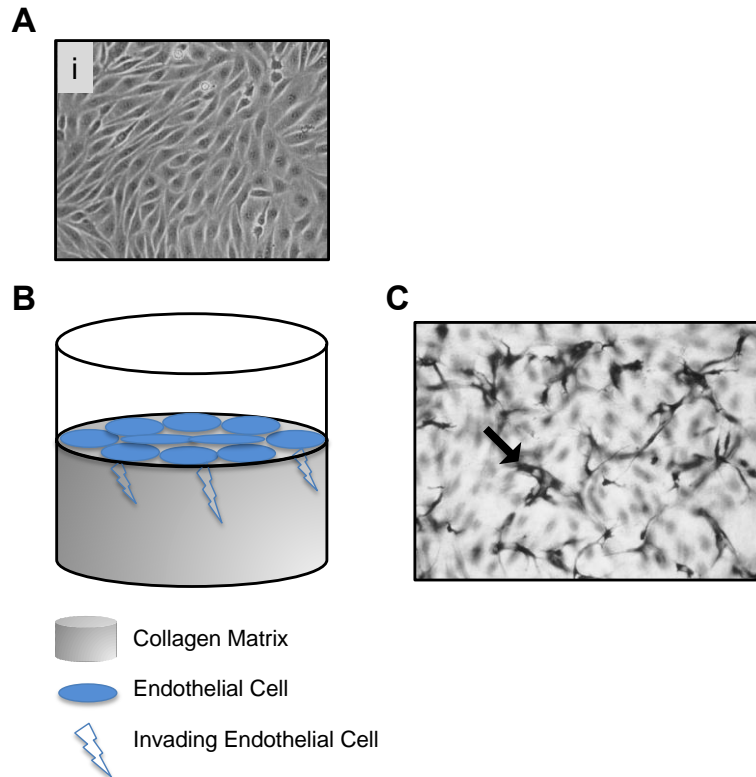
A plethora of knowledge exists about the growth factors and receptors that drive angiogenesis, and in recent years there has been an increase in knowledge about the signaling pathways downstream of these receptors. However, the transcription factors that govern new gene expression during this highly regulated process are far less studied. The following chapters provide evidence that Snail and Slug are two transcription factors expressed, regulated, and required during the angiogenic cascade. Although these transcription factors have been well documented as drivers of EMT and EndMT programs during developmental and pathological events, to our knowledge we are the first to identify that Snail and Slug are required during angiogenesis. Indeed, expression of these genes has been observed in angiogenic EC, however, a functional role for Snail and Slug during angiogenesis has not been elucidated until now.

Here we characterize the expression patterns of Snail and Slug in EC undergoing angiogenesis and provide preliminary data on their individual functions. In particular, we show that inhibition

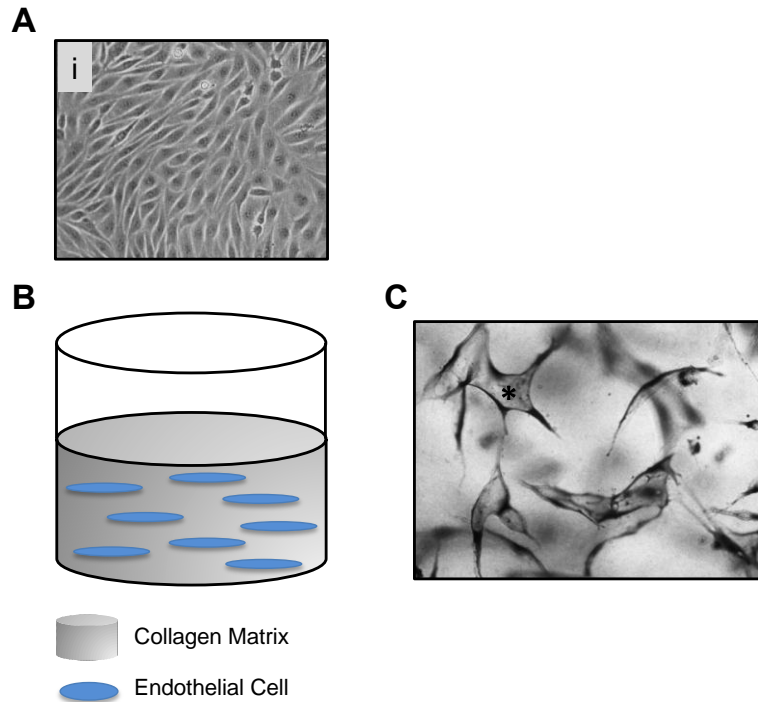
of either transcription factor reduces sprouting and lumen formation in several *in vitro* angiogenesis assays, indicating a lack of redundancy. Our data identify an underlying cellular mechanism in which Slug regulates sprouting angiogenesis through MT1-MMP, although this is unlikely due to direct transcriptional regulation. Furthermore, a decrease in MT1-MMP expression results in reduced activity of MMP-2 in the absence of Slug. As these two proteases are required for angiogenesis to proceed and due to the fact their expression is increased in Slug-expressing carcinomas, our findings are logical and in line with existing data. We also show that inhibition of Snail results in reduced activity of both MMP-2 and MMP-9 although additional studies are required to determine a definitive mechanism behind the actions of Snail in angiogenic EC.



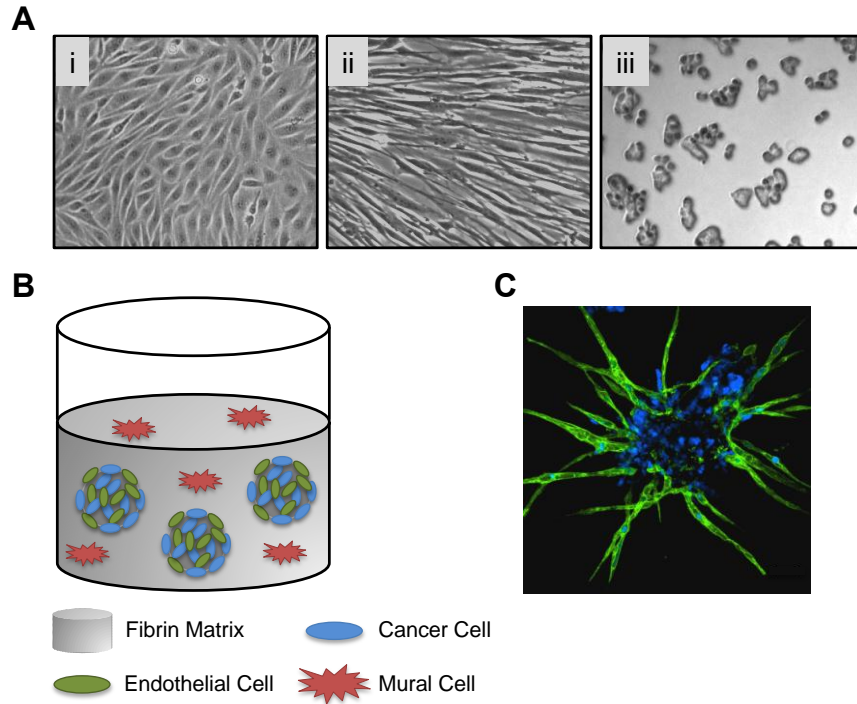
**Figure 1.1. Fibrin-gel angiogenesis assay.** (A) Monolayer culture images of cells used in the fibrin-gel angiogenesis assay. (i) Human umbilical vein endothelial cells (HUVEC) are the most common endothelial cells (EC) used in the assay. (ii) Normal human lung fibroblasts (NHLF) are the most common mural cell used in the assay. (B) Schematic representation of the fibrin-gel angiogenesis assay. (C) Representative images of the fibrin-gel angiogenesis assay over the course of ten days. On day three, early events of sprouting and migration are observed. During days four through eight, vessels continue to migrate, sprout, and branching begins. By day ten, patent lumens have formed, neighboring sprouts have anastomosed, and a mature network is observed.



**Figure 1.2. Collagen I invasion assay.** (A) Monolayer culture image of (i) human umbilical vein endothelial cells (HUVEC), the most common endothelial cells (EC) used in the assay. (B) Schematic representation of the collagen I invasion assay. (C) Representative image (top view) of the assay after 24 hours of EC invasion. Arrow indicates an invading EC.



**Figure 1.3. Collagen I lumen assay.** (A) Monolayer culture image of (i) human umbilical vein endothelial cells (HUVEC), the most common endothelial cells (EC) used in the assay. (B) Schematic representation of the collagen I lumen assay. (C) Representative image (top view) of the assay after 24 hours of EC lumenogenesis. Asterisk indicates an intercellular lumen.



**Figure 1.4. 3D vascularized tumor assay.** (A) Monolayer culture images of cells used in the 3D vascularized tumor assay. (i) Human umbilical vein endothelial cells (HUVEC) are the most common endothelial cells (EC) used in the assay. (ii) Normal human lung fibroblasts (NHLF) are the most common mural cell used in the assay. (iii) SW620s are one of many cancer cells lines that can be used in the assay (B) Schematic representation of the 3D vascularized tumor assay. (C) Representative image of one EC-SW620 spheroid on day seven of the assay; a point when EC sprouting and lumen formation are robust.

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## **CHAPTER 2**

### **Endothelial-mesenchymal transitions**

Katrina M. Welch-Reardon<sup>1,a</sup>, Nan Wu<sup>1,a</sup>, and Christopher C.W. Hughes<sup>1,2,3,v</sup>

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<sup>a</sup>These authors contributed equally to this work. <sup>1</sup>The Department of Molecular Biology and Biochemistry, <sup>2</sup>The Department of Biomedical Engineering, <sup>3</sup>Edwards Lifesciences Center for Advanced Cardiovascular Technology, University of California Irvine, Irvine, CA 92697

Accepted for publication in *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2014.

#### **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 (EndMT) contribute to heart valve formation, the generation of cancer-associated-fibroblasts, and the angiogenic sprouting that supports tumor growth and metastasis. A key regulatory point in these processes determines whether cells undergo a full or a partial EMT /EndMT, however, very little is known about how this switch is controlled. Here we discuss these two developmental/pathologic pathways, with a particular focus on their role in vascular biology.

## **INTRODUCTION**

Morphological changes in tissues are invariably associated with phenotypical 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 transcriptional reprogramming that leads to significant changes in morphology and function. One class of such changes is called the epithelial-to mesenchymal transition (EMT), and subsets of EMT include endothelial-to-mesenchymal transition (EndMT) as well as partial EMT/EndMT (pEMT/EndMT). Our focus will be to highlight the distinctions among the subsets, with an emphasis on angiogenesis as a unique example of pEndMT.

### **Epithelial-to-mesenchymal transitions**

EMT is a highly conserved cellular reprogramming event in which polarized, non-migratory epithelial cells transition into motile, mesenchymal-like cells. During this transition epithelial cells lose apical-basal polarity, sever intercellular junctions, degrade basement membrane components and migrate into the surrounding tissue. This transition is initiated by key EMT-inducing transcription factors including Snail (Snai1), Slug (Snai2), Twist, and Zeb1 and 2. EMT was first described as a necessary and critical tissue remodeling event that occurs during embryonic development, including the stages of mesoderm and neural crest formation<sup>1</sup>. Aside from its importance during development, EMT is also observed during physiological and pathological events such as wound healing, organ fibrosis and cancer. Specifically, EMT is implicated in promoting invasion and metastasis in many epithelial-derived cancers, and is thought to be critical for the early, intravasation stage of metastasis.

## **Endothelial-to-mesenchymal transitions**

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 (EndMT). During embryogenesis subsets of EC in the developing heart undergo EndMT, acquire mesenchymal markers, invade the surrounding tissue and form the valves and septa of the adult heart<sup>2</sup>, a process that involves transforming growth factor- $\beta$  (TGF- $\beta$ ), bone morphogenetic protein (BMP) and Notch signaling pathways<sup>3, 4</sup>. Pathologically, EndMT is reactivated in the adult heart, contributing to cardiac fibrosis<sup>5</sup>, 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 EndMT in this process<sup>5</sup>. Importantly, EndMT was TGF- $\beta$ 1-dependent, whereas BMP-7 preserved the EC phenotype and consequently reduced fibrosis<sup>5</sup>. EndMT has also been implicated as a source of fibroblasts in hypertrophic cardiomyopathy<sup>6</sup>, diabetes-induced cardiac fibrosis<sup>7</sup>, and chronic pulmonary hypertension<sup>8, 9</sup>, although these studies lacked definitive lineage-tracing analyses.

There is also evidence supporting a role for EndMT during both acute and chronic kidney injury<sup>10</sup>. In three distinct mouse models of chronic kidney disease approximately 30 to 50% of fibroblasts co-expressed the EC marker CD31 along with markers of myofibroblasts and fibroblasts, including fibroblast specific protein-1 (FSP-1) and  $\alpha$ -SMA. Lineage tracing experiments confirmed the EC origin of these cells<sup>10, 11</sup>. Thus, EndMT provides a source of fibroblasts in both damaged heart and kidney, and may function to facilitate tissue remodeling and fibrosis.

Finally, EndMT also has a significant role to play in cancer. For example, Zeisberg and colleagues, using two different mouse models of cancer, demonstrated that EndMT accounts for up to 40% of cancer associated fibroblasts (CAFs)<sup>12</sup>. A distinct population of fibroblasts co-expressed the EC marker CD31 along with either FSP-1 or  $\alpha$ -SMA. 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 EndMT is a significant source of CAFs in tumors.

### **Partial EMT and EndMT**

When epithelial/endothelial cells commit to a mesenchymal phenotype, as described above, the event is designated as a complete EMT/EndMT. Partial EMT/EndMT is also possible, and this occurs when one or more of the key characteristics of complete-EMT/EndMT is 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 (BM); however, these cells retain some intercellular junctions and migrate as a cohesive cell sheet<sup>13</sup>. This process has thus been termed a partial-EMT. Partial-EMT has also been implicated in branching morphogenesis during the formation of the mammary glands, kidneys and trachea<sup>14</sup>. Additional data point to a role for the EMT-transcription factor Slug in promoting a partial-EMT during Madin-Darby canine kidney (MDCK) cell tubulogenesis, at least in part by promoting cell survival. Furthermore, these chains of epithelial cells retained some cell-cell junctions and did not acquire full mesenchymal characteristics, confirming this morphogenesis process as a partial-EMT<sup>15</sup>. These findings in epithelial tubule-forming assays

have prompted speculation that endothelial sprouting and tube formation during angiogenesis may likewise be driven by a partial EndMT.

### **Angiogenesis: a partial EndMT**

Angiogenesis, the formation of new blood vessels from the pre-existing vasculature, is essential during development and many normal physiological processes, but is also important in numerous pathological processes, including tumor growth. Although much is known about the growth factors, receptors and signaling pathways that govern angiogenesis, the transcriptional changes that govern new gene expression patterns remain to be elucidated. Interestingly, comparison of angiogenesis and EMT reveals several similarities. Among these, the tip cells that lead emerging sprouts lack apical-basal polarity, degrade both BM and extracellular matrix (ECM) and, by definition, are migratory. However, angiogenic EC do not usually separate from their neighbors, suggesting that angiogenesis may involve a partial EndMT<sup>16, 17</sup>. Our lab has recently published preliminary evidence demonstrating that the transcription factors Snail and Slug are indeed expressed and regulated by angiogenic EC during *in vitro* angiogenesis<sup>16</sup>. We demonstrated that inhibition of Snail or Slug expression results in a reduced ability of angiogenic EC to invade and migrate through multiple ECM 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 MT1-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, breast<sup>18</sup> and ovarian carcinoma<sup>19</sup>. Finally, we have preliminary data suggesting that Slug deficiency in mice leads both to impaired developmental and pathological angiogenesis (KMWR, NW and CCWH, unpublished data). In aggregate, these data clearly point to a role for the Snail family of transcription factors during angiogenesis.

### **Signaling pathways governing EMT and EndMT**

Members of the TGF- $\beta$  superfamily are considered some of the major regulators of EMT and EndMT. The activation of TGF- $\beta$  signaling through Smad-dependent and independent pathways leads to direct transcriptional regulation of multiple genes, including several EMT-inducing transcription factors<sup>20</sup>. Expression of these transcription factors subsequently drives loss of cell-cell adhesion by repression of epithelial/endothelial tight junction gene transcription, regulation of cytoskeletal rearrangement, and increased expression and activity of both MT-MMPs and secreted MMPs<sup>21</sup>. Moreover, during EndMT, upregulation of EC Slug by TGF- $\beta$  and other growth factors results in increased migration and invasion into multiple ECM matrices, and this is due in part to the indirect activation of MT1-MMP, MMP-2 and MMP-9<sup>16</sup>. Interestingly, nuclear Smads form multi-protein complexes with EMT-transcription factors resulting in suppression or activation of promoters of epithelial (E-Cadherin, Occludin, ZO-1) or mesenchymal (Vimentin, N-Cadherin) genes, respectively<sup>22</sup>. TGF- $\beta$  can also activate Smad-independent pathways such as MAPK/ERK/JNK, all of which are implicated in driving EMT<sup>20</sup>.

Aside from TGF- $\beta$ , several other signaling pathways are also associated with EMT. 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  $\beta$ -Catenin correlates with the level of hypoxia-induced factor 1 alpha (HIF-1 $\alpha$ ), and HIF-1 $\alpha$ -induced EMT<sup>23</sup>. The degree of hypoxia-induced EMT can also be enhanced by Wnt3a-induced activation of  $\beta$ -catenin in hepatic carcinoma<sup>24</sup>. Furthermore, it has been demonstrated that canonical Wnt signaling stabilizes Slug expression through regulating glycogen synthase kinase 3- $\beta$  (GSK3- $\beta$ ) phosphorylation and  $\beta$ Tcrp-1-mediated ubiquitination, thereby inducing EMT in

triple-negative breast cancer<sup>25</sup>. In contrast, the role of non-canonical Wnt signaling in EMT and cancer metastasis remains controversial. While many studies demonstrate that expression of Wnt5a, a representative non-canonical Wnt ligand, promotes EMT in some cancers<sup>26-28</sup>, others provide evidence that Wnt5a attenuates canonical Wnt signaling, thus preventing EMT and consequently the ability to predict a better prognosis in colon cancer patients<sup>29, 30</sup>.

Notch activation is linked to both EMT and EndMT events. The cleavage and nuclear translocation of the Notch intracellular domain (NICD) can induce genetic reprogramming and hence a series of morphological and functional changes related to a mesenchymal transition<sup>31</sup>. Notch can suppress epithelial gene expression directly or through upregulation of Snail and Slug in both epithelial cells and EC, and thus initiate EMT and EndMT in both developmental and pathological conditions<sup>32-34</sup>. Notch ligands can also be induced by TGF- $\beta$  signaling to activate Notch receptors and enhance EMT synergistically<sup>35</sup>. Blockage of either Jagged-1, or its downstream signaling target Hey-1, can attenuate TGF- $\beta$ -induced EMT in mammary gland, kidney tubule and epidermal epithelial cells<sup>33, 36</sup>.

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 HIF pathways leads to increased “stemness” of cancer cells, self-renewal ability and a complete EMT<sup>33, 37</sup>. 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 pEndMT in angiogenic tumor

EC leading to the formation of unstable, leaky vessels<sup>38</sup>. Altered vessel integrity and permeability correlates with enhanced tumor cell dissemination to distant sites<sup>39</sup>.

Notch-mediated EMT is unusual, and somewhat paradoxical, as it is contact-dependent. Importantly, the ability of cells to retain cell-cell adhesion complexes while migrating as a group is crucial to tubulogenesis. As described above, processes involving tubulogenesis, such as angiogenesis and kidney tubule formation, both require a partial EMT/EndMT, during which the participating cells temporarily lose polarity and gain migratory capacity, but never fully acquire all mesenchymal phenotypes, nor completely lose cellular adhesion. While other signaling pathways such as TGF- $\beta$ , HGF and FGF are capable of promoting this process, it is intriguing to speculate that Notch activation is a crucial determinant of a partial versus full EMT/EndMT.

Aside from the major signaling pathways discussed above, miRNA, epigenetic regulation and histone modification have also recently emerged as regulators of EMT. 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 the expression and/or activity of the miRNA, or histone modifying enzymes<sup>30, 32</sup>. Clearly, the relationship(s) between the master regulators governing EMT are extremely complex<sup>20, 22</sup>.

### **Transcription factor interactions governing EMT and EndMT**

Snail, Slug, ZEB1/2 and Twist have been identified as the key transcriptional regulators of EMTs and EndMTs. A shared function of these proteins is their ability to repress the transcription of E-cadherin, however, numerous studies have demonstrated that they have overlapping but non-



redundant roles in EMT and tumor progression. In human carcinomas it is generally accepted that Snail plays a major role in inducing EMT, while Zeb1/2 and Twist are mainly involved in maintaining the invasive mesenchymal phenotype<sup>21</sup>. However, our recent study on EndMT suggests that at least in the case of sprouting angiogenesis, Slug is the primary initiator of this process while the induction of Snail occurs at a much later time<sup>16</sup>. It is therefore unclear if each of these transcription factors has a distinct and specific role during EMT/EndMT 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 non-linear map that suggests these transcription factors mostly act in concert. For example, Snail can upregulate Zeb1 and Zeb2 in oral squamous carcinoma and, at the same time, negatively regulate its own expression through direct promoter binding<sup>40, 41</sup>. Moreover, Slug indirectly upregulates Snail through EGF and/or HGF signaling, thereby promoting mammary gland branching morphogenesis<sup>42</sup>. Slug can also activate Zeb1 and its own expression through direct transcriptional regulation<sup>43, 44</sup>. In addition, many have shown that Twist1 can regulate the expression level of Snail and Slug by either directly influencing transcription<sup>45, 46</sup> or through post-translational regulation via the NF- $\kappa$ B/GSK-3 $\beta$  axis<sup>47</sup>.

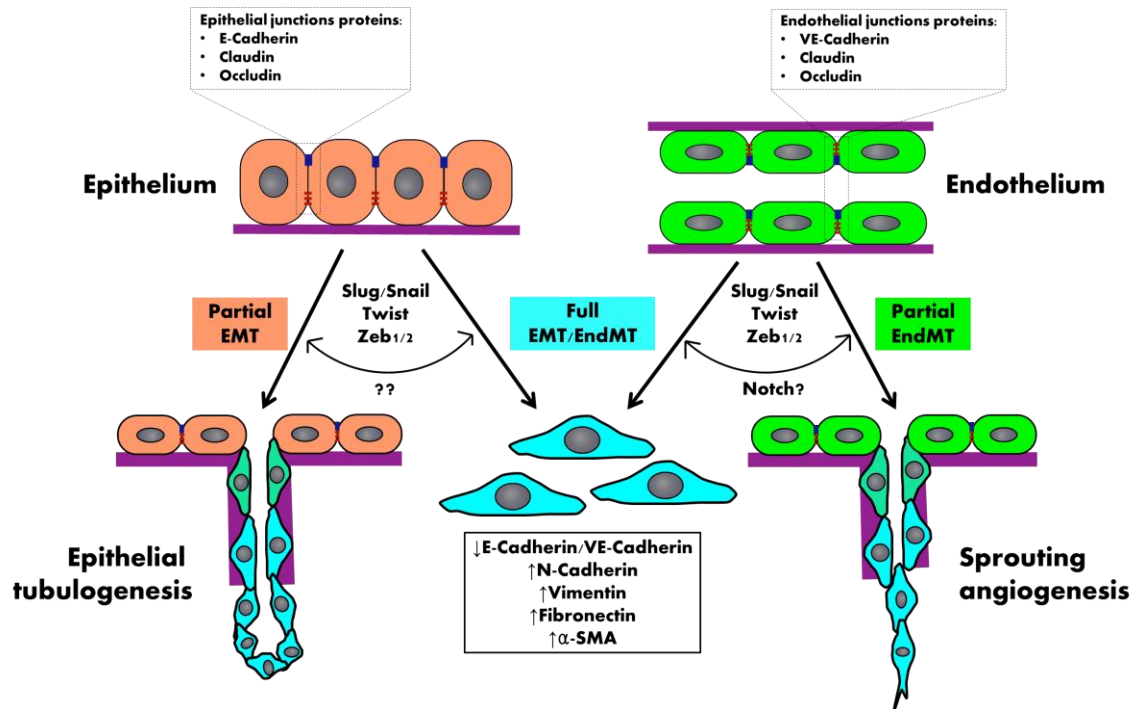
### **Dynamic functions of EMT and EndMT transcriptional regulators**

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/EndMT-like phenotypes in cells without complete loss of membrane E-cadherin – a partial EMT. Likewise, the deletion of E-cadherin alone is not sufficient to induce EMT. Interestingly, Shamir et al demonstrated that the induction

of Twist alone is sufficient to induce single cell dissemination/local invasion without the loss of epithelial identity, and that E-Cadherin expression is required for this process<sup>48</sup>. Moreover, in the absence of E-Cadherin, and despite a reduction in multiple classes of junction proteins, these rounded epithelial cells invade the surrounding matrix as a chain rather than single cells<sup>48</sup>. Similarly, we observed that overexpression of Slug in EC promotes EC sprouting, a process reminiscent of a partial EndMT without altering the mRNA levels or surface expression of vascular endothelial-cadherin (VE-Cadherin), the EC equivalent of E-Cadherin<sup>16</sup>. Finally, Leroy et al. and others have previously shown that Slug upregulation prevents apoptosis and promotes cell proliferation through p53<sup>49</sup>. Collectively these data suggest that master regulators of EMT serve more functions than simply acting as repressors of epithelial genes.

### **Remaining questions and perspectives**

The Snail/Slug, Zeb and Twist transcription factors all seem to have non-redundant roles in the developmental processes involving EMT/EndMT. However, whether one or all of these genes is required for cells to acquire and maintain a complete mesenchymal phenotype in pathological processes remains to be elucidated. Further characterization of the individual steps of EMT/EndMT, comparisons of specific functions, and observations of spatial and temporal expression patterns of each of these transcription factors will inevitably provide insight into their roles during both full and partial EMT/EndMT. It is plausible that the requirement for each transcription factor to induce full or partial EMT may be tissue or cell type specific, dependent on the lineage of the cells, or perhaps is preprogrammed during development. A still unanswered question is how important these genes are in angiogenesis and tubulogenesis and how they act to maintain a partial EndMT.



**Figure 2.1 Complete vs. Partial EMT/EndMT.** Epithelial and endothelial cells comprise the quiescent epithelium and endothelium respectively and utilize junctional proteins to maintain connections. Once transcriptional reprogramming is initiated, an event led by the EMT/EndMT-transcription factors Slug, Snail, Twist and Zeb1/2, the epithelial/endothelial cells lose apical-basal polarity, sever intercellular junctions and become motile cells. However, the regulatory signal(s) that determine whether these cells undergo a complete EMT/EndMT or partial EMT/EndMT remains unclear. In the case of sprouting angiogenesis, the unique contact-dependent nature of the Notch signaling pathway holds great potential to be a gateway for such decisions.

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## **CHAPTER 3**

### **Angiogenic sprouting is regulated by endothelial cell expression of Slug (Snai2)**

Katrina M. Welch-Reardon<sup>1</sup>, Seema M. Ehsan<sup>3</sup>, Kehui Wang<sup>4</sup>, Nan Wu<sup>1</sup>, Andrew C. Newman<sup>1</sup>, Monica Romero-Lopez<sup>2</sup>, Ashley H. Fong<sup>1</sup>, Steven C. George<sup>2,3,5</sup>, Robert A. Edwards<sup>4</sup>, and Christopher C.W. Hughes<sup>1,2,5</sup>

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<sup>1</sup>The Department of Molecular Biology and Biochemistry, <sup>2</sup>The Department of Biomedical Engineering, <sup>3</sup>The Department of Chemical Engineering and Materials Science, <sup>4</sup>The Department of Pathology and Laboratory Medicine, and <sup>5</sup>The Edwards Lifesciences Center for Advanced Cardiovascular Technology, University of California Irvine, Irvine, CA 92697

Published in the Journal of Cell Science. 2014 May 1;127(Pt 9):2017-28.

#### **ABSTRACT**

The Snail family of zinc-finger transcription factors are evolutionarily conserved proteins that control processes requiring cell movement. Specifically, they regulate epithelial-to-mesenchymal transitions (EMT) where an epithelial cell severs intercellular junctions, degrades basement membrane and becomes a migratory, mesenchymal-like cell. Interestingly, Slug expression has been observed in angiogenic endothelial cells (EC) *in vivo*, suggesting that angiogenic sprouting may share common attributes with EMT. Here we demonstrate that sprouting EC *in vitro* express Slug, and that siRNA-mediated knockdown of Slug expression inhibits sprouting and migration in multiple *in vitro* angiogenesis assays. We find that expression of MT1-MMP, but not VE-Cadherin, is regulated by Slug and that loss of sprouting as a consequence of reduced Slug expression can be reversed by lentiviral-mediated re-expression of MT1-MMP. Activity



of MMP-2 and MMP-9 are also affected by Slug expression, likely through MT1-MMP. Importantly, we find enhanced expression of Slug in EC in human colorectal cancer samples compared to normal colon tissue, suggesting a role for Slug in pathological angiogenesis. In summary, these data implicate Slug as an important regulator of sprouting angiogenesis, particularly in pathological settings.

## INTRODUCTION

Angiogenesis is a multi-step, tightly-regulated process that plays a critical role during embryogenesis and wound healing, as well as in pathological conditions such as tumor growth.<sup>1-3</sup> During sprouting angiogenesis, endothelial cells (EC) are activated in response to angiogenic stimuli, the best characterized being vascular endothelial growth factor (VEGF).<sup>2, 4</sup> EC-activation triggers a cascade of events including degradation of the adjacent basement membrane, migration of nascent sprouts into the surrounding extracellular matrix (ECM), formation of lumens, branching, anastomosis, and a return to quiescence once support cells have been recruited to the newly-formed vessel.<sup>1, 2, 4</sup> Initiation of sprouting requires generation of at least two distinct EC phenotypes – tip cells and trunk cells. Each assumes a different morphology and performs unique functions. A tip cell leads the sprout; it is polarized along its anterior-posterior axis, rarely proliferates and is highly migratory.<sup>5-8</sup> Trunk cells trail tip cells; they are proliferative, apically-basally polarized, and form the vessel lumen<sup>9</sup>. Gene expression profiles reveal tip cells to be highly enriched in vascular endothelial growth factor receptor-2 (VEGFR-2)<sup>7-10</sup>, platelet-derived growth factor-B (PDGFB)<sup>8, 9</sup>, neuropilin receptor-2 (NRP-2)<sup>8</sup>, Jagged-1 (Jag-1)<sup>8, 11</sup>, membrane type-1 matrix metalloproteinase (MT1-MMP)<sup>12, 13</sup>, and delta-like 4 (Dll4)<sup>6, 14</sup>. Expression of tip cell genes and induction of angiogenic sprouting are stimulated and regulated by pro-angiogenic cytokines including VEGF<sup>2, 9</sup>, tumor necrosis factor-alpha (TNF- $\alpha$ )<sup>8, 15</sup>, transforming growth factor-beta (TGF- $\beta$ )<sup>15</sup>, fibroblast growth factor (FGF)<sup>2, 15</sup>, and hepatocyte growth factor (HGF)<sup>16</sup>. During pathological events such as inflammation and tumor growth, several of

these growth factors induce expression of the transcription factor Slug (Snai2), and expression of this gene in tumor cells contributes to invasion and metastasis.<sup>17-19</sup>

The Snail family of zinc-finger transcription factors are evolutionarily conserved and involved in processes that require cell movement. Expression of these genes is essential during embryonic development in events such as mesoderm, neural crest, and heart cushion formation.<sup>20, 21</sup> During epithelial-to-mesenchymal transitions (EMT), Slug acts as a transcriptional repressor by binding E-box elements in target promoters. Under certain conditions Slug represses transcription of genes involved in formation of both adherens junctions (E-Cadherin), and tight junctions (claudins, occludins, ZO-1), and promotes disassembly of desmosomes.<sup>19, 21, 22</sup> Slug also indirectly induces expression of genes that degrade ECM such as matrix metalloproteinases (MMP).<sup>19, 23, 24</sup> A specialized form of EMT is an endothelial-to-mesenchymal-transition (EndMT). This event was first observed in developmental studies of heart formation<sup>25</sup>, and studies in the heart continue to reveal mechanistic insights, including a role for Notch signaling and induction of Slug during EndMT<sup>20</sup>. Interestingly, Slug expression is upregulated in tumor-associated EC<sup>26</sup> and EndMT has been identified as an origin of cancer associated fibroblasts<sup>27</sup>. Here we provide evidence that Slug is expressed in angiogenic EC and is a critical mediator of angiogenic sprouting. Interestingly, we find that Slug regulates expression of MT1-MMP but not VE-cadherin, and that while it promotes EC migration it does not lead to a loss of EC-EC junctions or the separation of EC from their neighbors. Collectively, these studies suggest that Slug expression in EC promotes only a partial EndMT during angiogenesis.

## RESULTS

### **Slug expression is temporally regulated during *in vitro* angiogenesis**

In order to study the mechanisms regulating EC morphogenesis we use an *in vitro* angiogenesis model<sup>28</sup> in which EC sprout into fibrin gels. The assay recapitulates several critical steps of angiogenesis, including sprouting, lumen formation, branching and anastomosis (Figure 2.1 A). Using this assay we analyzed Slug expression in angiogenic EC at several time points up to 10 days, a point at which extensive, lumenized sprouts are present. Slug mRNA expression is strongly induced on day 3 when sprouts first begin to emerge from the beads, and remains highly expressed up to day 6, the time at which protein expression is highest (Figure 2.1 B, C). At this point lumen formation begins to dominate the cultures, with fewer new sprouts emerging, and this correlates with a slow decline in Slug expression out to 10 days (Figure 2.1 B, C). Thus, in an *in vitro* assay that mimics pathologic and/or wound healing angiogenesis, Slug expression in EC correlates with neovessel sprouting.

### **Tumor-associated blood vessels in multiple cancers express Slug**

To examine whether Slug is expressed in EC during pathologic angiogenesis *in vivo* we first surveyed cancer tissues stained for Slug in the Human Protein Atlas Database ([www.proteinatlas.org](http://www.proteinatlas.org)). We observed Slug expression in vessels of gliomas (patient ID: 3120 and 3174), breast carcinomas (patient ID: 1882 and 2091), squamous cell lung carcinomas (patient ID: 1765, 1428, and 2231), liver carcinomas (patient ID: 2279, 2280, and 887), and colon adenocarcinomas (patient ID: 2060 and 2106), among others. Slug expression was not exclusive to vessels, however, as many of the tumor cells were also

Slug-positive. To confirm that Slug is expressed in the EC of pathologic vessels, we obtained samples of normal human colon and colorectal cancer (CRC) and used double-labeling immunohistochemistry to look for Slug expression in CD31 positive EC. As shown in Figure 2.1 D, EC lining normal vessels only rarely express Slug. In sharp contrast, we found numerous Slug positive EC in blood vessels in the reactive stroma, within and adjacent to colorectal tumor tissue. Some perivascular cells (possibly pericytes) were also positive in some vessels. Non-vascular cells expressing Slug, in both normal and tumor tissues are likely to be pericryptal myofibroblasts. We quantitated these findings and found less than 1% of vessels in normal tissues containing Slug-positive EC, whereas in two CRC tumors examined the proportion of Slug-positive vessels was 44% and 55%. We also examined vessels in an orthotopic, syngeneic (CT26) mouse colorectal cancer model, and here again we observed Slug staining in the vessels (Figure 2.1 Div).

### **Loss of Slug inhibits EC sprouting**

To determine if Slug is required for vessel formation we utilized small interfering RNA (siRNA) oligonucleotides to inhibit Slug expression in several *in vitro* angiogenesis assays. We first confirmed that targeting Slug with siRNA in EC resulted in robust inhibition of mRNA and protein expression (Figure 2.2 A, B). Next we examined the effect of Slug knockdown on the ability of EC to sprout into fibrin gels, and consistently observed a dramatic loss of sprout formation (Figure 2.2 Ci, Cii, D). In addition, those sprouts that did form appeared to have a reduced ability to form lumens (Figure 2.2 Ci, Cii, E), a finding we confirmed in a second assay<sup>29</sup> that specifically models lumen

formation (see below). Importantly, Slug knockdown was still >60% at the mRNA level on day 5, the latest time at which phenotypes were quantified (Figure 2.2 C).

To confirm the loss of sprouting in a second assay we looked at the ability of control or Slug knockdown EC to invade collagen I gels in response to pro-angiogenic chemokines<sup>29</sup>. Again, loss of Slug severely limited EC sprouting (Figure 2.2 Ciii, Civ, F). To rule out off-target effects of the siRNA, we obtained a second, independent sequence (Ambion) and repeated this assay. Once more, siRNA-mediated loss of Slug expression strongly inhibited EC sprouting (Figure S2.1 D, E). Thus, Slug expression is necessary for sprouting in both fibrin and collagen gels.

Our data showing a role for Slug during EC sprouting into fibrin gels suggest that it may be particularly important during pathologic angiogenesis – indeed, it is already known from mouse knockout studies to be dispensable for developmental angiogenesis<sup>30</sup>. We therefore turned to an *in vitro* 3D vascularized tumor model to explore the role of Slug further. Co-cultures of EC transfected with either control or Slug siRNA, and colon cancer SW620 cells transduced to express GFP, were formulated into multicellular spheroids and embedded in fibrin gels distributed with fibroblasts. After 7 days, tissue constructs were fixed and tumor vessel networks were assessed. In the absence of Slug expression we observed fewer sprouts compared to control cultures and, when EC did form sprouts, less than 20% of vascularized spheres had greater than 5 vessels; 70% less than control (Figure 2.2 Cv, Cvi, G, H). The average total vessel length was also significantly decreased in the absence of EC Slug expression (Figure 2.2 I). Collectively,

these data demonstrate that Slug is critical during angiogenesis in the pathological setting of an *in vitro* 3D tumor.

### **Slug regulates lumen formation**

Several mechanisms have been suggested for the formation of lumens during angiogenesis and the likelihood is that different mechanisms may pertain to large and small vessels, and developmental and pathologic processes.<sup>31, 32</sup> A widely accepted mechanism for lumen formation in small vessels involves formation of intracellular pinocytic vesicles, the fusion of these into larger intracellular vacuoles, and finally, the joining of these between neighboring EC to form a contiguous intercellular luminal space.<sup>31</sup> This is the process we see most often *in vitro*. To examine the role of Slug in EC undergoing lumen formation, we used an assay originally devised by the Davis lab in which EC are induced to form lumens in collagen gels.<sup>29</sup> As shown in Figure S2.2, knockdown of Slug reduced both mean luminal area as well as number of lumens per high-power field (Figure S2.2 A-C). Again, we confirmed this finding using a second, independent siRNA (Figure S2.2 A, D, E). We next assessed early stages of lumen formation by quantifying the number of intracellular vesicles in control and Slug knockdown-EC in the presence of FITC-dextran – FITC-dextran is incorporated into the newly formed pinocytic vacuoles.<sup>33</sup> We found no difference between control and Slug-knockdown EC suggesting that Slug's effects on lumen formation are downstream of the early, vesicle-forming stage, and likely at the stage of intercellular lumen formation (Figure S2.2 F-H).

## **Inducers of Slug expression in EC**

To gain insight into the induction of Slug expression, we tested several pro-angiogenic growth factors known to be present in our *in vitro* angiogenesis models. Some of these were added to the medium and the fibroblasts provide several more.<sup>34</sup> We therefore tested the ability of these individually, or in combination, to induce Slug mRNA and protein in monolayer cultures (Figure S2.3). Several factors induced moderate Slug expression when tested independently, and more robust expression when used in combination. These data suggest that the expression of Slug depends on integration of multiple signals, potentially including those derived from the 3D microenvironment.

## **Slug misexpression promotes sprouting**

To determine whether forced expression of Slug would promote sprouting and whether Slug-expressing EC sprout preferentially, EC were transduced with Slug lentivirus in which Slug was directly linked to copGFP via the self-cleaving peptide T2A permitting visualization of Slug expression (these cells are referred to as EC<sup>Slug/GFP</sup>). A second set of EC were transduced with copGFP lentivirus lacking Slug and these served as a control (referred to as EC<sup>GFP</sup>). EC<sup>Slug/GFP</sup> exhibited overexpression of Slug compared to EC<sup>GFP</sup> and untransduced EC (EC<sup>Control</sup>) as confirmed by Western blot (Figure 2.3A). We then tested these cells in the fibrin gel angiogenesis assay. Compared to EC<sup>GFP</sup> the EC<sup>Slug/GFP</sup> cells showed a dramatic increase in their ability to form sprouts (Figure 2.3 B, D). Thus, Slug expression can drive angiogenic sprouting.



To test whether this effect is cell-autonomous, we mixed EC<sup>Slug/GFP</sup> with EC<sup>Control</sup> at different ratios and again looked at sprouting in the fibrin gel angiogenesis assay, comparing this mixture to the same ratios of EC<sup>GFP</sup> with EC<sup>Control</sup>. As shown in Figure 2.3 D, at each ratio (10%, 25% and 100% EC<sup>Slug/GFP</sup>) there was more sprouting compared to the cultures containing 10%, 25% or 100% EC<sup>GFP</sup>. Interestingly, there were a disproportionate number of sprouts containing GFP-positive cells in 10% and 25% EC<sup>Slug/GFP</sup> cultures compared to EC<sup>GFP</sup> cultures of the same percentages (Figure 3 F). Indeed, almost all of the sprouts in 25% EC<sup>Slug/GFP</sup> cultures contained Slug-positive cells and almost all of the cells within the sprout were Slug-positive (Figure 2.3 F, C). While the expression of Slug clearly pre-disposes EC to sprout, these data also suggest that Slug-expressing cells may suppress neighboring cells from sprouting (see Discussion). We also noted a secondary phenotype resulting from Slug expression – the detachment of sprouts from the beads, which became progressively more apparent at higher ratios of Slug-expressing cells (Figure 2.3 E, C).

### **Loss of Slug reduces MT1-MMP expression but does not affect VE-Cadherin**

In epithelial cells, genes of the Snail family regulate expression of E-Cadherin, and thereby the ability of cells to release from each other (EMT). We therefore examined the expression of VE-Cadherin (the EC equivalent of E-Cadherin) in Slug knockdown-EC during sprouting into fibrin gels. Interestingly, we saw no change in the mRNA expression of this gene using either of the siRNAs (Figures 2.4 A, S2.1 C). In addition, we evaluated VE-Cadherin protein localization in EC undergoing vessel formation in the absence of Slug expression and saw no differences compared to control (Figure 2.4 C).

This is consistent with our finding that misexpression of Slug does not lead to a loss of EC junctional integrity (Figure 2.3).

An early, critical stage of angiogenesis is the establishment of a tip cell that leads migration of the nascent sprout.<sup>9</sup> In light of the sprouting defect observed in Slug knockdown cells, we hypothesized that Slug might regulate EMT-related genes and/or known tip cell genes. We therefore examined mRNA levels for the following genes in the presence or absence of Slug in the fibrin gel angiogenesis assay: VEGFR-2, PDGFB, NRP-2, Jag-1, Dll4, Integrin  $\alpha_v$ , Integrin  $\beta_3$ , Vimentin, N-Cadherin and MT1-MMP. Of these, only MT1-MMP (Figure 2.4 B) and Jag-1 (Figure 2.5 A) were consistently decreased in Slug knockdown EC. We chose to pursue further studies with MT1-MMP and confirmed regulation by Slug using a second, independent Slug siRNA (Figure S2.1 B). MT1-MMP, a membrane tethered MMP, is expressed in tip cells during angiogenesis<sup>12, 13</sup> and is required to facilitate migration through both fibrin and collagen matrices<sup>35-37</sup>. We therefore examined Slug regulation of MT1-MMP in the collagen gel invasion assay. Slug was strongly induced at 24 hours and this induction was completely blocked by Slug siRNA (Figure 2.4 D). In the same cells, MT1-MMP mRNA was also strongly induced at 24 hours and this induction was blocked 50% by loss of Slug (Figure 2.4 E). Flow cytometry analysis confirmed upregulated surface expression of MT1-MMP protein and a concomitant decrease in cells treated with siRNA (data not shown). These data were also confirmed with a second, independent siRNA to Slug (Figure S2.1 F, G). As further confirmation that the decreased sprouting seen with Slug knockdown cells is due (at least in part) to loss of MT1-MMP expression we performed a rescue experiment.

EC were transduced with lentivirus expressing either GFP or MT1-MMP, and then transfected with control or Slug siRNA and tested for their ability to invade collagen gels. Expression of transduced MT1-MMP was confirmed by western blot (Figure 2.4 F). Knockdown of Slug reduced invasion by over 50% and this was not affected by expression of GFP (Figure 2.4 G, H). However, expression of MT1-MMP completely rescued the loss of sprouting due to Slug knockdown, confirming that MT1-MMP is a critical downstream target of Slug during angiogenic sprouting.

### **Slug indirectly regulates activity of MMP-2 and MMP-9**

During sprouting angiogenesis, the enzymatic activity of several MMPs is required to degrade and remodel the surrounding 3D ECM.<sup>38</sup> MMP-2 is a secreted protease that is inactive in its native form, however, in the presence of TIMP-2 it is cleaved and activated by surface-expressed MT1-MMP.<sup>39</sup> Interestingly, several studies have reported that expression of Slug correlates with an increase in activity of several MMPs.<sup>19, 23, 24</sup> We therefore reasoned that the decrease of MT1-MMP expression observed in the absence of Slug might result in decreased enzymatic activity of MMP-2 and perhaps other MMPs such as MMP-9. Indeed, this was the case. Using gelatin zymography we found that knockdown of Slug in EC reduced both MMP-2 and MMP-9 activity by 50% when compared to control (Figure 2.6 A, B, D). This result was confirmed using a second independent siRNA targeting Slug (Figure S2.1 H-J). Interestingly, we saw no decrease in mRNA levels of either MMP-2 or MMP-9 at 24 hours, although we did see strong induction of MMP-9 in this assay (Figure 2.6 C, E). These data are consistent with Slug regulating the activity of MMP-2 through MT1-MMP, however the mechanisms

underlying the effects of Slug knockdown on MMP-9 activity are as yet unclear as MMP-9 does not require activation by MT1-MMP. Interestingly, TIMP1, which blocks MMP-2 and MMP-9 but not MT1-MMP, blocked sprouting (data not shown) suggesting that MMP-2 and MMP-9 may have a role in this process. In aggregate our data show that Slug regulates EC protease activity during angiogenic sprouting.

## DISCUSSION

In recent years there has been a dramatic increase in our understanding of the growth factors and receptors that drive angiogenesis, and a growing appreciation of the signaling pathways downstream of these receptors. Our understanding of the transcription factors that form the link between these signals and new gene expression is, however, much less complete. Here we define a role for the transcription factor Slug in sprouting angiogenesis. Slug expression drives sprouting through the induction of MT1-MMP and the regulation of MMP-2 activity. In the absence of Slug, EC sprouting is disrupted and this can be overcome by re-expression of MT1-MMP. Importantly, we also find Slug expression in tumor-associated vessels in multiple cancers. Our data therefore suggest that Slug potentially regulates pathologic angiogenesis in settings including cancer.

Slug is perhaps best characterized as a member of a family of transcription factors, including Snail, Twist, ZEB-1 and -2, that drive epithelial-to-mesenchymal transitions (EMT).<sup>40</sup> EndMT has been previously described during cardiac cushion morphogenesis<sup>20</sup>, and several studies have suggested that EndMT provides a source for cancer-associated myofibroblast cells<sup>40, 41</sup>. We therefore wondered whether Slug expression during angiogenesis was driving a partial EndMT, particularly affecting tip cells. Slug certainly drives migration and invasion, through MT1-MMP expression, however we saw no change in VE-Cadherin expression, nor did we see regulation of several genes, other than MT1-MMP and Jag-1, known to be upregulated in tip cells.<sup>12, 13</sup> Somewhat surprisingly, our hypothesis that Slug-expressing cells would localize preferentially to a tip location was not borne out. Instead, Slug-expressing cells were found throughout the sprout,

suggesting that Slug expression in EC may be a more general marker for an activated, angiogenic phenotype rather than a specific marker for EndMT-like processes occurring in tip cells. Strikingly, when EC were forced to express Slug by lentiviral-mediated transduction, and these were mixed 1 to 3 with untransduced-EC, the vast majority of cells locating to sprouts expressed Slug. In sharp contrast, when GFP-expressing EC were mixed 1 to 3 with untransduced EC, GFP-expressing cells were found both in and out of sprouts. The strong implication is that Slug-expressing cells not only preferentially localize to sprouts, but also actively suppress non Slug-expressing cells from sprouting. Without further experimentation we cannot be sure of the mechanism underlying this finding, however, data from our lab<sup>42</sup> and others<sup>6, 14</sup> may implicate Notch signaling. Notch ligand expression, especially Dll4, suppresses neighboring cells from sprouting both *in vitro* and *in vivo*<sup>6, 14, 42</sup>, however, our preliminary data did not show a loss of Dll4 expression in Slug-knockdown cells, although Jag-1 was suppressed. Further work will be required to determine the interactions between Slug and the Notch pathway in this process.

MMPs, including MT1-MMP, are critical mediators of angiogenesis, responsible for matrix degradation<sup>4, 37-39, 43</sup> as well as release of matrix-bound pro-angiogenic factors including bFGF and VEGF<sup>44</sup>. MT1-MMP directly degrades both fibrin and collagen<sup>35-37</sup> and acts in concert with TIMP2 to cleave pro-MMP-2 into its active form<sup>39</sup>. Several studies have also shown that MT1-MMP is required for both sprouting<sup>45</sup> and lumen formation *in vitro*<sup>46</sup> – a finding we suggest is linked to expression of Slug (Figure 2.4). These data are consistent with several previous reports that Slug regulates MMP

expression and activity in cancer cells. For example, Slug regulates both MT1-MMP and MMP-9 in pancreatic cancer<sup>24, 47</sup>, and has also been shown to regulate MT4-MMP<sup>23</sup>. Moreover, we find that Slug is upregulated in blood vessels adjacent to invasive tumors, but is largely absent in quiescent vessels (Figure 2.1 D). Finally, a previous report found Slug in invasive ovarian tumor-associated EC.<sup>26</sup> In aggregate, these data support a role for Slug-regulated MMP expression in both tumor cells, and their associated angiogenic vasculature.

Interestingly, Slug knockout mice are viable with no major phenotype<sup>30</sup>, although loss of the closely-related gene, Snail, causes early embryonic lethality due to problems with gastrulation<sup>48</sup>. It is therefore possible that Snail compensates for the loss of Slug during early development, masking a potential role for Slug in this process. In our *in vitro* studies, in contrast, we find that Snail cannot compensate for Slug in the pathological setting of invasion into fibrin gels. We have preliminary data showing that Snail is expressed under these conditions, although along a different time course than Slug, and that its expression is also required for proper sprouting (Chapter 3). It is likely, therefore, that under these conditions Slug and Snail regulate a separate but potentially overlapping suite of genes. We are currently investigating this possibility. Importantly, there are a number of precedents for genes being critical for pathological angiogenesis but dispensable for developmental angiogenesis including tetraspanin CD151<sup>49</sup>, Aminopeptidase N (CD13)<sup>50</sup> and TNFRI (CD120)<sup>51</sup>.

In summary, our data suggest a critical role for Slug expression in angiogenic EC upstream of MT1-MMP expression, and suggest that Slug may be a useful target for regulating angiogenic EC in multiple human tumor types.



## **MATERIALS AND METHODS**

### **Cell culture and small interfering RNA transfection**

Primary human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords obtained from local hospitals under University of California Irvine Institutional Review Board approval. HUVEC were routinely cultured in 1X M199 (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and endothelial cell growth supplement (ECGS; BD Biosciences) at 37°C and 5% CO<sub>2</sub>. Normal human lung fibroblasts (NHLF) were purchased from Lonza, routinely grown in 1X M199 supplemented with 10% FBS at 37°C and 5% CO<sub>2</sub>. HUVEC at 80% confluency were transfected with 50 nM siRNA purchased from Invitrogen or 16 nM siRNA purchased from Ambion using Lipofectamine 2000 in Opti-MEM (Invitrogen) for four hours with transfection mixture and recovered in endothelial growth media-2 (EGM-2; Lonza) overnight. The non-targeting stealth RNAi negative control high GC duplex #2 (Invitrogen) or the silencer select negative control #1 siRNA (Ambion) was used as a control for sequence independent effects of siRNA delivery. Transfection efficiencies were determined by qRT-PCR and western blot analysis. siRNA oligonucleotide sequences listed in Appendix I.

### **Lentiviral constructs and transductions**

Full-length human HA-tagged MT1-MMP or full-length human Slug was cloned into the lentiviral vector pCDH (CD521A-1; System Biosciences). Lentivirus was made by transfection of pCDH constructs along with the packaging lines psPAX2 and pCMV-VSV-G into 293T cells using Lipofectamine 2000 in Opti-MEM according to the

manufacturer's protocol. Viral supernatants were collected and precipitated using 50% polyethylene glycol (PEG) and passage 0 HUVEC were transduced with virus using polybrene (8 µg/ml; Santa Cruz Biotechnology).

### ***In vitro* fibrin gel angiogenesis assay**

Fibrin gel angiogenesis assays were performed as previously described.<sup>28</sup> Briefly, HUVEC were coated onto Cytodex 3 microcarrier beads (Amersham) at a concentration of 150 cells/bead for four hours and allowed to adhere overnight. HUVEC coated beads were then resuspended in a 2.5 mg/ml fibrinogen solution (MP Biomedicals) at a concentration of 250 beads/ml. Gels were formed by adding 500 µl of the fibrinogen/bead suspension to each well of a 24-well plate containing 0.5 U of thrombin (Sigma-Aldrich). Once gels clotted, 1 ml of EMG-2 containing 20,000-50,000 NHLF was added to each well. Assays were quantified between days 5-6 by live-culture imaging using bright field microscopy. Thirty beads per condition were quantified per experiment.

For RNA and protein isolation, HUVEC were isolated from the fibrin gels by removing fibroblasts with 3 mg/ml trypsin (Sigma-Aldrich) under gentle agitation. Residual fibroblasts were removed by washing the gels using 1X Hank's Balanced Salt Solution (HBSS; Cellgro). Fibrin gels were digested with 4 mg/ml trypsin and gels were dislodged from the wells of the 24-well plate. The entire contents of each well was transferred to a conical tube and placed under rotation at 37°C to achieve complete digestion. When harvesting cells for studies of Slug protein expression, the cells were pre-treated 10 µM MG-132 (Calbiochem) for 1 hour to retard proteasome-mediated degradation.

### ***In vitro* fibrin gel sandwich assay**

HUVEC were transfected with control or Slug siRNA (Ambion) as described above. 500  $\mu$ l of 2.5 mg/ml fibrinogen was mixed with 0.5 U thrombin (Sigma-Aldrich) in four wells of a 12-well plate and allowed to clot at 37°C. HUVEC were seeded on top of each gel at a concentration  $2.5 \times 10^5$  cells/ml in EGM-2 and allowed to adhere at 37°C for 3 hours. EGM-2 was aspirated and 500  $\mu$ l of 2.5 mg/ml fibrinogen pre mixed with 0.5 U thrombin was added to create a fibrin-sandwich, gels were allowed to clot at 37°C and 1 ml of EGM-2 containing 40,000 NHLF was added to each well. HUVEC were allowed to undergo morphogenesis for 3 days. HUVEC were isolated from the fibrin-gel-sandwich by removing fibroblasts using 3 mg/ml trypsin (Sigma-Aldrich). Gels were washed with 1X HBSS to remove residual NHLF. Fibrin gels were digested with 4 mg/ml trypsin and HUVEC isolation was monitored under a microscope. The contents of 4 wells/condition were combined, and digested product was centrifuged at 1,200 rpm. The resulting pellet containing HUVEC was resuspended in TRIZOL for qRT-PCR analysis as described below.

### **Human tissue and Immunohistochemistry**

Formalin fixed, paraffin-embedded sections of de-identified human colorectal cancer slides were obtained from the Experimental Tissue Resource in accordance with UCI Biorepository procedures. Deparaffinized human colorectal cancer tissue sections underwent citrate-based antigen retrieval, and blocking with 5% goat serum. Sections were incubated in rabbit anti-Slug (1:200; Cell Signaling, 9585) and biotinylated goat anti-rabbit antibody, followed by development with a peroxidase-based Vectastain ABC

kit. The stained slides were re-blocked with goat serum, and incubated in mouse anti-CD31 (1:100; Dako, IR610) followed by ImmPRESS alkaline phosphatase-conjugated anti-mouse IgG (Vector Laboratories, MP-54020) and developed with Vector-Blue substrate. Counterstaining was performed with tri-methyl green.

### ***In vitro* invasion assays and *in vitro* lumenogenesis assays in 3D collagen matrices**

Assays were performed as previous described.<sup>29</sup> For invasion assays, collagen gels were made with 30  $\mu$ l of rat-tail collagen I (3.75 mg/ml) supplemented with 200 ng/ml SDF-1 $\alpha$  (PeproTech) and 1  $\mu$ M S1P (Biomol). Gels were added to each well of a 4.5 mm diameter 96 microwell-plate (Corning) and incubated at 37°C until polymerized. HUVEC were then suspended in serum-free culture media of 1X M199 containing 1X ITS+3 (Sigma-Aldrich), 40 ng/ml VEGF (R&D Systems), 40 ng/ml FGF-2 (R&D Systems), 50  $\mu$ g/ml ascorbic acid (Fisher Scientific), and 50 ng/ml PMA (Calbiochem) at a concentration of  $1 \times 10^5$  cells/ml and 100  $\mu$ l of cell suspension was added to each well. HUVEC were allowed to invade for 24 hours at 37°C and 5% CO<sub>2</sub>. Cultures were fixed in 3% glutaraldehyde for 30 minutes, washed with sterile water and stained using 1% toluidine blue in 30% methanol for 1 hour. Assays were destained with water and bright field images (three gels/condition) were taken a few micrometers below the monolayer in order to quantify the number of invading HUVEC. To isolate HUVEC, 65 gels/condition were digested in 5 mg/ml collagenase (Worthington Biochemical) dissolved in dPBS (Gibco) and the cellular pellet was resuspended in 1 ml of TrizOL (Invitrogen).

Alternatively, HUVEC used in lumenogenesis assays were suspended in 30  $\mu$ l of rat-tail collagen I (3.75 mg/ml) gels at a final concentration of  $6 \times 10^5$  cells/ml, added to each well of a 4.5 mm diameter microwell-plate (Corning) and incubated at 37°C until polymerized. 100  $\mu$ l of serum-free culture media described above (omitting cells) was added to each well. HUVEC were allowed to undergo morphogenesis for 24-48 hours at 37°C and 5% CO<sub>2</sub> and fixed, stained and destained as described for invasion assays. Four bright field images were captured per well (three wells/condition) and intercellular lumens were manual traced using NIH ImageJ, converted from pixels to square micrometers and averaged for each condition. An EC lumen was defined as a multicellular luminal space in addition to intracellular lumen compartments.

Early stage lumen formation was assessed using the assay described above with the addition of 5 mg/ml FITC-dextran (Molecular Probes) to the culture media. After 4 hours of morphogenesis, gels were digested with 5 mg/ml collagenase type I for 10 minutes at 37°C and the contents of three microwells were added to 500  $\mu$ l phenol red free 1X M199. Cells were seeded onto glass coverslips coated with 50  $\mu$ g/ml type I collagen and allowed to adhere for 10 minutes at 37°C. Coverslips were mounted and the percent of cells containing fluorescent-labeled intracellular lumens/high power field (HPF) and the number of fluorescently labeled intracellular lumens/cell were quantified for each condition ( $n=400$  cells).

### **Methylcellulose production and 3D vascularized colon cancer spheroid assay**

Methylcellulose was generated by autoclaving 1.2 grams of powder in a 250 ml beaker at 120°C for 20 minutes. Under sterile conditions, 50 ml of preheated (60°C) endothelial basal media (EBM; Lonza) was added to the autoclaved methylcellulose and dissolved by stirring at 60°C for 20 minutes. Once dissolved, an additional 50 ml of EBM was added for a final volume of 100 ml. The methylcellulose solution was covered with foil and mixed for 2 hours at 4°C. The solution was centrifuged at 4,000 rpm for 2 hours. Supernatant was removed, approximately 90% of the volume, and the resulting methylcellulose was stored at 4°C. The PDMS-retaining rings used to generate tissues had a diameter of 8 mm and a height of 0.8 mm. For quantification, a total of 5 tissues/condition were quantified for each independent experiment ( $n=3$ ) and one tissue contained approximately eight vascularized spheroids.

HUVEC and SW620 were seeded into EGM-2 containing 15% methylcellulose at  $7.5 \times 10^4$  cells/ml and  $2.5 \times 10^4$  cells/ml respectively. Cellular suspensions were aliquoted (150  $\mu$ l/well) into a 96-well U-bottom plate (Greiner Bio-one, CellStar) and allowed to form spheres overnight. Spheroids were resuspended in fibrinogen (2.5 mg/ml; Sigma) containing NHLF at  $1 \times 10^6$  cells/ml. 50  $\mu$ l of spheroid/cell suspension was added onto a 12-mm circular glass cover slip with an affixed polydimethylsiloxane (PDMS)-retaining ring and mixed with  $5 \times 10^{-3}$  U thrombin (Sigma-Aldrich). Tissues were fed with EGM-2 and maintained at 37°C and 5% CO<sub>2</sub>. On day 7, tissues were fixed and immunofluorescent staining was performed.

### **Growth factor treatments**

HUVEC were cultured as previously described. At 100% confluency, HUVEC were serum starved in 1X M199 containing 2% FBS overnight. The following day HUVEC were treated with the following growth factors in 1X M199 containing 2% FBS for 24 hours; SDF1 $\alpha$  (200 ng/ml; PeproTech), VEGF (40 ng/ml; PeproTech), bFGF (40 ng/ml; PeproTech), S1P (1  $\mu$ M; Biomol), PMA (50 ng/ml; Calbiochem), TGF- $\beta$ 1 (5 ng/ml; PeproTech), TNF- $\alpha$  (10 ng/ml; PeproTech), TGF- $\alpha$  (50 ng/ml; PeproTech), HGF (100 ng/ml; PeproTech), ANG-1 (250 ng/ml; PeproTech), and Angiogenin (250 ng/ml; R&D Systems). HUVEC were then harvested and expression levels were detected via western blot or qRT-PCR analysis as described below.

### **Quantitative Real-Time PCR**

Total RNA was isolated from HUVEC using TrizOL reagent (Invitrogen) according to the manufacturer's protocol. Isolated RNA was treated with RQ1 DNase (Promega) for 1 hour. Total RNA was used for cDNA synthesis using an iScript cDNA Synthesis Kit (BioRad). A BioRad iCycler and HotStartTaq DNA Polymerase (Qiagen) was used to perform qRT-PCR with SYBR Green (Molecular Probes) as the readout. Average  $C_T$  values were normalized to GAPDH expression levels and all samples were measured in triplicate. Primers were synthesized by Integrated DNA Technologies and sequences can be found in Appendix I.

## **Western blot**

HUVEC isolated from the fibrin gel angiogenesis assay as described above were lysed on ice in RIPA buffer (50mM Tris-Cl pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 150mM NaCl) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, 5 mM DTT and 1X protease inhibitor cocktail. Lysates were sonicated twice at 10 Watts for 15 seconds, and cellular debris and beads were cleared by centrifugation - 14,000 rpm for 10 minutes at 4°C. Alternatively, protein lysates from monolayer HUVEC were extracted directly from culture dishes by adding supplemented RIPA buffer to culture dishes placed on ice for 10 minutes. Dishes were scrapped and the cellular contents were added to a microfuge tube and allowed to lyse for an additional 10 minutes on ice. Lysates were sonicated and spun at 14,000 rpm for 10 minutes at 4°C. Protein concentrations were determined using bicinchoninic acid assay (Sigma-Aldrich) according to manufacturer's instructions. Samples were mixed 3:1 with Laemmli 4X sample buffer (BioRad), boiled for 5 minutes at 95°C, and equal amounts of protein (40-100 µg) were loaded and electrophoresed in 4-20% Mini-PROTEAN TGX polyacrylamide gels (BioRad) under denaturing and reducing conditions. Proteins were transferred to a polyvinylidene fluoride membrane (Millipore). Membranes were blocked for 2 hours in TBS/0.1% Tween 20 (0.1% TBST) containing 5% non-fat dry milk. Membranes were then incubated overnight at 4°C in primary antibodies - primary rabbit monoclonal anti-Slug (1:750; Cell Signaling, 9585) or primary rabbit monoclonal anti-MMP14 (1:2000; Epitomics, 2010-1) were used. Anti-Slug antibody was diluted in 0.1% TBST containing 5% bovine serum albumin (BSA) and anti-MMP14 was diluted in 0.1% TBST containing 2% milk. The following day, membranes were washed with TBS/0.2%



Tween 20 (0.2% TBST) before secondary antibody was added. HRP-conjugated goat anti-rabbit secondary antibody (1:5000; Abcam) was diluted in 0.1% TBST containing 5% BSA and added to the blot for 2 hours at RT. Protein expression was detected using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) and membranes were imaged using a Nikon AF 50 mm f/1.4D camera (Nikon). To ensure equal loading, membranes were stripped using restore stripping buffer (Thermo Scientific), blocked for 1 hour in 0.1% TBST containing 5% BSA and re-probed for 2 hours with HRP-conjugated GAPDH (1:5000; Abcam, ab9482) antibody.

### **Gelatin zymography**

Supernatant/culture media from 3D collagen I invasion assays (see above) were collected from 20 wells/condition, combined and cellular debris was removed by centrifugation. Collected media was concentrated using ultra centrifugal devices with a 3,000 nominal molecular weight limit (Amicon) according to the manufacturer's protocol. 25-100 µg of protein was resolved on 10% polyacrylamide gels containing 1% (w/v) gelatin (BioRad). Zymogram reagents were purchased from BioRad and the manufacturer's protocol was followed. Briefly, gels were washed 4 times for 15 minutes in 25 ml of 1X Renaturation Buffer (BioRad), incubated in Development Buffer (BioRad) for 20 min at 37°C, stained with 0.1% amido black (Sigma-Aldrich) in 30% methanol (v/v) and 10% acetic acid (v/v), and then destained in 30% methanol (v/v) and 10% acetic acid (v/v). Zymograms were imaged using a Gel Doc 2000 equipped with an 8-bit CCD camera and Quantity One software (BioRad) and densitometry quantification was completed using NIH ImageJ.

## **Immunofluorescence**

Fibrin gel angiogenesis assays used for immunofluorescence were performed in Lab-Tek II 4-well chambered borosilicate coverglass system (No. 1.0; Thermo Fisher Scientific). Prior to staining, the NHLF monolayer was removed as described above. Assays were fixed in 4% PFA for 15 minutes and extensively washed in 1X PBS containing 0.3 M glycine to remove fixative. Assays were permeabilized and blocked for 2 hours at RT using 1X PBS supplemented with 0.3 M glycine, 5% BSA, 5% goat serum, 0.2% sodium azide, and 0.3% Triton X-100. Assays were treated with primary monoclonal rabbit anti-VE-Cadherin antibody (1:75; Enzo, ALX-210-232) diluted in blocking/permeabilization solution and incubated at 4°C overnight. The following day cultures were treated with secondary goat anti-rabbit 488-conjugated antibody (1:200; Invitrogen, A11008) overnight at 4°C. Cultures were extensively washed in 1X PBS. Nuclei were stained with 1 µg/ml DAPI (Sigma-Aldrich) and F-actin was stained with 0.2 µM Texas Red-X phalloidin (Invitrogen). All steps were completed under gentle agitation.

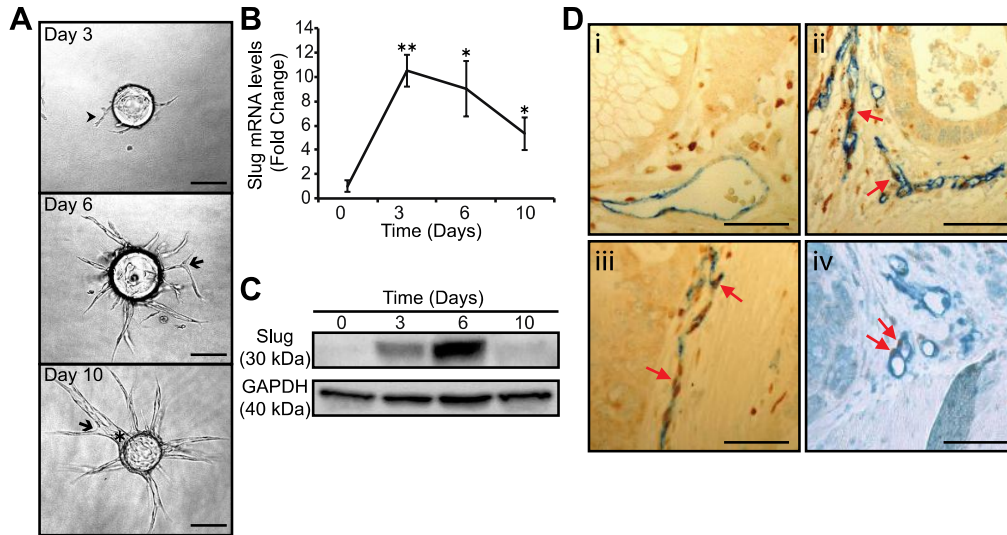
Vascularized 3D colon cancer spheroids were fixed in 10% formalin (Fisher Scientific). Tissues were permeabilized for 30 minutes at RT using 1X PBS supplemented with 0.5% Tween-20. Non-specific binding was blocked with 1X PBS containing 2% BSA and 0.1% Tween-20. Tissues were incubated overnight at 4°C using a mouse anti-CD31 antibody (1:100; Dako, IR610) diluted in blocking buffer followed by a goat anti-mouse 568-conjugated (1:500; Invitrogen, A11004) secondary antibody also diluted in blocking buffer. Tissues were extensively washed with 1X PBS containing 0.3 M glycine to remove background. All steps were completed under gentle agitation.

## **Microscopy**

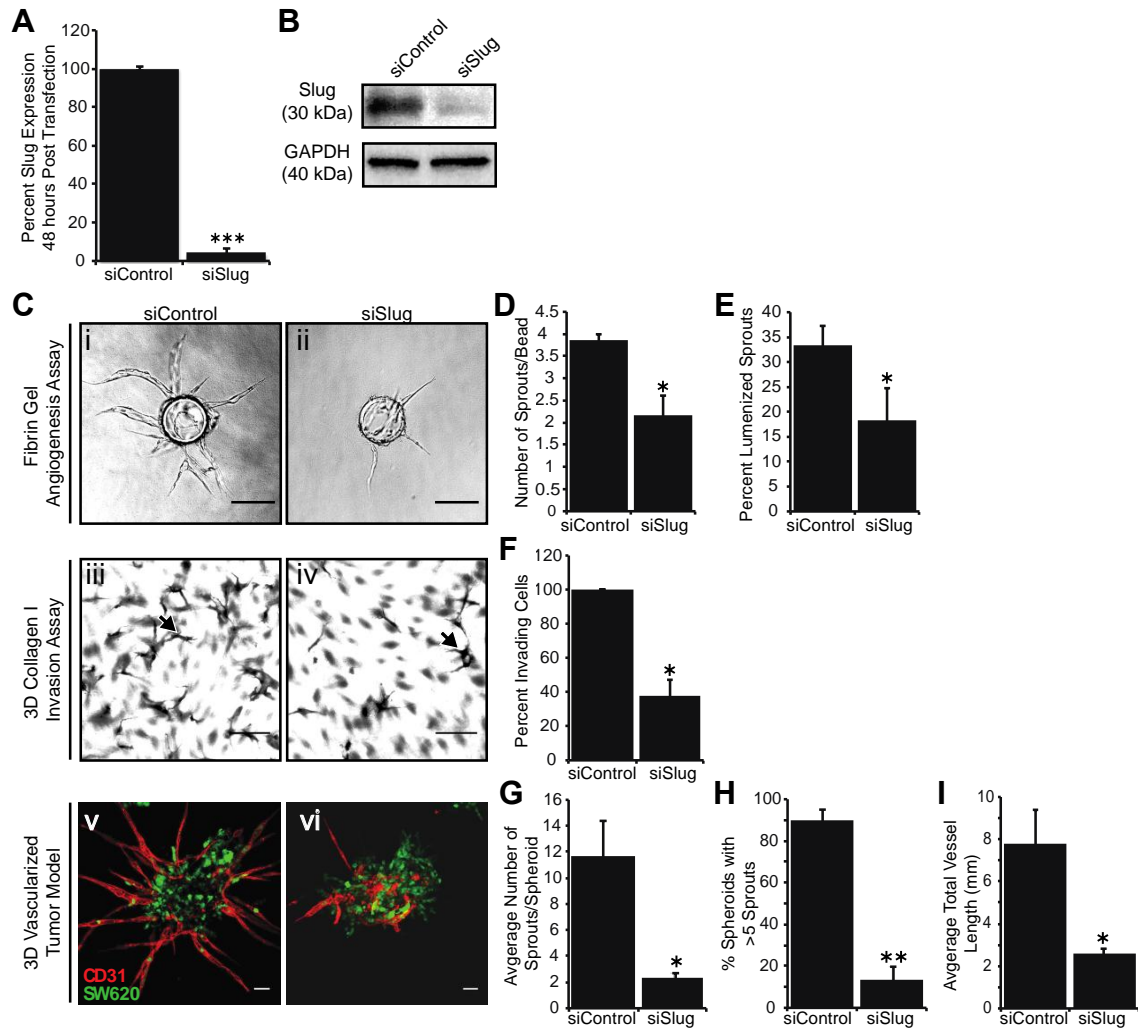
An inverted microscope (IX70; Olympus) was used for all conventional bright field images. Images were captured using a SPOT Idea 3.0 megapixel color mosaic camera and Spot acquisition software (Sport Imaging Solutions). For confocal microscopy, a Nikon Eclipse Ti inverted confocal microscope (Nikon) equipped with a CoolSNAP ES2 CCD camera (Photometrics) and EZ-C1 acquisition software (version 3.91; Nikon) was used. Confocal images were 12-bit (containing 1024x1024 pixels) and four scans were averaged per pixel. Adjustments to image brightness and/or contrast were performed using Adobe Photoshop software - images between difference conditions were treated identically.

## **Statistical analysis**

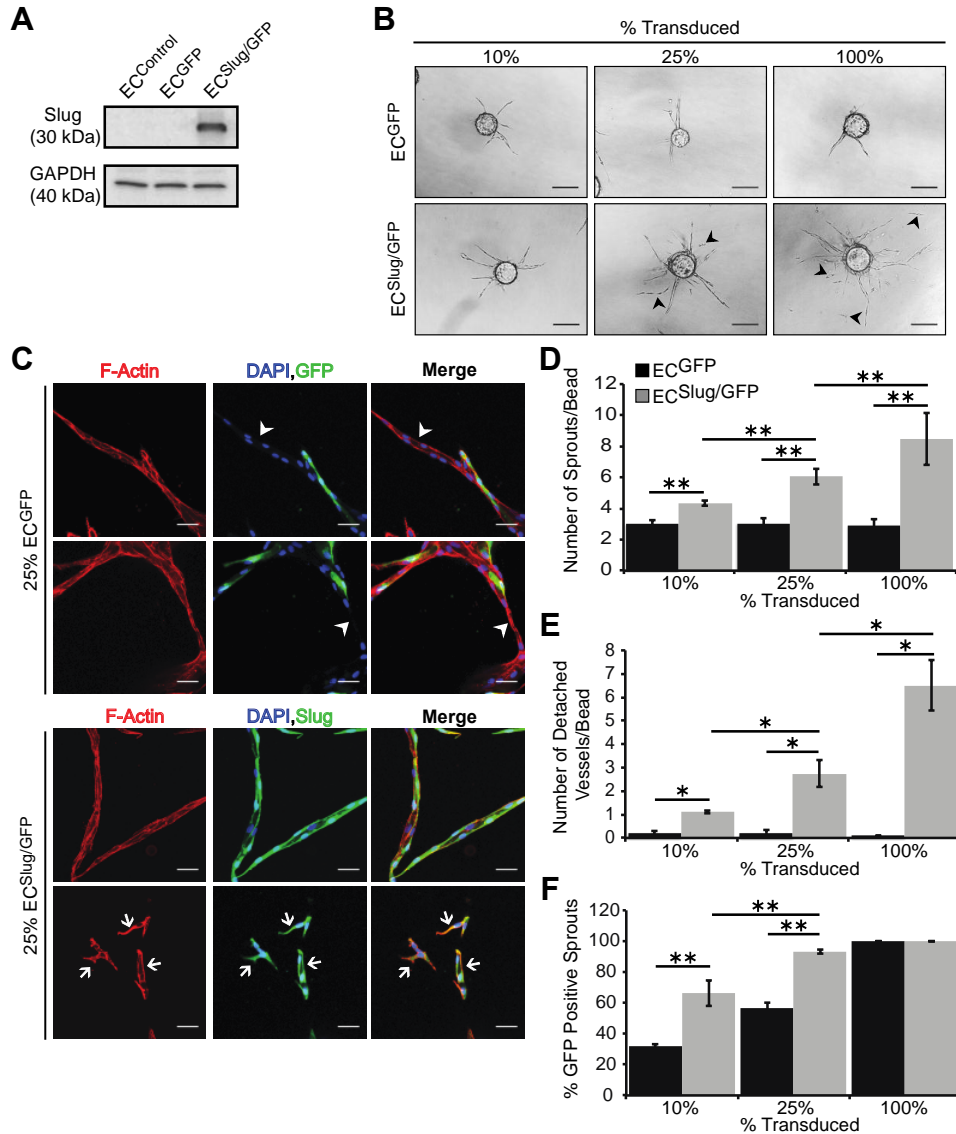
Researchers were blinded to experimental conditions prior to performing quantifications. All experiments were repeated at least three times. Data are reported as mean  $\pm$  standard error of the mean (SEM). A Student's t-test was used to analyze differences between experimental groups of equal variance when only two groups were being compared. For comparisons involving three or more conditions and/or two independent time points, a 2-way analysis of variance (ANOVA) with multiple comparisons was performed and the TukeyHSD probability value was used to determine significance. For analysis of Slug overexpression data (Fig. 3), a generalized linear mixed model (GLMM) was performed using SPSS software and an LSD pairwise contrast method was used to determine significance.



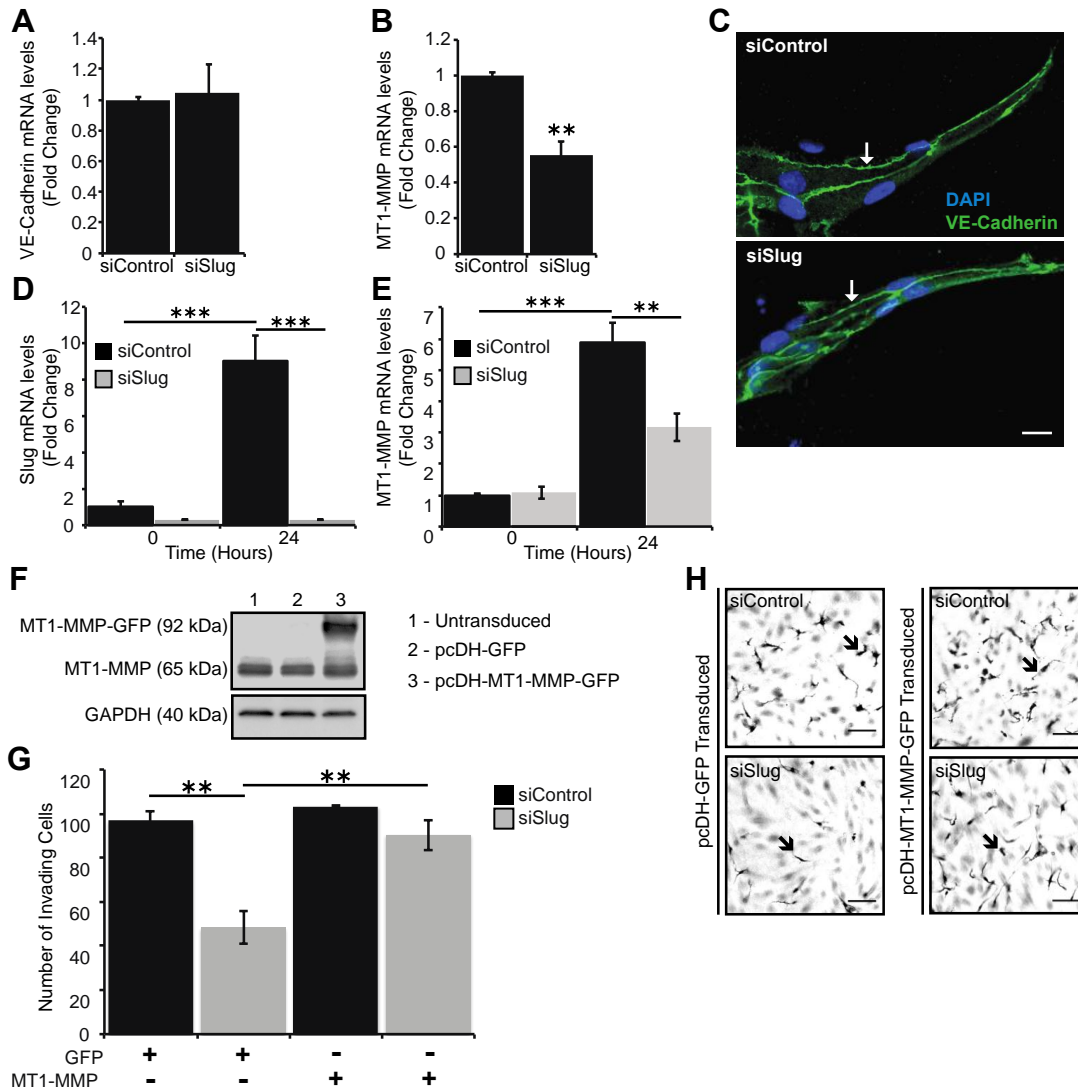
**Figure 3.1. Angiogenic EC express Slug.** (A) Representative images depicting EC morphogenesis during *in vitro* angiogenesis in fibrin gels. Nascent sprouts (arrowhead) are observed on day 3 and continue to proliferate, migrate, branch (arrow) and form lumens (asterisk) through days 6-10. Scale bars, 150  $\mu$ m. (B) EC were harvested on the indicated days from fibrin gels and Slug mRNA levels were assessed by qRT-PCR. Results conveyed as fold change over day 0  $\pm$  SEM ( $n=5$ ; \* $P<0.01$  and \*\* $P<0.0001$ ; Student's t-test). (C) Western blot analysis of Slug protein levels in EC isolated from fibrin gels on the indicated days. (D) Formalin fixed, paraffin-embedded sections of de-identified (i) normal human colon tissue, (ii-iii) human colorectal cancer tissue, and (iv) mouse colorectal cancer tissue, stained for Slug (brown) and CD31 (blue), and counterstained with tri-methyl green. Red arrows depict Slug positive EC. Scale bars, 20  $\mu$ m. Two representative images of five human patient samples analyzed.



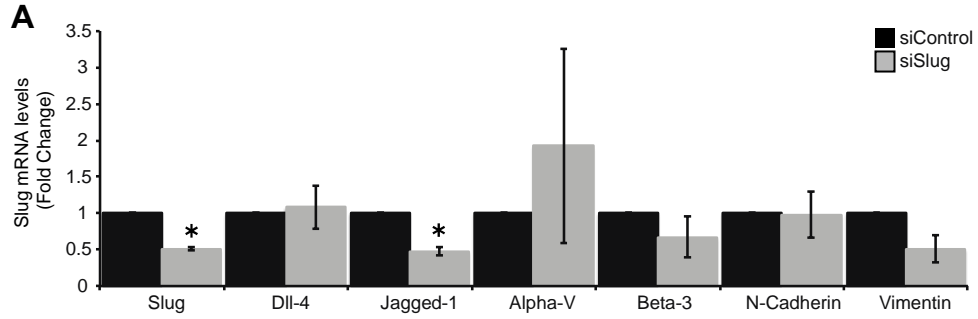
**Figure 3.2. Loss of Slug inhibits EC sprouting in multiple *in vitro* angiogenesis assays.** (A) EC were transfected with control or Slug siRNA and Slug mRNA levels were assessed by qRT-PCR 48 hours later. Results are shown as percent of control set to  $100 \pm \text{SEM}$  ( $n=3$ ;  $***P<0.0001$ ; Student's t-test). (B) EC were transfected with control or Slug siRNA and harvested at 72 hours for analysis of Slug protein levels by western blot. (C) EC transfected with control or Slug siRNA were used in fibrin gel sprouting assays (Ci,Cii), in 3D collagen I invasion assays (Ciii,Civ), and in 3D vascularized tumor spheroids (Cv,Cvi). Representative images from one of at least three similar experiments are shown. Scale bars,  $150 \mu\text{m}$  (Ci,Cii);  $100 \mu\text{m}$  (Ciii-vi). (D,E) Sprouting, defined as a vessel with length greater than or equal to the diameter of the bead ( $150 \mu\text{m}$ ), and lumen formation, defined as a vessel with a luminal space throughout the entire vessel, were quantified on day 5 of the fibrin-gel sprouting assay. Results are expressed as mean  $\pm$  SEM ( $n=3$ ;  $*P<0.05$ ; Student's t-test). (F) Sprout invasion into collagen gels was analyzed 24 hours after seeding. Results are shown as percent of control set to  $100 \pm \text{SEM}$  ( $n=3$ ;  $*P<0.05$ ; Student's t-test). (G-I) Sprouting phenotypes from 3D vascularized tumor spheroids were quantified on day 7 ( $n=3$ ;  $*P<0.05$ ,  $**P<0.001$ ,  $***P<0.0001$ ; Student's t-test).



**Figure 3.3. Slug misexpression in EC promotes angiogenic sprouting.** (A) EC were transduced with pCDH-T2A-copGFP (EC<sup>GFP</sup>), or pCDH-Slug-T2A-copGFP (EC<sup>Slug/GFP</sup>) lentivirus, or were left untransduced (EC<sup>Control</sup>), and then analyzed for Slug expression by Western blot. (B) Transduced EC (EC<sup>GFP</sup> or EC<sup>Slug/GFP</sup>) were mixed with EC<sup>Control</sup> and beads were then coated such that 10%, 25%, or 100% of the cells were transduced and the remainder were untransduced. Fibrin-embedded beads were then examined for sprouting on day 6. Arrowheads indicate detached sprouts. (C) Confocal microscopy of sprouts from 25% transduced-EC assays stained for nuclei (DAPI, blue) and F-actin (red). Arrowheads depict sprouts lacking GFP-expressing EC. Arrows indicate detached vessels; a detached vessel was defined as a sprout no longer attached to a Cytodex bead. Scale bars, 50  $\mu$ m. (D) Quantification of sprouts/bead at the indicated ratios of transduced cells. (E) Quantification of detached vessels at the indicated ratios of transduced cells. (F) Quantification of sprouts that contain at least one EC<sup>GFP</sup> or EC<sup>Slug/GFP</sup>-positive EC. All results expressed as mean  $\pm$  SEM ( $n=3$ ; \* $P<0.01$ , \*\* $P>0.001$ ; GLMM).

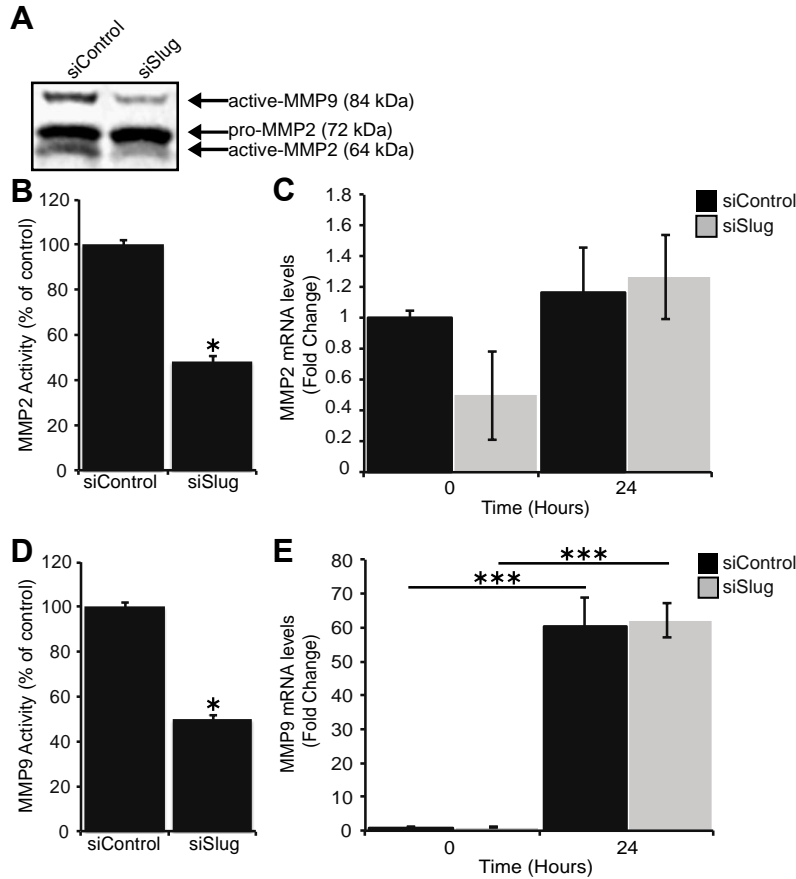


**Figure 3.4. Loss of Slug reduces MT1-MMP expression, but does not affect VE-Cadherin.** (A-C) EC were transfected with control or Slug siRNA and seeded into fibrin gels. (A,B) EC harvested on day 5 for analysis of VE-Cadherin or MT1-MMP expression by qRT-PCR. Results are expressed as mean  $\pm$  SEM ( $n=3$ ;  $**P<0.001$ ; Student's *t*-test). (C) Confocal microscopy of fibrin gels on day 5 stained for VE-Cadherin (green), nuclei visualized with DAPI (blue), arrows indicate VE-Cadherin-positive adherens junctions. Scale bar, 10  $\mu$ m. (D,E) EC transfected with control or Slug siRNA seeded on top of collagen I gels and stimulated to invade for 24 hours. EC were harvested at the indicated time points and mRNA levels of Slug and MT1-MMP were determined by qRT-PCR. Results shown as fold change over time  $0 \pm$  SEM ( $n=3$ ;  $**P<0.01$  and  $***P<0.001$ ; ANOVA). (F) EC were transduced with the indicated lentiviral vectors and examined for expression of MT1-MMP by western blot. (G) Transduced EC were subsequently transfected with control or Slug siRNA, seeded onto collagen gels. After 24 hrs, gels were fixed, stained, and invading cells were quantified ( $n=3$ ;  $**P<0.01$  and  $***P<0.001$ ; ANOVA). (H) Representative images from G captured at 24 hours. Arrows indicate invading cells. Scale bars, 100  $\mu$ m.

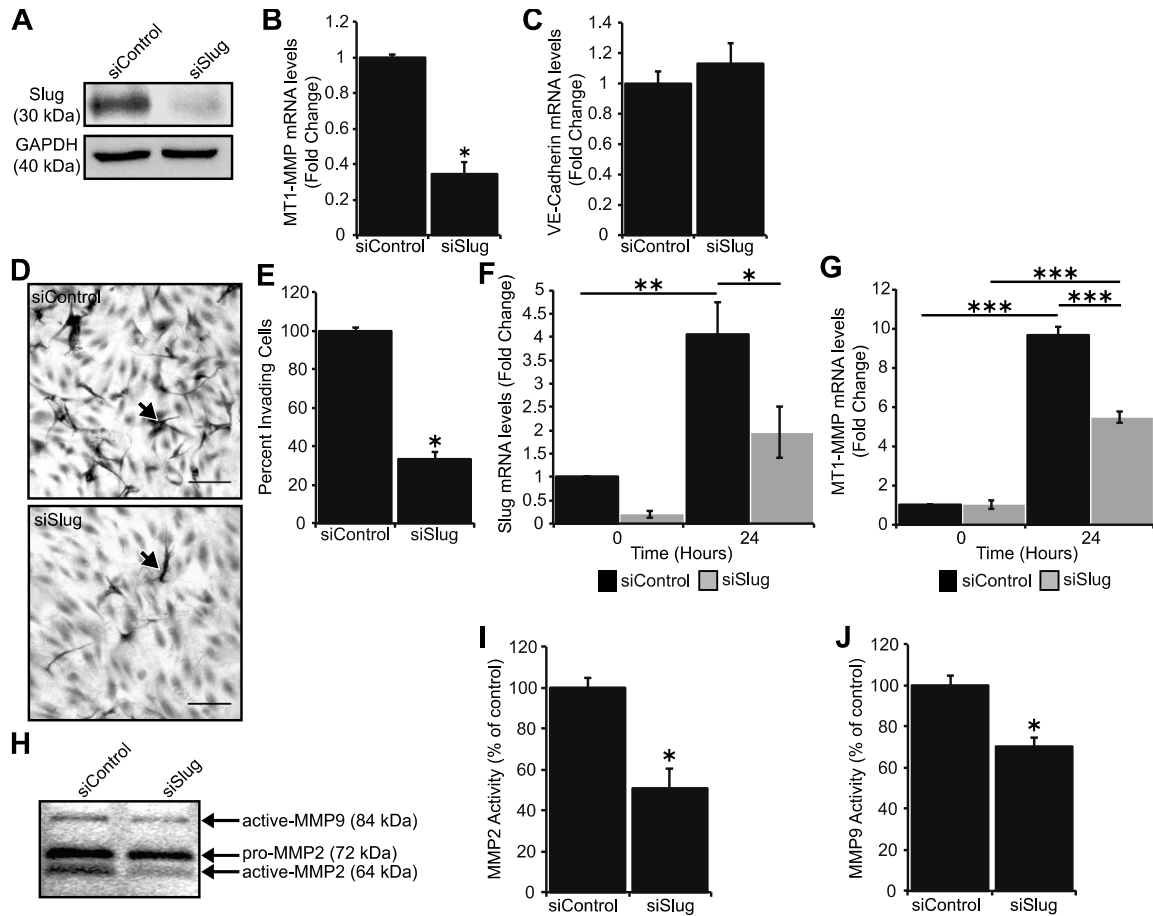


**Figure 3.5. EMT and tip-cell gene expression analysis in the absence of Slug.** (A) EC were transfected with control or Slug siRNA and seeded into fibrin gels. EC were then harvested on day 5 for analysis of expression levels of EMT and tip-cell genes by qRT-PCR. Results are expressed as mean  $\pm$  SEM ( $n=3$ ; \* $P<0.001$ ; Student's t-test)

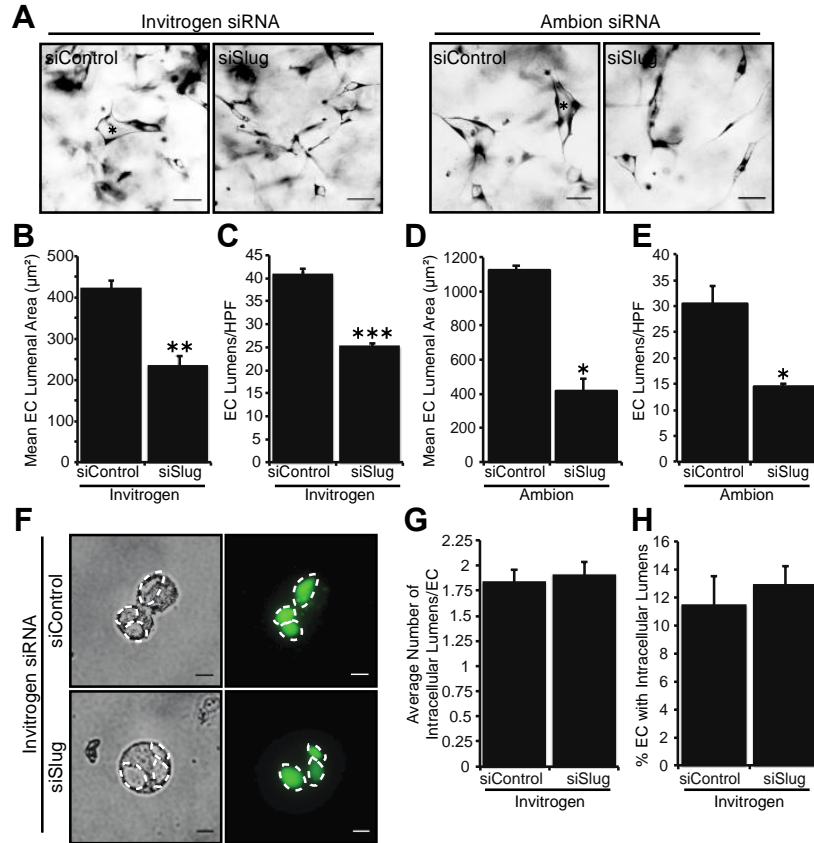




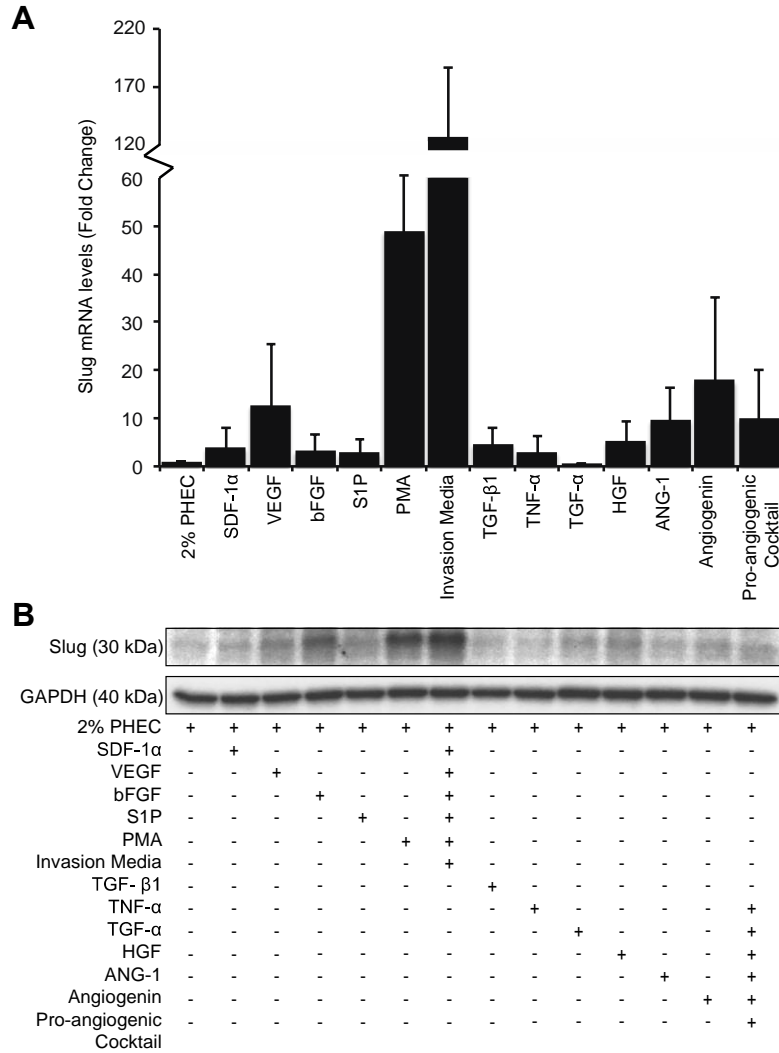
**Figure 3.6. MMP-2 and MMP-9 activity is indirectly regulated by Slug.** (A) EC were transfected with control or Slug siRNA, seeded on top of collagen gels, and stimulated to invade. After 24 hours, culture medium was collected and MMP activity assessed by gelatin zymography. (B,C) Quantitative analysis of MMP-2 activity and mRNA expression after Slug knockdown. Results of the zymography are shown as percent of control set to  $100 \pm \text{SEM}$  ( $n=3$ ;  $*P<0.05$ ; Student's t-test). Results of the qRT-PCR analysis are shown as fold change over time- $0 \pm \text{SEM}$  ( $n=3$ ;  $***P<0.001$ ; ANOVA). (D,E) Quantitative analysis of MMP-9 activity and mRNA expression after Slug knockdown. Details as for MMP-2.



**Figure S3.1. Slug is required during angiogenic sprouting and inhibition results in expression and activity alterations of several MMPs but not VE-Cadherin.** (A-J) HUVEC were transfected with control or Slug siRNA from Ambion. (A) Slug protein levels were assessed via western blot 72 hrs post transfection. (B,C) HUVEC were seeded in-between a fibrin sandwich, harvested on day 3 and mRNA levels for MT1-MMP (B) and VE-Cadherin (C) were assessed by qRT-PCR. Results are represented as fold change relative to control set to  $1 \pm \text{SEM}$  ( $n=3$ ; \* $P<0.01$ ; Student's t-test). (D,E) HUVEC were seeded on top of collagen matrices and stimulated to invade for 24 hours. Assays were fixed, stained, and invading cells (arrows) were quantified. Representative images captured at 24 hours. Results are represented as percent of control set to  $100 \pm \text{SEM}$  ( $n=3$ ; \* $P<0.05$ ; Student's t-test). Scale bars, 100  $\mu\text{m}$ . (F,G) HUVEC were seeded on top of collagen matrices and stimulated to invade for 24 hours. EC were harvested at the indicated time points and mRNA levels of Slug (F) and MT1-MMP (G) were determined by qRT-PCR. Results are conveyed as fold change over time  $0 \pm \text{SEM}$  ( $n=3$ ; \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ ; ANOVA). (H) Culture medium was also collected from each condition at 24 hours and MMP activity was assessed by gelatin zymography - representative zymogram. (I,J) Quantitative analysis of MMP-2 and MMP-9 activity calculated by densitometry using NIH ImageJ software. Results are represented as percent of control set to  $100 \pm \text{SEM}$  ( $n=3$ ; \* $P<0.05$ ; Student's t-test).



**Figure S3.2. Loss of Slug disrupts late-stage lumen formation in 3D collagen matrices.** (A-E) HUVEC transfected with control or Slug siRNA from Invitrogen or Ambion were seeded into collagen matrices (3.75mg/ml) and stimulated to invade for 24 hours. Assays were fixed and stained and representative images were captured. (A) Representative images captured at 24 hours. Scale bars, 50µm. (B,D) EC lumens were quantified by manual tracing intracellular and intercellular lumens (asterisk) using NIH ImageJ software. Results show mean EC luminal area ± SEM ( $n=3$ . \*\* $P<0.002$  and \* $P<0.01$ ; Student's t-test). (C,E) Lumens were also quantified by counting the total number of EC lumens per high power field (HPF) using NIH ImageJ software. Data are represented as EC lumens/HPF ± SEM ( $n=3$ ; \*\*\* $P<0.0001$  and \* $P<0.05$ ; Student's t-test). (F-H) HUVEC transfected with control or Slug siRNA from Invitrogen were seeded into collagen gels and allowed to undergo morphogenesis in the presence of soluble FITC-conjugated dextran. After 4 hours, collagen gels were digested to release the cells from the 3D matrix for live imaging on collagen-coated coverslips. (F) Bright field images and corresponding fluorescent images are shown. Scale bars, 10 µm. The average number of fluorescently labeled intracellular lumens (G) and the percent of cells containing fluorescently labeled intracellular lumens (H) were quantified and are expressed as mean ± SEM ( $n=2$ ).



**Figure S3.3. Slug expression is regulated by several angiogenic growth factors.** (A-B) HUVEC were grown to confluency in 2D and serum starved overnight in media containing 2% FBS. HUVEC were then treated with the indicated growth factors for 24 hours. (A) HUVEC were harvested and Slug mRNA levels were assessed by qRT-PCR. Results are conveyed as fold change over 2% FBS  $\pm$  SEM ( $n=2$ ). (B) Western blot analysis of Slug protein levels stimulated with the indicated growth factors. GAPDH is shown as the loading control.

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## **CHAPTER 4**

### **Snail (Snai1) is regulated by endothelial cells during *in vitro* angiogenesis.**

Katrina Welch-Reardon<sup>1</sup> and Christopher C.W. Hughes<sup>1,2,3</sup>

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<sup>1</sup>The Department of Molecular Biology and Biochemistry, <sup>2</sup>The Department of Biomedical Engineering, <sup>3</sup>The Edwards Lifesciences Center for Advanced Cardiovascular Technology, University of California Irvine, Irvine, CA 92697

#### **ABSTRACT**

An epithelial-to-mesenchymal transition (EMT) is an indispensable process during embryogenesis in which epithelial cells are converted into motile cells. In addition, EMT is a critical component of cancer progression and acts as an important mechanism of invasion and metastasis. Endothelial cells (EC), which are specialized epithelial cells, have also been found to undergo a similar transition termed an endothelial-to-mesenchymal transition (EndMT). First identified in developmental studies of heart formation, EndMT has more recently been identified in a variety of pathological states. Interestingly, Snail (Snai1), a key transcriptional regulatory of both EMT and EndMT, was found to be upregulated in tumor-associated EC when compared to normal endothelium suggesting a role for this gene during pathological angiogenesis. We have identified, for the first time, a critical role for the Snail gene, Snail (Snai1), in angiogenesis. We find that EC upregulate Snail expression during sprouting in an *in vitro* angiogenesis model, and that loss of expression inhibits sprouting, invasion and lumen formation *in vitro*. Moreover, we find the activity of MMP-2 and MMP-9 to be significantly decreased in the absence of Snail expression in angiogenic EC. In summary, these data implicate Snail as a novel regulator of angiogenesis.

## INTRODUCTION

Angiogenesis is a process that occurs during both physiological and pathological conditions including development and growth, tissue repair, inflammation and tumor progression.<sup>1-3</sup> This highly dynamic and tightly regulated process is initiated when endothelial cells (EC) respond to pro-angiogenic stimuli, the most well characterized being VEGF.<sup>2, 4</sup> Upon EC-activation, angiogenesis ensues resulting in degradation of the adjacent basement membrane, migration of nascent sprouts into the surrounding extracellular matrix (ECM), formation of lumens, branching, anastomosis, and a return to quiescence once support cells have been recruited to the newly-formed vessel.<sup>1, 2, 4</sup> Angiogenesis is a process involving EC migration, and proteolytic enzymes are therefore essential during the angiogenic cascade.<sup>5, 6</sup> These enzymes aid in EC invasion into the surrounding tissue by degrading the basement membrane, removing and releasing impeding matrix proteins, and by generating space in the ECM allowing EC tubules to form.<sup>7</sup> In addition, protease activity results in activation and modification of growth factors, cytokines and receptors, and generation of matrix protein fragments.<sup>5, 6, 8</sup>

Matrix metalloproteinases (MMP) are particularly well studied in angiogenesis and three members of this family, MMP-2, MMP-9 and membrane type 1-MMP (MT1-MMP) have been identified as critical regulators of this process.<sup>5, 6, 9</sup> Studies including knockout mouse models have identified MMP-2, MMP-9 and MT1-MMP as playing essential roles in the initiation of both tumor and developmental angiogenesis and angiogenic growth factors can induce expression of these proteases.<sup>10-12</sup> MMP-2 is a secreted protease that is inactive in its native form, however, in the presence of TIMP-2 it is cleaved and activated by surface-expressed MT1-MMP.<sup>13</sup> MT1-MMP is a membrane tethered MMP and is activated during transport from the

Golgi to the plasma membrane by removal of the propeptide by furin or furin-like serine protease.<sup>7</sup> Interestingly, evidence points to expression of MT1-MMP in angiogenic tip-cells<sup>14-16</sup> as well as playing an important role in lumen formation<sup>17</sup>. MMP-9, another secreted MMP, facilitates angiogenesis by degrading ECM components and by aiding in the release of ECM-bound pro-angiogenic growth factors include VEGF.<sup>18, 19</sup> It is clear that several MMPs are known and well-studied in the process of angiogenesis.

MMP activation is not only crucial during angiogenesis but upregulation of these proteases is found in nearly every tumor type. Of particular interest is their involvement in epithelial-to-mesenchymal transitions (EMT). EMT occurs during embryonic development and is involved in the progression of primary tumors towards metastases.<sup>20-22</sup> Key transcriptional regulators of this process belong to the Snail family of zinc-finger transcription factors including one of the most well studied, Snail (Snai1). Although well known for its role as a transcriptional repressor, expression of Snail during EMT also correlates with increased expression and activity of several MMPs including MMP-2<sup>23, 24</sup>, MMP-9<sup>25, 26</sup> and MT1-MMP<sup>27, 28</sup>. Given that Snail expression correlates with activity of proteases that are also required during angiogenesis, it is intriguing to speculate that Snail may also be regulated during this process. In fact, Snail expression was found to be upregulated in the endothelium of invasive breast carcinoma compared to the normal vasculature.<sup>29</sup> Here we provide evidence that Snail is regulated by EC during *in vitro* angiogenesis and is a critical mediator of angiogenic sprouting and late-stage lumen formation. In addition, we find that Snail indirectly regulates activity of MMP-2 and MMP-9 although the mechanism behind this action remains elusive. Collectively these studies suggest that Snail expression in EC may help to regulate several processes of angiogenesis.

## **RESULTS**

### **Snail expression is temporally regulated during *in vitro* angiogenesis**

To study the molecular mechanism governing EC morphogenesis, our lab utilizes the fibrin gel angiogenesis assay, a model in which EC are stimulated to sprout into fibrin gels.<sup>30</sup> This assay recapitulates critical stages of angiogenesis including sprouting, lumen formation, branching and anastomosis (Figure 3.1A). Utilizing this assay, we analyzed Snail expression in angiogenic EC at several time points. Snail mRNA expression is induced on day 6 when sprouts have formed and migration of these nascent sprouts through the fibrin gel is at its peak (Figure 3.1B). Expression then drastically declines through days 7 to 10 when sprouts begin to mature (Figure 3.1B). Thus, Snail expression correlates with migratory sprouts in an *in vitro* angiogenesis model.

To examine whether Snail is expressed in EC during pathologic angiogenesis *in vivo* we surveyed cancer tissues stained for Snail in the Human Protein Atlas Database ([www.proteinatlas.org](http://www.proteinatlas.org)). We observed Snail expression in vessels of colon adenocarcinoma (Figure 3.1C). Snail expression was not exclusive to vessels, however, as many of the tumor cells were also Snail-positive. Thus, in the pathologic setting of cancer, EC in angiogenic vessels express Slug and Snail, consistent with our *in vitro* model of pathologic angiogenesis.

### **Loss of Snail inhibits EC sprouting**

To elucidate a role for Snail during *in vitro* angiogenesis, we utilized small interfering RNA (siRNA) oligonucleotides to inhibit Snail expression. We then examined the effect of Snail knockdown on the ability of EC to undergo morphogenesis in the fibrin gel angiogenesis assay.

First, targeting Snail with siRNA resulted in 80% reduction of mRNA on day 3 of the fibrin gel angiogenesis assay and inhibition persisted through day 5 when mRNA was reduced by 20% (Figure 3.2A). Knockdown of Snail in angiogenic EC resulted in a dramatic phenotype, not only were fewer number of sprouts observed, but those sprouts that did form had reduced ability to form lumens (Figure 3.2A-C). Our data therefore demonstrate a role for Snail during multiple stages angiogenesis.

To better understand the role of Snail during angiogenesis, we next turned to an *in vitro* 3D collagen I invasion assay developed by the Davis lab.<sup>31</sup> This assay permits critical evaluation of processes required during initial stages of angiogenesis, namely sprouting and migration. As expect, inhibition of Snail resulted in reduction of EC invasion by 50% (Figure 3.3A,B). Surprisingly, Snail mRNA expression was not induced in this assay at 24 hours although knockdown was greater than 80% at both time of seeding (time 0) and 24 hours after EC invasion (Figure 3.3C).

### **Snail indirectly regulates activity of MMP-2 and MMP-9**

Angiogenesis requires the enzymatic activity of several MMPs. These proteinases are used by EC to degrade and remodel the surrounding 3D ECM during sprouting and also play an important role in the formation of patent lumens.<sup>7, 17, 32, 33</sup> MMP-2 is a secreted protease that is inactive in its native form, however, in the presence of TIMP-2 it is cleaved and activated by surface-expressed MT1-MMP.<sup>13</sup> MMP-9, yet another MMP secreted by EC, facilitates angiogenesis by degrading ECM components and by aiding in the release of ECM-bound pro-angiogenic growth factors including VEGF.<sup>18, 19</sup> Interestingly, several studies have reported that

expression of Snail correlates with an increase in activity of several proteases.<sup>25, 34-39</sup> We therefore reasoned that the inability to sprout and form lumens in the absence of Snail might be a result of decreased enzymatic activity of MMPs. Indeed, this was the case. Using gelatin zymography we found that knockdown of Snail in EC reduced both MMP-2 and MMP-9 activity by 40% and 20% respectively, when compared to control (Figure 3.3D-F). Interestingly, mRNA expression levels of these proteases were not altered after 24 hours of invasion (Figure 3.4A-B). However, at time zero (the time that EC are harvested from 2D culture) the mRNA expression levels of MMP-2 were significantly reduced in the absence of Snail suggesting direct transcriptional activation of MMP-2 by Snail (Figure 3.4A). However, given that this loss was not observed after 24 hours of invasion implicates that other transcription factors compensate for the loss of Snail induction of MMP-2 once in a 3D environment. In addition, MT1-MMP (a membrane-tethered protease known to activate MMP-2) mRNA expression was not changed before or after EC invasion into collagen gels (Figure 3.4C). These data therefore demonstrate that Snail regulates EC protease activity during angiogenic processes although not at the level of transcription.

### **Loss of Snail reduce late stage EC lumen formation**

A critical step in the angiogenic process is the formation of lumens. Although numerous mechanisms for this process have been proposed, it is most probably that different mechanisms occur in large and small vessels, and developmental and pathologic processes.<sup>17, 40</sup> In small vessels, one accepted mechanism for lumen development involves formation of intracellular pinocytotic vesicles, the fusion of these into larger intracellular vacuoles, and finally, the joining of these between neighboring EC to form a contiguous intercellular luminal space.<sup>17</sup> Using the

knowledge that inhibition of Snail resulted in fewer lumenized vessels in the fibrin gel angiogenesis assay (Figure 3.2A,C), we further examined the role of Snail in EC undergoing lumen formation by utilizing an assay originally devised by the Davis lab.<sup>41</sup> In this assay, EC are induced to form lumens in collagen gels. As shown in Figure 3.5, knockdown of Snail reduced both mean luminal area as well as number of lumens per high-power field (Figure 3.5A-C). Next, to assess early stages of lumen formation, we quantified the number of intracellular vesicles in control and Snail knockdown-EC in the presence of FITC-dextran – FITC-dextran is incorporated into the newly formed pinocytic vacuoles.<sup>42</sup> We found no difference between control and Snail-knockdown EC suggesting that Snail's effects on lumen formation are downstream of the early, vesicle-forming stage, and likely at the stage of intercellular lumen formation (Figure 3.5D-F).

## DISCUSSION

In this study, we show that Snail is regulated by EC during angiogenesis *in vitro*. We find that in the absence of Snail several steps of angiogenesis, including sprouting and early stage lumen formation, are disrupted. In addition, inhibition of Snail in EC results in decreased activity of both MMP-2 and MMP-9. Interestingly, although the primary function of Snail is that of a transcriptional repressor, mRNA levels of these two proteases remains unaltered. These data therefore suggest that Snail regulates an intermediate player required for MMP-2 and MMP-9 activation. Importantly, we also observe Snail expression in tumor-associated vessels in human colorectal cancer suggesting a possible role for Snail during pathological angiogenesis.

Snail, a member of the Snail family of zinc-finger transcription factors, drives epithelial-to-mesenchymal (EMT) transitions which occur during critical phases of embryonic development and carcinoma progression.<sup>43</sup> A specialized form of EMT, endothelial-to-mesenchymal (EndMT) transition, plays an important role during cardiac cushion morphogenesis, and several studies have proposed that EndMT is a unique source of cancer-associated myofibroblast cells.<sup>44-46</sup> Aside from acting as a transcriptional repressor of adheren junctions to drive these processes, Snail expression also correlates with upregulation of several MMPs including MT1-MMP, MMP-2, MMP-7 and MMP-9 in cancer cells.<sup>23-28, 37</sup> Noting that MMPs are required during sprouting angiogenesis, we hypothesized that Snail may be regulating proteolytic activity of these enzymes in angiogenic EC. Indeed, in the absence of Snail we observe decreased activity of both MMP-2 and MMP-9, however, mRNA levels remain unaltered.



MMPs are produced as zymogens and require proteolytic cleavage of the propeptide for activation. In the case of pro-MMP-2, the propeptide is cleaved by a cell-surface complex that consists of a homodimer of MT1-MMP and a single molecule of tissue inhibitor of metalloproteinases-2 (TIMP2).<sup>13</sup> We thus wondered if Snail might alter expression of MT1-MMP providing mechanistic insight into the regulation of MMP-2 by Snail. Interestingly, MT1-MMP mRNA levels did not change in the absence of Snail. Nonetheless, localization and levels of MT1-MMP surface expression will need to be examined before this mechanism is discounted. It is also intriguing to hypothesize that Snail may regulate expression of genes involved in recruiting MT1-MMP to the membrane, such as cortactin.<sup>27</sup> In fact, Snail deficient fibroblasts exhibit significant reduction in invadopodial clusters of cortactin and thus MT1-MMP.<sup>27</sup>

Snail deficient mouse embryos die early in gestation due to defects in gastrulation and mesoderm formation.<sup>47</sup> Snail not only influences cell behavior during development, but also during disease such as metastatic cancer. A plethora of studies have identified factors that induce expression of Snail and genes that are regulated by Snail in epithelial tumors. However, to our knowledge, no studies have identified a role for Snail in angiogenic EC. Interestingly, Snail was found to be upregulated in the endothelium of invasive breast carcinoma compared to the normal vasculature.<sup>29</sup> We too show Snail expression in EC in human colorectal cancer warranting further studies to better understand a role for Snail in pathologic EC.

In conclusion, our data clearly demonstrate that EC undergoing angiogenesis *in vitro* express Snail and that this transcription factor plays a critical role in several steps of this process. Preliminary findings suggest that MMPs are regulated by Snail providing a possible mechanism

behind loss of sprouting in the absence of this gene. However, additional gene expression analysis will need to be completed to reveal possible mechanistic action behind these findings. Moreover, and as discussed in chapter 2, it appears that Snail and its closely related family member Slug play independent but equally important roles during sprouting angiogenesis. The fact that inhibition of either Snail or Slug results in reduced sprouting and lumen formation suggests that these genes do not compensate for one another during sprouting angiogenesis. However, it is likely that these transcription factors work in concert to regulate specific stages of angiogenesis and may share some targets during the angiogenic cascade including MMPs (See Discussion).

## **MATERIALS AND METHODS**

### **Cell culture and small interfering RNA transfection**

Primary human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords obtained from local hospitals under University of California Irvine Institutional Review Board approval. HUVEC were routinely cultured in 1X M199 (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and endothelial cell growth supplement (ECGS; BD Biosciences) at 37°C and 5% CO<sub>2</sub>. Normal human lung fibroblasts (NHLF) were purchased from Lonza, routinely grown in 1X M199 supplemented with 10% FBS at 37°C and 5% CO<sub>2</sub>. HUVEC were used between P3 and P4 and NHLF were used between P6 and P13 for all experiments. HUVEC at 80% confluency were transfected with 50 nM siRNA (Invitrogen) using Lipofectamine 2000 in Opti-MEM (Invitrogen), incubated for four hours with transfection mixture and recovered in endothelial growth media-2 (EGM-2; Lonza) overnight. The non-targeting stealth RNAi negative control high GC duplex #2 (Invitrogen) was used as a control for sequence-independent effects of siRNA delivery. Transfected HUVEC were used in subsequent experiments 18 to 24 hours after transfection. Transfection efficiencies were determined by qRT-PCR. siRNA oligonucleotide sequences listed in Appendix I.

### **The fibrin gel angiogenesis assay**

Fibrin gel angiogenesis assays were performed as previously described.<sup>30</sup> Briefly, HUVEC were coated onto Cytodex 3 microcarrier beads (Amersham) at a concentration of 150 cells/bead for four hours and allowed to adhere overnight. The following day HUVEC coated beads were resuspended in a 2.5 mg/ml fibrinogen solution (MP Biomedicals) at a concentration of 250 beads/ml. Gels were formed by adding 500 µl of the fibrinogen/bead suspension to each well of a

24-well plate containing 0.5 U of thrombin (Sigma-Aldrich). Once gels clotted, 1 ml of EMG-2 containing 20,000-50,000 NHLF was added to each well. Assays were quantified between days 5-6 by live-culture imaging using bright field microscopy. Thirty beads per condition were quantified per experiment.

For expression analysis using qRT-PCR, HUVEC were isolated from the fibrin gels in 24-well plates by removing fibroblasts with 3 mg/ml trypsin (Sigma-Aldrich) for 6 minutes under gentle agitation. Residual fibroblasts were removed by washing gels two times using Hank's Balanced Salt Solution (HBSS, 1X; Cellgro) after trypsin treatments. Fibrin gels were then digested with 4 mg/ml trypsin and gels were dislodged from the wells of the 24-well plate after trypsin was added. The entire contents of each well was transferred to a conical tube and placed under rotation at 37°C for 6 minutes to achieve complete digestion. Isolated HUVEC were then resuspended in 1 ml of TrizOL (Invitrogen).

### **Quantitative Real-time PCR (qRT-PCR)**

Total RNA was isolated from HUVEC using TrizOL reagent (Invitrogen) according to the manufacturer's protocol. Isolated RNA was then treated with RQ1 DNase (Promega) for 1 hour. Total RNA was used for cDNA synthesis using an iScript cDNA Synthesis Kit (BioRad). A BioRad iCycler and HotStartTaq DNA Polymerase (Qiagen) was used to perform qRT-PCR with SYBR Green (Molecular Probes) as the readout. Average  $C_T$  values were normalized to GAPDH expression levels and all samples were measured in triplicate. Primers were synthesized by Integrated DNA Technologies and sequences can be found in Appendix I

### ***In vitro*, 3D Collagen I Invasion Assay**

Assays were performed as previous described.<sup>31</sup> Briefly, collagen gels were made with 30 µl of rat-tail collagen I (3.75 mg/ml) supplemented with 200 ng/ml SDF-1α (PeproTech) and 1 µM S1P (Biomol). Gels were added to each well of a 4.5 mm diameter 96 microwell-plate (Corning) and incubated at 37°C for 30 minutes to allow polymerization. HUVEC were then suspended in serum-free culture media of 1X M199 containing 1X ITS+3 (Sigma-Aldrich), 40 ng/ml VEGF (R&D Systems), 40 ng/ml FGF-2 (R&D Systems), 50 µg/ml ascorbic acid (Fisher Scientific), and 50 ng/ml PMA (Calbiochem) at a concentration of  $1 \times 10^5$  cells/ml and 100 µl of cell suspension was added to each well. HUVEC were allowed to invade for 24 hours at 37°C and 5% CO<sub>2</sub>. Cultures were fixed in 3% glutaraldehyde for 30 minutes, washed with sterile water and stained using 1% toluidine blue in 30% methanol for 1 hour. Assays were destained with water and bright field images (three gels/condition) were taken a few micrometers below the monolayer in order to quantify the number of invading HUVEC - NIH Image J was used to count invading cells

To isolate HUVEC, 65 gels/condition were digested in 5 mg/ml collagenase (Worthington Biochemical) dissolved in dPBS (Gibco) and the cellular pellet was resuspended in 1 ml of TrizOL (Invitrogen).

### **Gelatin Zymography**

Supernatant/culture media from 3D collagen invasion assays (see methods and above) were collected from 20 wells/condition, combined and cellular debris was removed by centrifugation. Collected media was concentrated using ultra centrifugal devices with a 3,000 nominal

molecular weight limit (Amicon) according to the manufacturer's protocol. 80 µg of protein was resolved on 10% polyacrylamide gels containing 1% (w/v) gelatin (BioRad). Zymogram reagents were purchased from BioRad and the manufacturer's protocol was followed. Briefly, gels were washed 4 times for 15 minutes in 25 ml of 1X Renaturation Buffer (BioRad), incubated in Development Buffer (BioRad) for 20 min at 37°C, stained with 0.1% amido black (Sigma-Aldrich) in 30% methanol (v/v) and 10% acetic acid (v/v), and then destained in 30% methanol (v/v) and 10% acetic acid (v/v). Zymograms were imaged using a Gel Doc 2000 equipped with an 8-bit CCD camera and Quantity One software (BioRad) and densitometry quantification was completed using NIH ImageJ.

#### ***In vitro*, 3D Collagen I Lumen Assay**

Assays were performed as previous described.<sup>41</sup> Briefly, HUVEC used in lumenogenesis assays were suspended in 30 µl of rat-tail collagen I (3.75 mg/ml) gels at a final concentration of  $6 \times 10^5$  cells/ml, added to each well of a 4.5 mm diameter microwell-plate (Corning) and incubated at 37°C for 30 minutes to allow polymerization. 100 µl of serum-free culture media, 1X M199, containing 1X ITS+3 (Sigma-Aldrich), 40 ng/ml VEGF (R&D Systems), 40 ng/ml FGF-2 (R&D Systems), 50 µg/ml ascorbic acid (Fisher Scientific), and 50 ng/ml PMA (Calbiochem) was added to each well. HUVEC were allowed to undergo morphogenesis for 24-48 hours at 37°C and 5% CO<sub>2</sub> at which point cultures were fixed in 3% glutaraldehyde for 30 minutes, washed with sterile water and stained using 1% toluidine blue in 30% methanol for 1 hour. Assays were destained with water and four bright field images were captured per well (three wells/condition) and intercellular lumens were manual traced using NIH ImageJ, converted from pixels to square

micrometers and averaged for each condition. An EC lumen was defined as a multicellular luminal space in addition to intracellular lumen compartments.

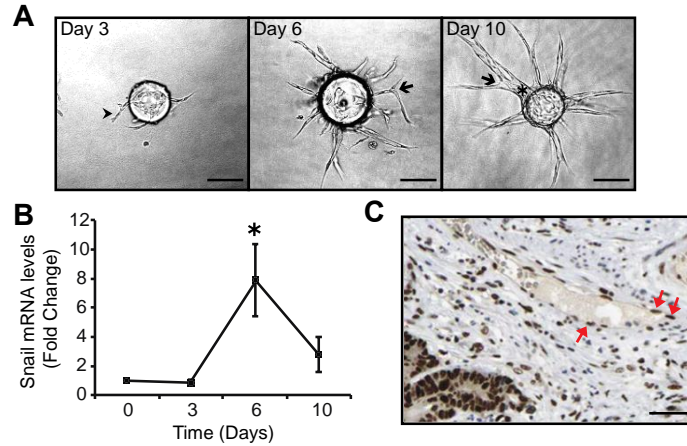
Early stage lumen formation was assessed using the assay described above with the addition of 5 mg/ml fluorescein-conjugated dextran (Molecular Probes) to the culture media. After 4 hours of morphogenesis, gels were digested with 5 mg/ml collagenase type I for 10 minutes at 37°C and the contents of three microwells were added to 500 µl phenol red free 1X M199. Cells were then seeded onto glass coverslips coated with 50 µg/ml type I collagen and allowed to adhere for 10 minutes at 37°C. Coverslips were mounted and the percent of cells containing fluorescent-labeled intracellular lumens/high power field (HPF) and the number of fluorescently labeled intracellular lumens/cell were quantified for each condition ( $n=400$  cells). All analysis was completed blinded.

### **Microscopy**

An inverted microscope (IX70; Olympus) was used for all conventional bright field images. Images were captured using a SPOT Idea 3.0 megapixel color mosaic camera and Spot acquisition software (Sport Imaging Solutions).

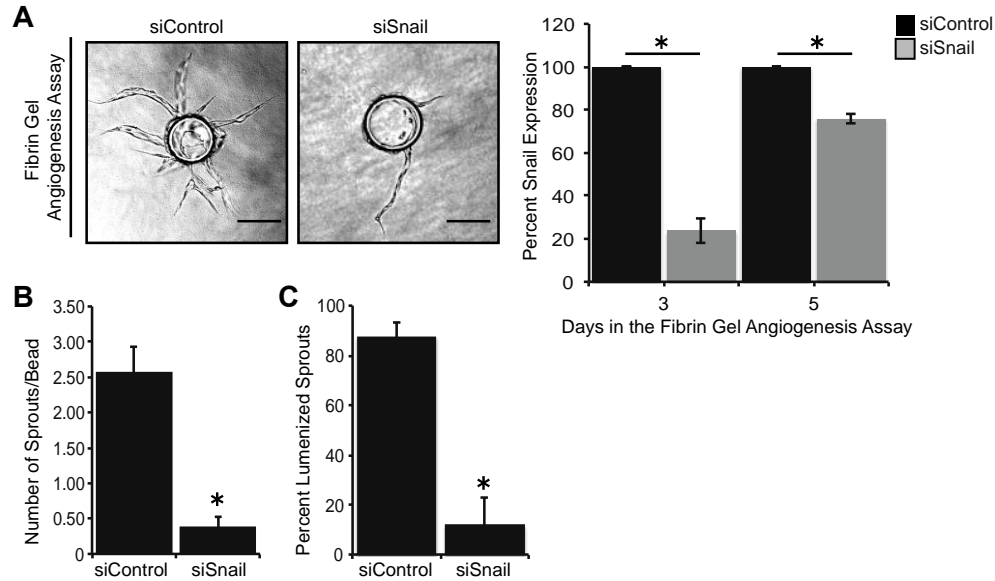
### **Statistical analysis**

Researchers were blinded to experimental conditions prior to performing quantifications. All experiments were repeated at least three times. Data are reported as mean  $\pm$  standard error of the mean (SEM). A Student's t-test was used to analyze differences between experimental groups of equal variance when only two groups were being compared.

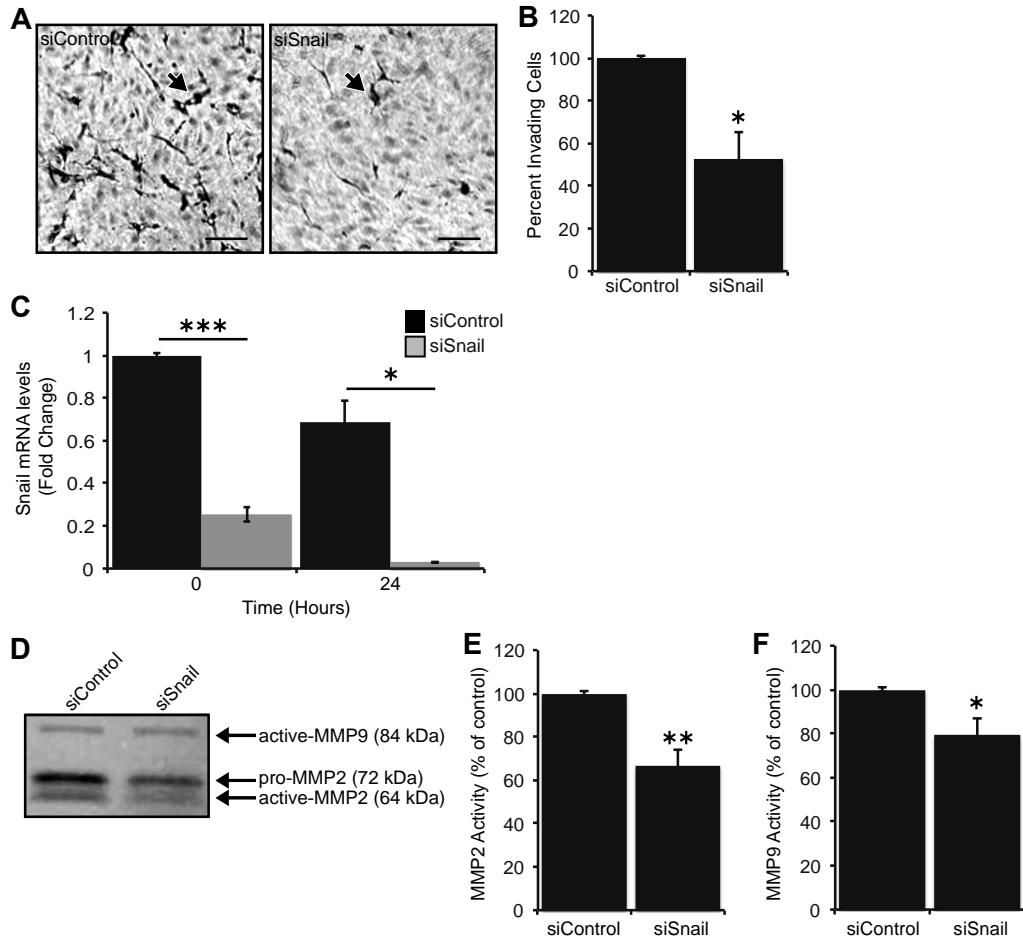


**Figure 4.1. Angiogenic EC express Snail.** (A) Representative images depicting EC morphogenesis during *in vitro* angiogenesis in fibrin gels. Nascent sprouts (arrowhead) are observed on day 3 and continue to proliferate, migrate, branch (arrow) and form lumens (asterisk) through days 6-10. Scale bars, 150  $\mu$ m. (B) EC were harvested on the indicated days from fibrin gels and Snail mRNA levels were assessed by qRT-PCR. Results conveyed as fold change over day 0  $\pm$  SEM ( $n=3$ ; \* $P<0.05$ ; Student's t-test). (C) Human colon cancer tissue sectioned and stained for Snail. Arrows depict Snail positive EC. Scale bars, 50  $\mu$ m. Image from The Human Protein Atlas, with permission ([www.proteinatlas.org](http://www.proteinatlas.org)).

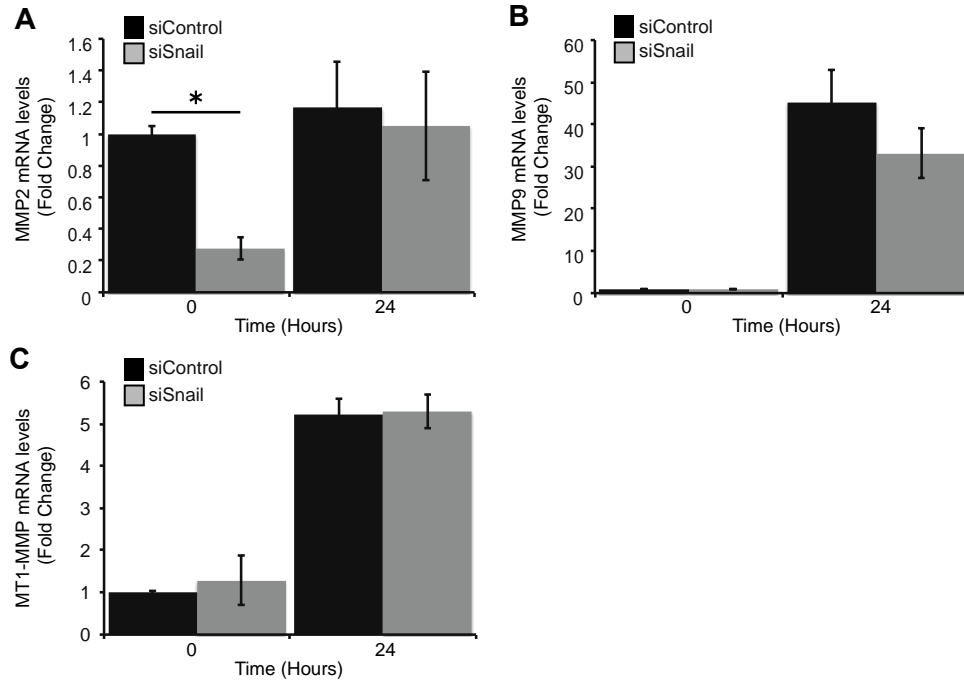




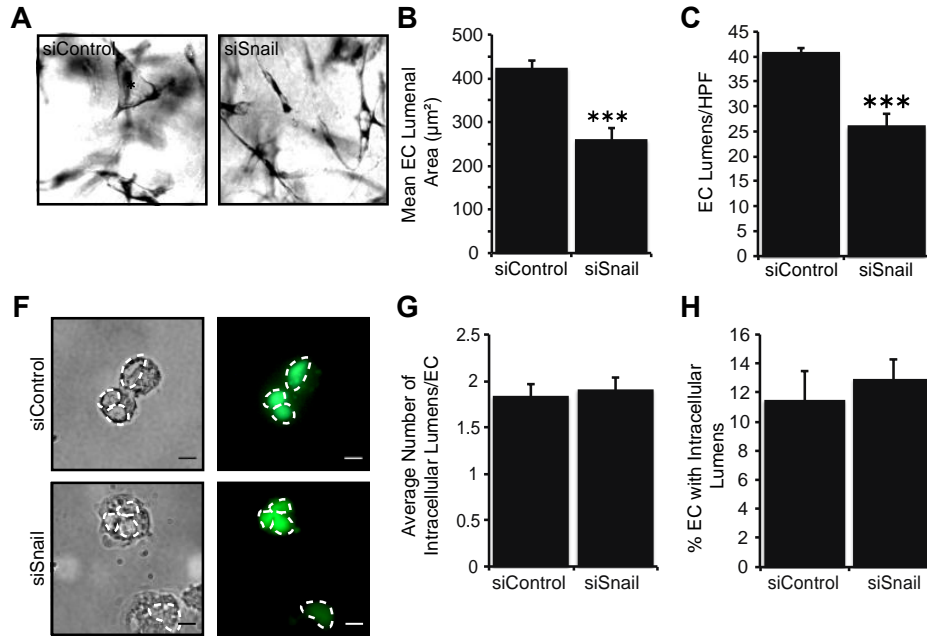
**Figure 4.2. Loss of Snail inhibits EC sprouting in an *in vitro* angiogenesis assay.** (A-C) EC were transfected with control or Snail siRNA and seeded into the fibrin gel angiogenesis assay. (A) Representative images from one of at least three similar experiments are shown from analysis on day 5. Scale bars, 150  $\mu$ m. Snail mRNA levels were assessed by qRT-PCR on day 3 and day 5 of the assay. Results are shown as percent of control set to  $100 \pm$  SEM ( $n=2$ ;  $*P<0.005$ ; Student's t-test). (B-C) Sprouting, defined as a vessel with length greater than or equal to the diameter of the bead (150  $\mu$ m), and lumen formation, defined as a vessel with a luminal space throughout the entire vessel, were quantified on day 5 of the fibrin gel angiogenesis assay. Results are expressed as mean  $\pm$  SEM ( $n=2$ ;  $*P<0.005$ ; Student's t-test).



**Figure 4.3. Loss of Snail inhibits EC invasion into 3D collagen matrices.** (A-C) EC transfected with control or Snail siRNA were seeded on top of collagen I gels and stimulated to invade for 24 hours. (A-B) Gels were fixed and stained, and representative images of each condition were captured at 24 hours and invading cells were quantified using NIH Image J. Arrows indicate invading cells. Scale bars, 100  $\mu$ m. Results are shown as percent of control set to  $100 \pm$  SEM ( $n=2$ ;  $*P<0.02$ ; Student's t-test). (C) Transfected EC were harvested at the indicated time points and mRNA levels of Snail were determined by qRT-PCR. Results shown as fold change over time  $0 \pm$  SEM ( $n=2$ ;  $***P<0.002$  and  $*P<0.02$ ; Student's t-test). (D-F) EC were transfected with control or Snail siRNA, seeded on top of collagen gels, and stimulated to invade. After 24 hours, culture medium was collected and MMP activity assessed by gelatin zymography. (E) Quantitative analysis of MMP2 activity after Snail knockdown. Results of the zymography are shown as percent of control set to  $100 \pm$  SEM ( $n=2$ ;  $**P<0.01$ ; Student's t-test). (F) Quantitative analysis of MMP9 activity after Snail knockdown. Details as for MMP2 ( $n=2$ ,  $*P<0.05$ ; Student's t-test).



**Figure 4.4. Loss of Snail does not affect MMP mRNA levels following EC invasion.** (A-C) EC transfected with control or Snail siRNA were seeded on top of collagen I gels and stimulated to invade for 24 hours. EC were harvested at the indicated time points and mRNA levels of MMP2 (A), MMP9 (B), and MT1-MMP (C) were determined by qRT-PCR. Results shown as fold change over time  $0 \pm \text{SEM}$  ( $n=2$ ;  $*P<0.01$ ; Student's t-test).



**Figure 4.5. Loss of Snail disrupts late-stage lumen formation in 3D collagen matrices.** (A-C) HUVEC transfected with control or Snail siRNA were seeded into collagen matrices and stimulated to invade for 24 hours. (A) Assays were fixed, stained, and representative images were captured at 24 hours. (B) EC lumens were quantified by manually tracing intracellular and intercellular lumens (asterisk) using NIH ImageJ software. Results show mean EC luminal area  $\pm$  SEM ( $n=2$ ; \*\*\* $P<0.007$ ; Student's t-test). (C) Lumens were also quantified by counting the total number of EC lumens per high power field (HPF) using NIH ImageJ software. Data are represented as EC lumens/HPF  $\pm$  SEM ( $n=2$ ; \*\*\* $P<0.004$ ; Student's t-test). (D-F) HUVEC transfected with control or Snail siRNA were seeded into collagen gels and allowed to undergo morphogenesis in the presence of soluble FITC-conjugated dextran. After 4 hours, collagen gels were digested to release the cells from the 3D matrix for live imaging on collagen-coated coverslips. (D) Bright field images and corresponding fluorescent images are shown. Scale bars, 10  $\mu\text{m}$ . The average number of fluorescently labeled intracellular lumens (E) and the percent of cells containing fluorescently labeled intracellular lumens (F) were quantified and are expressed as mean  $\pm$  SEM ( $n=2$ ).

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## **CHAPTER 5**

### **Summary and Conclusions**

#### **DISCUSSION**

The Snail superfamily of zinc-finger transcription factors occupies a central role in cellular morphogenesis during embryonic development and cancer progression. In particular, this protein family is critical in triggering epithelial-to-mesenchymal transition (EMT), a program fundamental to both normal development and many pathological conditions, such as cancer, fibrosis, and wound healing.<sup>1-3</sup> During EMT, epithelial cells alter their polarity, dissolve intercellular junctions, degrade basement member (BM), and transform into migratory, mesenchymal cells. However, these cellular alterations are not necessarily ordered nor do epithelial cells acquire all the characteristics associated with EMT. In fact, when epithelial cells lose genes that define them as epithelial, such as epithelial-cadherin (E-Cadherin), but do not fully commit to a mesenchymal phenotype or genotype, the event is termed a partial EMT.

Partial EMT has been observed in both developmental and pathological states. For example, partial EMT is implicated in the branching morphogenesis that occurs during the formation of several organs, including the mammary glands, kidneys and trachea.<sup>2</sup> In addition, partial EMT occurs during re-epithelialization of wounded skin. In this instance, although keratinocytes undergo a series of changes reminiscent of EMT including loss of polarity, alterations of the actin cytoskeleton, adjustments in cell-cell contacts, and



breakdown of basement membrane (BM), they do, however, retain some intercellular junctions and migrate as a cohesive cell sheet.<sup>4</sup>

In recent years, data indicate that other cell types, namely endothelial cells (EC) undergo endothelial-to-mesenchymal transitions (EndMT), a program reminiscent of EMT. In fact, it was speculated that EndMT might play a central role in angiogenic sprouting by enabling tip-cells to migrate into adjacent tissue.<sup>5</sup> Tip-cells certainly have phenotypes similar to mesenchymal cells and, as they are positioned at the angiogenic front, are exposed to a variety of pro-angiogenic stimuli including vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), transforming growth factor- $\beta$  (TGF- $\beta$ ), fibroblast growth factor (FGF), and epidermal growth factor (EGF). Knowing that these stimuli also induce expression of the EMT transcription factors Snail and Slug in epithelial cells, it is easy to hypothesize that these growth factors may also induce their expression in EC to promote angiogenesis. Furthermore, TGF- $\beta$ , the bone morphogenetic protein (BMP) pathway, as well as Wnt and Notch signaling cascades have also been found to induce Snail and Slug expression.<sup>6-</sup>  
<sup>8</sup> Importantly, these signaling pathways also play key roles during angiogenesis. Finally, angiogenesis is an invasive process that requires alterations in polarity, cytoskeletal rearrangement, and proteolytic degradation of extracellular matrix (ECM), all changes that enable a nascent sprout to migrate. It is therefore intriguing to consider that EC may require Snail family members during the formation of vascular tubes, thus suggesting angiogenesis as a partial EndMT process.

This thesis work identified Snail and Slug as being regulated by EC during *in vitro* angiogenesis. The unique and dynamic expression patterns of Snail and Slug suggest that these transcription factors regulate different steps, but perhaps overlapping genes, during the angiogenic cascade. To elucidate a role for these transcription factors during angiogenesis, we independently inhibited their expression using small interfering RNA (siRNA). We showed that inhibition of Snail or Slug expression prevents sprouting angiogenesis, invasion, and lumen formation in multiple *in vitro* angiogenesis assays. We next hypothesized that Snail and Slug were inducing a partial EndMT owing to the fact that EC maintain their junctional integrity and migrate together during tube formation. To no surprise, expression levels of the adherens junction protein vascular endothelial-cadherin (VE-Cadherin) were not altered in the absence of Slug expression (this experiment was not completed in the absence of Snail). Instead, inhibition of Snail or Slug led to reduced activity of both matrix metalloproteinase (MMP)-2 and MMP-9. Given that several MMPs are indirectly regulated subsequent to Snail or Slug expression, these findings were in line with published results. Furthermore, membrane type 1-MMP (MT1-MMP) was significantly decreased in the absence of Slug. Importantly, retroviral-mediated re-expression of this gene recovered the sprouting defect observed in the absence of Slug. Given that Slug did not regulate VE-Cadherin but did alter activity and expression of several MMPs, we hypothesize that angiogenesis is indeed a partial EndMT process. However, our findings also suggested that the partial EndMT event was not exclusive to tip-cells as previous hypothesized.

Although activity of MMP-2 and MMP-9 were altered in the absence of Snail or Slug, it is unlikely that this is a result of direct transcription repression. In fact, mRNA expression levels of MMP-2 and MMP-9 were not altered when Snail or Slug were inhibited. And, while mRNA levels of MT1-MMP were decreased in the absence of Slug providing an explanation for the decreased activity of MMP-2, it is yet to be determined if Slug directly regulates the transcription of MT1-MMP. Alternatively, if Slug does not directly regulate MT1-MMP transcription, studies show that Slug expression promotes formation of the  $\beta$ -catenin/T-cell factor (TCF) transcription complex.<sup>9</sup> Moreover, in an independent study it was demonstrated that MT1-MMP was a target of the  $\beta$ -catenin/TCF complex.<sup>10</sup> Together these findings provide a potential mechanism by which Slug indirectly regulates MT1-MMP in angiogenic EC: Slug induces formation of the  $\beta$ -catenin/TCF complex resulting in increased MT1-MMP transcript.

Worth noting is the low endogenous expression levels of Snail and Slug in EC culture in 2D. Even after addition of endothelial growth media (EGM) supplemented with an abundance of pro-angiogenic growth factors, Snail and Slug expression remain relatively low. However, when EC are placed in a 3D environment and stimulated with EGM, their expression levels dramatically increase. We therefore speculate that integrins may play a role in these observations. Indeed, integrin  $\alpha\beta 3$  upregulates Slug expression in cancer cells and expression of this same integrin is increased in tumor vessels.<sup>13, 14</sup> In addition, Slug expression results in down-regulation of integrins  $\alpha 3$ ,  $\beta 1$  and  $\beta 4$  in keratinocytes.<sup>15</sup> Although these data point to a potential link between Slug and integrins, it should also be mentioned that many of our 3D culture systems contain fibroblasts. These mural cells

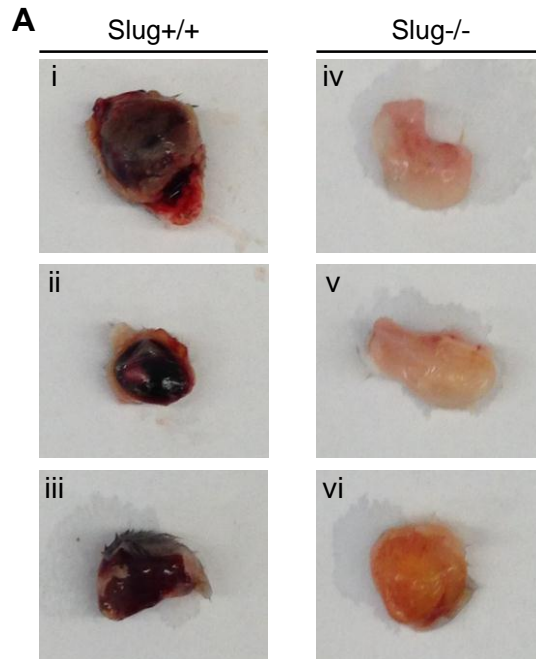
secrete additional pro-angiogenic factors that may synergize with signals from the 3D matrix (integrins) to facilitate induction of Snail and Slug. For example, we have shown that upon inhibition of HGF produced by fibroblasts, sprouting is dramatically reduced. Given that HGF induces Snail and Slug expression and that inhibition of Snail or Slug results in reduced sprouting, we speculate that HGF might be one factor inducing expression of these transcription factors in our 3D culture systems.

In addition to our own findings, *in vivo* work completed by others also points to a role for Snail and Slug during angiogenesis. In humans, Snail expression was increased in the vasculature of breast carcinoma and Slug expression was elevated in vessels of ovarian carcinoma suggesting that these two genes may be important during pathological angiogenesis.<sup>16, 17</sup> Since embryonic lethality occurs in mice homozygous for a null mutation in the Snail gene, few studies have been completed to identify a role for Snail beyond gastrulation. However, in one study it was observed that a conditional deletion of Snail mid-gestation resulted in embryonic lethality in part due to severe cardiovascular defects.<sup>18</sup> This finding hints that Snail also plays a role in blood vessel formation and studies on an EC-specific conditional Snail knockout may help to reveal this function.

In sharp contrast to mice homozygous for a null mutation in Snail, mice homozygous for a null mutation of Slug are viable suggesting this gene is not critical for vascular developmental. However, we cannot rule out the involvement of Slug in pathological angiogenesis. In fact, as discussed in chapter 2, there are several examples of genes being dispensable during developmental angiogenesis but required for pathological

angiogenesis.<sup>19-21</sup> We therefore hypothesized that Slug may be one of these genes. Excitingly, preliminary findings suggest that Slug is indeed important during pathological angiogenesis. Using a Matrigel plug angiogenesis assay, we observed less blood vessel infiltration into Matrigel plugs in Slug knockout mice compared to control mice (Figure 4.1). Further studies including immunohistochemistry analysis will be necessary to evaluate the precise number of blood vessels invading the gels and to rule out that increased vessel infiltration in control mice Matrigel plugs was not simply due to increased invasion of fibroblasts. Regardless, these are promising findings that our lab will continue to pursue.

In summary, the findings presented in this thesis begin to reveal mechanistic actions behind Snail and Slug expression in angiogenic EC. However, more research is warranted to complete these stories. In particular, several questions still remain about the molecular mechanisms behind the actions of Snail. Specifically, we plan to uncover additional downstream targets of Snail family members using RNA-sequencing. With these data we hope to identify additional cellular and molecular functions for Snail and Slug during *in vitro* angiogenesis and confirm these findings in pathological angiogenesis models *in vivo*.



**Figure 5.1. Pathological angiogenesis is visual reduced in Slug knockout mice.** (A) Matrigel was spiked with VEGF (400 ng/ $\mu$ l), bFGF (400 ng/ $\mu$ l), or heparin (20U/ml) and injected subcutaneously into the ventral side of control (Slug+/+; Ai-iii) or knockout (Slug-/-; Aiv-vi) mice that were 24 weeks of age. The Matrigel plug was then harvested seven days post injection and visualized for vessel infiltration. ( $n=3$ )

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## APPENDIX I

### Oligonucleotides used in this study

#### qRT-PCR Primers

Gene Name	Sequence 5' → 3'	
α <sub>v</sub>	GCAGTCAGAGATGGATACT	(forward)
	GACTCGAGACTCCTCTTATC	(reverse)
β <sub>3</sub>	CCCTGCTCATCTGGAAACTC	(forward)
	CGGTACGTGATATTGGTGAAGG	(reverse)
DII4	GTAACGAATGCATCCCCACAAT	(forward)
	CTCCCCAGCCCTCATCACAAGTA	(reverse)
GAPDH	TCGACAGTCAGCCGCATCTTC	(forward)
	GCGCCCAATACGACCAAATCC	(reverse)
Jagged-1	CTGCCCAACCCCTGCCATAAT	(forward)
	GCACCGGTACCAGTTGTGTCCAT	(reverse)
MMP2	CGCAGTGACGGAAAGATGTGG	(forward)
	AGAGCTCCTGAATGCCCTTGA	(reverse)
MMP9	ACCAAGTGGGCTACGTGACCTATG	(forward)
	GTATCCGGCAAACCTGGCTCCTT	(reverse)
MT1-MMP	CATGGCCACGGTGTCAAAGTT	(forward)
	CTCCCGGCCTTCTGTTCCCTG	(reverse)
N-Cadherin	CAGTATCCGGTCCGATCTGC	(forward)
	GTCCTGCTCACCACCACTAC	(reverse)
Slug (Snai2)	AGATGCATATTCGGACCCAC	(forward)
	CCTCATGTTTGTGCAGGAGA	(reverse)
Snail (Snai1)	GCCTTCAACTGCAAATACTGC	(forward)
	CTTCTTGACATCTGAGTGGGT	(reverse)
VE-Cadherin	CGCCCGGCCTTCCCTCTA	(forward)
	CGTGGTCCGCCTCGTCCTT	(reverse)
Vimentin	GACGCCATCAACACCGAGTT	(forward)
	CTTTGTCGTTGGTTAGCTGGT	(reverse)

#### siRNA Oligos

Gene Name – Company	Sequence 5' → 3'	
Slug (Snai2) – Invitrogen	GGCUCAUCUGCAGACCCAUUCUGAU	(sense)
	AUCAGAAUGGUCUGCAGAUGAGCC	(antisense)
Slug (Snai2) – Ambion	CAAUAAGACCUAUUCAACUtt	(sense)
	AGUUGAAUAGGUCUUAUUGca	(antisense)
Snail (Snai1) – Invitrogen	CCUCGCUGCCAAUGCUCaucUGGGA	(sense)
	UCCCAGAUGAGCAUUGGCAGCGAGG	(antisense)

## **APPENDIX II**

### **List of Abbreviations**

2D	Two-dimensional
3D	Three-dimensional
$\alpha$ -SMA	$\alpha$ -smooth muscle actin
Ang-1	Angiopoietin-1
ANOVA	Analysis of variance
BM	Basement membrane
BMP	Bone morphogenetic protein
CAF	Cancer associated fibroblast
CRC	Colorectal cancer
CXCR-4	C-X-C chemokine receptor-4
Dll-4	Delta-like ligand-4
EBM	Endothelial basal media
E-Cadherin	Epithelial-cadherin
EC	Endothelial cell
ECGS	Endothelial growth supplement
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
EGM-2	Endothelial growth media-2
EMT	Epithelial-to-mesenchymal transition

EndMT	Endothelial-to-mesenchymal transition
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGF-2	Fibroblast growth factor-2
FITC	Fluorescein isothiocyanate
FSP-1	Fibroblast specific protein-1
GFP	Green fluorescent protein
GLMM	Generalized linear mixed model
GSK-3 $\beta$	Glycogen synthase kinase 3 $\beta$
HBSS	Hank's balanced salt solution
HGF	Hepatocyte growth factor
HLF	Hepatic leukemic factor
HPF	High power field
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cell
Jag-1	Jagged-1
MDCK	Madin-darby canine kidney
MMP	Matrix metalloproteinase
MT1-MMP	Membrane type-1 matrix metalloproteinase
NHLF	Normal human lung fibroblast
NICD	Notch intracellular domain
NRP-2	Neuropilin receptor-2
PAI-1	Plasminogen activator inhibitor-1

Pak-1	P21-activated kinase-1
PBS	Phosphate buffer solution
PC	Pericytes
PDMS	Polydimethylsiloxane
PDGFB	Platelet-derived growth factor B
PDGFR- $\beta$ .	Platelet-derived growth factor receptor- $\beta$
PEG	Polyethylene glycol
PIGF	Placental growth factor
PMA	Phorbol 12-myristate 13-acetate
qRT-PCR	Quantitative real time polymerase chain reaction
RTK	Receptor tyrosine kinase
sFlt1	Soluble Flt1
S1P	Sphingosine-1 phosphate
SDF-1	Stromal cell-derived factor-1
SEM	Standard error of the mean
siRNA	Small interfering RNA
SMC	Smooth muscle cells
TCF	T-cell factor
TGF- $\alpha$	Transforming growth factor-alpha
TGF- $\beta$	Transforming growth factor-beta
TIMP	Tissue inhibitor of matrix metalloproteinase
TNF- $\alpha$	Tumor necrosis factor-alpha
TNFR1	Tumor necrosis factor receptor-1

TSP-1	Thrombospondin-1
UNC5B	Unc-5 homolog B
VE-Cadherin	Vascular endothelial-cadherin
VEGF	Vascular endothelial growth factor