Breast cancer affects the lives of nearly 200,000 women every year. Progression of this disease is facilitated by the deregulation of molecular networks that create and maintain the mammary gland. Therefore, it is critical to identify these genetic networks because elucidating these pathways will identify therapeutic targets that may one day lead to better diagnostic markers, treatments, or methods to prevent this disease.

In my thesis research on breast development, I have uncovered a relationship between two important signaling networks that have been viewed as independent from each other. Both of these pathways suppress branching morphogenesis, a poorly understood, but critical, process that establishes the structure of the breast. The genetic pathways that operate during normal and disease states are numerous; consequently, genomics provide a powerful approach capable of identifying signaling pathways. In this application I propose to define the components of signaling networks and their relationship to each other by employing SOLiDTM high throughput sequencing.

The mouse mammary gland is a bi-layered, branching ductal network. It begins as a small mass of epithelial cells that differentiate, elongate and hollow to create a bi-layered duct with a central lumen. This bi-layer comprises an inner layer of luminal epithelial cells that is surrounded by an outer layer of myoepithelial cells. The gland is encompassed by a basal lamina that separates it from the surrounding fat pad (Figure 1). During development, the gland undergoes repeated branching to create a tree-like structure (Silberstein, 2001.) Branching is induced by growth factors such as scatter factor (HGF) and fibroblast growth factor (FGF). In contrast, one master inhibitory regulator
of branching has been identified, transforming growth factor beta 1 (TGF-β1.) Little is understood about how these opposing factors orchestrate the complex events required in branching morphogenesis.

SLIT2 and ROBO1 are a receptor-ligand pair expressed in the mammary gland. Our laboratory has recently established a role for these proteins in maintaining adhesion between luminal and myoepithelial cell layers (Strickland, 2006.) I undertook an analysis of gross morphology in Slit2-/- or Robo1-/- mammary glands and discovered a precocious branching phenotype during early development (Figure 2). This defect led me to hypothesize that the SLIT2/ROBO1 signaling axis acts as an inhibitor of mammary gland branching morphogenesis during early development.

TGF-β1 has long been recognized as a powerful inhibitor of mammary gland branching. Insertion of plastic beads, loaded with TGF-β1, in front of the developing mammary gland results in an inhibition of branching (Daniel, 1989.) I have repeated this experiment and found that SLIT2 beads have a similar inhibitory effect on branching. To investigate the relationship between TGF-β1 and SLIT2/ROBO1 signaling, we examined Robo1 levels in primary mammary cells after treatment with TGF-β1. We separated the mammary gland into luminal and myoepithelial fractions and observed upregulation of Robo1 specifically in the myoepithelial population, confirming previous expression analysis of the developing gland.

This increase in Robo1 levels upon addition of TGF-β1 led me to believe that SLIT2/ROBO1 signaling functions downstream of TGF-β1. My model predicts that increased ROBO1 expression on the cell surface after TGF-β1 addition will provide more receptors for SLIT2. As a result, I expect to observe enhanced effects, for example enhanced inhibition of branching upon addition of both TGF-β1 and SLIT2. I recently demonstrated this synergistic effect by using an in vitro branching assay. Primary
mammary epithelial cells form simple branched aggregates \textit{in vitro}. When treated with TGF-\(\beta\)1 or SLIT2, alone, branching of these epithelial aggregates is inhibited. Together, the inhibition is 3-fold greater. Thus, addition of both factors resulted in a stronger inhibition than addition of either factor alone. Together with our data showing upregulation of \textit{Robo1} by TGF-\(\beta\)1, this synergistic effect of the factors suggests that SLIT2/ROBO1 signaling functions downstream of TGF-\(\beta\)1 to inhibit branch formation during mammary gland development.

To elucidate the molecular mechanisms behind this inhibition, comparative mRNA profiling will be performed on the transcriptome, the set of all messenger RNAs produced in one or a population of cells. Insight into molecular pathways is derived from the analysis of the transcriptome upon perturbation by genetic or pharmacological methods. I propose to use SOLiD\textsuperscript{TM} high throughput sequencing, a next-generation sequencing technology available at UCSC. My strategy will be to use immortalized human myoepithelial cells lines (HME50 and HMS1), rather than primary cells that are subject to variability and difficult to obtain in sufficient quantities. I will analyze the transcriptome of these cells lines after treatment with SLIT2 and TGF-\(\beta\)1 alone, and both together.

Pathway analysis of the gene expression profiles under these various conditions will allow me to identify specific genes. An example of the anticipated results is illustrated in Figure 3. We expect that each factor, alone, will induce changes in many gene networks. However, we anticipate there will be a subset of genes, those that inhibit branching via SLIT2/ROBO1 signaling, that will be regulated in the same manner after

![Figure 3: Cartoon illustrating results from pathway analysis](image)

A) Treatment with TGFb or SLIT2 regulates numerous genes (TGFb: a-g; SLIT2: d-j), a subset of which overlap (d, g) and are putative inhibitors of branching.

B) Treatment with both SLIT2 and TGFb regulates the same numerous genes (TGFb: a-g; SLIT2: d-j), but overlapping genes are more dramatically changed (D,G)
treatment with either SLIT2 or TGF-β1. Moreover, due to the synergistic effect we observe in our branching assay, we predict these regulatory changes will be enhanced with the treatment of both SLIT2 and TGF-β1. Thus, the synergistic relationship between these pathways, revealed in my functional assays, will aid the bioinformatic elucidation of the gene network that is downstream of SLIT2/ROBO1 signaling and controlled by master regulator, TGF-β1. The UCSC genome sequencing center will have a full-time bioinformatics specialist to help users such as me analyze their data. After I have identified candidates, I will select a small number to evaluate in my primary cell assays.

In sum, I believe, as do many researchers, that the molecular mechanisms regulating mammary gland development are deregulated during breast cancer and the identification of these genes will aid our understanding of this disease. Our laboratory has uncovered an exciting, novel mechanism of mammary gland growth regulation. To further define this mechanism, we propose a genomic analysis. By receiving this aid I will have the ability to conduct this analysis.


