ABSTRACT

GASIOR, KELSEY ILENE. A Computational and Experimental Model of the Epithelial Mesenchymal Transition (Under the direction of Marlene Hauck and Sudin Bhattacharya).

After a carcinoma forms, neoplastic cells are able to metastasize by undergoing the epithelial mesenchymal transition (EMT). EMT, triggered by cues from inflammatory and stromal cells in the surrounding microenvironment, allows epithelial cells to lose their highly adhesive nature and, instead, adopt the spindle-like appearance, as well as the invasive and migratory behavior, of mesenchymal cells. It is our hypothesis that there is a bistable switch between the epithelial and mesenchymal phenotypes governing EMT—where the cell can maintain its mesenchymal phenotype and behavior after it leaves the primary tumor microenvironment and extracellular signal.

By establishing a system of ordinary differential equations (ODEs), we present a simple mathematical model of EMT using the Wnt pathway and the roles played by Dvl, E-cadherin, β-catenin, and Slug as a case study. With the activation of the Wnt pathway, Dvl moves to the cell membrane to inhibit the β-catenin degradation complex. The model presented here suggests that there is a threshold level of membrane bound Dvl that the cell must overcome in order for it to transition to the mesenchymal state, a state that the cell is able to maintain once it has migrated away from the tumor. Additionally, the use of sensitivity analysis suggests that the steady state behavior of E-cadherin and Slug are more sensitive to changes in the rate at which β-catenin translocates to the nucleus and activates Slug after Wnt has been activated. These results highlight the importance of the behavior of β-catenin within the cell.

The TGF-β signaling pathway is also capable of activating EMT. Experiments using ELISAs, flow cytometry, qPCR, and immunocytochemistry techniques show that MCF7 breast carcinoma cells can occupy two distinct states, epithelial or mesenchymal. In contrast, SW480
colon carcinoma cells must pass through a transitional phase to become mesenchymal cells. Taken together, these data suggest that EMT activation via TGF-β is not a universal process between cell lines.

Using the data obtained from the MCF7 cells and the techniques developed in mathematically modeling the Wnt pathway, we mathematically explored the dynamics between E-cadherin, Slug, and cell-to-cell adhesion when attempting to upregulate EMT via the TGF-β signaling pathway. This work suggests that cells with many neighbors could only undergo the bistable switch from the epithelial to the mesenchymal phenotype if they were to lose cell-to-cell contact due to the migration of neighboring cells in the presence of TGF-β. The model also suggests that, with the application of TGF-β, the MCF7 cells comprising the mesenchymal state are those with fewer than two neighboring cells, a result that is supported by the immunocytochemistry experiments.

The insights gained from the feedback between developing modeling techniques and experimental data, as well as understanding the possible sources of error that exist between the two, are essential for understanding the activation of EMT in carcinomas. The mathematical model presented in this work can be used to guide future in vitro experiments, specifically those capable of providing further insights on the behavior of cells as they undergo EMT. Additionally, this work could be beneficial to the creation of more detailed models capable of guiding the development of future therapies.
A Computational and Experimental Model of the Epithelial Mesenchymal Transition

by
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DEDICATION

I was never the little girl who thought a career in science was an option for her. Thank you, Mr. Steven Thumser, for not only showing me that this path was possible, but for giving me the future that is yet to come.
BIOGRAPHY

Kelsey Gasior was born in the state of Michigan after blatantly refusing to exit the womb. Twice. Raised in the small towns of Milford and Brighton, MI, young Kelsey wanted to be a chef, a lawyer, a writer, a senator, and everything else in between before graduating from Brighton High School in 2007 with a passion to study mathematics. While attending the University of Michigan, Kelsey obtained a Bachelor of Science with a major in Applied Mathematics and a minor in Physics and, ironically, discovered her passion for Mathematical Biology while working at her first job in astrophysics research. In 2011, Kelsey followed her love of Mathematical Biology all the way to Raleigh, North Carolina where she spent time working on her PhD at North Carolina State University. In the winter, Kelsey will continue to pursue her quest for an unconventional career path by entering a postdoctoral position in the Department of Biology at the University of North Carolina.
ACKNOWLEDGMENTS

First of all, thank you to my parents, Kim and Diane, for every late night phone call answered, every care package sent, every hug, every word of encouragement…. The list could go on for forever. Thank you for being my rock. I love you both so dearly. To my brother, Zach, thank you for teaching me to be thick skinned and, to put it kindly, ‘unapologetically persistent’. To the rest of my family and friends- you have all been my biggest cheerleaders since Day One. In particular, thank you to Lindsey, Angela, Sarah, Nusrat, Alex, Sergio, Caitlin, Jake, Greg, Cassidy, Katelyn, Bruce, the Drs. Hartman, and my Aunt Laurie and Uncle Jeff– I cannot put into words how much your positivity and support has lifted me up during this entire process. A special thank you to Natalie for making sure I didn’t starve in lab during my all-nighters.

To my wonderful lab family, Linda, Nikki, and Taylor, thank you for all of the wonderful memories and support. The long days would have been incredibly dull without your friendship, your smiles, your support, and our time together. Thank you to the fantastic team at Starbucks that I visited every morning for 3 years on my way to work. During the toughest of times, it was the free shots of espresso, the doodles on my cups, and the fact that everyone knew my order before I made it to the register that truly put a smile on my face and helped me through the day.

Finally, a special thank you to my committee, Dr. Marlene Hauck, Dr. Sudin Bhattacharya, Dr. Alyson Wilson, and Dr. Sharon Lubkin, and my department chair, Dr. Alun Lloyd. Thank you so much for your support throughout the past 5 years- I would not be here without your guidance.
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In each subfigure, Dvl (d) is varied along the x-axis while, along the y-axis, a different non-dimensional parameter is varied. For the parameter values in this model, the cell begins in Region II in the epithelial state. If Dvl is increased and the cell crosses L2 into Region III, the cell will switch to the mesenchymal steady state. If the cell were to begin in the mesenchymal steady state in Region II and crosses L1 into Region I with changes in Dvl (d), the cell will switch to the epithelial steady state.

In this set of experiments, cells were grown to three different confluences (30%, 60%, and 100%) and three different concentrations of exogenous TGF-β (0 ng/mL, 3 ng/mL and 9.33 ng/mL) were added to each confluence. In order to understand if the process of EMT and the effects of confluence and exogenous signal are more universal across different tissues, these experimental conditions were carried out in two different cell lines: SW480 cells (colorectal carcinoma) and MCF7 cells (breast carcinoma).

(A) shows an image of MCF7 nuclei stained with DAPI once the background noise has been subtracted using ImageJ. (B) shows the final post processed image corresponding to (A) where each cell was given a corresponding numerical identity using ImageJ.

Images of MCF7 cells at 30% confluence were post-processed using ImageJ. Brightness and contrast was adjusted using a control image for each stain, which are shown on the left.

Images of MCF7 cells at 100% confluence were post-processed using ImageJ. Brightness and contrast was adjusted using a control image for each stain, which are shown on the left.

Images of SW480 cells at 30% confluence were post-processed using ImageJ. Brightness and contrast was adjusted using a control image for each stain, which are shown on the left.

Images of SW480 cells at 100% confluence were post-processed using ImageJ. Brightness and contrast was adjusted using a control image for each stain, which are shown on the left.

Concentrations for cytosolic TGF-β1 for each cell line are derived from 1x10^6 cells. As exogenous TGF-β1 is added and confluence is reduced, the cytosolic concentration of TGF-β1 rises, until it saturates at a concentration of ~ 25 pg/mL for SW480 cells and ~ 9 pg/mL for MCF7 cells. Both confluence and exogenous TGF-β1, as well as the interaction of the two
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The relative expression of E-cadherin and Slug in different treatment groups when compared to 60% confluent MCF7 cells + 0 ng/mL TGF-β (A), 30% confluent MCF7 cells + 0 ng/mL TGF-β (B), 100% confluent MCF7 cells + 3 ng/mL TGF-β (C), and 100% confluent MCF7 cells + 9.33 ng/mL TGF-β (D).

The number of neighbors was determined for each cell line using different thresholding nuclei distances. A and B show the neighbor number for 30% confluent SW480 (A) and MCF7 (B) cells at a threshold of the average nuclei distance while C-F show the two cell lines’ neighbor numbers at a threshold of the average nuclei distance + 1 standard deviation and 2 standard deviations.

The number of neighbors was determined for each cell line using different thresholding nuclei distances. A and B show the neighbor number for 100% confluent SW480 (A) and MCF7 (B) cells at a threshold of the average nuclei distance while C-F show the two cell lines’ neighbor numbers at a threshold of the average nuclei distance + 1 standard deviation and 2 standard deviations.

The percentage of SW480 cells that stained for E-cadherin only, vimentin only, both markers, and neither marker in cell populations that are 30% confluent are shown. The number of neighbors a cell had was determined using a distance threshold of the average distance between nuclei.

The percentage of SW480 cells that stained for E-cadherin only, vimentin only, both markers, and neither marker in cell populations that are 30% confluent are shown. The number of neighbors a cell had was determined using a distance threshold of the average distance between nuclei + 1 standard deviation.

The percentage of SW480 cells that stained for E-cadherin only, vimentin only, both markers, and neither marker in cell populations that are 30% confluent are shown. The number of neighbors a cell had was determined using a distance threshold of the average distance between nuclei + 2 standard deviations.

The percentage of MCF7 cells that stained for E-cadherin only, vimentin only, both markers, and neither marker in cell populations that are 30% confluent are shown. The number of neighbors a cell had was determined using a distance threshold of the average distance between nuclei.
Figure 2.19  The percentage of MCF7 cells that stained for E-cadherin only, vimentin only, both markers, and neither marker in cell populations that are 30% confluent are shown. The number of neighbors a cell had was determined using a distance threshold of the average distance between nuclei + 1 standard deviation.

Figure 2.20  The percentage of MCF7 cells that stained for E-cadherin only, vimentin only, both markers, and neither marker in cell populations that are 30% confluent are shown. The number of neighbors a cell had was determined using a distance threshold of the average distance between nuclei + 2 standard deviations.

Figure 2.21  Examples of staining in the MCF7 cell line. A and B are 30% confluent cells with 0 ng/mL of exogenous TGF-β while C and D are 30% confluent cells with 9.33 ng/mL of exogenous TGF-β. Arrows are color coordinated: blue arrows indicate a cell with a low number of neighbors, green indicates cells with a medium number of neighbors, and orange indicates cells with a high number of neighbors, as determined using a threshold distance of the average nuclei distance + 1 standard deviation. A scale bar of 100 μm is shown in each image.

Figure 2.22  Examples of staining in the MCF7 cell line. Figures A and B are 100% confluent cells + 0 ng/mL of exogenous TGF-β and 100% confluent cells + 9.33 ng/mL of TGF-β in figures C and D. Yellow arrows point to appearance of vimentin in images. A scale bar of 100 μm is shown in each image.

Figure 2.23  Examples of staining in the SW480 cell line. A and B are 30% confluent cells with 0 ng/mL of exogenous TGF-β while C and D are 30% confluent cells with 9.33 ng/mL of exogenous TGF-β. Arrows are color coordinated: blue arrows indicate a cell with a low number of neighbors, green indicates cells with a medium number of neighbors, and orange indicates cells with a high number of neighbors, as determined using a threshold distance of the average nuclei distance + 1 standard deviation. A scale bar of 100 μm is shown in each image.

Figure 2.24  Examples of staining in the SW480 cell line. A and B are 100% confluent + 0 ng/mL of exogenous TGF-β while C and D are 100% confluent + 9.33 ng/mL of exogenous TGF-β. A scale bar of 100 μm is shown in each image.

Figure 3.1  Chapter 1 explored the dynamics between E-cadherin, β-catenin, and Slug. In a carcinoma cell, E-cadherin sequesters β-catenin at the membrane, giving the cell its characteristic adhesive
quality. Free β-catenin in the cytosol is degraded. Upon activation of the Wnt pathway, the degradation complex is disabled and β-catenin is then free to accumulate, translocate to the nucleus, and upregulate Slug. Slug inhibits the production of E-cadherin, thus reducing the concentration of E-cadherin available to sequester β-catenin and thus completing the feedback loop [1, 2].

**Figure 3.2**

(A) Focuses on three key components to EMT (E-cadherin, β-catenin, and Slug) but now in response to TGF-β and cell-to-cell contact. In the presence of neighboring cells, E-cadherin in a carcinoma cell will exist at the membrane to form intercellular bonds. The cytoplasmic tail of E-cadherin is bound to β-catenin while, in the nucleus, Slug is kept at low levels. Once activated due to the presence of exogenous TGF-β, Slug will bind to the E-box element of the E-cadherin promoter, inhibiting E-cadherin production and resulting in a decrease in E-cadherin available to sequester β-catenin. β-catenin can now build up and translocate to the nucleus to activate Slug further, thus completing the feedback loop. (B) For modeling purposes, the feedback loop is simplified so that the contributions of β-catenin to the loop are included in the $k_1$ term.

**Figure 3.3**

(A&B) In the absence of TGF-β, cells with $\mu = 2.3$ begin in the epithelial steady state with E-cadherin active at the membrane and Slug kept at a low value. If $\mu$ is reduced, the membrane bound E-cadherin level is reduced, allowing Slug to accumulate. When the cell overcomes $L_M$, it will become a mesenchymal cell. If the cell were to regain enough cellular neighbors, E-cadherin would translocate to the membrane and it would switch back to the epithelial steady state at $L_E$. When TGF-β is added to the system ($\theta=0.135$ (C&D) and $\theta=0.42$ (E&F)), cells with $\mu = 2.3$ still begin in the epithelial steady state and are still capable of switching to the mesenchymal state as $\mu$ is reduced. However, due to the presence of TGF-β, it is now easier for the cell to transition. If the cell regains cell-to-cell contact, it would require more neighbors in order to force the epithelial phenotype. (G&H) With $\theta=0.75$, the two separate states and the bistable switch have disappeared.

**Figure 3.4**

At $\theta = 0$, cells with $\mu = 2.3$ begin in Region I in the epithelial steady state. As $\mu$ is reduced, the cell passes through Region II and, at $L_2$, transitions to the mesenchymal steady state. If the cell regains neighbors and crosses $L_1$ from Region II to Region I, the cell will reenter the mesenchymal steady state. The black dashed line indicates the parameter value for the model while the blue dashed line shows that, for a certain range of each parameter, it is possible for the cell to begin in Region I, and remain there. Note the absence of a blue dashed line in both (E) and (I), which correspond to parameters that are involved in the term surrounding the input of $\theta$. 
At \( \theta = 0.60 \), the bistable Region II shrinks. Cells with \( \mu = 2.3 \) begin in Region I in the epithelial steady state. The cell on the dashed black line can still undergo a bistable switch but the range of \( \mu \) values that this can occur is much smaller. In all figures, the blue dashed line shows that, for a certain range of each parameter, it is possible for the cell to begin in Region I, and remain there. Note that, with the addition of TGF-\( \beta \), the behavior of the switch at the black line is approaching the behavior of the switch at the blue line: the lack of a bistable switch.

In the absence of TGF-\( \beta \) (\( \theta = 0 \)), cells without any neighbors (\( \mu = 0 \)) begin in the mesenchymal steady state, meaning that membrane-bound E-cadherin is low and Slug is high, while cells with at least 1 neighbors (\( \mu \geq 0.46 \)) begin in the epithelial steady state, indicated by the high value of membrane-bound E-cadherin and the low value of Slug. (A&B) If a small amount of TGF-\( \beta \) (\( \theta = 0.10 \)) is added \( \tau = 100 \), the values of E-cadherin and the Slug family do not significantly deviate from their values without TGF-\( \beta \). With \( \theta = 0.40 \) (C&D) and \( \theta = 0.70 \) (E&F), more TGF-\( \beta \) is required for cells with a higher number of neighbors to be able to both transition to the mesenchymal steady state and maintain the mesenchymal steady state, even after the exogenous signal is removed.

(A&B) In the absence of TGF-\( \beta \), cells with \( \mu = 0 \) begin as mesenchymal cells and, as TGF-\( \beta \) is added, the cells maintain their mesenchymal phenotype. Note the different y-axis scale on (A). Cells with 1+ neighbors begin as epithelial cells in the absence of TGF-\( \beta \) (C-H). (C and D) As TGF-\( \beta \) is added to cells with 1 neighbor, the cell must reach a value of \( \theta = 0.147 \) (blue vertical dashed line) before it can transition to the mesenchymal steady state. (E&F) For cells with 2 neighbors, as TGF-\( \beta \) is added, the cell reach a value of \( \theta = 0.517 \) (blue vertical dashed line) before it can transition to the mesenchymal state. (G&H) For cells with 3 neighbors, no matter how much TGF-\( \beta \) is added to the system, the cell cannot undergo the bistable switch and transition to the mesenchymal steady state.

The black dashed line indicates the parameter value for the model. At \( \mu = 0.43 \) and \( \theta = 0 \), cells begin in Region II in the epithelial state. As TGF-\( \beta \) is increased, the cells pass through Region II, cross L2, and transition to the mesenchymal steady state. If the value of TGF-\( \beta \) in the system is reduced and a cell crosses L2 back into Region II, the cell would maintain its mesenchymal state. In all figures, the blue dashed line shows that, for a certain range of each parameter, it is possible for the cell to begin in Region I and remain there.

When \( \mu = 1.0 \) (G-L), the bistable Region II shrinks. The cell on the black line can still undergo a switch but the range of \( \theta \) values that it can occur at is much smaller. Also, with this increase
in $\mu$, the switch has now changed from an irreversible to a reversible switch: if TGF-β in the system was reduced and the cell reentered Region II and crossed L1 into Region I, it would be capable of reentering the epithelial steady state. Note that, with the addition of TGF-β, the behavior of the switch at the black line is approaching the behavior of the switch at the blue line: the lack of a bistable switch. Additionally, with $\theta > 0$, there is not a parameter value for $n_4$ where the (newly) reversible switch does not exist.

Figure 3.10

Sensitivity analysis was carried out to understand the relationship between steady state behavior of E-cadherin ($e$) and Slug ($s$) and the 6 nondimensional parameters. (A) $(\theta, \mu) = (0, 1.38)$ (B) $(\theta, \mu) = (0.42, 1.38)$ (C) $(\theta, \mu) = (0, 0.92)$ (D) $(\theta, \mu) = (0.42, 0.92)$ (E) $(\theta, \mu) = (0, 0.46)$ (F) $(\theta, \mu) = (0.42, 0.46)$ (G) $(\theta, \mu) = (0, 0)$ (H) $(\theta, \mu) = (0.42, 0)$. For both levels of $\theta$, the steady state behaviors of E-cadherin and Slug were sensitive to changes in their own degradation rates, $G_1$ and $G_3$, respectively, as $\mu$ was decreased, the steady state behavior of E-cadherin gained sensitivity to changes in $G_3$ and lost sensitivity to $G_1$. Additionally, with an increase in $\theta$, at all levels of cell-to-cell contact, the sensitivity of Slug to changes in $A_3$ decreases while, for cells with at least 3 neighbors, the sensitivity of E-cadherin to changes in $F_2$ increases.

Figure 3.11

The cell indicated in (A) begins at point 1 with 3 neighbors ($\mu = 1.38$). When a uniform field of TGF-β is added ($\theta = 0.70$) at point 2, two of its neighbors are capable of transitioning to the mesenchymal steady state. Once these cells migrate away from the cell, the cell will be left with 1 neighbor ($\mu = 0.46$) at point 3, allowing it to transition to the mesenchymal state. The cell can detach from its final neighbor at point 4 and then migrate away from the TGF-β field. Even after the cell has left the field of TGF-β, it maintains its mesenchymal phenotype, thus indicating that it has undergone the bistable switch. The bifurcation diagrams for the cell indicated in (A) during this process for E-cadherin and Slug are in (B) and (C) respectively.

Figure 3.12

(A) Relative expression when cells are 30% confluent (A), 60% confluent (B), and 100% confluent (C). From Chapter 2, the relative expression of E-cadherin mRNA to $\theta = 0$ and its standard error is shown in blue and the model-predicted relative expression of E-cadherin protein to $\theta = 0$ is shown in yellow. The relative expression of Slug mRNA to $\theta = 0$ and the standard error is shown in red while the model-predicted relative expression of Slug to $\theta = 0$ is shown in green.

Figure A1

Figures A1A-A1EN show the monotonic behavior for each of the three nondimensional variables ($e$, $b$, $s$) in response to changes in the 8 nondimensional parameters at select values of Dvl $(d)$ in Chapter 1. Figures in the left column show the parameter varied over its parameter

xvi
space in a system with epithelial initial conditions while figures in the right column show the individual parameter varied over its parameter space with mesenchymal initial conditions.

**Figure A2.**
The monotonic behavior for $\epsilon$ and $s$ in response to changes in the 6 nondimensional parameters at select values of both TGF-β ($\theta$) and cell-to-cell contact ($\mu$).
INTRODUCTION

Cancer is the second most common cause of death in the United States, with 500,000+ cancer related deaths occurring in 2015 [3]. Of all cancer related deaths occurring in the Western Hemisphere, 80% are attributed to carcinomas. Arising from epithelial tissue, carcinomas are the most common type of cancer seen in humans [4] and, for some tissues, the majority of deaths do not occur because a tumor has formed, but rather from the metastases that result [5, 6].

Epithelial cells are characterized by their highly adhesive nature with each other, a characteristic that inhibits individual cell movement [7-9]. In order for a carcinoma to metastasize, epithelial cells must acquire the ability for individual cells to migrate away from the tumor via the epithelial mesenchymal transition (EMT) [10]. A cellular process that is vital to embryogenesis and development past the blastula phase [11], EMT allows carcinoma cells to gain the spindle-like phenotype [12] and individual cell movement that is characteristic of mesenchymal cells that ultimately allows them to migrate and invade the surrounding tissue.

Carcinoma cells rely on external signals from the microenvironment in order to activate the transition into mesenchymal-like cells [13, 14]. However, once the cell gains the migratory properties attributed to mesenchymal cells, it will break through the membrane and into the blood stream, thus leaving its initial microenvironment and the extracellular signal that activated the transition [10]. In order to complete the trek to a different tissue and ultimately form a metastasis, the cell must maintain its mesenchymal phenotype and behavior even after it has lost initial extracellular cues. The maintenance of this behavior is crucial to the formation of a metastasis, raising the question of exactly how an initially adhesive cell is capable of
retaining this invasive behavior. Understanding the driving force behind the maintenance of the newly acquired phenotype and behavior could ultimately lead to a path towards treatment and prevention: how to force the cell back to its epithelial phenotype or prevent it from acquiring the mesenchymal phenotype so as to prevent a metastasis.

Mathematical models and laboratory generated data are excellent tools in understanding the driving force behind these changes, as well as the relationships between intracellular proteins and extracellular signals. By using the two methodologies together, scientists can gain a clearer picture of how carcinoma cells are able to metastasize but, more importantly, mathematical models and laboratory experiments can highlight the limitations of each individual method and potential sources of error. In this work, we attempt to take advantage of the unique perspective associated with using mathematical modeling techniques and generating our own laboratory data in order to create relevant mathematical models that could help us understand the dynamics underlying EMT and provide a guiding scaffold upon which future work could be built.
CHAPTER 1: A Theoretical Model of the Wnt Signaling Pathway and the Epithelial Mesenchymal Transition (EMT)

Background

Epithelial tumors, or carcinomas, are the most common type of neoplasia found in humans, accounting for roughly 80% of all cancer related deaths in the Western Hemisphere [4]. Epithelial cells are characterized by their apical-basal polarity and high cell-cell adhesion [8]. When two epithelial cells are in contact with one another, the E-cadherin protein is sequestered at the membrane of each cell and bound across the intercellular space. Simultaneously, E-cadherin is bound to intracellular catenin members on its cytoplasmic tail [15]. This E-cadherin complex is what gives the epithelial cells in both normal tissue and carcinomas their strong adhesive bonds and inhibits the movement of individual cells [8, 9].

In carcinomas, it is possible for neoplastic cells to undergo the epithelial mesenchymal transition (EMT) [15]. Triggered by cues from the surrounding stroma that lead to intracellular changes [16], EMT allows epithelial cells to alter their physical structure so as to acquire the spindle-like appearance of mesenchymal cells, as well as their invasive and migratory properties [12]. While this process plays a key role in embryogenesis [17], occurrence of EMT in tumors allows transformed cells to invade the extracellular matrix (ECM) around the primary tumor, penetrate the basement membrane of the blood vessel, and travel via the bloodstream to other locations in the body where they ultimately form a metastasis [10].

There are a multitude of different pathways that can activate EMT, with significant crosstalk between these signaling systems. One of them is the Wnt signaling pathway, which has long been the focus of studies attempting to understand cellular behavior. Lee et al. [18]
proposed a fifteen-equation mathematical model centered upon the formation of the protein complexes involved in β-catenin phosphorylation and degradation, a process that, when disrupted, leads to the accumulation of the β-catenin/TCF complex in the nucleus. In particular, the authors attempt to explain the distinct roles that different members of the degradation complex carry out. In addition, these authors carried out experiments in *Xenopus* egg extracts to support their hypothesized interactions [18]. Ramis-Conde et al. [19] built on the work of Lee and others with a multiscale mathematical model that examined the involvement of E-cadherin and β-catenin in the adhesion of epithelial cells to one another. Like Lee et al., Ramis-Conde et al. included the involvement of β-catenin in the degradation complex in the cytosol. However, Ramis-Conde et al. also focused on the behavior of E-cadherin. The authors considered the concentration of E-cadherin to be a constant that is then divided by sub-cellular localization: free E-cadherin at the membrane, free E-cadherin in the cytosol, and the E-cadherin-β-catenin membrane complex [19]. Basan et al. [20] also explored the adhesion complex relationship. However, where Ramis-Conde et al. considered the key component of cellular adhesion to be the binding of β-catenin and E-cadherin, which they modeled using ordinary differential equations (ODEs), Basan et al. examined the relationship between β-catenin and E-cadherin and the involvement of α-catenin in the adhesion complex by using reaction-diffusion equations [20].

Other authors have continued to explore the dynamics of the Wnt signaling pathway, as well as its crosstalk with other pathways. Shin et al. [21] used six ordinary differential equations to examine the link between ERK and Wnt pathways. In addition to the involvement
of the adhesion complex and the degradation complex, these authors included the shuttling of β-catenin to the nucleus and its involvement with the transcription factor Slug. They also examined the changes in E-cadherin in response to different oncogenic stimuli: EGF and Wnt, and proposed a switch-like behavior in E-cadherin, allowing the cell to transition from the epithelial to mesenchymal state [21]. Shin et al. are not alone in discussing the possibility of a switch in cellular behavior underlying EMT. Indeed, MacLean et al. [22] also proposed a 19-equation model of the bistable switch between the epithelial and mesenchymal steady states, highlighting the importance cytoplasmic-nuclear shuttling of β-catenin.

Due to the complex nature of the Wnt signaling pathway and intracellular signals as a whole, the models presented by these authors are quite intricate, featuring large sets of equations, and most only focus on a few of the molecular interactions β-catenin is involved in. In this work, we, like Shin et al. and MacLean et al., hypothesize that the mechanism underlying EMT in a primary solid tumor is a bistable switch between the epithelial and the mesenchymal phenotypes: once transitioned the cell will maintain its mesenchymal phenotype, even in the absence of sustained extracellular signaling. With regards to the Wnt pathway, we hypothesize that the bistable switch is centered around the behavior and interactions of β-catenin. Unlike the previous works mentioned, the model put forth here incorporates all three primary interactions β-catenin is involved in: the adhesion complex with E-cadherin, its degradation via the complex formed by GSK-3β, and its translocation to the nucleus to activate the transcription factor Slug. Additionally, even though we are examining all three relationships involved in the switch-like behavior of the cell, we propose a simplified theoretical model of only three ODEs to describe the ultrasensitive feedback loop in the Wnt-
β-catenin signaling pathway. By creating a simple model of the primary interactions of β-catenin in the Wnt pathway, we are able to show how activation of the Wnt pathway alone can drive EMT in a carcinoma cell, as well as explain the intracellular β-catenin interactions that may be causing the bistable switch. Our model could provide a scaffold upon which other, more complex models, of EMT and the bistable switch could be built, as well as a guide for experimental exploration into which interactions could be targeted for prevention of EMT and metastasis.

Methods

In a Wnt-absent environment, E-cadherin binds with intracellular catenin members such as α-, β-, γ-catenin [23]. Any free β-catenin that is not bound in this complex is degraded in the cytosol by a protein complex [23]. Axin, a scaffold protein that is present in epithelial cells [24], mediates the formation of this cytosolic degradation complex along with the proteins adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK-3β), casein kinase 1 (CK1), and protein phosphatase 2A (PP2A) [25]. When β-catenin comes in contact with Axin and APC, the casein kinase 1α (CK1α) component of Axin and GSK-3β work together to phosphorylate β-catenin and mark it for degradation. Due to this constant degradation, the cytosolic β-catenin is maintained at a low concentration in the absence of Wnt [23].

Carcinomas are associated with an oncogenic promoting stroma that is considered ‘reactive’, which is characterized, in part, by an increase in the number of fibroblasts that now associate with the ECM [26, 27]. Secreted from these fibroblasts is the Wnt protein, a morphogen that relies on short-range signaling in order to activate the Wnt pathway in
carcinoma cells [16, 28]. Once the Wnt ligand binds to its receptor, Frizzled (Frz) [18] on
epithelial cells, LRP5-LRP6 receptors are phosphorylated by CK1γ and GSK-3β [23]. The
messenger protein Disheveled (Dvl) is brought to the plasma membrane where it associates
with Frz. Dvl then binds with Axin at the membrane to deactivate and dismantle the Axin/APC/
GSK-3β complex, allowing β-catenin to accumulate in the cytosol and subsequently
translocate to the nucleus [25]. In the absence of Wnt signaling, the T-cell factor and lymphoid
enhancer factor (TCF/LEF) works in the nucleus with Groucho to repress Wnt target genes
[29]. However, once Wnt signaling is activated, β-catenin replaces the Groucho factors and is
able to form a complex with TCF and LEF1 [23], upregulating the expression of transcription
factors, such as Slug [21]. Slug, a zinc-finger transcription factor, then binds to E-boxes in the
promoter regions of the E-cadherin gene and prevents transcription of E-cadherin [30]. This
suppression of E-cadherin production limits the concentration of E-cadherin available to bind
with β-catenin, further encouraging the translocation of β-catenin to the nucleus as it
accumulates in the cytosol [31], thus creating a Wnt-driven feedback loop. The continued
suppression of E-cadherin production by Slug and other transcription factors ultimately causes
the epithelial cell to lose its adhesion with the other surrounding epithelial cells and undergo
EMT.

Within these complex dynamics, we identify 3 key relationships centered around β-
catenin, which are shown in Figure 1.1. In a primary carcinoma cell, β-catenin is sequestered
into a complex with E-cadherin. Any β-catenin that is not in this complex is marked for
degradation. With the activation of the Wnt pathway, the degradation complex is inactivated
due to the movement of Dvl to the cellular membrane, allowing β-catenin to accumulate, translocate to the nucleus, and upregulate Slug. In order to model these β-catenin relationships at the core of the canonical Wnt pathway and the manner in which they affect the key proteins involved, we employed a system of three ODEs. The changes over time in the concentration of E-cadherin (E), free cytosolic β-catenin (B), and Slug (S) are described in Equations (1.1)-(1.3) below:

\[
\frac{dE}{dt} = \frac{\alpha_1}{1 + \left(\frac{E}{IC_E}\right)^{n_1}} - \beta_1 \cdot E \quad (1.1)
\]

\[
\frac{dB}{dt} = \alpha_2 - \frac{k_1 \cdot \left(\frac{E}{IC_E}\right)^{n_2}}{1 + \left(\frac{E}{IC_E}\right)^{n_2}} - \left(\beta_2 \cdot B - \frac{k_0 \cdot \left(\frac{D}{IC_D}\right)^{n_4}}{1 + \left(\frac{D}{IC_D}\right)^{n_4}}\right) \quad (1.2)
\]

\[
\frac{dS}{dt} = \alpha_3 + \frac{k_2 \cdot \left(\frac{B}{IC_B}\right)^{n_3}}{1 + \left(\frac{B}{IC_B}\right)^{n_3}} - \beta_3 \cdot S \quad (1.3)
\]

A complete list of the parameters and their definitions are found in Table 1.1. In the epithelial steady state, the concentration of cytosolic β-catenin is very low: most β-catenin exists in complex form with other members of the catenin family and E-cadherin. This relationship is represented in (1.2) with the term \(\frac{k_1 \cdot \left(\frac{E}{IC_E}\right)^{n_2}}{1 + \left(\frac{E}{IC_E}\right)^{n_2}}\), where \(k_1\) is the rate at which E-cadherin sequesters β-catenin. In the model presented by Ramis-Conde et al. [19], the authors discuss two rates to describe this interaction: the rates at which β-catenin and E-cadherin bind and unbind to form
this complex. However, the authors also estimate that the rate at which the two proteins bind to form the complex is 50x faster than the rate at which they unbind: hence we have used a single term to model the reduction in free cytosolic β-catenin due to the presence of E-cadherin. Any β-catenin that is not bound to E-cadherin is subjected to degradation by the GSK-3β complex. In this model, we assume that once β-catenin is bound to the GSK-3β complex in the epithelial steady state, it will be phosphorylated and marked for degradation. Thus, the basal degradation of β-catenin is represented by the term β₂ · B in Equation (1.2), where β₂ is the rate at which β-catenin binds to the GSK-3β complex. However, once Wnt signaling is activated, Dvl works to break apart the GSK-3β complex by binding with Axin at the membrane, sparing β-catenin from phosphorylation and degradation. With Dvl (D) > 0, the term \( \frac{k_0 \left( \frac{D}{K_D} \right)^{n_4}}{1 + \left( \frac{D}{K_D} \right)^{n_4}} > 0 \) in (1.2) where \( k_0 \) is the rate at which Dvl binds to Axin and deactivates the degradation complex.

Once Wnt signaling is turned on and Dvl is upregulated, β-catenin can accumulate and translocate to the nucleus, enhancing the expression of Slug in Equation (1.3). Slug then binds to the E-box elements in the promoter of the E-cadherin gene, which inhibits E-cadherin production, as modeled in (1.1) with the term \( \frac{\alpha_1}{1 + \left( \frac{S}{K_S} \right)^{n_1}} \). These interactions constitute the core of the double-negative feedback loop driven by changes in the behavior of β-catenin. This double-negative feedback loop, combined with ultra-sensitivity in the interactions (represented by Hill coefficients \( n_1 - n_4 \) in Equations (1.1) - (1.3)), creates the potential for a bistable switch.

Due to the number of parameters we were required to estimate for this model, as well
Figure 1.1A

Figure 1.1B

Figure 1.1: (A) β-catenin – E-cadherin relationship in a primary carcinoma tumor cell, pre-EMT. E-cadherin sequesters cytosolic β-catenin at the cell membrane where it forms a complex with other members of the catenin family to help E-cadherin attach to the cell’s cytoskeleton. (B) The upregulation of Dvl via Wnt signaling inhibits the degradation of β-catenin by deactivating the GSK-3β/Axin complex, allowing β-catenin to translocate to the nucleus and upregulate the transcription factor Slug. Slug suppresses the transcription of E-cadherin, which means there is less E-cadherin to sequester β-catenin at the membrane. β-catenin can continue to accumulate and translocate to the nucleus, thus completing the feedback loop.
<table>
<thead>
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<th>Definition</th>
<th>Units</th>
<th>Assumed Value</th>
<th>Ref</th>
</tr>
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<td>$\alpha_1$</td>
<td>Basal production of E-cadherin</td>
<td>$\frac{nM}{min}$</td>
<td>0.01</td>
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<tr>
<td>$\alpha_2$</td>
<td>Basal production of $\beta$-catenin</td>
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<td>$\alpha_3$</td>
<td>Basal production of Slug</td>
<td>$\frac{nM}{min}$</td>
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<td>$\beta_1$</td>
<td>Basal degradation of E-cadherin</td>
<td>$\frac{1}{min}$</td>
<td>0.03</td>
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<td>$\beta_2$</td>
<td>Rate at which $\beta$-catenin binds to the GSK-3$\beta$/Axin/APC complex</td>
<td>$\frac{1}{min}$</td>
<td>0.03</td>
<td>[19]</td>
</tr>
<tr>
<td>$\beta_3$</td>
<td>Basal degradation of Slug</td>
<td>$\frac{1}{min}$</td>
<td>0.03</td>
<td>estimated</td>
</tr>
<tr>
<td>$k_0$</td>
<td>Rate at which Dvl inactivates the GSK-3$\beta$/Axin/APC complex</td>
<td>$\frac{nM}{min}$</td>
<td>$3.7\times10^{-3}$</td>
<td>estimated</td>
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<tr>
<td>$k_1$</td>
<td>Rate at which E-cadherin sequesters $\beta$-catenin at membrane</td>
<td>$\frac{nM}{min}$</td>
<td>0.01</td>
<td>estimated</td>
</tr>
<tr>
<td>$k_2$</td>
<td>Rate at which $\beta$-catenin upregulates Slug in cell nucleus</td>
<td>$\frac{nM}{min}$</td>
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<td>$IC_S$</td>
<td>Half maximal Slug concentration required to inhibit E-cadherin transcription</td>
<td>$nM$</td>
<td>3.3</td>
<td>estimated</td>
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<tr>
<td>$IC_B$</td>
<td>Half maximal $\beta$-catenin concentration for Slug upregulation</td>
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<tr>
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<td>0.033</td>
<td>estimated</td>
</tr>
<tr>
<td>$IC_D$</td>
<td>Half maximal Dvl concentration needed to inhibit $\beta$-catenin degradation by GSK-3$\beta$</td>
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<td>estimated</td>
</tr>
<tr>
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<tr>
<td>$n_3$</td>
<td>Hill Coefficient</td>
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</tr>
<tr>
<td>$n_4$</td>
<td>Hill Coefficient</td>
<td>--</td>
<td>5</td>
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as to continue our theoretical exploration into how these rates contribute to the underlying bistable switch, we nondimensionalized the system in Equations (1.1) – (1.3) using the relationships:

\[ E = \varepsilon \cdot e \quad B = \lambda \cdot b \quad S = \sigma \cdot s \quad D = \delta \cdot d \quad t = T \cdot \tau \]

where \( e, b, s, d, \) and \( \tau \) are dimensionless variables. Substituting in these relationships and defining the constants as

\[ \varepsilon = IC_E \quad \lambda = IC_B \quad \sigma = IC_S \quad \delta = IC_D \quad T = \frac{IC_B}{k_1} \]

we are left with the nondimensional system

\[ \frac{de}{d\tau} = \frac{A_1}{1 + s^{n_1}} - C_1 \cdot e \quad (1.4) \]

\[ \frac{db}{d\tau} = A_2 - \frac{e^{n_2}}{1 + e^{n_2}} - \left( C_2 \cdot b - \frac{F_1 \cdot d^{n_4}}{1 + d^{n_4}} \right) \quad (1.5) \]

\[ \frac{ds}{d\tau} = A_3 + \frac{F_2 \cdot b^{n_3}}{1 + b^{n_3}} - C_3 \cdot s \quad (1.6) \]

While the range of values for each dimensionless parameter was explored (see Figure 1.5), the values that were ultimately used in this model are defined in Table 1.2. Analysis of this model was carried out with MATLAB software and XPPAUT [32] using the initial conditions of \( e = 10, b = 0, \) and \( s = 0 \) when the cell begins in the epithelial steady state.
<table>
<thead>
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<th>Definition</th>
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<td>$\frac{\alpha_2}{k_1}$</td>
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</tr>
<tr>
<td>$A_3$</td>
<td>$\frac{\alpha_3 \cdot IC_B}{k_1 \cdot IC_S}$</td>
<td>0.01</td>
</tr>
<tr>
<td>$C_1$</td>
<td>$\frac{\beta_1 \cdot IC_B}{k_1}$</td>
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<tr>
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<td>$\frac{\beta_2 \cdot IC_B}{k_1}$</td>
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</tr>
<tr>
<td>$C_3$</td>
<td>$\frac{\beta_3 \cdot IC_B}{k_1}$</td>
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<td>$\frac{k_0}{k_1}$</td>
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<td>$n_1$</td>
<td>--</td>
<td>3</td>
</tr>
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<td>$n_3$</td>
<td>--</td>
<td>2</td>
</tr>
<tr>
<td>$n_4$</td>
<td>--</td>
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</tbody>
</table>
Results and Discussion

To test the response of the epithelial cell to different concentrations of Wnt signaling, Figure 1.2 shows the changes in protein concentrations with respect to time. In both Figures 1.2A and 1.2B, the cell exists in the epithelial steady state over the time range $\tau = [0, 20]$: E-cadherin level is high ($e = 10$) whereas both $\beta$-catenin ($b$) and Slug ($s$) are low ($< 0.015$). At time $\tau = 20$, Dvl is turned on, which implicitly models the release of Wnt protein from the surrounding environment and consequent movement of Dvl from the cytosol to the membrane. If Wnt signaling is very low, membrane Dvl will not be sufficiently high to induce EMT. This is illustrated in Figure 1.2A by setting $d = 1.2$. The proteins do not experience much change in the presence of low levels of membrane Dvl: E-cadherin dips to 6.7, while Slug and $\beta$-catenin are only slightly increased with $\text{Slug} \approx 0.8$ and $\text{\(\beta\)-catenin} \approx 0.3$. If the microenvironment was to then stop releasing the Wnt signal at time $\tau = 50$, Dvl would detach from the membrane and return to the cytosol, meaning that the (nondimensional) concentration of membrane Dvl would return to its initial value of 0 (this is modeled by setting $d = 0$ at time $\tau = 50$). Because the epithelial steady state was maintained even with $d = 1.2$, the proteins all go back to their original, pre-signaling epithelial levels.

If, however, the tumor microenvironment releases sufficient Wnt protein, the amount of Dvl that accumulates at the membrane may be enough to induce significant changes in the proteins, as shown in Figure 1.2B. We model this scenario by setting intracellular Dvl to a value of 5.4 at time $\tau = 20$, which induces the canonical intracellular Wnt pathway. Free $\beta$-catenin accumulates and translocates to the nucleus, upregulating Slug. Ultimately, intracellular $\beta$-catenin reaches a steady state value of 1.37 in the cytosol and Slug reaches a
**Figure 1.2:** For $\tau = [0, 20)$, the cell exists in the epithelial steady state where E-cadherin level is high ($e = 10$) and both $\beta$-catenin ($b$) and Slug ($s$) are low. (A) If a small amount of Wnt signal is released at $\tau = 20$, Dvl ($d = 1.2$) will accumulate at the membrane. E-cadherin will decrease slightly ($e = 6.85$) and both $\beta$-catenin and Slug will not rise enough to induce EMT ($b = 0.291$, $s = 0.77$). If, at $\tau = 50$, the Wnt signal stops, Dvl will detach from the membrane and the proteins return to their initial epithelial values. (B) If enough Wnt signal is released at $\tau = 20$ so that $d = 5.4$, the concentrations of $\beta$-catenin and Slug will rise ($b = 1.37$, $s = 6.54$) and E-cadherin ($e$) will reach a low steady state value of 0.036. The lack of E-cadherin means that the cell will no longer be adhesive with the surrounding cells, allowing it to move away from the primary tumor. If the concentration of membrane Dvl returns to 0 due to the cellular distance from the Wnt signal at $\tau = 50$, E-cadherin, $\beta$-catenin, and Slug will stabilize at values that allow the cell to maintain its mesenchymal state.
steady state value of $\approx 6.5$. The activation of the Wnt pathway and the subsequent changes in Slug and $\beta$-catenin result in a significant decrease in E-cadherin level to $\approx 0.04$. This drop in E-cadherin with a rise in active membrane-bound Dvl means that $\beta$-catenin is not being as quickly sequestered into the adhesion complex, nor is it being as quickly degraded by the GSK-3$\beta$ complex, and is thus able to translocate to the nucleus. Even if membrane Dvl returns to 0 (as shown in Figure 1.2B at $\tau = 50$), the cell does not revert to the protein concentrations pertaining to the initial epithelial steady state, but has instead stabilized and maintains the protein levels of the converted mesenchymal state. In Figure 1.2B, with $d = 0$ at $\tau = 50$, E-cadherin is still maintained at a low value of 0.08. Slug and $\beta$-catenin dip only slightly to $\approx 5.0$ and $\approx 1.0$ respectively. These results suggest that, epithelial cells cannot transition to the mesenchymal steady state below a threshold level of membrane Dvl ($d$) because not enough Dvl will have accumulated at the membrane to inactivate the GSK-3$\beta$ complexes. If the extracellular Wnt signal were to subside, the remaining active GSK-3$\beta$ complexes could phosphorylate the small accumulated amount of $\beta$-catenin, marking it for degradation. For higher values of Dvl above the threshold, there is sufficient $\beta$-catenin accumulated in the cytosol and not enough active GSK-3$\beta$ complex to degrade it, meaning that that $\beta$-catenin can translocate to the nucleus. Now, even if the Wnt signal is reduced or subsides completely, $\beta$-catenin will have stabilized to a concentration that the GSK-3$\beta$ complexes are unable to degrade.

This biphasic cell behavior is suggestive of a threshold-driven bistable switch between the epithelial and mesenchymal states. The bifurcation diagrams in Figures 1.3A-C give further
insight into this switch between the epithelial and mesenchymal states, and the manner in which the protein levels of E-cadherin, β-catenin, and Slug change with respect to alterations in membrane Dvl. As such, these figures can be thought of as concentration-response curves of protein levels with respect to membrane Dvl. If the cell starts in the epithelial steady state, such as our system does in Figure 1.2, and the level of membrane Dvl is steadily increased, the cell remains in the epithelial state, with high E-cadherin and low β-catenin and Slug levels, until Dvl reaches a threshold level of about 1.33 (blue vertical dashed line, Figures 1.3A-C). At this point, the cell undergoes EMT and transitions into a mesenchymal-like state with low E-cadherin and high β-catenin and Slug levels. For further increases of Dvl beyond 1.33, the cell remains in the switched mesenchymal state. However, when we start from the newly attained mesenchymal steady state and move leftward in Figures 1.3A-C, reducing the level of membrane Dvl, the cell remains in the mesenchymal steady state and does not switch back to the epithelial state. In fact, in this instance, with $d = 0$, the values that the proteins maintain are $e = 0.08$, $b = 0.99$, and $s = 4.98$, which are the steady state mesenchymal concentrations of the proteins at $\tau = 80$ in Figure 1.2B. There is thus a range of levels of Dvl ($d = 0$ to 1.33) for which the cell can exist in one of two distinct steady states – epithelial or mesenchymal – depending on its history. This “cellular memory” mechanism generated by the bistable switch could explain how the EMT-derived mesenchymal cell is able to retain its invasive phenotype and not revert to an epithelial state even in the absence of sustained pro-EMT signaling from the microenvironment, for example in blood or lymphatic vessels. The switch mechanism also suggests that it is not the lack of extracellular signal per se that forces the cells to revert to the epithelial phenotype in the metastatic environment (the mesenchymal to epithelial transition,
Figure 1.3: These bifurcation diagrams show a concentration-response curve of E-cadherin (A), β-catenin (B), and Slug (C) with respect to membrane Dvl (d). If the cell starts in the epithelial steady state (d = 0), and Dvl is increased, the cell remains in the epithelial steady state until $d = 1.33$ (vertical blue dashed line). At this point, the cell undergoes EMT and transitions into a mesenchymal-like state. Once in the mesenchymal state, the cell will stay there, even after the level of membrane Dvl is decreased back to 0.
MET), but rather a different extracellular signal in the new microenvironment is required to render the switch reversible.

To assess the sensitivity of the model to its various parameters, we carried out sensitivity analysis using a Latin Hypercube Design [33] and Partial Ranked Correlation Coefficient (PRCC). Latin Hypercube Design (LHC) is a methodology that allows us to divide the range for each of our K parameters into N intervals, where \( N > \frac{4}{3} \cdot K \), and randomly sample a value from each interval [34]. Sampled values for each parameter are then randomly assembled together into N parameter sets and the model is run for each different set of parameters [35]. Once the LHC has been carried out, PRCC analysis permits us to transform our parameter input values and our outcomes into ranked values and measure the correlation between the rank-transformed input parameters and the rank-transformed outcomes [35]. As the Hill coefficients are all greater than 1 and are thus ultrasensitive terms in the model, by using LHC and PRCC, we sought to understand how the other 8 nondimensional parameters influenced the steady state behaviors of E-cadherin (e), β-catenin (b), and Slug (s). Uniform distributions were used for each of the 8 parameters, with 1000 intervals per parameter, and sensitivity analysis was carried out at different values of Dvl (d). The ranges of the parameter values are found in Table 1.3. Parameter A2 begins at a value of 1 due to the lack of monotonic behavior on the range of \([0,1)\) of the variable. After examining the partial correlation coefficients between each of the individual ranked parameter values at different values of Dvl (d), the parameters were found to be uncorrelated with each other. Additionally, scatterplots of the steady state values for E-cadherin (e), β-catenin (b), and Slug (s) at different values of Dvl (d) in response to the nondimensional
parameters are given in Appendix A. These plots show the monotonic behavior of the variable steady states in response to changes in the different parameters, which is required to apply PRCC.

Figure 1.4 shows the PRCC for the different nondimensional parameters with the steady state values for E-cadherin (e), β-catenin (b), and Slug (s) beginning in the epithelial steady state at Dvl (d) = 0 (Figure 1.4A), and again at Dvl (d) = 6 (Figure 1.4B). For both

<table>
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<th>Maximum Value</th>
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<tr>
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Table 1.3: Nondimensional Parameter Ranges for Sensitivity Analysis: Wnt Model

low and high values of Dvl (d), parameters A₁, A₂, A₃, and C₁ are not significantly correlated with the steady state values at any of the three variables, while parameter C₂ is significantly correlated with the steady state value of β-catenin (b) and C₃ is significantly correlated with the steady state value of E-cadherin (e) and Slug (s). From Table 1.2, we know that C₂ and
Figure 1.4: Sensitivity analysis was carried out to understand the relationship between the steady state behavior of E-cadherin (e), β-catenin (b), and Slug (s) and the 8 nondimensional parameters (Table 1.2) at different levels of Dvl (d). (A): Only C\textsubscript{2} is significantly correlated (correlation coefficient < -0.5) with the steady state behavior of b, while only C\textsubscript{3} is significantly correlated (correlation coefficient < -0.5 or correlation coefficient > 0.5) with the steady state behavior of e and s. (B): With the introduction of Dvl (d) > 0, the nondimensional rate at which b translocates to the nucleus and activates s, F\textsubscript{2}, becomes significantly correlated with the steady state values of e and s.
C_3 only differ in composition by one term: C_2 contains \( \beta_2 \) and C_3 contains \( \beta_3 \). This small change in composition would suggest that it is actually the rate at which \( \beta \)-catenin is binding to the GSK-3\( \beta \) complex that is influencing the steady state concentration of free cytosolic \( \beta \)-catenin, whereas the rate of degradation of Slug significantly influences the steady state concentrations of E-cadherin and Slug. Additionally for an epithelial cell prior to Wnt signaling, \( F_1 \) and \( F_2 \) are not significantly correlated with the steady state levels of the proteins. But, with an increase in active membrane-bound Dvl (\( d = 6 \) in Figure 1.4B), the sensitivity of the steady state value of \( \beta \)-catenin (\( b \)) to changes in \( F_1 \) grows, as does the sensitivity of the steady state values of both E-cadherin (\( e \)) and Slug (\( s \)) to \( F_2 \). Note that, if the newly transformed mesenchymal cell were to then migrate away from the extracellular Wnt source and Dvl returned to 0, the steady state value of \( \beta \)-catenin would lose sensitivity to \( F_1 \) and the steady state values of E-cadherin and Slug would lose sensitivity to \( F_2 \) with this decrease in Dvl.

These sensitivity relationships suggest that the cellular phenotype relies on a delicate balance of intracellular protein concentrations and compartmentalization. Without an extracellular signal, both as an epithelial and a mesenchymal cell, the pathway relies on the GSK-3\( \beta \) degradation complex, as well as the degradation of Slug to maintain the cellular phenotype. Additionally, note that the steady state behavior of E-cadherin is influenced by the degradation rate of Slug, not its own, suggesting the events in the nucleus as possible targets for maintaining the appropriate epithelial levels of E-cadherin. In the presence of the Wnt signal and possibility of a switch with respect to membrane-bound Dvl, the sensitivity
of the steady state levels of Slug and membrane-bound E-cadherin to the rate of translocation of \( \beta \)-catenin (\( F_2 \)) to the nucleus changes, as does the sensitivity of \( \beta \)-catenin to the rate at which Dvl can inactivate the GSK-3\( \beta \) complex (\( F_1 \)). Now, with an external stimulus, the accumulation and translocation of \( \beta \)-catenin from the cytosol to the nucleus influence changes in the steady state behavior of both Slug and E-cadherin, behavior that is key for switching of behavior and phenotype. The influence of the movement of \( \beta \)-catenin to the nucleus in an epithelial cell exposed to an extracellular Wnt signal gives credence to the importance of \( \beta \)-catenin translocation to the bistable switch suggested by MacLean et al [22]. These changes in significance also highlight the possibility of therapeutically targeting nucleus-involved events, the half maximal Dvl concentration needed to inhibit \( \beta \)-catenin degradation, or the rate at which Dvl inactivates the GSK-3\( \beta \) complex for prevention of the change from the epithelial to the mesenchymal phenotype.

To further understand how changes in the eight non-dimensional parameters and the four Hill coefficients influence the system, particularly the bistable switch behavior, two-parameter bifurcation diagrams were generated using XPPAUT. Shown in Figure 1.5, these plots depict the steady state phenotype of the simulated cell over the parameter space. Along the x-axis of each plot, Dvl (\( d \)) is varied. Along the y-axis, the eight non-dimensional parameters \( A_1, A_2, A_3, C_1, C_2, C_3, F_1, \) and \( F_2 \) (described in Table 1.2) are varied one at a time. In each of the figures, the cell can only be in the epithelial steady state for parameter values in Region I and can only be in the mesenchymal steady state in Region III. In Region II, either steady state is possible depending on the history of the cell. If the system begins in the epithelial
steady state (Region I) and crosses the L1 boundary into Region II, the cell will remain in the epithelial steady state. Similarly, if the cell begins in the mesenchymal steady state (Region III) and crosses the L2 boundary into Region II, it will remain in the mesenchymal steady state.

If, based on the parameter values, the system begins in Region II, it will depend upon the initial conditions whether or not the cell assumes an epithelial or mesenchymal phenotype. Beginning as an epithelial cell in Region II, once the concentration of Dvl ($d$) is increased such that the L2 boundary is crossed into Region III, the cell will make the transition from epithelial to mesenchymal. Conversely, if the cell begins as a mesenchymal cell in Region II and the concentration of Dvl ($d$) is decreased so that the L1 boundary is crossed, the cell will transition from a mesenchymal cell into an epithelial cell as the system transitions from Region II to Region I. The region of bistability (i.e. Region II) is seen to be fairly robust to parameter variation.

Note that Region I does not appear in Figures 1.5C and 1.5G because the values in Region I are negative and are thus not biologically relevant to this model. Also, in Figure 1.5G and 1.5H, once $F_1$ and $F_2$ overcome a certain threshold value in their respective parameter spaces, the system is destined to switch when the Wnt signaling pathway is activated. This is particularly interesting in light of what we now know about the sensitivity of the Slug and E-cadherin steady state concentrations to changes in $F_2$ and the sensitivity of the $\beta$-catenin steady state concentration to changes in $F_1$. With the upregulation of the Wnt signal and the activation of Dvl at the membrane ($d > 0$), if the rate at which Dvl disrupts the GSK-3$\beta$ complex or the rate at which $\beta$-catenin moves to the nucleus and binds with TCF is fast enough, the cell will have no choice but to switch from the epithelial to the mesenchymal steady state.
Figure 1.5: In each subfigure, Dvl ($d$) is varied along the x-axis while, along the y-axis, a different non-dimensional parameter is varied. For the parameter values in this model, the cell begins in Region II in the epithelial state. If Dvl is increased and the cell crosses L2 into Region III, the cell will switch to the mesenchymal steady state. If the cell were to begin in the mesenchymal steady state in Region II and crosses L1 into Region I with changes in Dvl ($d$), the cell will switch to the epithelial steady state.
Conclusions

There is a consensus in the literature that the epithelial mesenchymal transition is triggered in a tumor by external signals from the microenvironment and the mesenchymal phenotype is maintained until a cell reaches a new site. This suggests that the switch from the epithelial to the mesenchymal state is bistable \textit{in situ} and the maintenance of this mesenchymal phenotype means that the cell can conserve its invasive behavior during metastasis.

While multiple intracellular signaling pathways can stimulate EMT, we consider Wnt signaling as a case study. The Wnt signaling pathway has been under intense scrutiny from both mathematicians and biologists in order to understand how it contributes to changes in cellular behavior. Mathematical models attempting to describe the intracellular pathway are often complex and limited to one group of protein interactions. Instead of examining one subset of the Wnt pathway, the model presented in this work examines the three key relationships centered around $\beta$-catenin that comprise the Wnt signaling pathway and drive the change in cellular phenotype. By studying the system as a whole, it allows for a better understanding of which interactions are likely to be responsible for the bistable switch.

This model opens up many avenues for possible theoretical and practical exploration. With the theory of a bistable switch underlying EMT, it would be prudent to examine how the switch is affected by the presence of neighboring cells and the translocation of the E-cadherin-$\beta$-catenin complex to the membrane. The stabilization of these bonds could ultimately work against the loss of adhesion in the epithelial cell and the resulting transformation. Additionally, sensitivity analysis and exploration of the response of the system to changes in parameter values as Wnt signaling is upregulated suggests that, with the external upregulation of the Wnt
pathway, two excellent candidates for further investigation are the disruption of the GSK-3β complex via Dvl and the shuttling of β-catenin to the nucleus. These insights, coupled with the dependence of the steady state value of E-cadherin on changes in Slug, suggest possible avenues for practical exploration would be therapeutically raising the half maximal concentration of Dvl needed to to inhibit the degradation of β-catenin by GSK-3β to prevent pathway activation, as well as lowering the rate at which β-catenin translocates to the nucleus once the pathway is activated. Therapies targeted at reducing the concentration of cytosolic β-catenin or inhibiting the movement of β-catenin to the nucleus could prevent the activation of Slug and ultimately work to maintain the E-cadherin-β-catenin complex at the membrane. Pharmacological exploration of these components of the Wnt pathway could help prevent EMT prior to intravasation and metastasis, the primary cause of cancer-related mortality.
CHAPTER 2: The Influence of Cell-to-Cell Contact and TGF-β on the Epithelial Mesenchymal Transition

Background

*In vivo*, epithelial cells in both normal tissue and carcinomas form strong intercellular bonds [36]. These intercellular connections, which utilize transmembrane protein complexes involving E-cadherin and members of the catenin family [37], must be lost in order for carcinoma cells to metastasize [15]. Epithelial mesenchymal transition (EMT) is the process by which epithelial cells lose their adhesion with other cells and adopt the invasive and migratory behavior of mesenchymal cells [38]. Characterized by their spindle-like phenotype [39], these newly formed mesenchymal cells then invade the microenvironment surrounding the tumor, enter the blood stream (by breaking through the basement membrane), and travel to a distant site in the body [40]. Once at this distant site, these mesenchymal cells can undergo the reverse process, the mesenchymal epithelial transition (MET), and proliferate—thus forming a metastasis [10].

Many pathways induce EMT in carcinoma cells, one of which is the transforming growth factor β (TGF-β) pathway [41, 42]. When TGF-β binds to receptors on the carcinoma cell, it activates a protein cascade that involves the Smad proteins [43, 44] and ultimately upregulates the Snail transcription factor family [41]. Members of the Snail family then bind with E-boxes in the E-cadherin promoter and prevent the production of further E-cadherin by the cell [45]. The suppression of E-cadherin production decreases the supply of E-cadherin, ultimately resulting in a loss of the adhesion complex forming cell-to-cell bonds [46].
As outlined in Figure 2.1, by using cellular cultures grown to three different confluencies (30% confluent, 60% confluent, and 100% confluent) and by applying three different concentrations of exogenous TGF-β (0 ng/mL, 3 ng/mL, and 9.33 ng/mL) to each confluence, we aim to understand and explore the dynamic between cell-to-cell contact and the activation of intracellular signaling pathways when attempting to activate EMT. It is our hypothesis that existing adhesive cell-cell contacts in epithelial cells inhibit the activation of EMT via the TGF-β pathway. By using qPCR, we are able to understand the influence of these two factors on the cells on a genetic level, and by employing flow cytometry and immunocytochemistry, we are able to understand how the genetic changes translate to phenotypic and behavioral changes. Additionally, by carrying out this set of experiments on two different cell lines, colon (SW480) [47-49] and breast (MCF7) [50, 51] carcinoma, we aim to understand if this competition between exogenous factors and cell-cell contacts is a more universal relationship, independent of the tissue from which the cell originates.

**Materials and Methods**

*Cell Culture:*

SW480 (human colorectal adenocarcinoma) and MCF7 (human breast adenocarcinoma) cells were cultured and maintained in Dulbecco’s Modification of Eagle’s Medium (DMEM: Mediatech, Inc., Manassas, VA) supplemented with penicillin-streptomycin, L-glutamine (Mediatech, Inc., Manassas, VA), Plasmocin prophylactic (InvivoGen, San Diego, CA), and 10% heat inactivated fetal bovine serum (FBS: Mediatech, Inc., Manassas, VA).
Figure 2.1: In this set of experiments, cells were grown to three different confluences (30%, 60%, and 100%) and three different concentrations of exogenous TGF-β (0 ng/mL, 3 ng/mL and 9.33 ng/mL) were added to each confluence. In order to understand if the process of EMT and the effects of confluence and exogenous signal are more universal across different tissues, these experimental conditions were carried out in two different cell lines: SW480 cells (colorectal carcinoma) and MCF7 cells (breast carcinoma).

SW480 Confluence: In order to obtain 30% confluent, $2.7 \times 10^5 - 1.4 \times 10^6$ were seeded into a 143.0 cm$^2$ tissue culture treated dish and grown to an estimated 20-40% confluent. For 60% confluent, $5.4 \times 10^5 - 2.7 \times 10^6$ cells per dish were grown to an estimated 50-70% confluent. $5.4 \times 10^6 - 2.7 \times 10^7$ cells per dish were grown to 90-100% confluent.

MCF7 Confluence: Cells were grown in 143.0 cm$^2$ tissue culture treated dishes: $2.4 \times 10^5 - 7.9 \times 10^5$ cells were seeded to each dish and grown to an estimated 30% confluent.
7.9x10^5 – 1.6x10^6 cells were seeded to each dish and grown to an estimated 50-70% confluent, and 2.4x10^6 – 1.2x10^7 cells were grown to 90-100% confluent.

TGF-β: Both cell lines were seeded for the different confluences as described above. Once cells reached 15-20% confluent (for 30% confluent), 30-50% confluent (for 60% confluent), or 80-90% confluent (for 100% confluent), their media was removed and replaced with fresh media containing 1% FBS and incubated for 24 hours. After 24 hours, the cells were given fresh media containing 1% FBS and either 3 ng/mL or 9.33 ng/mL of TGF-β (R&D Systems, Minneapolis, MN) and incubated for 48 additional hours, allowing the cells to reach the desired confluence.

Protein Extractions:

Cells were collected from tissue culture dishes using a cell scraper. For 100% confluent treatment groups, cells were collected from the entire dish. For cells of lower confluence, cells increased in confluence as they grew closer to the edge of the dish. Therefore, for dishes that were part of the 30% and 60% treatment groups, cells were only taken from the center of the plate where confluence was truly 20-40% or 50-70% respectively. Cells were placed into single cell suspension using a 22-gauge needle, counted, and 2.5 – 5.5x10^7 cells were collected and pelleted. Differential detergent fractionation (DDF) protein extraction adapted from McCarthy et al. [52] was performed to sequentially extract protein from different parts of the cell while maintaining cellular organization. Base Buffers 1 & 2, as well as a 0.1 M EDTA stock solution and a 0.1 M phenylmethylsulfonyl fluoride (PMSF) stock solution, were prepared ahead of time as outlined in McCarthy et al [52]. For each extraction, SDE Buffers and the 10% Triton X-100 stock solution were prepared fresh.
SDE#1: SDE Buffer 1 was prepared as described in McCarthy et al. [52] with a final concentration of 25% Base Buffer 1. One mL of SDE Buffer 1 was added to the cell pellet. The cells were resuspended in the buffer using gentle pipetting and then incubated on ice for 30 min with gentle mixing using a rocker. Samples were centrifuged for 5 min at 4°C at 550xg. The supernatant was removed and then centrifuged for 10 min at 4°C at 10600xg. The supernatant was then collected and held on ice. These steps were repeated 9 times for a total of 10 extractions. All supernatants were combined together, vortexed to ensure mixing, and then aliquoted for storage at -80°C.

SDE#2: SDE Buffer 2 was prepared and extraction was performed as described in McCarthy et al [52]. Cells were resuspended in 100 µL of buffer using gentle pipetting, incubated and gently mixed on ice for 30 minutes, and then centrifuged at 5000xg for 10 min at 4°C. This extraction process was carried out once and stored at -80°C.

SDE#3: SDE Buffer 3 was prepared and extraction was performed as described in McCarthy et al [52]. Buffer 3 (100 µL) was added to cells and nuclei were disrupted using a pellet pestle before cells were centrifuged at 6780xg for 10 min at 4°C. The process was repeated for a total of two extractions. These extractions were mixed together into one aliquot and stored at -80°C.

_TGF-β1 Enzyme-linked Immunosorbent Assay (ELISA):_

Cytosolic TGF-β1: TGF-β1 concentration was measured in SDE#1 using the Quantikine ELISA Human TGF-β1 Kit from R&D Systems. TGF-β1 was activated as described in the kit directions using 1N HCl and a solution of 1.2 N NaOH/0.5M HEPES. Serial dilutions of each sample of 1:2 and 1:4 were then carried out using the supplied diluent,
Calibrator Diluent RD5-53 (diluted 1:4) and all samples and serial dilutions were measured in triplicate. Optical density for each well was determined using a 450 nm wavelength and wavelength correction at 570 nm.

**Flow Cytometry:**

Cells were collected in the same manner as described in our Protein Extraction section and were stained in a protocol adapted from several eBioscience staining protocols. For each sample, $4 \times 10^5 - 1 \times 10^6$ cells were collected and resuspended in azide-free and serum-free PBS. 1 µL of eBioscience Fixable Viability Dye was added per $1 \times 10^6$ cells suspended in 1 mL of PBS and were allowed to fix for 30 minutes at 4°C in the dark. Cells were then centrifuged at 500xg at room temperature for 5 minutes, washed with FACS buffer (PBS containing 0.05% azide and 2% FBS), and centrifuged again at room temperature for 5 minutes. The supernatants were discarded. Cells were then resuspended in 100 µL FACS buffer and 5 µL of cellular surface BD Biosciences PE mouse anti-human E-cadherin antibody per $1 \times 10^6$ cells for 30 minutes at 4°C in the dark. Cells were washed with FACS buffer, centrifuged for 500xg for 5 minutes, twice, with the supernatant discarded in between washes. Cells were then resuspended in 100 µL of eBioscience IC Fixation Buffer and 100 µL FACS buffer for 20-60 minutes. During this time, a 1X dilution of the eBioscience permeabilization buffer was made using dH₂O. Two mL of a 1X dilution of the eBioscience permeabilization buffer was added to the cells and the diluted fixation buffer and were centrifuged again at 500xg at room temperature for 5 minutes. After discarding the supernatant and resuspending the cell pellet in 2 mL of the 1X permeabilization buffer, the samples were centrifuged again at 500xg at room temperature. The supernatants were then discarded and cells were resuspended in 100 µL of the 1X
permeabilization buffer and 5 μL of BD Biosciences Alexa 488 mouse anti-human vimentin antibody per 1x10^6 cells for 20 minutes at room temperature in the dark. Two mL of the 1X permeabilization buffer was then added to each sample and the cells were centrifuged at 500xg for 5 minutes. After discarding the supernatant, 2 mL of FACS buffer was added to each sample and the cells were centrifuged at 500xg for 5 minutes at room temperature. Finally, cells were resuspended in 300 μL of FACS buffer. In addition to cells, compensation beads (eBioscience, San Diego, CA) were also stained with PE mouse anti-human E-cadherin stain and Alexa 488 mouse anti-human vimentin stain, and fixed following staining. All 1x10^4 cells (or events) per sample were then analyzed on the BD LSRII flow cytometer (BD Biosciences, San Jose, CA).

**Quantitative Polymerase Chain Reaction (qPCR):**

RNA Extraction and cDNA Preparation: Cells were collected in the same manner as described in our Protein Extraction section and RNA extractions were carried out following the OMEGA bio-tek E.Z.N.A. Total RNA Kit I Animal Cell Protocol. During preparation 1.5x10^6- 3x10^6 cells were collected and centrifuged at 500xg for 5 minutes. The supernatant was then removed and the pellet was then resuspended in 700 μL of β-mercaptoethanol (BME)-TRK Lysis Buffer (20 μL BME per 1 mL TRK buffer). DNA was sheared by passing the cells through a 20-gauge needle 5-10x, 700 μL of 70% ethanol (EtOH) was added, and the cells were vortexed. Cells were then transferred to a HiBind RNA mini column in a 2 mL collection tube and spun at 10000xg for 1 min. Flow through was discarded and the process was repeated until sample was transferred to column. RNA Wash Buffer I (500 μL) was added and the column was centrifuged at 10000xg for 30 seconds, after which flow through was
discarded. RNA Wash Buffer II diluted with EtOH (500 µL) was added and the column was
centrifuged at 10000xg for 1 minute, after which flow through was discarded. RNA Wash
Buffer II step was carried out twice. The column was then centrifuged at maximum speed for
2 minutes to dry it before the column was transferred to a clean 1.5mL tube. To the column,
40 µL DEPC H₂O was added and it was incubate at room temperature for 5 minutes before it
was centrifuged at the maximum speed for 2 minutes to elute RNA. A concentration of 2 µg/
20 µL of cDNA was then synthesized from the RNA using the SuperScript VILO cDNA
Synthesis Kit Protocol for First-Strand cDNA Synthesis (Invitrogen) and was stored at -20°C.
RNA quality was checked using a NanoDrop and Agilent Bioanalyzer.

qPCR: Primer sequences for both reference genes (RPS17 and PUM1) [53-56] and both
genes of interest (E-cadherin and Slug) [57] are listed in Table 2.1. Specificity of primers was
confirmed using the NCBI Basic Local Alignment Search Tool (BLAST). The optimal primer
concentration for this assay was 200 nM for each primer and primer efficiencies were
determined using a standard curve of diluted DNA. Calculated primer efficiencies ranged from
103% to 112%.

cDNA was diluted to 6.25 ng/µL. qPCR reactions were run in triplicate. Each reaction
had a total volume of 20 µL that consisted of 10 µL Power SYBR Green PCR Master Mix
(Thermo Fisher Scientific, Waltham, MA), 12.5 ng of diluted cDNA, and 200 nM each of the
forward and reverse primers. qPCR reactions were carried out on the Step One RT PCR System
(Applied Biosystems, Foster City, CA). Reactions were heated to 95.0°C and remained at such
for 10 minutes during the holding stage before then cycling for 40 cycles between 95.0°C for
15 seconds and 60.0°C for 1 minute. Following cycling, a melt curve was generated. Cycle
threshold (Ct) values with a standard deviation > 0.5 were repeated and relative expression for both E-cadherin and Slug were calculated using their Ct values for the target and the reference genes.

*Immunocytochemistry:*

SW480 Cells: Four well glass slides were used. For all treatment groups 2x10^3 cells were seeded per well and grown. Staining was carried out for the SW480 treatment groups of cells at 30% confluent + 0 ng/mL TGF-β, 30% confluent + 9.33 ng/mL TGF-β, 100% confluent + 0 ng/mL TGF-β, and 100% confluent + 9.33 ng/mL TGF-β. Technical replicates were carried out for each of the 4 treatment groups.

<table>
<thead>
<tr>
<th>Table 2.1: qPCR Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene</strong></td>
</tr>
<tr>
<td>RPS17</td>
</tr>
<tr>
<td>PUM1</td>
</tr>
<tr>
<td>E-CADHERIN</td>
</tr>
<tr>
<td>SLUG</td>
</tr>
</tbody>
</table>

MCF7 Cells: Four well glass slides were used. For low confluence treatment groups, 5x10^3 cells were seeded per well and grown. For high confluence treatment groups, 5x10^3-5x10^4 cells were seeded per well and grown to the desired confluence. Staining was carried out for the MCF7 treatment groups of cells at 30% confluent + 0 ng/mL TGF-β, 30% confluent...
+ 9.33 ng/mL TGF-β, 100% confluent + 0 ng/mL TGF-β, and 100% confluent + 9.33 ng/mL TGF-β. Technical replicates were carried out for each of the 4 treatment groups.

TGF-β: For the 30% confluent treatment group without exogenous TGF-β, cells were grown until they reached 30% confluent. For the 30% confluent treatment group with exogenous TGF-β, cells were grown until they reached 15-20% confluent, at which time their media was removed and replaced with fresh media containing 1% FBS and incubated for 24 hours. After 24 hours, the cells were given fresh media containing 1% FBS and 9.33 ng/mL of TGF-β (R&D Systems, Minneapolis, MN) and incubated for 48 additional hours, allowing the cells to reach ~30% confluent. For the 100% confluent treatment group without exogenous TGF-β, cells were grown until they reached 80-100% confluent. For the 100% confluent treatment group with exogenous TGF-β, cells were grown until they reached 80-90% confluent, at which time their media was removed and replaced with fresh media containing 1% FBS and incubated for 24 hours. After 24 hours, the cells were given fresh media containing 1% FBS and 9.33 ng/mL of TGF-β (R&D Systems, Minneapolis, MN) and incubated for 48 additional hours, allowing the cells to reach the desired confluence.

Immunocytochemistry: Cell slides were washed 1x with PBS and then fixed for 30 minutes at room temperature in 4% paraformaldehyde. Cells were then washed 3x with PBS and then permeabilized and blocked with a permeabilization solution of Dako Serum-free Protein Block and 0.1% saponin for 1 hour at room temperature. The purified mouse anti-human E-cadherin antibody (clone 67A4, BD Biosciences, San Jose, CA) was diluted in the permeabilization solution. For SW480 cells, based on optimization studies, 11 µL of antibody
was diluted to 220 µL while, for the MCF7 cells, 5 µL of antibody was diluted to 220 µL. Cells were then incubated with the purified E-cadherin antibody overnight at 4°C. Following the incubation, the antibody solution was removed and cells were washed quickly twice in PBS and then given two 10 minute washes with PBS. The secondary goat anti-mouse Immunoglobulin G (IgG H&L) (ab6787, Abcam, Cambridge, MA) Texas Red labeled antibody was diluted to 220 µL. Cells were then incubated with the secondary antibody solution for 90 minutes. Following this incubation, cells were washed 3x quickly and then given two 8 minute washes with PBS. The conjugated BD Biosciences Alexa 488 mouse anti-human vimentin (clone RV202, BD Biosciences, San Jose, CA) was diluted in the permeabilization solution to a ratio of 11 µL antibody to 220 µL for both cell lines and cells incubated overnight in the dark at 4°C. Following the incubation with the anti-vimentin antibody, the antibody was removed and cells were washed 3x quickly and then twice for 8 minutes with PBS. DAPI was diluted in PBS to a ratio of 2 drops per 1 mL of diluent and cells were stained for 10 minutes. Cells were given a final set of washes: 2 quick washes and then two 10 minute washes in PBS. Cells were mounted with Prolong Gold and a cover slide and set for at least 4 hours before imaging. For each treatment group (and each technical replicate), 15 sets of non-overlapping images were taken at 20x magnification: one image of the blue DAPI filter, one image of the Texas Red E-cadherin filter, one image of the Alexa 488 vimentin filter, and one bright field image using the Olympus IX81 microscope and the Hamamatsu Orca-Flash4.0 V2 Digital CMOS camera 22cu.

Neighbor Number: Opening the DAPI image using ImageJ, the background was subtracted and the image was then converted to an 8.0 bit image. Using the threshold and
watershed features, nuclei were isolated as shown in Figure 2.2A. Analyzing particles that ranged in size from 500-infinity pixels\(^2\) and had a circularity of 0-1.0, the individual cellular nuclei were numbered and a drawing of the nuclei, as well as their corresponding numbers for each image was produced, as shown in Figure 2.2B. Additionally, using the Analyze Particles feature, ImageJ recorded the X and Y coordinates of the center of mass of each nuclei.

![Figure 2.2A](image1)

![Figure 2.2B](image2)

**Figure 2.2:** (A) shows an image of MCF7 nuclei stained with DAPI once the background noise has been subtracted using ImageJ. (B) shows the final post processed image corresponding to (A) where each cell was given a corresponding numerical identity using ImageJ.

In order to gauge the distance between nuclei of neighboring cells, 5% of the cells from each of the 30% confluent + 0 ng/mL TGF-β treatment group were chosen for each cell line. Using the DAPI nuclei filtered image and the corresponding bright field image, the numeric neighbor of each cell and the distance between the center of mass of their two nuclei was recorded. The average distance between the neighboring nuclei in the 30% confluent + 0 ng/mL TGF-β treatment group was then found individually for each cell line. For the SW480 cells,
the neighboring nuclei were found to have an average distance of 26.6 µm and a standard deviation of 13.0 µm, while, in the MCF7 cells, the average distance between the nuclei of neighboring cells was 29.9 µm with a standard deviation of 12.5 µm. Using the MATLAB software and a threshold value based on the average distance and the standard deviation for each cell line, for a given cell, it was determined if the other cells in an image was considered to be within the neighbor threshold radius and were thus considered a neighbor.

Cellular Staining Determination: Once neighbor number was established for the threshold distance, cells were divided into three categories: low number of neighbors (0-2), medium number of neighbors (3-5), and high number of neighbors (6+). Using the MATLAB software, 25 cells (or the maximum number of cells if less than 25) were randomly selected from each category for each treatment group. Using a control image for each stain, the brightness and contrast for each image were adjusted in ImageJ. Example images of staining for each cell line are shown in Figures 2.3-2.7. The staining for the randomly selected cells was then determined for these images.

Statistics:

TGF-β ELISA: Due to the three levels of cellular culture confluence and the three concentrations of TGF-β that were added to the cultures, a two-way ANOVA test was employed to analyze whether changes in confluence and changes in exogenous TGF-β influenced the change in cytosolic TGF-β concentration for each cell line. Using $\alpha = 0.05$, we found that the individual effects of confluence and exogenous TGF-β, as well as the interaction between the two factors, significantly influenced the concentration of cytosolic TGF-β in both
Figure 2.3: Images of MCF7 cells at 30% confluence were post-processed using ImageJ. Brightness and contrast was adjusted using a control image for each stain, which are shown on the left.

Figure 2.4: Images of MCF7 cells at 100% confluence were post-processed using ImageJ. Brightness and contrast was adjusted using a control image for each stain, which are shown on the left.
**Figure 2.5:** Images of SW480 cells at 30% confluence were post-processed using ImageJ. Brightness and contrast was adjusted using a control image for each stain, which are shown on the left.

**Figure 2.6:** Images of SW480 cells at 100% confluence were post-processed using ImageJ. Brightness and contrast was adjusted using a control image for each stain, which are shown on the left.
SW480 and MCF7 cell lines, which is summarized in Tables 2.2 and 2.3, respectively.

### Table 2.2: Two-way ANOVA for Cytosolic TGF-β in SW480 Cells

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>DF</th>
<th>MS</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>302.0</td>
<td>4</td>
<td>75.50</td>
<td>6.508</td>
<td>0.0003</td>
</tr>
<tr>
<td>TGF-β</td>
<td>3859</td>
<td>2</td>
<td>1929</td>
<td>166.3</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Confluence</td>
<td>374</td>
<td>2</td>
<td>187.0</td>
<td>16.12</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>510.5</td>
<td>44</td>
<td>11.60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2.3: Two-way ANOVA for Cytosolic TGF-β in MCF7 Cells

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>DF</th>
<th>MS</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>66.00</td>
<td>4</td>
<td>16.50</td>
<td>12.19</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>TGF-β</td>
<td>331.3</td>
<td>2</td>
<td>165.70</td>
<td>122.4</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Confluence</td>
<td>59.09</td>
<td>2</td>
<td>29.54</td>
<td>21.82</td>
<td>&lt; 0.0001</td>
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<tr>
<td>Residual</td>
<td>59.57</td>
<td>44</td>
<td>1.354</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Flow Cytometry: With flow cytometry, the four different types of staining that a cell can exhibit (unstained, E-cadherin only, vimentin only, or dual staining), all add up to 100%
and are dependent upon each other: the percentage of cells stained for one subpopulation cannot increase or decrease without respective changes in the other subpopulations. Therefore, instead of using an ANOVA test, the flow cytometry data was converted into contingency tables with three rows (levels of confluence) three columns (concentration of exogenous TGF-β), and four sub-cells (staining) per treatment group and a Chi Squared Test was carried out.

Operating under the null hypothesis that the A columns, B rows, and C layers of the contingency table are independent, for a Chi Squared Test on a three-way contingency table, the test statistics using the observed value ($O$) and the expected value ($E$) are defined as [58]:

\[
X^2_{ABC} = \sum_{i,j,k} \frac{(O_{ijk} - E_{ijk})^2}{E_{ijk}} \quad \text{df}_{ABC} = (A-1)(B-1)(C-1) + (A-1)(B-1) + (A-1)(C-1) + (B-1)(C-1) \tag{2.1}
\]

\[
X^2_{AB} = \sum_{i,j} \frac{(O_{ij} - E_{ij})^2}{E_{ij}} \quad \text{df}_{AB} = (A-1)(B-1) \tag{2.2}
\]

\[
X^2_{BC} = \sum_{j,k} \frac{(O_{jk} - E_{jk})^2}{E_{jk}} \quad \text{df}_{BC} = (B-1)(C-1) \tag{2.3}
\]

\[
X^2_{AC} = \sum_{i,k} \frac{(O_{ik} - E_{ik})^2}{E_{ik}} \quad \text{df}_{AC} = (A-1)(C-1) \tag{2.4}
\]

For the flow cytometry data, $A = 3$ confluences, $B = 3$ concentrations of exogenous TGF-β, and $C = 4$ subpopulations of staining. The results of this statistical test are denoted in
Tables 2.4 for the SW480 cells and Table 2.5 for the MCF7 cells. A value of $\alpha = 0.05$ was used for analysis.

qPCR: Statistical analysis was carried out via the REST© software which uses a Pair Wise Fixed Reallocation Randomisation Test© [59] to determine whether or not there was a difference in expression between the control and treatment groups. Tables 2.6 and 2.8 show the analysis produced for SW480 cells and MCF7 cells, respectively, using 100% confluent + 0 ng/mL TGF-β as the control group. Tables 2.7 and 2.9 relied on other treatment groups as the control in order to produce further analysis for the SW480 cells and MCF7 cells, respectively.

Immunocytochemistry: In order to analyze whether or not neighbor number, concentration of added TGF-β, and cellular staining are independent, contingency tables and Chi-squared tests were used and the full analysis is listed in Tables 2.10-2.15. Tables 2.10-2.15 show that the only time neighbor number and cellular staining in SW480 cells were not found to be independent was when cells were classified using the average nuclei distance as the neighbor number threshold. In particular, by holding the level of exogenous TGF-β constant, it is the neighbor number and the cellular staining at 0 ng/mL TGF-β that are not independent. Other than this instance, for SW480 cells at all three distance thresholds, the statistical analysis suggests that neighbor number, concentration of added TGF-β, and cellular staining do not depend on each other. Additionally, for all treatment groups of the MCF7 cell line, no matter what neighbor number threshold distance was used, neighbor number, concentration of exogenous TGF-β, and the cellular staining were not found to depend on each other.
<table>
<thead>
<tr>
<th>Interaction</th>
<th>Test Statistic</th>
<th>df</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>confluence x TGF-β x staining</td>
<td>89.89</td>
<td>28</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>confluence x staining</td>
<td>47.40</td>
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</tr>
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<td>TGF-β x staining</td>
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</tr>
<tr>
<td>confluence x TGF-β</td>
<td>0</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
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<td>11.24</td>
<td>6</td>
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<td>confluence x staining @ 3 ng/mL TGF-β</td>
<td>22.44</td>
<td>6</td>
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</tr>
<tr>
<td>confluence x staining @ 9.33 ng/mL TGF-β</td>
<td>27.30</td>
<td>6</td>
<td>0.0001</td>
</tr>
<tr>
<td>TGF-β x staining @ 100% confluence</td>
<td>19.31</td>
<td>6</td>
<td>0.004</td>
</tr>
<tr>
<td>TGF-β x staining @ 60% confluence</td>
<td>8.59</td>
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<td>0.20</td>
</tr>
<tr>
<td>TGF-β x staining @ 30% confluence</td>
<td>13.52</td>
<td>6</td>
<td>0.03</td>
</tr>
<tr>
<td>Interaction</td>
<td>Test Statistic</td>
<td>df</td>
<td>p-value</td>
</tr>
<tr>
<td>-----------------------------------------</td>
<td>----------------</td>
<td>----</td>
<td>---------</td>
</tr>
<tr>
<td>confluence x TGF-β x staining</td>
<td>81.65</td>
<td>28</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>confluence x staining</td>
<td>20.06</td>
<td>6</td>
<td>0.003</td>
</tr>
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<td>TGF-β x staining</td>
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<td>0.28</td>
</tr>
<tr>
<td>confluence x TGF-β</td>
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<td>1</td>
</tr>
<tr>
<td>confluence x staining @ 0 ng/mL TGF-β</td>
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</tr>
<tr>
<td>confluence x staining @ 3 ng/mL TGF-β</td>
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<td>0.05</td>
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<td>confluence x staining @ 9.33 ng/mL TGF-β</td>
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<tr>
<td>TGF-β x staining @ 100% confluence</td>
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<td>0.006</td>
</tr>
<tr>
<td>TGF-β x staining @ 60% confluence</td>
<td>4.12</td>
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<td>0.66</td>
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<td>TGF-β x staining @ 30% confluence</td>
<td>5.98</td>
<td>6</td>
<td>0.42</td>
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Table 2.6: REST Analysis for Gene Expression in SW480 Cells

<table>
<thead>
<tr>
<th>Control</th>
<th>Treatment Group</th>
<th>Gene</th>
<th>Relative Expression</th>
<th>Std. Error Range</th>
<th>p-value</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% CF 0 ng/mL TGF-β</td>
<td>60% CF 0 ng/mL TGF-β</td>
<td>E-cadherin</td>
<td>0.55</td>
<td>(0.4-0.8)</td>
<td>&lt; 0.0001</td>
<td>Down</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Slug</td>
<td>0.89</td>
<td>(0.2-2.7)</td>
<td>0.747</td>
<td>--</td>
</tr>
<tr>
<td>100% CF 0 ng/mL TGF-β</td>
<td>30% CF 0 ng/mL TGF-β</td>
<td>E-cadherin</td>
<td>0.44</td>
<td>(0.3-0.6)</td>
<td>&lt; 0.0001</td>
<td>Down</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Slug</td>
<td>2.01</td>
<td>(0.4-5.6)</td>
<td>0.140</td>
<td>--</td>
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<tr>
<td>100% CF 0 ng/mL TGF-β</td>
<td>100% CF 3 ng/mL TGF-β</td>
<td>E-cadherin</td>
<td>0.43</td>
<td>(0.3-0.7)</td>
<td>&lt; 0.0001</td>
<td>Down</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Slug</td>
<td>0.31</td>
<td>(0.1-1.4)</td>
<td>0.023</td>
<td>Down</td>
</tr>
<tr>
<td>100% CF 0 ng/mL TGF-β</td>
<td>60% CF 3 ng/mL TGF-β</td>
<td>E-cadherin</td>
<td>0.41</td>
<td>(0.2-0.7)</td>
<td>&lt; 0.0001</td>
<td>Down</td>
</tr>
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<td></td>
<td></td>
<td>Slug</td>
<td>0.58</td>
<td>(0.1-1.8)</td>
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<td>100% CF 0 ng/mL TGF-β</td>
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<td>E-cadherin</td>
<td>0.17</td>
<td>(0.1-0.4)</td>
<td>&lt; 0.0001</td>
<td>Down</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Slug</td>
<td>2.25</td>
<td>(0.8-10.1)</td>
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<td>100% CF 0 ng/mL TGF-β</td>
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<td>E-cadherin</td>
<td>0.81</td>
<td>(0.6-1.0)</td>
<td>0.124</td>
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<td></td>
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<td>0.36</td>
<td>(0.1-2.3)</td>
<td>0.055</td>
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<td>100% CF 0 ng/mL TGF-β</td>
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<td>E-cadherin</td>
<td>0.39</td>
<td>(0.2-0.8)</td>
<td>&lt; 0.0001</td>
<td>Down</td>
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<td></td>
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<td>Slug</td>
<td>0.76</td>
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<td>100% CF 0 ng/mL TGF-β</td>
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<td>E-cadherin</td>
<td>0.21</td>
<td>(0.2-0.3)</td>
<td>&lt; 0.0001</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Slug</td>
<td>3.6</td>
<td>(1.0-10.9)</td>
<td>0.016</td>
<td>Up</td>
</tr>
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</table>
Table 2.7: Further REST Analysis for Gene Expression in SW480 Cells

<table>
<thead>
<tr>
<th>Control</th>
<th>Treatment Group</th>
<th>Gene</th>
<th>Relative Expression</th>
<th>Std. Error Range</th>
<th>p-value</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>60% CF 0 ng/mL TGF-β</td>
<td>60% CF 3 ng/mL TGF-β</td>
<td>E-cadherin 0.75</td>
<td>(0.5-1.3)</td>
<td>0.122</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Slug 0.65</td>
<td>(0.5-1.0)</td>
<td>0.008</td>
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<tr>
<td>60% CF 0 ng/mL TGF-β</td>
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<td>E-cadherin 0.72</td>
<td>(0.4-1.4)</td>
<td>0.129</td>
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<td>Slug 0.85</td>
<td>(0.5-1.5)</td>
<td>0.404</td>
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<tr>
<td>30% CF 0 ng/mL TGF-β</td>
<td>30% CF 3 ng/mL TGF-β</td>
<td>E-cadherin 0.39</td>
<td>(0.2-0.9)</td>
<td>0.002</td>
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<td></td>
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<td>(0.4-3.1)</td>
<td>0.809</td>
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</tr>
<tr>
<td>30% CF 0 ng/mL TGF-β</td>
<td>30% CF 9.33 ng/mL TGF-β</td>
<td>E-cadherin 0.49</td>
<td>(0.4-0.6)</td>
<td>0.001</td>
<td>Down</td>
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</tr>
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<td></td>
<td>Slug 1.79</td>
<td>(1.0-3.3)</td>
<td>0.036</td>
<td>Up</td>
<td></td>
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<tr>
<td>100% CF 3 ng/mL TGF-β</td>
<td>60% CF 3 ng/mL TGF-β</td>
<td>E-cadherin 0.95</td>
<td>(0.5-1.9)</td>
<td>0.704</td>
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<td></td>
<td></td>
<td>Slug 1.87</td>
<td>(0.6-5.5)</td>
<td>0.189</td>
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<tr>
<td>100% CF 3 ng/mL TGF-β</td>
<td>30% CF 3 ng/mL TGF-β</td>
<td>E-cadherin 1.83</td>
<td>(0.6-5.9)</td>
<td>0.159</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Slug 7.27</td>
<td>(1.2-39.3)</td>
<td>0.116</td>
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<td></td>
</tr>
<tr>
<td>100% CF 9.33 ng/mL TGF-β</td>
<td>60% CF 9.33 ng/mL TGF-β</td>
<td>E-cadherin 0.49</td>
<td>(0.3-0.8)</td>
<td>0.001</td>
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Table 2.11: Chi Squared Test for MCF7 Cell Staining at Average Distance

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Results and Discussion

*TGF-β ELISA*

When exogenous TGF-β binds to the cellular surface receptors, epithelial cells are able to endocytose the TGF-β into the cytosol [60]. Additionally, it has been shown that, with the activation of the TGF-β pathway, epithelial cells are able to produce their own TGF-β [61], continuing the positive TGF-β feedback loop. Therefore, measuring the concentration of cytosolic TGF-β helps confirm the activation of the TGF-β pathway in the cells. Figure 2.7 shows the cytosolic concentration of TGF-β derived from 1x10^6 cells for both SW480 (Figure 2.7A) and MCF7 (Figure 2.7B) cells. Initially, prior to the addition of exogenous TGF-β, both cell lines have a low level of cytoplasmic TGF-β present. When 3 ng/mL of TGF-β is added, there is an increase in the concentration of cytosolic TGF-β across all three confluences. In the SW480 cells, as the confluence is reduced in the 3 ng/mL of TGF-β treatment groups, there is an increase in the concentration of cytosolic TGF-β present. However, this does not hold for the MCF7 cells: As confluence is reduced in this same set of treatment groups, the concentration of cytosolic TGF-β remains constant. With the addition of 9.33 ng/mL TGF-β to each tissue culture, the reduction in confluence from 100% to 60% results in an increase in the concentration of cytosolic TGF-β, which then plateaus as the confluence is reduced further to 30% in both cell lines. Note that while their behavior is similar, the SW480 cells have 2-3x as much cytosolic TGF-β present as the MCF7 cells in the 30% confluent + 9.33 ng/mL exogenous TGF-β treatment group.
Figure 2.7: Concentrations for cytosolic TGF-β1 for each cell line are derived from 1x10⁶ cells. As exogenous TGF-β1 is added and confluence is reduced, the cytosolic concentration of TGF-β1 rises, until it saturates at a concentration of ~ 25 pg/mL for SW480 cells and ~ 9 pg/mL for MCF7 cells. Both confluence and exogenous TGF-β1, as well as the interaction of the two factors, were found to significantly influence the concentration of cytosolic TGF-β1 in both cell lines. The results of this analysis can be found in Tables 2.2 and 2.3.
This ELISA analysis can be taken one step further in order to understand the cytosolic level of TGF-β on an individual cell basis. If the concentrations in Figure 2.7 are reduced to describe the average TGF-β concentration in 1 cell, using the cellular volume of 2.0x10⁻⁹ mL [62] and the molecular weight of TGF-β of 25 kDa [63], the average number of molecules in an SW480 cell in the 100% confluent + 0 ng/mL TGF-β culture is 3.1x10⁻⁸ molecules per cell of cytosolic TGF-β. As confluence is reduced and exogenous TGF-β is applied, this cytosolic TGF-β value increases to 1.2x10⁻⁶ molecules per cell in the 30% confluent + 9.33 ng/mL TGF-β. Likewise, for the MCF7 cells, using the values from Figure 2.7, a cell in the 100% confluent + 0 ng/mL TGF-β treatment group begins with 3.0x10⁻⁸ molecules per cell, which is then increased to 4.5x10⁻⁷ molecules per cell in the 30% confluent + 9.33 ng/mL TGF-β treatment group. The fractional number of molecules per cell in each cell line would suggest that many of the cells in each treatment group either (1) did not have the TGF-β bind to its cellular receptors, (2) did not endocytose the TGF-β that did bind to its receptors, or (3) endocytosed the exogenous TGF-β but it was simply degraded and no additional TGF-β was produced by the cell, all of which would mean that the TGF-β pathway was not activated in these cells. However, as Figure 2.7 showed, the overall concentration of cytosolic TGF-β in the cell cultures did significantly increase as confluence was reduced and TGF-β was applied, suggesting that this significant change in cytosolic TGF-β was driven by a small subset of the population that did see their TGF-β pathways activated and a significant increase in their cytosolic TGF-β.
Figure 2.8: The fluorescence intensity of E-cadherin and vimentin on 4 treatment groups are shown, with SW480 cells in A-D and MCF7 cells in E-H. For 100% confluent, the SW480 population did have high E-cadherin fluorescent intensity, as indicated by the dark blue group of cells in the upper left hand quadrant in A and B. As confluence is reduced and TGF-β is added, there is a population of cells in the lower right quadrant, indicating an increase in cells that stain vimentin-only, and thus exhibit the mesenchymal phenotype. In E-H, MCF7 cells stained mostly for E-cadherin-only. But, as confluence is reduced and TGF-β is added, the portion of the population that stain vimentin-only grew.
Flow Cytometry

Examples of the two-stain plots for each cell population are shown in Figure 2.8. Using single color controls, the results for each cell line were gated to determine which cells were unstained, which cells stained for only E-cadherin, an epithelial marker [64, 65], or vimentin, a mesenchymal marker [64], and which cells stained for both. The results of the gating and the subpopulation percentages for each cell line are summarized in Tables 2.16-2.21 and shown in Figure 2.9.

From the plots in Figures 2.8 and 2.9, it is clear that the two cell types have different characteristics and exhibit different behavior when subjected to external factors, leading to a different division in the cells that stain for each grouping. For all treatment groups with the SW480s, there is still a distinct subpopulation of cells that are staining for both E-cadherin and vimentin. Conversely, the MCF7 cells have a very low percentage of cells that double stain. Instead, the MCF7s mostly stained for either E-cadherin or vimentin.

As shown in Figure 2.9, without the addition of exogenous TGF-β, as confluency is decreased, the SW480s maintain roughly the same percentage of cells in each of the four subpopulations. When 3 ng/mL of exogenous TGF-β is added to the SW480 cells, there is a slight decrease in the percentage of cells that have E-cadherin-only expression for all three confluences. Additionally, within the 3 ng/mL TGF-β treatment cluster, as confluency is reduced, the percentage of cells that are expressing vimentin-only increased, while the percentage of cells that dually express both E-cadherin and vimentin decreased as confluency decreased. If 9.33 ng/mL TGF-β is applied, there is a dramatic decrease in the expression of E-cadherin-only stained cells (Figure 2.9C) and a rise in the cell subpopulation that stained
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Figure 2.9: (A&B) There were cells that did not stain for either marker for both cell lines across all treatment groups. (C&D) Both cell lines contained cells that stained for only E-cadherin, with, on average, < 60% of the total cell population of SW480s and > 60% of the total MCF7 cell population occupying this subpopulation. (E&F) For both cell lines, with the addition of exogenous TGF-β and a reduction in confluence, the population of cells that stained for vimentin alone grew. (G&H) While the SW480 cells maintain a large portion of the cells that stain for both E-cadherin and vimentin across all treatment groups, there is only a very small subpopulation of MCF7 cells that stain for both E-cadherin and vimentin.
vimentin-only as confluence is decreased. The dually stained SW480 subpopulation, however, does not decrease for this concentration of exogenous TGF-β.

Like the SW480s, without exogenous TGF-β, the MCF7s also maintain the same percentage of cells in each of the subpopulations as confluence is decreased, as shown in Figure 2.9. When 3 ng/mL of exogenous TGF-β is added to the MCF7 cells, the percentage of cells that stain E-cadherin-only stays roughly the same, even as confluence is reduced or more TGF-β is added. However, there is an increase in the subset of cells that stained for vimentin-only as confluence is reduced in the sets of cells that are exposed to 3 ng/mL and those exposed to 9.33 ng/mL of exogenous TGF-β. It is important to note that, even though a portion of every treatment group did stain for vimentin-only as confluence is reduced in the sets of cells that are exposed to 3 ng/mL and those exposed to 9.33 ng/mL of exogenous TGF-β. It is important to note that, even though a portion of every treatment group did stain for vimentin-only, the majority of the cells in each treatment group stained for E-cadherin-only, indicating that the majority of the cells in each treatment group maintained their epithelial qualities.

From the summary of the flow cytometry data analysis, with a Chi-Squared test statistic ($\chi^2$) of 89.89 and a p-value < 0.0001, it is apparent that, for the SW480 cells, the changes in confluence, exogenous TGF-β, and the number of cells that stain for each subset are not independent of each other. Additionally, from Table 2.4 we can see that changes in confluence influences changes in cellular staining, as does the changes in exogenous TGF-β.

As shown in Table 2.5, with a Chi-Squared test statistic ($\chi^2$) of 81.65 and a p-value < 0.0001, like the SW480 cells, the effects of the changes in confluence, the changes in exogenous TGF-β application, and cellular staining are not independent in the MCF7 cells.
Also, for the MCF7 cells, changes in confluence alone influences the changes in cellular staining. However, unlike the SW480 cells, in the MCF7 cells, changes in the cellular staining is independent of changes in the exogenous TGF-β concentration alone. Note that, for both cell lines, confluence and exogenous TGF-β concentration have a test statistic of 0 and a p-value = 1, meaning that the two do not influence each other. This is to be expected, considering these are two independent external factors applied to the cells.

In addition to analyzing and determining the dependence of the two treatments and the staining, several sub-contingency tables were created over one specific treatment level, which are also shown in Tables 2.4 and 2.5. Without the addition of any exogenous TGF-β, we fail to reject the null hypothesis that changes in cellular staining are independent of changes in cellular confluence in both SW480 (p-value = 0.08) and MCF7 (p-value = 0.66) cells. With the application of 3 ng/mL of exogenous TGF-β, we are able to reject the null hypothesis for SW480 cells (p-value = 0.001), but are unable to reject the null hypothesis for MCF7 cells (p-value = 0.05), leaving us to conclude that, in SW480 cells, changes in cellular staining are not independent of changes in confluence but, in MCF7 cells, they are. Finally, with the addition of 9.33 ng/mL exogenous TGF-β to the cell cultures, we are able to reject the null hypothesis for both SW480 and MCF7 cells and conclude that changes in confluence in both cell lines significantly effect changes in cellular staining.

With these results, we can see that, without the application of exogenous TGF-β, changes in confluence alone are not enough to change the staining of the cells nor push the cells from epithelial to mesenchymal for either cell line. However, with the addition of
exogenous TGF-β, we see a decrease in the p-value for the test statistic of both of these treatment groups in both cell lines, with changes in the SW480 confluence becoming significant at 3 ng/mL of TGF-β and changes in the MCF7 confluence becoming significant at 9.33 ng/mL of TGF-β. Thus, we can conclude that it is only with the addition of the exogenous TGF-β that changes in confluence significantly impact changes in cellular staining for flow cytometry but, a different concentration of exogenous TGF-β is required for the different cell lines in order to have this effect.

**qPCR**

Figure 2.10 shows the log relative expression of different treatment groups of the SW480 cells (Figure 2.10A) and the MCF7 cells (Figure 2.10B) to their respective 100% confluent + 0 ng/mL exogenous TGF-β treatment groups. For the SW480 cells (Figure 2.10A), as confluence was decreased and exogenous TGF-β concentration was increased, the mean expression of E-cadherin relative to the 100% confluent + 0 ng/mL TGF-β control grew smaller. This change in E-cadherin expression was statistically significant for all treatment groups, except for the 100% confluent + 9.33 ng/mL TGF-β, as shown in Table 2.6. Additionally, for all treatment groups at 60% and 100% confluent, the mean expression of Slug relative to the 100% confluent + 0 ng/mL exogenous TGF-β control also decreased while, for all treatment groups at 30% confluent, the mean expression of Slug relative to the 100% + 0 ng/mL exogenous TGF-β control increased. Most of the relative changes in SW480 Slug expression, however, were not statistically significant, except at the 100% confluent + 3 ng/mL TGF-β and the 30% confluent + 9.33 ng/mL TGF-β treatment groups when compared to the
Figure 2.10: The log relative gene expression of different treatment groups when compared to the 100% confluent + 0 ng/mL TGF-β treatment group is shown, with SW480 cells in (A) and MCF7 cells in (B). The log relative expression of E-cadherin is shown in blue and the log relative expression of Slug is shown in red for both cell lines. Note that, for both the SW480 and MCF7 cells, the relative expression of E-cadherin decreased as confluence was reduced and exogenous TGF-β was added. However, when compared to the 100% confluent + 0 ng/mL TGF-β treatment group, the relative expression of Slug only increased for the 30% confluent treatment groups in the SW480s, but increased for all treatment groups except for the 100% confluent + 9.33 ng/mL treatment group in the MCF7s. The relative expression analysis is summarized for both cell lines in Tables 2.6 – 2.9.
100% confluent + 0 ng/mL TGF-β control, as is reported in Table 2.6. Conversely, for the MCF7 cells (Figure A10B), the mean expression of E-cadherin, relative to the 100% confluent + 0 ng/mL TGF-β control, decreased for all treatment groups. Additionally, all mean relative expression levels of E-cadherin were considered statistically significant, as shown in Table 2.7. Unlike the SW480 cells, the mean expression of Slug relative to the control increased for all MCF7 treatment groups. The only two treatment groups whose Slug relative expression was not considered statistically significant were the 100% confluent treatment groups.

Figure 2.11 shows further log relative expressions of different treatment groups when the control is changed in SW480 cells. As shown in Figure 2.11A, when confluence is maintained at 60% but exogenous TGF-β is added to the culture, there is a very small change to the relative expression of E-cadherin and Slug when compared to the 60% confluent + 0 ng/mL TGF-β treatment group. When confluence of SW480 cells is reduced to 30% and exogenous TGF-β is added, as shown in Figure 2.11B, there is a greater decrease in E-cadherin expression when compared to the 30% confluent + 0 ng/mL TGF-β treatment group. Additionally, as more TGF-β is added to the SW480 cells, there is a greater increase in Slug expression when the treatment groups are compared to the control 30% confluent + 0 ng/mL TGF-β. When confluence of the SW480s is changed but the concentration of exogenous TGF-β is maintained at 3 ng/mL, we see an increase in the relative expression of Slug when compared to the control of 100% confluent + 3 ng/mL TGF-β for both treatment groups (Figure 2.11C), as well as an increase in the relative expression of E-cadherin when compared to the control. However, none of these changes are considered statistically significant, as shown in
Table 2.6. Finally, when exogenous TGF-β is maintained at 9.33 ng/mL and confluence of the SW480s is changed (Figure 2.11D), there is a decrease in the relative expression of E-cadherin and an increase in the relative expression of Slug for both treatment groups when compared to the 100% confluent + 9.33 ng/mL TGF-β control. Again, however, note that the increase in Slug expression is not considered statistically significant in Table 2.6.

**Figure 2.11:** The relative expression of E-cadherin and Slug in different treatment groups when compared to 60% confluent SW480 cells + 0 ng/mL TGF-β (A), 30% confluent SW480 cells + 0 ng/mL TGF-β (B), 100% confluent SW480 cells + 3 ng/mL TGF-β (C), and 100% confluent SW480 cells + 9.33 ng/mL TGF-β (D).
Figure 2.12: The relative expression of E-cadherin and Slug in different treatment groups when compared to 60% confluent MCF7 cells + 0 ng/mL TGF-β (A), 30% confluent MCF7 cells + 0 ng/mL TGF-β (B), 100% confluent MCF7 cells + 3 ng/mL TGF-β (C), and 100% confluent MCF7 cells + 9.33 ng/mL TGF-β (D).

Figure 2.12 shows further log relative expressions of different treatment groups when the control is changed in MCF7 cells. Figure 2.12A examines the log relative expression of E-cadherin and Slug mRNA in the 60% confluent treatment groups with added exogenous TGF-β. The relative expression of both E-cadherin and Slug increase in both treatment groups when compared to the control of 60% confluent + 0 ng/mL TGF-β. When the confluence is reduced to 30% and kept constant but the exogenous TGF-β is changed as shown in Figure 2.12B, the
relative expression of E-cadherin decreases and the relative expression of Slug increases for both treatment groups when compared to their control of 30% confluent + 0 ng/mL TGF-β. However, note that the relative changes in E-cadherin for both treatment groups, as well as the relative change in Slug for the 30% confluent + 9.33 ng/mL TGF-β treatment group are not considered statistically significant due to the high deviation in the data. When exogenous TGF-β is kept constant but the confluence is reduced, as in Figures 2.12C and 2.12D, the relative expression of E-cadherin decreases and the relative expression of Slug increases for all treatment groups when compared to their respective controls (100% confluent + 3 ng/mL TGF-β in 2.12C and 100% confluent + 9.33 ng/mL TGF-β in 2.12D). Note that all 4 sets of these relative expression changes in 2.12C and 2.12D are considered statistically significant, as shown in Table 2.7.

**Immunocytochemistry**

Once neighbor number was established using the nuclei threshold distance, 100 cells were randomly selected from each treatment group and technical replicate using MATLAB software. The results of this selection are shown in Figures 2.13 and 2.14. Due to the large standard deviation, this process was carried out at a threshold of the average distance, the average distance + 1 standard deviation, and the average distance + 2 standard deviations for each cell line. As shown in Figure 2.13, for both cell lines at 30% confluence, at all three threshold distances, the population of cells was skewed right, meaning that the majority of the cells had a lower number of neighbors. For the SW480 cells, at its average threshold distance (Figure 2.13A), the majority of cells at 30% confluence without the addition of exogenous TGF-β had 2 neighboring cells while, for the MCF7 cells in this same treatment group (Figure
2.13B), the majority of cells had 0 neighbors. With the addition of 9.33 ng/mL exogenous TGF-β, the SW480 cells still have the majority of the cells with 1 or 2 neighbors (Figure 2.13A), while most MCF7 cells have 0-2 neighbors (Figure 2.13B).

When the nuclei threshold was extended to include cells at the average distance + 1 standard deviation, both cell lines remained skewed right, as shown in Figures 2.13C and 2.13D. For the SW480 cells in Figure 2.13C, the neighbor number count for the majority of both 30% confluent populations shift toward 3-5 neighbors, while in 2.13D, at a threshold of its average nuclei distance + 1 standard deviation, the majority of the MCF7 cells in the 30% confluent treatment groups have 0-5 neighbors.

With the nuclei threshold extended again to include cells at the average nuclei distance + 2 standard deviations, both cell lines remained skewed right and saw an increase in their maximum neighbor number, as shown in Figures 2.13E and 2.13F. Additionally, as was shown in Figures 2.13C and 2.13D, with the lengthening of the threshold distance, the higher maximum neighbor number was accompanied by a lower frequency of the neighbor numbers for each cell line.

Applying this same process to the 100% confluent cells for both cell lines, Figure 2.14 shows that, for a threshold distance of the average nuclei distance, both cell lines have a bell curve shape with the most SW480 cells having 6 neighbors at both concentrations of TGF-β and most MCF7 cells having 10-11 neighbors at both concentrations of TGF-β. As the threshold distance is extended to include 1 and 2 standard deviations, both cell lines see a shift towards a higher number of neighbors for the majority of the cells, as well as a lower frequency
Figure 2.13: The number of neighbors was determined for each cell line using different thresholding nuclei distances. A and B show the neighbor number for 30% confluent SW480 (A) and MCF7 (B) cells at a threshold of the average nuclei distance while C-F show the two cell lines’ neighbor numbers at a threshold of the average nuclei distance + 1 standard deviation and 2 standard deviations.
Figure 2.14: The number of neighbors was determined for each cell line using different thresholding nuclei distances. A and B show the neighbor number for 100% confluent SW480 (A) and MCF7 (B) cells at a threshold of the average nuclei distance while C-F show the two cell lines’ neighbor numbers at a threshold of the average nuclei distance + 1 standard deviation and 2 standard deviations.
peak, resulting in both populations skewed left at the threshold distance of the average nuclei distance + 2 standard deviations.

Due to the varying maximum number of neighbors for each cell line and TGF-β treatment group, cellular neighbor number was divided into 3 categories: low neighbor number (0-2 neighbors), medium neighbor number (3-5 neighbors), and high neighbor number (6+ neighbors). For each of the 30% confluent treatment groups, 25 cells from each of the neighbor number categories were randomly selected and categorized based on their staining: neither, E-cadherin-only, vimentin-only, and dual staining. For treatment groups where a neighbor number category contained less than 25 cells, the total number of cells in that category was counted. Staining was determined for cells from different neighbor number categories using threshold distances of the average nuclei distance, the average distance + 1 standard deviation, and the average distance + 2 standard deviations.

Figure 2.15 shows the cellular staining of the SW480 cells using a distance threshold of the average nuclei distance. Cells with a low number of neighbors and a medium number of neighbors include cells that did not stain for either marker (16-28% of the subpopulation), cells that stained for only E-cadherin (32-58% of the subpopulation), cells that stained for only vimentin (8-20% of the subpopulation), and cells that stained for both markers (18-26% of the subpopulation). For cells with a high number of neighbors, both with and without the addition of exogenous TGF-β, 70+% of the cells stained for E-cadherin only. Additionally, without the addition of any exogenous TGF-β, 0% of the cells stained for vimentin alone. Instead 12% of the cells stained for both E-cadherin and vimentin markers while 6% of the cells did not stain for either. However, with the addition of 9.33 ng/mL of TGF-β, the cell staining shifts so that,
**Figure 2.15:** The percentage of SW480 cells that stained for E-cadherin only, vimentin only, both markers, and neither marker in cell populations that are 30% confluent are shown. The number of neighbors a cell had was determined using a distance threshold of the average distance between nuclei.
Figure 2.16: The percentage of SW480 cells that stained for E-cadherin only, vimentin only, both markers, and neither marker in cell populations that are 30% confluent are shown. The number of neighbors a cell had was determined using a distance threshold of the average distance between nuclei + 1 standard deviation.
Figure 2.17: The percentage of SW480 cells that stained for E-cadherin only, vimentin only, both markers, and neither marker in cell populations that are 30% confluent are shown. The number of neighbors a cell had was determined using a distance threshold of the average distance between nuclei + 2 standard deviations.
while the majority of the cells still stained for E-cadherin only, 5.41% of the cells stained for vimentin-only. In Figures 2.16 and 2.17, the distance threshold for the SW480 cells is increased to include one and two standard deviations, respectively. With the exception of cells at a high neighbor number + 0 ng/mL TGF-β at a distance threshold of the average nuclei distance + 1 standard deviation, all high neighbor number subgroups had 50+% of the cells stain for E-cadherin only, indicating a majority of the subpopulation has epithelial characteristics. Additionally, with the exception of the cells with a high number of neighbors and 9.33 ng/mL TGF-β at a threshold distance of the average nuclei + 1 standard deviation, all cellular groups had cellular subsets that stained for only vimentin, indicating that it was possible for cells in all neighbor number subpopulations to acquire mesenchymal characteristics.

Figure 2.18 shows the cellular staining of the MCF7 cells using a distance threshold of the average nuclei distance. While the SW480 neighbor number categories in Figure 2.15 had cells that exhibited all four staining classifications, the MCF7 cells in all three neighbor number groupings had an overwhelmingly positive staining of E-cadherin with 86+% of the cells staining for E-cadherin only. The MCF7 cells with the low neighbor number that did not have exogenous TGF-β added to them had the highest percentage of cells that did not stain for either E-cadherin or vimentin. Additionally, when 9.33 ng/mL of TGF-β was added to the 30% confluent cells with a low neighbor number, this was the only treatment group that expressed a subset of cells that exhibited any vimentin expression, as 8% of the cells at a low neighbor number dually stained for E-cadherin and vimentin. When the threshold distance was extended to include one and two standard deviations, as shown in Figures 2.19 and 2.20, respectively, at least 75% of the cells in each neighbor number subset stained positively for E-cadherin only.
Figure 2.18: The percentage of MCF7 cells that stained for E-cadherin only, vimentin only, both markers, and neither marker in cell populations that are 30% confluent are shown. The number of neighbors a cell had was determined using a distance threshold of the average distance between nuclei.
Figure 2.19: The percentage of MCF7 cells that stained for E-cadherin only, vimentin only, both markers, and neither marker in cell populations that are 30% confluent are shown. The number of neighbors a cell had was determined using a distance threshold of the average distance between nuclei + 1 standard deviation.
Figure 2.20: The percentage of MCF7 cells that stained for E-cadherin only, vimentin only, both markers, and neither marker in cell populations that are 30% confluent are shown. The number of neighbors a cell had was determined using a distance threshold of the average distance between nuclei + 2 standard deviations.
whether or not TGF-β is present. Like the MCF7 neighbor groupings that were determined using a distance threshold of the average nuclei distance, when the distance is increased in Figures 2.19 and 2.20, the cells with the highest percentage of non-E-cadherin stained subpopulation are the cells with a low neighbor number and without any exogenous TGF-β added (Figures 2.19A and 2.20A). When 9.33 ng/mL of TGF-β was added to the 30% confluent cells, the MCF7 cells with few neighbors at these higher thresholding distances are the cellular populations that see the appearance of vimentin stained cells or dual E-cadherin and vimentin expressing cells (Figure 2.19B and 2.20B).

Figure 2.21 shows several examples of the MCF7 cells stained for E-cadherin, vimentin, and DAPI. Using a neighbor threshold distance of the MCF7 nuclei average distance + 1 standard deviation, cells from all three groupings of neighbor number are indicated by different arrow colors: blue (low), green (medium), and orange (high). Figures 2.21A and 2.21B are two images of MCF7 cells in the 30% confluent + 0 ng/mL exogenous TGF-β treatment group. In Figure 2.21A, arrows 1 and 2 point out two examples of the different staining and phenotype that cells in the same treatment group can express: the cell at arrow 1 has a low neighbor number and has stained faintly for E-cadherin while the cell at arrow 2 also has a low neighbor number and has stained slightly more for E-cadherin, despite the fact that it is beginning to adopt a spindle-like phenotype. Additionally, E-cadherin is also expressed in the cell at the green arrow, which has a medium number of neighbors, as well as the cell at the orange arrow, which has a high number of neighbors. In Figure 2.21B, several of the cells are beginning to slightly express vimentin, even though they are categorized differently: the blue
Figure 2.21: Examples of staining in the MCF7 cell line. A and B are 30% confluent cells with 0 ng/mL of exogenous TGF-β while C and D are 30% confluent cells with 9.33 ng/mL of exogenous TGF-β. Arrows are color coordinated: blue arrows indicate a cell with a low number of neighbors, green indicates cells with a medium number of neighbors, and orange indicates cells with a high number of neighbors, as determined using a threshold distance of the average nuclei distance + 1 standard deviation. A scale bar of 100 µm is shown in each image.
arrow indicating a cell with a low neighbor number and the green arrow indicating a cell with a medium number of neighbors. Figures 2.21C and 2.21D are images of MCF7 cells at 30% confluent + 9.33 ng/mL exogenous TGF-β. In Figure 2.21C, note that for cells at all categories of neighbor number, the medium neighbor number cells and the high neighbor number cells number are positively stained for E-cadherin, as shown by the green and orange arrows, respectively. Arrow 1 indicates a cell with a low neighbor number that has not stained for either distinctively express E-cadherin. For the low neighbor number cells, cell 1 lacks any stain while cell 2 is stained with E-cadherin, despite adopting the spindle-like phenotype. In Figure 2.21D, there is again a variation in protein expression. Cells with a medium and high neighbor E-cadherin or vimentin, or even just faintly E-cadherin, while arrow 2 points to a cell that also has a low neighbor number that has positively stained for vimentin.

For comparison, Figures 2.22A and 2.22B show two images of MCF7 cells at 100% confluence + 0 ng/mL of exogenous TGF-β and Figures 2.22C and 2.22D show two images of MCF7 cells at 100% confluence + 9.33 ng/mL of exogenous TGF-β. The staining for E-cadherin in all 4 of these images is much stronger and more uniform than in Figures 2.21A-D. Additionally, because these cells grow on top of each other and thus form multiple layers, some cells in underlying layers have stained for vimentin in both 100% confluent treatment groups, as indicated by the arrows in Figures 2.22B and 2.22D.

Figure 2.23 shows several examples of the SW480 cells stained for E-cadherin, vimentin, and DAPI. Again, using a neighbor threshold distance of the SW480 nuclei average distance + 1 standard deviation, cells from all three groupings of neighbor number are indicated by different arrow colors: blue (low), green (medium), and orange (high). In contrast to the
Figure 2.22: Examples of staining in the MCF7 cell line. Figures A and B are 100% confluent cells + 0 ng/mL of exogenous TGF-β and 100% confluent cells + 9.33 ng/mL of TGF-β in figures C and D. Yellow arrows point to appearance of vimentin in images. A scale bar of 100 μm is shown in each image.
MCF7 cells, cells in all 30% confluent treatment groups for the SW480 cells exhibit a wide variety of staining, as shown in Figures 2.23A-D. Figures 2.23A and 2.23B show two images from SW480 cells at 30% confluent + 0 ng/mL of exogenous TGF-β. In Figure 2.23A, arrows 1, 2, and 3 all indicate three different cells that were classified as having a high number of neighbors that all stained differently: arrow 1 has stained solely for vimentin, arrow 2 for E-cadherin only, and arrow 3 has stained for both vimentin and E-cadherin simultaneously. In Figure 2.23B, as indicated by the arrows, all three cells have a different neighbor number classification but all three have stained positively for vimentin. Figures 2.23C and 2.23D show the SW480 30% confluent + 9.33 ng/mL TGF-β treatment group. In Figure 2.23C, again, there are cells that are classified as having different neighbor numbers all staining dually for E-cadherin and vimentin. Additionally, in Figure 2.23D, both arrows 1 and 2 are cells classified as having a high number of neighbors but stain differently: the cell at arrow 1 is stained positively for E-cadherin only while the cell at arrow 2 is stained for both E-cadherin and vimentin.

For comparison, Figures 2.24A-D show the SW480 cells at 80-100% confluent at both levels of added exogenous TGF-β. Cells without exogenous TGF-β are shown in Figures 2.24A and 2.24B while cells with 9.33 ng/mL of exogenous TGF-β are shown in Figures 2.24C and 2.24D. The variety of staining that was exhibited in the 30% confluent cells is carried over as confluence is increased, whether or not the treatment group includes the addition of exogenous TGF-β.
Figure 2.23: Examples of staining in the SW480 cell line. A and B are 30% confluent cells with 0 ng/mL of exogenous TGF-β while C and D are 30% confluent cells with 9.33 ng/mL of exogenous TGF-β. Arrows are color coordinated: blue arrows indicate a cell with a low number of neighbors, green indicates cells with a medium number of neighbors, and orange indicates cells with a high number of neighbors, as determined using a threshold distance of the average nuclei distance + 1 standard deviation. A scale bar of 100 µm is shown in each image.
Figure 2.24: Examples of staining in the SW480 cell line. A and B are 100% confluent + 0 ng/mL of exogenous TGF-β while C and D are 100% confluent + 9.33 ng/mL of exogenous TGF-β. A scale bar of 100 µm is shown in each image.
Conclusion

There were many similarities and differences in the behavior of the MCF7 and SW480 cells when exposed to the same experimental treatments. As confluence was reduced at 0 ng/mL TGF-β, both cell lines saw a slight increase in the concentration of cytosolic TGF-β but it was not sufficient to influence the phenotype of the cells in either cell line. As shown by the flow cytometry results, the majority of the MCF7 cells remained epithelial as the confluence was reduced and the majority of SW480 cells occupying either the epithelial or the intermediate state.

When 3 ng/mL TGF-β was added to the cells, the SW480 cells had an increase in the cytosolic TGF-β concentration at the population level, which then increased further as confluence was reduced. With the reduction in confluence, at 3 ng/mL TGF-β, these cells saw a significant change in staining for epithelial and mesenchymal markers, which was accompanied by an increase in the number of cells that stained only for the vimentin marker but did not result in a significant change in the expression of E-cadherin and Slug when compared to the 100% confluent + 3 ng/mL TGF-β treatment group. If the expression of E-cadherin and Slug in the treatment groups are compared to the 100% confluent + 0 ng/mL TGF-β treatment group, 60% and 30% confluent + 3 ng/mL TGF-β treatment groups saw a significant drop in E-cadherin expression but an insignificant change in Slug expression. This suggests that, while the changes in confluence at this concentration of TGF-β are encouraging EMT, variability in the tissue cultures or even the fact that the cells must travel through an intermediate phase prior to becoming fully mesenchymal may be contributing to the lack of
increase in Slug expression in the population as a whole. In contrast to the SW480 cells, the MCF7 cells saw an initial increase in concentration of TGF-β at the population level but, as confluence of the cells was reduced at 3 ng/mL TGF-β, the concentration of cytosolic TGF-β in the population remained stagnant. As confluence was reduced at 3 ng/mL TGF-β, the expression of E-cadherin significantly decreased and the expression of Slug significantly increased. Despite these significant changes in E-cadherin and Slug mRNA expression, the overall population did not significantly change their staining patterns, with the majority of cells remaining in the epithelial state. The absence of significant change in both concentration of cytosolic TGF-β and staining for epithelial and mesenchymal markers shows that the addition of TGF-β and change in confluence was enough to begin to force a significant change in mRNA expression but which was not yet apparent in protein expression. This lack of change in the protein expression suggests that either the collection of data was possibly at a time point that could not capture the changes or that the changes in TGF-β and confluence were not enough to activate the intracellular pathway and induce EMT.

With the application of 9.33 ng/mL TGF-β, both cell lines saw an increase in cytosolic concentration of TGF-β as confluence was reduced. Accompanying this increase in cytosolic concentration of TGF-β, the overall population of both cell lines saw a significant change in staining for epithelial and mesenchymal markers as confluence was reduced from 100% to 30%. Additionally, when compared to the 100% confluent + 9.33 ng/mL treatment group, both cell lines saw a decrease in the expression of E-cadherin mRNA and an increase in expression of Slug mRNA as confluence was reduced at this concentration of exogenous TGF-β.
Although, note that the SW480 60% confluent + 9.33 ng/mL treatment group saw an increase in the expression of Slug mRNA when compared to the 100% confluent + 9.33 ng/mL treatment group, the change was not considered statistically significant, possibly due to variability in the tissue cultures and subsequent data collected. These changes with the reduction of confluence suggest that, at this concentration of exogenous TGF-β, reducing the confluence of the culture allows for more cells to undergo the transition from the epithelial to the mesenchymal state. Reduction in confluence meant creating more space within the culture for cells to move about.

For both cell lines, the reduction in confluence was not enough to encourage cells to progress towards adopting the mesenchymal phenotype and behavior. Even when compared to a sheet of epithelial cells at 100% confluence, cells at lower confluencies, as a whole, would not move towards a more mesenchymal phenotype for the population until enough TGF-β was applied. However, the threshold level of TGF-β required to force the cells to begin transforming into mesenchymal cells differed between the two cell lines, with the SW480 cells requiring a lower amount of exogenous TGF-β to result in a significant change in the cellular staining and phenotype.

Despite the increase in TGF-β in the overall population, for both cell lines, on average, there was less than one molecule of TGF-β per cell in all of the treatment groups, suggesting that the rise in concentration of TGF-β in the population was due to a small subset of cells in which the TGF-β pathway was activated. When confluence was held constant and the concentration of exogenous TGF-β was increased from 0 ng/mL to 9.33 ng/mL, the number of
neighbors that cells had at low confluencies were examined in an attempt to understand where this small subpopulation of transforming cells was originating. For the SW480 cells, the lack of dependence between TGF-β, the number of neighbors a cell had, and the staining for epithelial and mesenchymal markers can be attributed to the fact that all four levels of fluorescent staining were apparent at all different neighbor number levels and both concentrations of TGF-β. These cells often grew on top of each other in culture, even at the lowest confluence, prohibiting cells below from receiving as much TGF-β and pulling certain cells towards the epithelial steady state, even when TGF-β was applied. With this limited data, it appears that the SW480 cells do not support our initial hypothesis that cell-to-cell contact inhibits activation of the TGF-β pathway, but further testing would need to be carried out to understand this relationship further. For the MCF7 cells, even though the change in neighbor number, exogenous TGF-β concentration, and staining were not found to depend on each other, examining the staining of different levels of neighbor number did provide insight into where the small subset of transforming cells was originating from. At all three thresholds for measuring the number of neighbors of any given cell, the population that was most likely to contain non-E-cadherin only stained cells was those cells with the fewest neighbors, suggesting that the subpopulation that is undergoing EMT in the MCF7 cells are those that have 0-2 neighbors in culture. Unlike the SW480 cells, the observations in the MCF7 cells do support our initial hypothesis by suggesting that cells with a higher amount of cell-to-cell contact are being pulled towards the epithelial phenotype.
Taken collectively, the data in this work offers insight into the upregulation of EMT in two different cell lines. The MCF7 cell data suggests that, during low exposure to TGF-β, treatment should focus on cells at the edge of a tumor, as that is where non-epithelial cells are most likely to exist. By preventing these cells from transitioning, it could be possible to keep cells closer to the center of the tumor from transitioning due to their initial epithelial nature and their adhesion with surrounding cells. In contrast, to the MCF7 cells, the SW480 cells must travel through a transition phase where both epithelial and mesenchymal traits are expressed, before they become fully mesenchymal. Treatment focused on this in-between transitional state for the SW480 cells could be aimed at preventing cells from becoming fully mesenchymal and force them back towards the epithelial phenotype. The differences between the two cell lines studied in this work highlight that there is no universal process by which EMT is upregulated, nor is there a universal response to exogenous TGF-β or cell-to-cell contact and the need for further experimentation. Extension of this work to include higher concentrations of exogenous TGF-β or lower cell culture confluencies could help to show the progression of both cell lines as they respond to different environmental factors and ultimately highlight a clearer pathway to translate these findings to clinical applications.
CHAPTER 3: Modelling the Influence of Cellular Contact and TGF-β on the Epithelial Mesenchymal Transition in MCF7 Breast Carcinoma Cells

Background

Activation of the epithelial mesenchymal transition (EMT) allows epithelial cells to gain the invasive and migratory properties of mesenchymal cells [12, 66]. In carcinoma cells, EMT can occur due to exposure to extracellular signals and, upon activation, a cell can lose its adhesive property, a hallmark of an epithelial cell [9], and migrate away from the tumor [10]. Chapter 1 explored the hypothesis of a bistable switch underlying EMT in response to activation of the Wnt signaling pathway. As shown in Figure 3.1, this work examined changes in the protein levels of E-cadherin, β-catenin, and Slug as Wnt signaling was activated. In order to explore these dynamics and their relationship with the bistable switch underlying EMT, we presented a simple, three-equation model that generated similar conclusions as previous, more complicated models [22].

While the general modeling techniques from Chapter 1 can be applied for more detailed analysis of EMT, the models created using these techniques must be parametrized specifically to the tissue of origin. The work presented in Chapter 2 showed that EMT is not a universal process but, rather, cell type specific. In particular, Chapter 2 suggested the presence of a bistable switch in the breast carcinoma MCF7 cell line. The majority of MCF7 cells stained positively for either the E-cadherin protein or the vimentin protein, characteristic of the epithelial or the mesenchymal phenotype [67, 68], respectively, indicating that MCF7 cells are capable of occupying two distinct states. Even without the addition of an exogenous factor, some MCF7 cells are still capable of expressing vimentin and occupying the mesenchymal
phenotype. By using immunocytochemistry staining, it was found that cells most likely to express markers other than E-cadherin are those with few (0-2) neighbors in low confluence cultures and, with the addition of TGF-β to the cell cultures, the subpopulation of MCF7 cells expressing the mesenchymal marker grew. Together, these results suggest that EMT in MCF7 cells is potentially activated due to changes in cell-to-cell contact, as well as the addition of exogenous TGF-β.

**Figure 3.1:** Chapter 1 explored the dynamics between E-cadherin, β-catenin, and Slug. In a carcinoma cell, E-cadherin sequesters β-catenin at the membrane, giving the cell its characteristic adhesive quality. Free β-catenin in the cytosol is degraded. Upon activation of the Wnt pathway, the degradation complex is disabled and β-catenin is then free to accumulate, translocate to the nucleus, and upregulate Slug. Slug inhibits the production of E-cadherin, thus reducing the concentration of E-cadherin available to sequester β-catenin and thus completing the feedback loop [1, 2].
Thus, it is our hypothesis that the transition between the epithelial and the mesenchymal steady states in MCF7 cells is a bistable switch, a mechanism that allows the cell to maintain its invasive and migratory properties even after it has lost its extracellular signal. Using the general modeling techniques established in Chapter 1 and the cell line-specific data from Chapter 2, the work in this chapter seeks to understand the dynamics of MCF7 breast carcinoma cells as they integrate various extracellular cues, such as TGF-β signaling and cell-to-cell contact, during EMT activation.

**Methods**

In the presence of cell-to-cell contact, the E-cadherin in a carcinoma cell translocates to the membrane to form a complex with the members of the catenin family [19, 69]. It is this cadherin-catenin complex that gives the cell its characteristic adhesion [70, 71]. With the formation of this cadherin-catenin complex, β-catenin is sequestered at the membrane [72, 73], unable to translocate to the nucleus where it would upregulate transcription factors, such as Slug [1, 2]. While the model in Chapter 1 assumed that this adhesion complex was already present at the membrane, here we seek to understand the role of E-cadherin translocation to the membrane due to the presence of cell-to-cell contact. In order to model the recruitment of E-cadherin to the membrane of the cell due to the presence of neighboring cells, the term 

$$k_b \frac{(C)^{n_4}}{1+(C)^{n_4}}$$

is used in Equation (3.1) below, where $C$ is the number of cells in contact with the membrane of the cell in question. Rather than assume that the contact of two cells is an all-or-nothing occurrence, in this model, the possibility of “partial cellular neighbors” is considered, modeling the situation where a neighboring cell progressively “peels away”.
When the large latent complex (LLC) is released from the extracellular matrix (ECM) [74, 75], changes in the microenvironment pH, integrin interactions, or proteolysis [76] can result in the release of TGF-β from latency associated peptide (LAP) [77, 78]. The newly active TGF-β is now free to bind with the type II TGF-β receptor (TGFBRII) of the carcinoma cell. The TGF-β-TGFBRII complex will then bind with the type I TGF-β receptor (TGFBRI) [79], activating TGFBRI and allowing it to phosphorylate R-Smads. Once phosphorylated, the R-Smads bind with the co-Smad and translocate to the nucleus [80-82]. Inside the nucleus, the R-Smad-co-Smad complex accumulates and upregulates the expression of transcription factors, such as Slug [83-85]. When Slug binds to E-boxes in the E-cadherin gene promoter, it suppresses the transcription of E-cadherin [86, 87], thus reducing the amount of E-cadherin that can translocate to the cell membrane in the presence of neighboring cells. With the reduction in available E-cadherin, β-catenin would then be able to accumulate in the cytosol, translocate to the nucleus, and continue activating Slug, thus completing the feedback loop. This dynamic is highlighted in Figure 3.2A.

As in the Wnt pathway, within the TGF-β signaling pathway, there is a mutually-repressive feedback loop between membrane-bound E-cadherin and Slug. But, while the Wnt pathway directly targets the behavior of β-catenin for the upregulation of EMT, the TGF-β pathway relies on the Smad proteins to activate the Slug transcription factors, as represented by the term $\frac{k_2 \cdot \left( \frac{T_{IT}}{I_{CT}} \right)^{n_3}}{1 + \left( \frac{T_{IT}}{I_{CT}} \right)^{n_3}}$ in Equation (3.2) below. Therefore, for simplicity, β-catenin was not included here when modeling the dynamics between membrane-bound E-cadherin and Slug.
Figure 3.2: (A) Focuses on three key components to EMT (E-cadherin, β-catenin, and Slug) but now in response to TGF-β and cell-to-cell contact. In the presence of neighboring cells, E-cadherin in a carcinoma cell will exist at the membrane to form intercellular bonds. The cytoplasmic tail of E-cadherin is bound to β-catenin while, in the nucleus, Slug is kept at low levels. Once activated due to the presence of exogenous TGF-β, Slug will bind to the E-box element of the E-cadherin promoter, inhibiting E-cadherin production and resulting in a decrease in E-cadherin available to sequester β-catenin. β-catenin can now build up and translocate to the nucleus to activate Slug further, thus completing the feedback loop. (B) For modeling purposes, the feedback loop is simplified so that the contributions of β-catenin to the loop are included in the $k_1$ term.
Instead, as shown in Figure 3.2B, the suppression of Slug due to the presence of E-cadherin at the membrane was simplified and the suppression of Slug due to the sequestration of β-catenin to the membrane by E-cadherin is represented by the term \( \frac{k_1 \cdot \left( \frac{E}{IC_E} \right)^{n_2}}{1 + \left( \frac{E}{IC_E} \right)^{n_2}} \) in Equation (3.2).

Using these terms, as well as the term \( \frac{\alpha_4}{1 + \left( \frac{S}{IC_S} \right)^{n_2}} \) to indicate the suppression of E-cadherin production by Slug, we create the feedback loop between membrane-bound E-cadherin (E) and Slug (S) as they respond to changes in cellular neighbors (C) and the addition of exogenous TGF-β (T).

\[
\frac{dE}{dt} = \alpha_1 \frac{E}{IC_E} \frac{C}{IC_C}^{n_4} \left( \frac{C}{IC_C} \right)^{n_4} - \beta_1 \cdot E \quad (3.1)
\]

\[
\frac{dS}{dt} = \alpha_2 \left( \frac{E}{IC_E} \right)^{n_2} + \beta_2 \cdot S \quad (3.2)
\]

The parameters for Equations (3.1) and (3.2) are given in Table 3.1. If we define the variables (E, S, and t) and the input parameters (C and T) in terms of a constant with the same dimensions and a dimensionless variable, we have the following relationships:

\[
E = \varepsilon \cdot e \quad S = \sigma \cdot s \quad C = \zeta \cdot \mu \quad T = \gamma \cdot \theta \quad t = \varphi \cdot \tau
\]

Defining the constants in terms of the parameters as:

\[
\varepsilon = IC_E \quad \sigma = IC_S \quad \zeta = IC_C \quad \gamma = IC_T \quad \varphi = \frac{IC_S}{k_1}
\]

the system described in Equations (3.1) and (3.2) becomes the nondimensional system:
\[
\frac{de}{d\tau} = \frac{A_1}{1 + s^{n_1}} + F_2 \cdot \frac{\mu^{n_4}}{1 + \mu^{n_4}} - G_1 \cdot e 
\] (3.3)

\[
\frac{ds}{d\tau} = A_3 - \frac{e^{n_2}}{1 + e^{n_2}} + F_1 \cdot \frac{\theta^{n_3}}{1 + \theta^{n_3}} - G_3 \cdot s 
\] (3.4)

While a range of each dimensionless parameter in Equations (3.3) and (3.4) was explored (see Figures 3.4-3.5 and Figures 3.8-3.9), the values that were ultimately used in this model are defined in Table 3.2. Analysis was carried out using the MATLAB software and XPPAUT [32] with the initial conditions \( e = 7 \) and \( s = 0 \).

**Results and Discussion**

**Cell-to-Cell Contact**

Figure 3.3 shows the bifurcation diagrams of E-cadherin \( (e) \) and Slug \( (s) \), with respect to the number of cell neighbors \( (\mu) \). Using the relationship \( C = \zeta \cdot \mu, \mu = 0.46 \) represents 1 cellular neighbor in the nondimensional system. As shown in Figures 3.3A and 3.3B, in the absence of TGF-\( \beta \) \( (\theta = 0) \), cells with 5 cellular neighbors \( (\mu = 2.30) \) begin in the epithelial steady state. In this state, E-cadherin would exist at the membrane, bound to other cells, and Slug would exist at a low level in the nucleus. As the neighbor number is decreased, E-cadherin is endocytosed, freeing members of the catenin family capable of activating Slug in the nucleus. When enough E-cadherin has moved to the cytosol, the cell switches its phenotype from an epithelial cell to a mesenchymal cell. The switch-like behavior is marked in Figures 3.3A and 3.3B by the vertical blue dashed line \( L_M \) at \( \mu = 0.43 \). Once the cell has transitioned into the mesenchymal state due to the loss of neighbor contact it would be able to migrate away from its microenvironment. If the newly transformed mesenchymal cell were to regain its cell-to-cell contact, E-cadherin would move back to the membrane to bind with neighboring cells.
<table>
<thead>
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<th>Parameter</th>
<th>Definition</th>
<th>Assumed Value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
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<td>$\alpha_1$</td>
<td>Basal E-cadherin production</td>
<td>0.19</td>
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<td>$\alpha_2$</td>
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<td>Basal E-cadherin degradation</td>
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<td>$k_1$</td>
<td>Rate at which Slug is suppressed due to E-cadherin activation at the membrane</td>
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<td>$k_2$</td>
<td>Rate at which TGF-β upregulates Slug</td>
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<td>$\frac{ng}{mL \cdot hr}$</td>
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<td>$IC_S$</td>
<td>Half maximal concentration of Slug required to inhibit E-cadherin production</td>
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<td>$\frac{ng}{mL}$</td>
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<tr>
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<td>Half maximal concentration of E-cadherin required to inhibit Slug upregulation</td>
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<td>$IC_T$</td>
<td>Half maximal concentration of TGF-β required to activate Slug</td>
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<tr>
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<td>--</td>
<td>3</td>
<td>--</td>
</tr>
<tr>
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<td>--</td>
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<tr>
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<tr>
<td>$F_1$</td>
<td>$\frac{k_2}{k_1}$</td>
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<tr>
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<tr>
<td>$n_1$</td>
<td>--</td>
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<td></td>
</tr>
<tr>
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<td>--</td>
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</tr>
<tr>
<td>$n_3$</td>
<td>--</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>$n_4$</td>
<td>--</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>
The movement of E-cadherin to the membrane and the reassembly of the adhesion complex would allow E-cadherin to sequester members of the catenin family, again reducing their ability to activate Slug. Once the cell has gained sufficient cell-to-cell contact, it will undergo the transition to become an epithelial cell again. Note that the switch of the mesenchymal cell back to the epithelial cell is located at $L_E$ in Figure 3.3, where $\mu = 0.923$ in Figures 3.3A and 3.3B. $L_E \neq L_M$ indicates that there is a second and separate threshold of cell-to-cell contact that the cell must overcome to regain its epithelial properties.

In Figures 3.3C and 3.3D, the cell is exposed to a higher level of TGF-β, with $\theta = 0.135$. In the model, due to the increase in TGF-β, Slug is upregulated a small amount and binds to the E-boxes of the E-cadherin promoter, resulting in a decreased level of E-cadherin. As such, a cell with $\mu = 2.3$ has a lower steady state value of E-cadherin active at the membrane but, due to the cell-to-cell contact, is still able to maintain its epithelial phenotype. As the cellular neighbor number is reduced at this value of TGF-β, the cell must still overcome the $L_M$ threshold in order to transition into a mesenchymal cell. However, with the addition of this small amount of TGF-β, the cell now requires the loss of fewer neighbors to be able to transition into the mesenchymal steady state, with a threshold value of $L_M$ at $\mu = 0.45$. If the cell were to begin to regain neighbors, due to this increased value of TGF-β, the cell now requires higher cell-to-cell contact in order to regain its epithelial steady state ($L_E = 0.924$), indicating that the addition of exogenous TGF-β promotes the mesenchymal phenotype. When
Figure 3.3: (A&B) In the absence of TGF-β, cells with $\mu = 2.3$ begin in the epithelial steady state with E-cadherin active at the membrane and Slug kept at a low value. If $\mu$ is reduced, the membrane bound E-cadherin level is reduced, allowing Slug to accumulate. When the cell overcomes $L_M$, it will become a mesenchymal cell. If the cell were to regain enough cellular neighbors, E-cadherin would translocate to the membrane and it would switch back to the epithelial steady state at $L_E$. When TGF-β is added to the system ($\theta = 0.135$ (C&D) and $\theta = 0.42$ (E&F)), cells with $\mu = 2.3$ still begin in the epithelial steady state and are still capable of switching to the mesenchymal state as $\mu$ is reduced. However, due to the presence of TGF-β, it is now easier for the cell to transition. If the cell regains cell-to-cell contact, it would require more neighbors in order to force the epithelial phenotype. (G&H) With $\theta = 0.75$, the two separate states and the bistable switch have disappeared.
the value of TGF-β is increased further to $\theta = 0.42$ in Figures 3.3E and 3.3F, the cell again sees a reduced value of E-cadherin bound to the membrane but, in the presence of 5 neighbors ($\mu = 2.3$), it is still able to occupy the epithelial steady state. As neighbor number is reduced, the cell is more easily able to enter the mesenchymal state, as the threshold of cell-to-cell contact required to force the cell from the epithelial to the mesenchymal steady state is $L_M$ at $\mu = 0.76$, and the cell now requires a higher amount of cell-to-cell contact in order to regain its epithelial properties, with $L_E$ at $\mu = 0.97$. Finally, when enough TGF-β is added to the system, such as is the case in Figures 3.3G and 3.3H with $\theta = 0.75$, the model suggests that the cell will only have an E-cadherin value of $e = 3.32$ at the membrane. When the number of neighbors is reduced, while the cell will see a decrease in membrane bound E-cadherin and an increase in the activity of Slug, it cannot undergo the bistable switch. Instead, the cell will now occupy a stable state where it is able to experience a fluctuation in its steady state values of E-cadherin and Slug, but is unable to switch its behavior.

Figures 3.4 and 3.5 further explore how changes in the parameters influence the system dynamics as the number of neighbors is decreased. In both figures, the cell begins with 5 neighbors ($\mu = 2.3$) along the x-axis and thus exists in the epithelial steady state which, at this confluence, corresponds to the single stable state region marked as Region I in all figures. The value of the model parameter is marked with a black dashed line in each figure. When TGF-β = 0 (Figure 3.4), as the cell loses more neighbors, it crosses into Region II, in which there are two stable steady states that the cell can occupy, depending on whether it entered the region by crossing over L1 or L2. In this particular case, crossing L1 indicates that the cell has
maintained its epithelial phenotype in Region II. But, as the cell continues to lose neighbors, it crosses over L2 and reenters Region I, meaning that the cell has now entered the mesenchymal steady state. Parameter adjustments corresponding to the blue dashed line in Figures 3.4 indicate that the model now has a parameter value such that a cell beginning in Region I would remain in Region I and cannot undergo the bistable switch due to a loss of cell-to-cell contact. Note that the blue dashed line does not exist in Figures 3.4E and 3.4I. For both the $F_1$ parameter and the $n_3$ Hill coefficient, all values greater than or equal to zero will result in a bistable switch due to the fact that $\theta = 0$.

When the value of TGF-β added to the system increases to $\theta = 0.60$ in Figure 3.5, the region of bistability in the system with respect to cell-to-cell contact shrinks. The bistable region still exists for certain parameter values but, for the parameters in this model (corresponding to the black dashed line), the range of values of $\mu$ that allow the cell to exist in the bistable region gets smaller, meaning that the system behavior at the black dashed line begins to resemble the system behavior at the blue dashed line. Even with the loss of cell-to-cell contact, the cell is losing the ability to undergo a switch and is approaching the possibility of being stuck in the single steady state due to the increase in TGF-β.

**TGF-β Activation**

Figures 3.6A-F show how cells with varying cell-to-cell contact respond to the addition of exogenous TGF-β. In all figures, a cell without any neighbors ($\mu = 0$) is shown in red and begins in the mesenchymal steady state with a low level of membrane-bound E-cadherin ($e \approx 0.03$) and a high level of Slug ($s \approx 5$) at $\tau = 0$. However, as was shown in Figures 3.3A
Figure 3.4: At $\theta = 0$, cells with $\mu = 2.3$ begin in Region I in the epithelial steady state. As $\mu$ is reduced, the cell passes through Region II and, at L2, transitions to the mesenchymal steady state. If the cell regains neighbors and crosses L1 from Region II to Region I, the cell will reenter the mesenchymal steady state. The black dashed line indicates the parameter value for the model while the blue dashed line shows that, for a certain range of each parameter, it is possible for the cell to begin in Region I, and remain there. Note the absence of a blue dashed line in both (E) and (I), which correspond to parameters that are involved in the term surrounding the input of $\theta$. 
Figure 3.5: At $\theta = 0.60$, the bistable Region II shrinks. Cells with $\mu = 2.3$ begin in Region I in the epithelial steady state. The cell on the dashed black line can still undergo a bistable switch but the range of $\mu$ values that this can occur is much smaller. In all figures, the blue dashed line shows that, for a certain range of each parameter, it is possible for the cell to begin in Region I, and remain there. Note that, with the addition of TGF-β, the behavior of the switch at the black line is approaching the behavior of the switch at the blue line: the lack of a bistable switch.
and 3.3B, cells with $\mu \geq 0.43$ exist in the epithelial steady state from $\tau = [0,100)$ with a high level of membrane bound E-cadherin and a low level of Slug: a cell with one neighbor ($\mu = 0.46$) is shown in blue and has $e \approx 2.8$ and $s \approx 0.56$, a cell with two neighbors ($\mu = 0.92$) is shown in black and has $e \approx 3.99$ and $s \approx 0.30$, and a cell with five neighbors ($\mu = 2.3$) is shown in purple and has $e \approx 5.19$ and $s \approx 0.18$. When a small amount of TGF-\(\beta\) ($\theta = 0.10$) is added to the system at $\tau = 100$ in Figures 3.6A and 3.6B, the levels of E-cadherin ($e$) and Slug ($s$) barely change from their resting values, to which they will return if the microenvironment ceases producing TGF-\(\beta\) at $\tau = 300$. If the dose of TGF-\(\beta\) $\tau = 100$ is increased ($\theta = 0.40$), as in Figures 3.6C and 3.6D, all of the cells show a drop in the level of the membrane-bound E-cadherin and an increase in Slug levels, meaning that enough TGF-\(\beta\) has been added to activate Slug and suppress production of E-cadherin. While all cells see changes in their E-cadherin and Slug values, it is the cell with 1 neighbor ($\mu = 0.46$) that has the greatest change in its values, with $e \approx 0.11$ and $s \approx 5.09$. When the TGF-\(\beta\) is removed and $\theta = 0 \tau = 300$, indicating a cell has shifted away from the signal source or the microenvironment has stopped producing a signal, the only epithelial cell that is able to retain its newly mesenchymal state is the cell with 1 neighbor ($\mu = 0.46$). Finally, when TGF-\(\beta\) is increased again ($\theta = 0.70$), such as in Figures 3.6E and 3.6F, although cells with 1, 2, and 5 neighbors all begin as epithelial cells, it is now the cells with 1 and 2 neighbors that are able to transition to the mesenchymal phenotype and maintain it even after TGF-\(\beta\) is removed. These cells have activated enough Slug to suppress production of E-cadherin.
Figure 3.6: In the absence of TGF-β ($\theta=0$), cells without any neighbors ($\mu=0$) begin in the mesenchymal steady state, meaning that membrane-bound E-cadherin is low and Slug is high, while cells with at least 1 neighbors ($\mu \geq 0.46$) begin in the epithelial steady state, indicated by the high value of membrane-bound E-cadherin and the low value of Slug. (A&B) If a small amount of TGF-β ($\theta=0.10$) is added $\tau = 100$, the values of E-cadherin and the Slug family do not significantly deviate from their values without TGF-β. With $\theta=0.40$ (C&D) and $\theta=0.70$ (E&F), more TGF-β is required for cells with a higher number of neighbors to be able to both transition to the mesenchymal steady state and maintain the mesenchymal steady state, even after the exogenous signal is removed.
Figures 3.7A-3.7H show the bifurcation diagrams of E-cadherin ($e$) and Slug ($s$) with respect to changes in TGF-β ($\theta$). As shown in Figures 3.7A and 3.7B, in the absence of TGF-β, cells that have 0 neighbors ($\mu = 0$) begin in the mesenchymal steady state, indicating that the membrane-bound E-cadherin is low and Slug remains at a higher value. With the addition of TGF-β, the cell maintains its mesenchymal phenotype. However, as shown in Figures 3.6C and 3.7D, once a cell acquires one full neighbor ($\mu = 0.46$), in the absence of TGF-β ($\theta = 0$), E-cadherin moves to the membrane to form intercellular bonds and Slug is now kept at a low level. When $\theta = 0.135$, as indicated by the red vertical dashed line, $\theta_1$, cells with 1 full neighbor have failed to undergo EMT. It is not until $\theta = 0.147$ that these cells overcome a threshold concentration of Slug necessary to inhibit E-cadherin production significantly so that the cell can transition into a mesenchymal cell and maintain the phenotype even after it loses its exogenous TGF-β signaling. This threshold value is indicated by the vertical blue dashed line in Figures 3.6C and 3.6D.

If the neighbor number of the cell is then increased to two full neighbors ($\mu = 0.92$), as is the case in Figures 3.7E and 3.7F, the cell still begins as an epithelial cell without the addition of TGF-β. When TGF-β is now added to the system, due to the additional cell-to-cell contact recruiting E-cadherin to the membrane, the cell continues to exist as an epithelial cell at both $\theta = 0.135$ ($\theta_1$) and $\theta = 0.42$ ($\theta_2$). It is not until $\theta = 0.517$ that the cell is able to overcome its threshold value of Slug necessary to transition to the mesenchymal phenotype (marked, again, by the vertical blue dashed line). At $\theta = 0.517$, once it does transition into a mesenchymal cell, the cell is able to maintain this phenotype and behavior once its source of TGF-β is removed.
Figure 3.7: (A&B) In the absence of TGF-β, cells with $\mu = 0$ begin as mesenchymal cells and, as TGF-β is added, the cells maintain their mesenchymal phenotype. Note the different y-axis scale on (A). Cells with 1+ neighbors begin as epithelial cells in the absence of TGF-β (C-H). (C and D) As TGF-β is added to cells with 1 neighbor, the cell must reach a value of $\theta = 0.147$ (blue vertical dashed line) before it can transition to the mesenchymal steady state. (E&F) For cells with 2 neighbors, as TGF-β is added, the cell reach a value of $\theta = 0.517$ (blue vertical dashed line) before it can transition to the mesenchymal state. (G&H) For cells with 3 neighbors, no matter how much TGF-β is added to the system, the cell cannot undergo the bistable switch and transition to the mesenchymal steady state.
Also, note that, similar to the bifurcation diagrams with respect to cell-to-cell contact and their relationship with increased TGF-β, the change in the protein levels due to the addition of TGF-β is less dramatic as cell-to-cell contact is increased. Although the cell is able to undergo the bistable switch with two full neighbors, the mesenchymal level of E-cadherin is higher than it was for transitioned cells with 1 neighbor. The existence of the epithelial steady state without the addition of TGF-β is still present as the number of neighbors is increased further, as is the case in Figures 3.7G and 3.7H where the cell has 3 full neighbors (μ = 1.38). However, due to the cell-to-cell contact, even if the value of TGF-β is increased to θ = 0.75 (θ₂), the cell is unable to switch to the mesenchymal state: the influence of the cell-to-cell contact is so strong that, even though the values of E-cadherin and Slug fluctuate, the cell is stuck in that stable state.

Figures 3.8 and 3.9 examine how changes in the parameters influence the bistability of the system due to changes in exogenous TGF-β. As indicated by the black dashed line, cells with the parameters given in this model begin in Region II, the bistable region where the state of the cell depends on its initial conditions. With μ = 0.43 (Figure 3.8), the cell has almost one full neighbor attached to it and thus begins as an epithelial cell. As TGF-β is applied, the cell crosses over L2 and undergoes a switch as it moves into Region I, thus becoming a mesenchymal cell. If the parameters are adjusted at (θ, μ) = (0, 0.43), the cell moves out of the bistable region and remains in Region I as TGF-β is added to the system, as shown by the blue dashed line. Note that the cell at the black dashed line, indicating the model parameter, begins at the base of Region II. In a cell with few neighbors (μ = 0.43), Region II is quite large,
Figure 3.8: The black dashed line indicates the parameter value for the model. At $\mu = 0.43$ and $\theta = 0$, cells begin in Region II in the epithelial state. As TGF-β is increased, the cells pass through Region II, cross L2, and transition to the mesenchymal steady state. If the value of TGF-β in the system is reduced and a cell crosses L2 back into Region II, the cell would maintain its mesenchymal state. In all figures, the blue dashed line shows that, for a certain range of each parameter, it is possible for the cell to begin in Region I and remain there.
Figure 3.9: When $\mu = 1.0$ (G-L), the bistable Region II shrinks. The cell on the black line can still undergo a switch but the range of $\theta$ values that it can occur at is much smaller. Also, with this increase in $\mu$, the switch has now changed from an irreversible to a reversible switch: if TGF-\(\beta\) in the system was reduced and the cell reentered Region II and crossed L1 into Region I, it would be capable of reentering the epithelial steady state. Note that, with the addition of TGF-\(\beta\), the behavior of the switch at the black line is approaching the behavior of the switch at the blue line: the lack of a bistable switch. Additionally, with $\theta > 0$, there is not a parameter value for $n_4$ where the (newly) reversible switch does not exist.
indicating that there is a robust parameter range in which the system can maintain its bistable behavior under these cellular conditions.

As more neighbors are added to the cell and $\mu$ is increased to $\mu = 1$ (Figure 3.9), the bistable Region II moves and changes shape so that there is a smaller range of TGF-β for the bistable switch to become activated. Additionally, as indicated by the black line, the behavior of the cell in the model is now a reversible bistable switch with respect to TGF-β due to the presence of the threshold, L1. The cell at the dashed black line must still cross L2 in order to change from epithelial to mesenchymal with the addition of TGF-β, but now, if the concentration of TGF-β was reduced far enough, the cell would be able to revert back to its epithelial state once it crossed L1. If the cell-to-cell contact is increased further, the cell would lose its ability to undergo the bistable switch with respect to TGF-β and begin to resemble the behavior at the blue dashed line, which indicates parameter values that produce a single steady state system with respect to TGF-β. Note the absence of a blue dashed line for the Hill coefficient $n_4$, indicating that the reversible switch will always exist at $\mu = 1$, despite any changes to this parameter.

**Cell-to-Cell Contact & TGF-β Activation Together**

In order to understand the sensitivity of the steady state behavior of E-cadherin and Slug to changes in the 6 nondimensional parameter values, sensitivity analysis via Latin Hypercube [33, 35, 88] construction and Pearson’s Ranked Correlation Coefficient (PRCC) [34, 35, 89] was carried out for various levels of added TGF-β and cell-to-cell contact. Note, as the Hill coefficients are all greater than 1, they are considered ultrasensitive terms in the
model. Parameter ranges were established based on the two parameter bifurcation diagrams in Figures 3.4-3.5 and Figures 3.7-3.8 and are listed in Table 3.3. Using a Uniform distribution, each parameter range was divided into N = 1000 intervals. Examining the partial correlation coefficients between each of the individual ranked parameter values at different values of cell-to-cell contact and TGF-β, the parameters were found to be uncorrelated with each other. Additionally, scatterplots of the steady state values for e and s at different levels of cell-to-cell contact and TGF-β are given in Appendix D. These plots show the monotonic behavior of the variable steady states in response to changes in the different parameters. Note that the range of $A_3 = [0.81, 5]$ in both Table 3.3 and Appendix D. This truncated parameter range is due to the lack of monotonic behavior from $A_3 = [0, 0.81)$.

<table>
<thead>
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<th>Parameter</th>
<th>Minimum Value</th>
<th>Maximum Value</th>
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<tr>
<td>$A_3$</td>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</table>

As shown in Figure 3.10A, for a cell in the epithelial steady state at $\mu = 1.38$ and $\theta=0$, the steady state behavior of Slug is sensitive to changes in its own production ($A_3$) and
degradation ($G_3$) rates. As TGF-β is added, for a small amount of TGF-β ($\theta = 0.42$), as shown in Figure 3.10B, the sensitivity of Slug to both parameters remains. However, after enough TGF-β is added, Slug will lose sensitivity to changes in $A_3$. This loss in sensitivity with TGF-β indicates that once enough TGF-β has been added to the system, the steady state concentration of Slug is no longer dependent on only its basal production rate. As cell-to-cell contact is reduced, the steady state value of Slug remains sensitive to changes in both $A_3$ and $G_3$ for both $\theta = 0$ and $\theta = 0.42$. If TGF-β is increased further, Slug still loses its sensitivity to $A_3$ but, with the lack of cell-to-cell contact, it takes less TGF-β for this to happen.

Although the steady state value of Slug is sensitive to its own degradation rate ($G_3$) in the presence of cell-to-cell contact and TGF-β, the steady state value of E-cadherin gains sensitivity to $G_3$ as it loses neighbors and, simultaneously, loses sensitivity to its own degradation rate ($G_1$). For both $\theta = 0$ and $\theta = 0.42$ in Figure 3.10, as the cell loses neighbors, the steady state value of membrane-bound E-cadherin becomes less sensitive to its own degradation rate. With a higher number of neighbors, as is the case in Figures 3.10A and 3.10B, it is the presence of E-cadherin at the membrane that gives the cell its epithelial phenotype in response to cell-to-cell contact. If the degradation rate of membrane-bound E-cadherin was increased, the cell would lose its cell-to-cell contact and, subsequently, its epithelial phenotype. At a lower number of neighbors, such as $\mu = 0$ in Figures 3.10G and 3.10H, the cell is already in the mesenchymal steady state so an increase in the degradation of membrane-bound E-cadherin would not change the cellular phenotype. Additionally, as $\mu$ is reduced and the cell approaches the mesenchymal steady state, an increase in the degradation rate of Slug ($G_3$)
Figure 3.10: Sensitivity analysis was carried out to understand the relationship between steady state behavior of E-cadherin (e) and Slug (s) and the 6 nondimensional parameters. (A) \((\theta, \mu) = (0, 1.38)\) (B) \((\theta, \mu) = (0.42, 1.38)\) (C) \((\theta, \mu) = (0, 0.92)\) (D) \((\theta, \mu) = (0.42, 0.92)\) (E) \((\theta, \mu) = (0, 0.46)\) (F) \((\theta, \mu) = (0.42, 0.46)\) (G) \((\theta, \mu) = (0, 0)\) (H) \((\theta, \mu) = (0.42, 0)\). For both levels of \(\theta\), the steady state behaviors of E-cadherin and Slug were sensitive to changes in their own degradation rates, \(G_1\) and \(G_3\), respectively, as \(\mu\) was decreased, the steady state behavior of E-cadherin gained sensitivity to changes in \(G_3\) and lost sensitivity to \(G_1\). Additionally, with an increase in \(\theta\), at all levels of cell-to-cell contact, the sensitivity of Slug to changes in \(A_3\) decreases while, for cells with at least 3 neighbors, the sensitivity of E-cadherin to changes in \(F_2\) increases.
could result in an increase in the amount of E-cadherin available for the cell to use in intracellular bonds and force the epithelial phenotype.

Finally, while the steady state concentration of membrane-bound E-cadherin is not sensitive to changes in $F_2$ for either $\theta = 0$ and $\theta = 0.42$, if the amount of TGF-β in the system increased, the sensitivity of E-cadherin to changes in $F_2$ would grow. At high levels of $\theta$, TGF-β is working to encourage the mesenchymal phenotype and, therefore, the steady state value of E-cadherin and, subsequently, the epithelial phenotype, depends on how quickly E-cadherin can move to the membrane to secure adhesion with neighboring cells. Note, the changes in sensitivity of the steady state values of E-cadherin and Slug to the parameters in the model is not retained if the extracellular signals ($\mu$ and $\theta$) are returned to their initial values: the changes in sensitivity to the model parameters only exist if the system is changing due to alterations in its microenvironment.

The change in sensitivity of the steady state behavior of E-cadherin and Slug in response to cell-to-cell contact and TGF-β raises the point that the behavior of the system can change when both external factors work together. In Figures 3.3-3.9, the loss of bistability and the emergence of a single stable state system relies on the assumption that cells in the tumor are immobile: a cell with 3 neighbors will always have 3 neighbors. However, because EMT encourages cellular mobility, these cells can migrate away from their neighbors and their extracellular TGF-β signal. With the understanding that this is a dynamic system, the question then becomes, are there circumstances under which a cell that begins with a higher number of neighbors could ultimately undergo the bistable switch and transition into the mesenchymal steady state? Figure 3.11 shows how a cell in a small carcinoma with three direct neighbors
would respond to the addition of exogenous TGF-β. For the carcinoma cell indicated in Figure 3.11A, Figures 3.11B and 3.11C show the bifurcation diagrams of E-cadherin and Slug with respect to TGF-β, respectively. If the cell begins with 3 neighbors (μ = 1.38) in the absence of TGF-β at point 1 and then a uniform field of TGF-β (θ = 0.70) is added to the system (point 2), the exogenous TGF-β will increase the steady state level of Slug in the cell from $s \approx 0.2$ and $s \approx 1.2$. This increased Slug will then inhibit E-cadherin production, reducing the steady state value of E-cadherin from $e \approx 4.8$ to $e \approx 2.8$. However, despite the changes caused by TGF-β, the cell still has too many neighbors to relinquish its epithelial phenotype. At point 2, while the cell in question has not undergone the bistable switch, cells with fewer neighbors can switch, allowing them to gain the migratory properties associated with mesenchymal cells. As shown in Figure 3.11A, if these newly transformed cells are the neighbors of the cell indicated by the arrow, their transition into the mesenchymal steady state will allow them to migrate away and thus reduce the number of neighbors surrounding the cell in question. Point 3 in Figure 3.11 signifies the loss of 2 neighboring cells as a result of the activation of EMT in their systems and a reduction in $\mu$ from $\mu = 1.38$ to $\mu = 0.46$ for the cell indicated, meaning that the cell now has 1 neighbor and would be able to switch to the mesenchymal state. Once the cell has switched, it acquires the migratory properties associated with mesenchymal cells and, at point 4, is capable of detaching from its remaining neighbor. Following the detachment from its neighbor, the cell the migrates away from the source of exogenous TGF-β. With this movement, the cell maintains its phenotype, even upon reaching point 5 at $\theta = 0$ in Figure 3.11, thus signifying that the cell has finally overcome its single state behavior and undergone the
**Figure 3.11**: The cell indicated in (A) begins at point 1 with 3 neighbors ($\mu = 1.38$). When a uniform field of TGF-β is added ($\theta = 0.70$) at point 2, two of its neighbors are capable of transitioning to the mesenchymal steady state. Once these cells migrate away from the cell, the cell will be left with 1 neighbor ($\mu = 0.46$) at point 3, allowing it to transition to the mesenchymal state. The cell can detach from its final neighbor at point 4 and then migrate away from the TGF-β field. Even after the cell has left the field of TGF-β, it maintains its mesenchymal phenotype, thus indicating that it has undergone the bistable switch. The bifurcation diagrams for the cell indicated in (A) during this process for E-cadherin and Slug are in (B) and (C) respectively.
bistable switch.

**MCF7 Experiments**

The model presented in this work is to be used to specifically describe EMT as it pertains to breast carcinoma. The work in Chapter 2 explored the dynamics of the MCF7 breast carcinoma cell line at three different confluencies (30%, 60%, and 100%) and showed that, within the low confluence treatment group, the majority of cells had 0-2 neighbors. With this skewed neighbor number distribution, the MCF7 cells at 30% confluence had room on the limited sized plate to move once they transitioned into mesenchymal cells, making the low confluence cells most indicative of an environment that would allow for EMT and cell motility.

The flow cytometry data from Chapter 2 shows that, at 30% confluence, the MCF7 cells are comprised of two distinct subpopulations: cells that stain for E-cadherin only, the epithelial marker, and cells that stain for vimentin only, the mesenchymal marker. In each of the 30% confluent MCF7 populations, less than 10% of each of the cell populations did not stain for either marker and less than 2% stained for both. Thus, for simplicity, these small fractions were not included and, instead, percentages of the population staining for E-cadherin only and vimentin only were adjusted accordingly and are shown in Table 3.4. The staining for these two markers indicates the presence of two different steady states existing within the population for all levels of TGF-β added to the system. If the adjusted staining percentages in Table 4 are taken to be weights of the contribution of epithelial cells and mesenchymal cells to expression of our proteins of interest, E-cadherin ($e$) and Slug ($s$), it is possible to estimate the relative expression of each protein after TGF-β is added to the system by using the relationship in Equation (3.5) below for each protein:
where \( x_0 \) and \( y_0 \) are the average values of the protein contributed by the epithelial cells and the mesenchymal cells, respectively, in the 30% confluent + 0 ng/mL TGF-\( \beta \) system and \( x_F \) and \( y_F \) are the average values of the protein contributed by the epithelial cells and the mesenchymal cells, respectively, in the 30% confluent treatment group with TGF-\( \beta \) (\( \theta \) > 0).

In Chapter 2, it is reported that the area of a tissue culture plate is 143 cm\(^2\) or 1.43x10\(^{10}\) \( \mu \)m\(^2\). At 30% confluent, the area of the plate that is covered by cells is reduced to 4.29x10\(^9\) \( \mu \)m\(^2\). Additionally, the neighbor number work carried out in Chapter 2 reported that the average distance between centers of mass of MCF7 nuclei was 29.9 \( \mu \)m, meaning that the average radius of the MCF7 cell was 14.95 \( \mu \)m and the average area covered by an individual cell on the plate was 702.15 \( \mu \)m\(^2\). Using these values and assuming that all cells on a plate took up an equal amount of TGF-\( \beta \), we find that for the 3 ng/mL exogenous TGF-\( \beta \) per plate, each cell used 4.9x10\(^{-7}\) ng/mL TGF-\( \beta \). Additionally, for the 9.33 ng/mL exogenous TGF-\( \beta \) per plate, each cell used 1.5x10\(^{-6}\) ng/mL TGF-\( \beta \). Using the relationship \( T = \gamma \cdot \theta \) relationship with \( \gamma = 3.64x10^{-6} \), these values equal \( \theta = 0.135 \) and \( \theta = 0.42 \) in the nondimensional model, respectively.

As reported in Table 3.4, at 0 ng/mL TGF-\( \beta \), or \( \theta = 0 \), 16.70% of the population stained for vimentin-only, indicating that \( M_0 = 0.1670 \), with the remaining population staining positively for E-cadherin (\( E_0 = 0.8330 \)). When the concentration of exogenous TGF-\( \beta \) was increased to 3 ng/mL, or \( \theta = 0.135 \), the epithelial subpopulation decreased to comprise 73.67% of the overall population (\( E_F = 0.7367 \)) while the mesenchymal cells rose to 26.33% of the
population \( (M_F = 0.2633) \). Using these weights on the average protein values from the model for cells in the epithelial and mesenchymal steady states \( (x\text{ and } y\text{, respectively}) \) at these values of \( \theta \), Equation (3.5) suggests that the overall cell population would have a relative E-cadherin expression of \( R = 0.885 \) and a relative Slug expression of \( R = 1.46 \), when compared to their values at \( \theta = 0 \). Increasing the concentration of exogenous TGF-\( \beta \) to \( 9.33 \text{ ng/mL} \) or \( \theta = 0.42 \) causes 30.48\% of the population to exist in the mesenchymal steady state \( (M_F = 0.3048) \) and 69.52\% of the population to exist in the epithelial steady state \( (E_F = 0.6952) \). Using these weights on the average protein values from the model for cells in the epithelial and mesenchymal steady states at this new value of \( \theta \), our model finds that at \( \theta = 0.42 \), the relative expression of E-cadherin is \( R = 0.84 \) and the relative expression of Slug is \( R = 1.78 \) when compared to the values at \( \theta = 0 \).

<table>
<thead>
<tr>
<th>TGF-( \beta ) Concentration (ng/mL)</th>
<th>( \theta )</th>
<th>Percent Population E-cadherin Stained (Avg ± 1 Std Deviation)</th>
<th>Percent Population Vimentin Stained (Avg ± 1 Std Deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>83.30% ± 11.87%</td>
<td>16.70% ± 11.87%</td>
</tr>
<tr>
<td>3</td>
<td>0.135</td>
<td>73.67% ± 4.6%</td>
<td>26.33% ± 4.6%</td>
</tr>
<tr>
<td>9.33</td>
<td>0.42</td>
<td>69.52% ± 17.15%</td>
<td>30.48% ± 17.15%</td>
</tr>
</tbody>
</table>

The model set forth in Equations (3.3) and (3.4) examines the nondimensional change in E-cadherin and Slug protein expression with nondimensional time. If it is assumed that there is a direct correlation between gene expression and protein expression, it is possible to compare the relative changes in protein values found using this model to the relative changes in mRNA expression found in MCF7 cells in Chapter 2. The relative changes in mRNA expression for MCF7 breast carcinoma cells are reported in Table 3.5. The model presented here, at \( \theta = 0.135 \),
has a 20.20% error from the actual experimental value of E-cadherin mRNA at 3 ng/mL of TGF-β and a 22.03% error from the experimental value of Slug reported. As $\theta = 0.42$, the predicted value of E-cadherin has a 5.39% error from the experimental value of E-cadherin mRNA and that Slug has a 6.14% error from the experimental value of Slug mRNA reported in Chapter 2 for 9.33 ng/mL of TGF-β. Figure 3.12A shows the relative expression at both levels of TGF-β and the standard error for both E-cadherin and Slug from Chapter 2, as well as the model-predicted relative expression of E-cadherin and Slug at 30% confluent. The predicted values at both levels of $\theta$ are still within the standard error of the data reported in Chapter 2, but the sources of these errors must be explored in order to understand where the model may fall short in predicting future experiments.

<table>
<thead>
<tr>
<th>Control</th>
<th>Treatment Group</th>
<th>Gene</th>
<th>Relative Expression</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% CF 0 ng/mL TGF-β ($\theta=0$)</td>
<td>30% CF 3 ng/mL TGF-β ($\theta=0.135$)</td>
<td>E-cadherin</td>
<td>0.736</td>
<td>(0.465-1.143)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Slug</td>
<td>1.874</td>
<td>(1.188-2.865)</td>
</tr>
<tr>
<td>30% CF 0 ng/mL TGF-β ($\theta=0$)</td>
<td>30% CF 9.33 ng/mL TGF-β ($\theta=0.42$)</td>
<td>E-cadherin</td>
<td>0.797</td>
<td>(0.503-1.225)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Slug</td>
<td>1.899</td>
<td>(0.638-4.746)</td>
</tr>
</tbody>
</table>

The assumptions made with this model could be the reason for the error found between the predictions of relative expression levels and the actual MCF7 data in Chapter 2. One assumption that was made with this model was that, under the right conditions, all cells would undergo EMT and that, with regards to the data, the cells expressing the mesenchymal marker thus are those occupying the low neighbor number subpopulation. Instead, the possibility that
not all cells are equally capable of undergoing EMT, as well as the possibility that cells with a higher number of neighbors could be transitioning and contributing to changes in the culture, are two possible contributions to the error of the model. Another possible source of error between the model and the data could be due to the assumption that there is a one-to-one correlation between mRNA expression and protein expression. The model presented here is examining changes in protein expression, whereas relative expression in Chapter 2 relies on changes in mRNA expression. Thus, the error between the model and the data could be due to a rate-limiting step between changes in mRNA expression and changes in protein expression that the model does not account for.

A final assumption that could be causing error between the model and the 30% confluent data is the basic assumption that EMT in breast carcinoma is a bistable switch. The data from Chapter 2 suggests that, for colon carcinoma cells (SW480), the transition from epithelial to mesenchymal is actually a three state system, with some cells staining clearly for E-cadherin only, some for vimentin only, and some cells that stained for both markers simultaneously. The dually stained cells may constitute an intermediate transitional state from epithelial to mesenchymal. The induction of a three state system has also been suggested by the literature [90]. Tian et al. [91] modeled the activation of EMT via the TGF-β pathway in MDCK and MCF10A cells and suggested the possibility that there is an intermediary, pEMT state that the cells must pass through before they can undergo the irreversible switch and transform into mesenchymal cells. In contrast to these results, the MCF7 breast carcinoma cells at 30% confluence in Chapter 2 stained very clearly for either the epithelial marker or the mesenchymal marker, with less than 10% of the cells lacking stain and less than 2% of the
cells staining for both. These low percentages are what justified the bistability assumption. However, it is possible that the cells that did not stain for either marker are representative of the intermediate transitional state between the epithelial and the mesenchymal phenotypes. Note that, while the percentage of cells that did not stain stays low, it does grow as more TGF-β is added to the system. The data from Chapter 2 did suggest that there was not a significant change in marker expression in the flow data at 30% confluence when TGF-β was added to the cells. Perhaps with the addition of more TGF-β to future experiments, this subpopulation of unstained cells would grow as more cells transition out of the epithelial steady state.

When confluence of the culture is increased to 60%, the area of the tissue culture plate that is occupied by cells is increased to $8.58 \times 10^9 \text{ µm}^2$, meaning that there are now $1.22 \times 10^7$ cells per plate that must share the exogenous TGF-β equally. With this increased cell number, 3 ng/mL TGF-β and 9.33 ng/mL TGF-β are represented by $\theta = 0.07$ and $\theta = 0.21$ in the nondimensional model, respectively.

At a confluence of 60%, the flow cytometry data from Chapter 2 was again adjusted to exclude the cells that did not stain for either marker, as well as the small percentage of cells that stained for both markers and was reported in Table 3.6. Using this data in Equation (3.5), the model predicts that at 60% confluence and $\theta = 0.07$, the relative expression of E-cadherin is 1.04 when compared to the expression at $\theta = 0$, which has an error of 53.48% from the relative expression of E-cadherin mRNA reported in Table 3.7. Similarly, at $\theta = 0.07$, the
Figure 3.12: (A) Relative expression when cells are 30% confluent (A), 60% confluent (B), and 100% confluent (C). From Chapter 2, the relative expression of E-cadherin mRNA to $\theta = 0$ and its standard error is shown in blue and the model-predicted relative expression of E-cadherin protein to $\theta = 0$ is shown in yellow. The relative expression of Slug mRNA to $\theta = 0$ and the standard error is shown in red while the model-predicted relative expression of Slug to $\theta = 0$ is shown in green.
model suggests that the relative expression of Slug, when compared to their value at $\theta = 0$ will be 0.90, a 55.38% error from the relative expression of Slug mRNA reported in Table 3.7. When $\theta$ is increased so that $\theta = 0.21$, using the data from Table 3.6 and Equation (3.5), the model predicts that E-cadherin will have a relative expression of 1.15 and that the Slug family will have a relative expression of 0.69 when compared to $\theta = 0$. When compared to the relative expression of E-cadherin mRNA and Slug mRNA in Table 3.7, the model-predicted E-cadherin expression has a 40.32% error, whereas the model prediction of Slug transcription factor expression has a 53.22% error from the MCF7 data. Figure 3.12B shows the relative expression at both levels of TGF-β and the standard error for both E-cadherin and Slug from Chapter 2, as well as the model-predicted relative expression of E-cadherin and Slug at 60% confluent. Note that for both values of $\theta$, with the increase in the error between the model and the data, the predicted relative expression of E-cadherin is now at the lower limit to the standard error of the data, while the predicted relative expression of Slug is now outside the standard error for the relative expression of the Slug data.

<table>
<thead>
<tr>
<th>TGF-β Concentration (ng/mL)</th>
<th>$\theta$</th>
<th>Percent Population E-cadherin Stained (Avg ± 1 Std Deviation)</th>
<th>Percent Population Vimentin Stained (Avg ± 1 Std Deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>72.53% ± 10.86%</td>
<td>27.47% ± 10.86%</td>
</tr>
<tr>
<td>3</td>
<td>0.07</td>
<td>75.63% ± 1.4%</td>
<td>24.37% ± 1.4%</td>
</tr>
<tr>
<td>9.33</td>
<td>0.21</td>
<td>82.85% ± 4.01%</td>
<td>17.15% ± 4.01%</td>
</tr>
</tbody>
</table>
If confluence of the culture is increased to 100%, the entire tissue culture plate is now occupied by cells, meaning that there are now $2.04 \times 10^7$ cells per plate that must share the exogenous TGF-β equally. With this increased cell number, 3 ng/mL TGF-β and 9.33 ng/mL TGF-β are represented by $\theta = 0.04$ and $\theta = 0.13$ in the nondimensional model, respectively.

At 100% confluence, the flow cytometry data from Chapter 2 can again be used in conjunction with the model to predict the change in expression of E-cadherin and Slug upon the addition of exogenous TGF-β. Just as was done with the lower confluent treatment groups, the flow cytometry data from Chapter 2 was adjusted to exclude the cells that did not stain for either marker, as well as the small percentage of cells that stained for both, and was reported in Table 3.8. However, with this increase in confluence, the errors between the model predictions and the MCF7 data reported in Table 3.9 are exacerbated even further. With $\theta = 0.04$, using Equation (3.5), the model predicts that the relative expression of E-cadherin should be 1.2 and the relative expression of the Slug family of transcription factors should be 0.48 when compared to their expression at $\theta = 0$, meaning that there is a 123.36% error between the model predictions and the MCF7 data reported in Table 3.9 for E-cadherin and a 65.38% error
between the model-suggested expression of Slug and the actual Slug mRNA data in Table 3.9. This behavior is repeated when $\theta = 0.13$ and, when we use the flow cytometry data in Equation (3.5), our model predicts that the relative expression of E-cadherin will be 1.45 and the relative expression of the Slug transcription factors will be 0.62 when compared to their expression at $\theta = 0$. These predicted relative expressions mean that there is a 276.32% error between the model and the reported data for E-cadherin expression and a 28.36% error for Slug in the model and the reported expression of Slug in MCF7 cells. Figure 3.12C shows the relative expression at both levels of TGF-β and the standard error for both E-cadherin and Slug from Chapter 2, as well as the model-predicted relative expression of E-cadherin and Slug at 100% confluent. Note that for both values of $\theta$, with the increase in the error between the model and the data, the predicted relative expression of E-cadherin is now outside the standard error of the data.

<table>
<thead>
<tr>
<th>TGF-β Concentration (ng/mL)</th>
<th>$\theta$</th>
<th>Percent Population E-cadherin Stained (Avg ± 1 Std Deviation)</th>
<th>Percent Population Vimentin Stained (Avg ± 1 Std Deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>75.73% ± 4.90%</td>
<td>24.27% ± 4.90%</td>
</tr>
<tr>
<td>3</td>
<td>0.04</td>
<td>90.83% ± 5.71%</td>
<td>9.17% ± 5.71%</td>
</tr>
<tr>
<td>9.33</td>
<td>0.13</td>
<td>86.83% ± 2.54%</td>
<td>13.17% ± 2.54%</td>
</tr>
</tbody>
</table>

Thus, what started as small errors within the range of the standard error in the data at the 30% confluent level, increase as the model is used to predict the relative expression of E-cadherin and Slug at higher confluencies. In conjunction with the errors associated with the assumptions made in the model, the increase in errors between the model and the data could be due to the error associated with the MCF7 data at the 60% and 100% confluencies. One such error is the lack of space: the data comes from cells grown in a dish in vitro, not in vivo.
This lack of space could mean that when the cells at 60% confluence move away from one cluster of neighbors, they could actually move into contact with another cluster. Additionally, at 100% confluence, the MCF7 cells grew in sheets, as well as on top of each other. The formation of cellular sheets in tissue culture could prevent cells from migrating away from their neighbors, and thus prevent the transition to the mesenchymal state.

<table>
<thead>
<tr>
<th>Control</th>
<th>Treatment Group</th>
<th>Gene</th>
<th>Relative Expression</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% CF 0 ng/mL TGF-β (θ=0)</td>
<td>100% CF 3 ng/mL TGF-β (θ=0.04)</td>
<td>E-cadherin</td>
<td>0.536</td>
<td>(0.337-0.882)</td>
</tr>
<tr>
<td>100% CF 0 ng/mL TGF-β (θ=0)</td>
<td>100% CF 9.33 ng/mL TGF-β (θ=0.13)</td>
<td>E-cadherin</td>
<td>0.304</td>
<td>(0.209-0.464)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Slug</td>
<td>1.374</td>
<td>(0.583-3.109)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Slug</td>
<td>1.014</td>
<td>(0.432-2.330)</td>
</tr>
</tbody>
</table>

Conclusion

In response to extracellular cues, the epithelial mesenchymal transition allows epithelial cells to acquire the invasive behavior characteristic of mesenchymal cells in order to migrate away from a tumor and metastasize. The work presented here focuses on the activation of EMT in MCF7 breast carcinoma cells and the roles played by cell-to-cell contact and the TGF-β signaling pathway. By using cell-to-cell contact to recruit E-cadherin to the membrane and TGF-β signaling to encourage the mesenchymal phenotype, the model suggests that it is more difficult for epithelial cells with more neighbors to undergo the bistable switch into the mesenchymal steady state, while cells with fewer neighbors require only a low dose of TGF-β to transition. With the application of TGF-β, it is only if a cell with a high amount of cell-to-
cell contact overcomes both its neighbor contact threshold and the necessary threshold of TGF-β present that it will be able to undergo the switch. Applying this observation to an in situ tumor suggests that the cells towards the border of the tumor, those on or near the edge, are the ones that will initially undergo the bistable switch and transition into mesenchymal cells. Only once these border cells move away are the cells closer to the center of the tumor able to undergo the bistable switch into the mesenchymal steady state so that they too can migrate away from the tumor. Using this dynamic, the results of the model suggest that the cells that are the greatest threat to undergoing EMT are those at the edge of the tumor and treatment could thus be focused on preventing those cells from transitioning. By securing the cells at the border and preventing them from leaving the tumor, the cells closer to the center of the tumor would be inhibited from undergoing EMT as well.

The in situ suggestions raised by this model, however, must be considered carefully, as the true value of this model comes from its ability to highlight the gaps in data from the experiments performed in Chapter 2, as well as a possible guide for future scientific experiments. Figure 3.6 shows that the changes attributed to the MCF7 cells in Chapter 2 would be a result of cells with fewer than two full neighbors undergoing the bistable switch at the highest concentration of TGF-β. Additionally, due to the lack of data, the model predicts that a cell with many neighbors will enter into a single steady state behavior that is unable to transition unless its neighbors transition and move away. To build a better model that could be shaped towards therapeutic intervention, the experiments carried out in Chapter 2 need to be performed again at higher values of TGF-β. Doing qPCR analysis and flow cytometry at higher concentrations of TGF-β would allow for better estimates of the parameters of the model and
combining this work with further investigations into pathway overlap could help create a more realistic model of the activation of EMT in breast carcinoma in tissue cultures. Additionally, the attempts to apply the model to cells at different conditions, such as 60% and 100% confluent tissue cultures show the limitations of the model and the inability to accurately describe the results at these higher confluencies suggests future experimental paths. In particular, it would be pertinent to grow the cells at lower confluencies (< 30% confluent) and carry out the qPCR and flow cytometry experiments of Chapter 2 in order to understand the behavior of the cells on an individual cell level. Additionally, using low confluence cultures for time course experiments and fluorescent imaging could help us understand how long it takes for cells with a certain number of neighbors to gain mesenchymal properties and move away from a cluster. Finally, by carrying out experiments at different concentrations of TGF-β that measure mRNA expression and quantify the amount of specific proteins in each cell, we would be able to understand if our assumption that there is a one-to-one correlation between mRNA and protein levels is problematic. Thus the model here is better suited to highlighting the limitations in the data collected, as well as presenting a guide for future experiments that could ultimately lead to the development of a more sophisticated model capable of suggesting therapeutic interventions.
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APPENDIX A: Monotonic Parameter Relationship Plots for Chapter 1 Sensitivity Analysis

Figure A1A

Figure A1B

Figure A1C

Figure A1D

Figure A1E

Figure A1F

Figure A1G

Figure A1H
Figure A1Y

Figure A1Z

Figure A1AA

Figure A1AB

Figure A1AC

Figure A1AD

Figure A1AE

Figure A1AF
Figure A1: Figures A1A-A1EN show the monotonic behavior for each of the three nondimensional variables \((e, b, s)\) in response to changes in the 8 nondimensional parameters at select values of Dvl \((d)\) in Chapter 1. Figures in the left column show the parameter varied
over its parameter space in a system with epithelial initial conditions while figures in the right column show the individual parameter varied over its parameter space with mesenchymal initial conditions.
APPENDIX B: Description of Code Used in Chapter 1

MATLAB TIME COURSE AND BIFURCATION CODE: CHAPTER 1

With a cell in the epithelial state and membrane Dvl \((d)\) set to 0, the MATLAB script EMT_wnt_nondimensional.m will call upon the ODE solver, ode15s, to solve our system of ordinary differential equations (ODEs), defined in the script EMTode_wnt_nondimensional.m and return values for the protein levels \(e, b, s\), repeating this process for values of Dvl up to \(d = 5.45\) and saving the steady state values of all three proteins. The code will then call upon ode15s to solve our system of ODEs, defined in EMTode_nondimensional_1.m, with the cell beginning in the mesenchymal state and \(d = 5.45\), repeating this process as Dvl is decreased in value down to \(d = 0\), saving the steady state levels of all three proteins for each value of Dvl. Finally, when finished, the code will plot all three proteins and show how their steady state concentrations change with respect to Dvl. In order to generate the time course plots in Figures 1.2A and 1.2B, the script wnt_pathway_plots.m can be used. This file uses ODE solver ode15s to solve our equations defined in EMTode_wnt_nondimensional.m for different concentrations of Dvl and then plots the variables, as well as the variation in Dvl, over \(\tau\).

XPPAUT CODE: CHAPTER 1

The script wnt_nondim.ode contains the initial conditions for each variable, the values for all parameters and the ODEs for all three proteins. In this file, \(d = 0\) and is listed under parameters. A time course for the system needs to be run with the model in XPPAUT so as to initialize the system and make sure that it reaches its steady state values. The parameter space can then be explored in the Auto module of XPPAUT. With Par1 = \(d\) and Par2 = the parameter of choice, two parameter bifurcation diagrams were created by first generating a bifurcation diagram of
$e$ vs. $d$, with $d = [-2, 5.45]$ along the x-axis and Par Min = -2 and Par Max = 5.45 specifically set under the Numerics tab, and then switching to a two-parameter view once the limit points of the system were established. Detailed instructions for running XPP and AUTO can be found at http://www.math.pitt.edu/~bard/xpp/xpp.html.

**MATLAB MONOTONICITY AND LATIN HYPERCUBE CODE: CHAPTER 1**

Setting Dvl ($d$) = 0 and using epithelial initial conditions, the scripts EMT_wnt_nondim_*.m where *=A1, A2, A3, C1, C2, C3, F1, and F2 call upon the ODE solver ode15s to solve the system of ordinary differential equations defined in the script EMTode_wnt_nondim.m, returning the steady state values of $e$, $b$, and $s$. This process was then repeated for all values in each parameter range defined by the two parameter XPPAUT diagrams and the protein steady state values were each plotted against the varying parameter to check for monotonicity. Dvl ($d$) was then increased incrementally and the process was repeated until Dvl ($d$) reached 5.45. The protein steady state values against the varying parameter for each value of Dvl were plotted to check for a monotonic relationship between the steady state behavior of the proteins and the 8 parameters as Dvl was increasingly applied to an epithelial cell. Dvl ($d$) being incrementally decreased to $d = 0$. The script LHC_wnt_nondim.m uses the parameter ranges for A1, A2, A3, C1, C2, C3, F1, and F2 defined by the two parameter XPPAUT diagrams to generate a Latin Hypercube where each of the 8 parameters is divided up into 1000 equal intervals. For each of the 1000 different parameter sets, the script calls upon ODE solver ode15s to solve the system of ODEs defined in the script EMTode_wnt_nondim.m for $d = 0$ and epithelial initial conditions. The 1000 values of each individual parameter were then ranked, as were the steady state protein levels of $e$, $b$, and $s$, using the MATLAB function tiedrank. The correlation
between each parameter and each of the steady state protein levels was then determined using the MATLAB function partialcorr. Maintaining epithelial initial conditions, Dvl was then incrementally increased and the process was repeated until $d = 5.45$. Then, Dvl was set to 5.45 with mesenchymal initial conditions and the process was repeated with Dvl being incrementally decreased until $d = 0$. 
APPENDIX C: Description of Code Used in Chapter 2

MATLAB CODE FOR NEIGHBOR NUMBERS: CHAPTER 2

The file imagej_coords.m imports the data files that were saved for each image post processed in ImageJ. For each cell in the image, the function pdist2 calculates the distance between the center of mass of the cell and the center of mass of each cell in the image using a specified threshold distance. If a cell is within that threshold, a value of ‘1’ is marked in the matrix neighbor_count, otherwise a value of ‘0’ is used. This process is repeated for all cells in the image and the tally is summed to produce the total number of neighbors each cell has. The data was then recorded in a master treatment group excel file and the process was repeated for all images in the data set, updating the excel file after every image. For each treatment group, technical replicate, and threshold distance, a different excel file was used. The excel file was saved as a .csv file when all images for the data were passed through imagej_coords.m.

In order to determine the distribution of the number of neighbors of each treatment group, the .csv file was then read into the MATLAB file histogramplots.m. From the .csv file, 100 cells were were then randomly chosen without replacement and their neighbor number was recorded. The number of cells in every neighbor number category were summed and the data was plotted using the software Prism 6. To determine which cells to examine for staining patterns, the .csv file produced by imagej_coords.m was then loaded into the MATLAB file tallies_cellstaining.m. The code finds that cells in the 30% treatment groups with (0-2) neighbors, (3-5) neighbors, and 6+ neighbors and chooses 25 cells from each group. The code returns the cell numbers for each subgroup in the .csv file, which were then found and marked by hand. Note, for the .csv files corresponding 30% confluence and neighbor number
determined using a threshold of the average distance between the centers of mass, the code could only produce the total number of cells with 6+ neighbors, as this value was < 25 cells.
APPENDIX D: Monotonic Parameter Relationship Plots
for Chapter 3 Sensitivity Analysis

Figure A2A

Figure A2B

Figure A2C

Figure A2D

Figure A2E

Figure A2F

Figure A2G

FIGURE A2H
Figure A2BM

Figure A2BN

Figure A2BO

Figure A2BP

Figure A2BQ

Figure A2BR

Figure A2BS

Figure A2BT
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Figure A2: The monotonic behavior for $e$ and $s$ in response to changes in the 6 nondimensional parameters at select values of both TGF-$\beta$ ($\theta$) and cell-to-cell contact ($\mu$).
APPENDIX E: Description of Code Used in Chapter 3

XPPAUT CODE: CHAPTER 3

The script confluent_tgfb.ode contains the nondimensional ordinary differential equations (ODEs) for both $e$ and $s$, the initial conditions for each variable, and the values for the nondimensional parameters. In this file, $\mu = 2.3$ and $\theta = 0$ and both are listed under parameters. First, a time course must be run for the model so as to initialize the model and confirm that the variables reach their steady state values. The bifurcation with respect to $\theta$ can be explored in the Auto module by setting Par1 = $\theta$, Par Min = -1, and Par Max = 1. Once the bifurcation plot has been created, two-parameter bifurcations can be generated by setting Par2 = the parameter of choice and then switching to a two-parameter view after the limit points of the system are established. This process can then be repeated for $\mu$. Detailed instructions for running XPP and AUTO can be found at http://www.math.pitt.edu/~bard/xpp/xpp.html.

MATLAB TIME COURSE CODE: CHAPTER 3

In order to generate the time-course plots in Figure 3.5, the script EMT_tgfb_nondim_timecourse.m can be used. This file uses ODE solver ode15s to solve the equations defined in EMTode_tgfb_nondim.m for cells with a different number of neighbors and a different amount of TGF-$\beta$ ($\theta$). The script then plots the variables, as well as the variation in $\theta$ over $\tau$.

MATLAB MONOTONICITY AND LATIN HYPERCUBE CODE: CHAPTER 3

The scripts EMT_tgfb_con_*.m where *=A1, A3, G1, G3, F1, and F2 call upon the ODE solver ode15s to solve the system of ordinary differential equations defined in the script
EMTode_tgfb_con_paramono.m for set values of both $\mu$ and $\theta$, returning the steady state values of $e$ and $s$. This process was then repeated for values in the parameter range defined by the two-parameter XPPAUT diagrams and the protein steady state values were plotted against the varying parameter to check for monotonicity. The input variables, $\mu$ and $\theta$, were independently increased and the monotonic plots were generated. The process was then repeated as both $\mu$ and $\theta$ were incrementally decreased. The script LHC_tgfb_con_nondim.m uses the parameter ranges for A1, A3, G1, G3, F1, and F2 defined by the two-parameter XPPAUT diagrams to generate a Latin Hypercube where each of the 6 parameters is divided into 1000 equal intervals. For each of the 1000 different parameter sets, the script calls upon ODE solver ode15s to solve the system defined in EMTode_tgfb_con_paramono.m for different combinations of $\mu$, $\theta$, and initial conditions. The 1000 values of each individual parameter were then ranked, as were the steady state protein levels of $e$ and $s$ using the MATLAB function ‘tiedrank’. The correlation between each parameter and each of the steady state protein levels was then determined using the MATLAB function ‘partialcorr’ and the process was repeated for different changes to $\mu$, $\theta$, and the initial conditions.

MATLAB MCF7 DATA CODE

In order to understand how well the model describes the data presented in Chapter 2, the script EMT_tgfb_nondim_fluxc.m uses the ODE solver ode15s to solve the system defined in EMTode_tgfb_nondim_fluxc.m and build matrices for the steady state values of $e$ and $s$ at different values of $\mu$ and $\theta$. Once these matrices are established, the script then calculates the
relative expression of both $e$ and $s$ to their values at $\theta = 0$, as well as the percent error of the model to the data in Chapter 2.