GAS6 ALTERS MAMMARY GLAND DEVELOPMENT AND BREAST CANCER PROGRESSION

AN ABSTRACT SUBMITTED ON THE TWENTY-FIRST DAY OF JULY 2016 TO THE GRADUATE PROGRAM IN BIOMEDICAL SCIENCES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE SCHOOL OF MEDICINE OF TULANE UNIVERSITY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

C. STANDLEE

COURTNEY ROSE STANDLEE R.S., MPH

APPROVED: ROBERT GARRY Ph.D.
Director

Heather Machado Ph.D.

Haitao Zhang Ph.D.

Yan Dong Ph.D.

Sarah Lindsey Ph.D.

Cindy Morris Ph.D.
ABSTRACT

Development of the mammary gland requires controlled apoptosis and the involvement of the innate immune system for the clearance of apoptotic cells. The studies described herein assess the effects of growth arrest–specific protein6 (Gas6) in the development of mammary glands and associated in vitro studies of Gas6. Gas6 is a secreted protein that binds Axl, a member of a family of receptor tyrosine kinases (RTKs) found on cell surfaces\(^1\text{-}^4\). Axl has previously been shown to mediate numerous biological functions including cell proliferation, migration, invasion, survival, clearing of apoptotic cells, and cytokine secretion\(^1\text{-}^4\). Most of these functions are also important in mammary gland development\(^5\text{-}^8\). However, the role of Gas6/Axl in mammary gland development remains unknown. Our lab previously showed that Gas6 expression is elevated in early-stage mammary lesions with high tumor-forming potential, suggesting it may also be important for early breast cancer progression. We hypothesize that Gas6 regulates mammary gland development and the progression of early stage breast cancer. To test this hypothesis, we will employ a Gas6 knock mouse to study development of the mammary gland, and we will study Gas6/Axl in vitro to investigate if the pathway’s activation will alter the cellular functions of proliferation, migration, motility, 3D morphogenesis, invasion and survival. Our studies show that germline deletions of Gas6 lead to impaired mammary gland development with possible consequences during lactation and involution. In addition, Gas6 stimulation of Axl may signal via invasion and anti-apoptotic pathways in vitro.
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DEDICATION

This work is dedicated to Theresa Eva Maciag Daly, my Nanna, who lost her battle with breast cancer in May 2016
ACKNOWLEDGEMENTS

I could not have completed this work without the support of my family! To my amazing husband David Wayne, for giving me the courage and support to strive for this degree. To my parents, siblings, and in-laws, I cannot thank them enough for the visits, cards, phone calls, texts, photos, hugs, kisses, and encouragement to keep going. Without all of you watching my progress and loving me, I most certainly would not have been so successful.

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CHAPTER 1: INTRODUCTION

Breast Cancer and Ductal Carcinoma in situ

The US has the highest rate of Ductal Carcinoma in situ (DCIS), which is attributed to increased screening. In 2016, approximately 246,660 invasive breast cancers and 61,000 non-invasive breast cancers will be newly identified and 40,450 women were expected to perish in 2015 from breast cancer. Despite increased early diagnosis of cancers in screening by mammograms, there has not been a significant reduction in the number of associated incidences of invasive breast cancer. DCIS, recently described clinically as Stage 0 cancer, is the premalignant non-obligated precursor to invasive ductal carcinoma (IDC) and is the most commonly diagnosed type of premalignant breast cancer. DCIS occurs when cells lining the duct of a mammary gland replicate aberrantly but the intact basement membrane encompassing the duct of the mammary gland constrains the premalignancy from expanding into the surrounding stroma. DCIS has the ability to remain non-progressive and confined to the mammary gland duct of the breast for years. In the U.S., incidents of cancer that are classified as DCIS make up 24.9% of all cancers detected every yearly screening. DCIS may be the non-obligate precursor to IDC and, regardless of it potential to remain DCIS, it is clinically treated the same as IDC, with surgery and radiation. Despite the recent attention to DCIS research, literature shows that there remains a lack of prognostic factors that can
define the potential invasiveness of newly diagnosed DCIS patients. Such prognostic factors that could delineate which incidents would require the most aggressive therapy and which cases could be treated with limited but effective therapy are a critical need in both defining DCIS and successfully treating it. While DCIS has entered the limelight of public familiarity, there remains a disconnect between the term DCIS and the severity of the disease, implicating a crucial need for specific diagnostic factors to better define cancer progression capacity.

The goal of this research is to understand what cellular factors regulate DCIS progression. These studies will yield insights that will allow us to identify molecular pathways that may predict which patients will progress to invasive breast cancer and which will remain non-invasive. Overtreatment increases the risk of patients having to undergo unnecessary physical and emotional pain and grief, as well as having to ensure the physical and financial cost of treatment. Understanding the aspects of DCIS progression will be imperative for intercepting malignancy before advancement to invasive or metastatic disease while also leading to a reduction in unnecessary surgeries and harsh treatments for newly diagnosed DCIS patients.

**Normal Mammary Gland Development**

Mammary gland development and function is similar in rodents and humans and consist of three stages: embryonic, pubertal, and adult\textsuperscript{17}. The epithelial bud, or rudimentary ductal tree, present at birth is the collection of epithelial cells whose development is arrested, but is where the mammary tissue
begins (Figure 1A)\textsuperscript{5,6,18,19}. At birth, epithelial expansion stalls, leaving only a rudimentary epithelial bud at the nipple until puberty, weeks 3-6 in mice (Figure 1B). During puberty, extensive ductal morphogenesis and ductal elongation of the mammary epithelium into the empty fatty stroma occurs (Figure 1C). Paracrine hormonal cues determine how often side branches will form and diverge from the primary duct (Figure 1C)\textsuperscript{20}. The process of ductal elongation, where the epithelium is driven through the empty fat pad, is facilitated and directed by terminal end buds (TEBs). TEBs are club-like structures that exist at the distal ends of the ducts farthest from the nipple (Figure 1C)\textsuperscript{21}. The TEB is made up of an outer layer of cap cells that surrounds the multilayered inner core of body cells\textsuperscript{20}. The body cell layer itself has the following two regions: the region adjacent to the cap cells, which is a zone of proliferation, and the second region, an apoptotic zone that will make the continuous lumen of the duct\textsuperscript{22}. The TEBs bifurcate, splitting a single branch into two, and allow the gland to grow proportionally. In mice, TEBs are no longer present at approximately 10-12 weeks and the adult gland is then observable as the fat pad is filled (Figure 1D). The process of side-branching, also known as secondary and tertiary branching, occurs during the estrous cycle (Figure 1E) and leads to the expansion of the mammary gland for adult development. This dynamic process of ductal morphogenesis is a constant process of proliferation, invasion, branching, differentiation, remodeling and apoptosis.

The normal adult mammary gland has expanded to fill the mammary fat pad that was present at birth (Figure 1)\textsuperscript{20}. A cross-section of a normal mammary
gland duct has two layers of cells, see Figure 2A\textsuperscript{20}. The entire mammary gland within the stroma encompasses the epithelial ductal network and is comprised of adipocytes, vascular endothelial cells, fibroblasts, and a variety of infiltrating innate immune cells\textsuperscript{20,23}. The epithelium is of two types, the outer most is the basement membrane and the myoepithelial cells that encompass the luminal epithelial cells that boarder the hollow lumen of the duct (Figure 2)\textsuperscript{20,23}.

If pregnancy occurs, ductal morphogenesis initiates the formation of tertiary branches with terminal alveolar buds that undergo proliferation to expand the luminal epithelium and that has alveolar cells that undergo lactogenic transition to produce milk that is expressed into the lumen to feed offspring\textsuperscript{20}. This lactation is followed by regression and involution that is a dynamic process of regulated apoptosis where about 80\% of the epithelium is removed within days after suckling offspring are removed due to weaning\textsuperscript{20}.

In summary, the changes that occur in the mammary gland are temporal and occur because of extrinsic and intrinsic systemic hormones, systemic steroids, niche paracrine hormones like estrogen, local growth factors like epidermal growth factors (EGF), and local proteins like matrix metalloproteases (MMPs)\textsuperscript{20,22}. When all of these processes are taken together, we see that mammary development requires extensive proliferation, migration, invasion, programmed cell death, and clearing of dead cells in order to build a normal functional mammary gland. The innate immune system is also important during mammary gland development. Innate immune cells invade the stroma and aid in clearing of apoptosis cells and cellular debris\textsuperscript{24–26}. This clearance is important
during puberty for lumen formation, after diestrus for the regression of branching, and following lactation associated with pregnancy during involution. The dynamic expansion and remodeling that occurs in the mammary gland utilizes a variety of pathways, one of which we believe will be Gas6/Axl.

**Development of DCIS**

When aberrant cellular replication occurs in a human mammary gland, a clinical pathologist can see multi-layered ductal luminal cells due to abnormal proliferation, known as ductal hyperplasia (Figure 2B)\(^{11,14,15}\). The malignancy progression can be classified as atypical ductal hyperplasia (ADH) and there is abnormal nuclear/cytoplasmic ratio (Figure 2C)\(^{11,14,15}\). When the duct is full or filling with cells but the basement membrane surrounding the milk duct of the mammary gland is still intact, this stage is known as ductal carcinoma in situ (DCIS) (Figure 2D)\(^{11,14,15}\). DCIS is defined as non-invasive, non-palpable, non-obligatory cancer that is confined by the ducts of the mammary gland and, thus, can be detected by mammogram due to the microcalcifications but is diagnosed by a core biopsy\(^{9}\). DCIS that ruptures the mammary gland basement membrane is called DCIS with microinvasion (Figure 2)\(^{11,14,15}\). Malignant cells that were previously contained egress into the surrounding stroma, continue to replicate, and may eventually metastasize\(^{11,14,15}\).

The hallmarks of cancer as outlined by Hanahan and Weinberg contend that cells must acquire functions to facilitate the development of cancer. This occurs in DCIS and these properties include sustained proliferation, avoiding
growth suppression, evading cell death, immortality by continued replication, angiogenesis, invasion, metastasis, genomic instability and inflammation\textsuperscript{27}.

**DCIS Treatment**

Patients diagnosed with DCIS receive a common standard of care including a surgical lumpectomy accompanied by radiation therapy\textsuperscript{9,11,28}. A lumpectomy is the surgical removal of the concentrated DCIS cells with a clear margin of healthy tissue remaining\textsuperscript{29}. Depending upon the case, a mastectomy is undertaken, which involves the surgical removal of all of the breast tissue, areola, and nipple\textsuperscript{30}. A mastectomy is recommended when the patient has a large area of DCIS, multiple regions of DCIS, or if the tissue biopsy reveals that the cells within the margin are cancerous\textsuperscript{30}. In the US, there has been an observed reduction in unilateral mastectomies that corresponds to an increase in lumpectomy procedures, but what is unexpected is the increase in bilateral mastectomies of DCIS patients\textsuperscript{9}.

**Roles of Gas6/Axl**

Gas6, growth arrest-specific gene 6, binds and activates a receptor tyrosine kinase (RTK) that is being examined as a therapeutic target in cancer treatments\textsuperscript{31}. Gas6 was detected in NIH3T3 fibroblast as a gene that was upregulated during serum starvation-induced growth arrest\textsuperscript{4}. Gas6 is a secreted extracellular protein that binds RTKs that include receptors: Tyro3 (also known as Tyro, Sky, Brt, Rse, DTK, Tif), Axl (also known as UFO, Ark, Tyro7) and MerTK (also known as Mer, c-mer, Eyk, Nym, Tyro12); this family is also called TAMR. The Gas6 signaling pathway is activated by the binding of the ligand
Gas6 to TAMR, which causes the extracellular signal to be transduced intracellularly\(^\text{32}\). Gas6 binds TAMRs with varying affinities; the strongest interaction is between Gas6 and Axl and it binds at a 1:1 ratio (\textbf{Figure 3A})\(^\text{33}\). Once bound extracellularly by Gas6, Axl can dimerize and autophosphorylate to activate its kinase activity thus associating with signaling pathways. However, Gas6 may also be bound by Phosphatidylserine (PtdSer), which is found extracellularly on cells that are undergoing apoptosis\(^\text{34,35}\). This binding of Gas6 to PtdSer is thought to allow for apoptotic cells to interact more readily with cells expressing TAMRs\(^\text{35}\). Cook et al. found that MerTK is important for homeostasis and protects the gland from damage by clearing of apoptotic cells within the mammary gland\(^\text{24}\). When Gas6 is knocked out in mice, it was found they were protected from arterial thrombosis because platelet aggregation function was altered\(^\text{36}\). Gas6 knockout mice also had increased vascular permeability\(^\text{36}\).

TAMRs are found ubiquitously expressed on the cell surfaces of an assortment of cells, for example epithelial, endothelial, and innate immune cells, and in a variety of organs and tissues within the body, such as the heart, testes, and breast, among others\(^\text{1,32}\). Axl is conserved evolutionarily among vertebrate species\(^\text{1}\) and was isolated and cloned in 1991 from two patients with chronic myelogenous leukaemia\(^\text{1}\). The family of TAMRs have similar structural domains, comprised of both the intracellular signaling domains and the extracellular ligand binding domain (\textbf{Figure 3A})\(^\text{33}\). Both Axl and Mer have an extracellular cleavage site, allowing the soluble receptor to bind its ligand in circulation (\textbf{Figure 3D})\(^\text{33}\). TAMRs function as kinases, meaning that when the receptor is activated it...
phosphorylates secondary proteins intracellularly, which subsequently cause signaling cascades (Figure 4). TAMRs have multiple autophosphorylation sites that signify activation. RTKs must dimerize to have a functional kinase activity by: binding Gas6 (Figure 3), the extracellular domain of adjacent Axl receptors (Figure 3B), interacting with radical oxygen species (Figure 3C), or by forming heterodimers with other cell surface receptors (Figure 3E)\textsuperscript{33}.

Much has been learned about the function of TAMRs through the generation of TAMR knockout mice. When all three TAMRs are knocked out, a state of hyper-inflammation occurs and autoimmune disease with lymphoproliferation leading to impaired homeostasis and impaired natural killer cell development occurs\textsuperscript{32,35}. Additionally, defective spermatogenesis and smaller testes size has been observed in male mice with triple TAMR knockout\textsuperscript{32}. Finally, TAMR knockout in an aged animal has been correlated with neural degeneration and subsequent paralysis and seizures\textsuperscript{32}. With this knowledge, it may be concluded that TAMRs play a critical role in many processes of homeostasis in the body.

The Gas6 TAMRs have previously been described in various microenvironments to be involved in many cellular functions including: cytoskeletal function, intracellular signaling, proliferation, migration, invasion, survival, cytokine secretion, and the clearing and efferocytosis of apoptotic cells\textsuperscript{35}. Several downstream signaling pathways have also been identified (Figure 4), but exactly how these pathways are important in DCIS progression is unknown. Studies using Axl\textsuperscript{−/−} mice to investigate its normal function have shown
that when Axl is removed, mice have both reduced thrombus development and elevated vascular permeability\textsuperscript{32}.

Classical signaling effects via Axl binding result by the MAPK/ERK and PI3K/AKT signaling, which lead to alterations in cell survival, proliferation, and/or migration\textsuperscript{37}. Roles for Axl signaling include modulating the innate immune response during inflammation and effecting natural killer cell responses, both of which are functions that are also important in mammary gland development during ductal morphogenesis\textsuperscript{35}.

The innate immune system protects us from harmful organisms or accumulation of cellular debris. Stimulation of the innate immune system occurs by cytokines released when macrophages and natural killer (NK) engulf cells and/or cellular debris\textsuperscript{38–40}. Macrophages can act as antigen-presenting cells (APCs) to educate and stimulate a more robust immune response\textsuperscript{38–40}. TAMR knockout mice’s causes an inability to have functional macrophages, and this has been associated with leads to autoimmune disease\textsuperscript{41}. Axl signaling has recently been called an immune modulator because it has the capacity to limit the response of the immune system by controlling cytokine production\textsuperscript{38,42}. It has also been found that Axl signaling is necessary for differentiation and development of innate immune cells. NK cells and macrophages work to maintain homeostasis and remove pathogens or cellular debris associated with apoptosis.
Functions of Gas6/Axl in Cancer

Research on a variety of cancers have identified the Gas6/Axl as factors that can alter progression and influence patient outcome, and the role of Gas6/Axl seems to be dependent on the cell, tissue, and microenvironment\textsuperscript{43}. For instance, Axl is an prognostic marker and target in acute myeloid leukemia, where it appears to promote growth and proliferation by inhibiting AKT and MAPK pathways (AML)\textsuperscript{37}, while higher Axl expression is associated with greater frequency of metastasis in prostate cancer\textsuperscript{37}. It has been shown that Axl is a regulator of breast cancer and a poor prognostic indicator of patient survival\textsuperscript{44}. Examination of invasive breast cancer has demonstrated that inhibition of Axl leads to a reduction in migration and invasion\textsuperscript{45}. However, the role of Axl in premalignant non-invasive DCIS is unknown. The influence of Axl on invasive breast cancer has been examined and it has been observed that Axl is correlated with poor prognosis and that Axl has been implicated in metastasis\textsuperscript{4,46}.

Building upon this knowledge, there are current and ongoing studies of Axl as a therapeutic target in treating human cancer\textsuperscript{31}. Rankin \textit{et al}. have demonstrated that the application of soluble Axl receptor inhibits the metastasis of ovarian cancer\textsuperscript{47}. With regard to these studies and considering that Gas6/Axl signaling has been associated with differential patient outcomes in a variety of human cancers, understanding the specific functions of Gas6/Axl signaling in DCIS is critically important.
Mouse model for in vivo identification of Gas6

For our initial findings, that spurred this line of inquiry, we used a novel mouse model that can recapitulate DCIS-like lesions in vivo. While there are some differences between the mouse and human mammary gland, in the field of breast cancer, the mouse model is similar enough in its developmental process to be a good model for in vivo research as both human and mouse mammary glands undergo similar processes of development to build a functional gland.

The model was established by removing the mammary gland epithelium from a p53-null Balb/c mouse; these epithelial cells were transplanted to mammary glands that were previously cleared of endogenous epithelium, allowing the preneoplastic (PN) tissue to form hyperplasia48. Serial passage of these cells formed transplantable lines with persistent tumorigenic phenotypes48. For our studies, we selected two lines with different tumor-forming potential. Experiments were designed to compare the two transplantable lines and it was observed that they differ in their ability to progress, from DCIS-like lesion to invasive cancer. Using this model, we have been able to determine potential upregulated components including Gas6/Axl that may be important for DCIS progression.

Summary with Hypothesis & Specific Aims

Breast cancer is the second leading cause of mortality for women in the United States12,13 and it has social and economic impacts14,49. Our lab investigates what influences DCIS to rupture the mammary gland duct, spurring microinvasion, and leading to progression of breast cancer14. Discovering what
influences DCIS progression can lead to potential biomarkers or therapeutic targets.

The findings of this research could also significantly impact new and current DCIS patients, as there are currently novel inhibitors in clinical trials that target the Gas6/Axl pathway. Some of these novel ways of targeting Gas6/Axl include: soluble Axl (sAxl) which would bind for Gas6 in circulation, R-428 which is a small molecule inhibitor of Axl, and yet other groups are endeavoring to target the kinase activity of Axl45.

The work described herein will investigate the role of Gas6 in mammary gland development and how it may contribute to breast cancer progression. We hypothesize that Gas6 will regulate mammary gland development and will be critical for breast cancer progression. In order to test this hypothesis, we developed two Specific Aims as described here.

**Specific Aim 1** is to determine the influence of Gas6 on mammary gland development. Here, we will utilize a Gas6 germline knockout mouse model to investigate the function of Gas6 signaling *in vivo* and to study its role in mammary gland development.

**Specific Aim 2** examines the roles of Axl and Gas6 *in vitro* as regulators of multiple cellular functions: proliferation, migration, motility, 3D morphogenesis, invasion, and survival. The Gas6/Axl pathway will be studied *in vitro* in a group of human breast cell lines that range from normal to malignant (MCF10A, MCF10AT, MCF10-DCIS.com) in order to determine the role of Gas6/Axl signaling axis in progression of breast cancer.
Figure 1 Mammary gland development from embryo to adult

The dynamic process of mouse mammary gland ductal morphogenesis from embryo to adult. (A) The ectoderm bud (mammary placode) that grows during embryonic development. (B) At birth, we can observe the nipple (N) and a rudimentary epithelial ductal tree that has not yet reached the lymph node (L), while the rest of the mouse mammary gland is empty fat pad (FP). (C) At 5 weeks of age, puberty has spurred ductal elongation to begin. We see the epithelium approaches the centrally located lymph node (L) and the distal ends of the epithelium are bulbous TEBs that bifurcate, splitting one branch into two branches. The TEBs are also the driving force that degrade the stroma and facilitate the epithelium to penetrate the fat pad. (D) As time passes and ductal morphogenesis continues, at about 8 weeks of age, almost the entire mouse fat pad is filled with epithelium and there are few to no TEBs and the ducts have elongated and formed secondary branches that have filled the majority of the mammary gland. (E) At 12 weeks of age, the mammary gland undergoes secondary and tertiary branching and cycles of epithelium expansion and regression occur with subsequent estrus cycles.

Adapted with permission from “The mammary cellular hierarchy and breast cancer” Oakes et al.50
Figure 2 Changes in the cross-section of a mammary gland duct

The cross section of a duct is seen here as it undergoes aberrant replication of the ductal luminal cells that line the mammary gland. This maybe considered a step-wise process where the duct changes from its normal state as it becomes malignant. (A) The normal duct cross-section depicts the outer most basement membrane, green, surrounding the duct that is surrounded by the stroma. Next is a layer of myoepithelial cells, orange, that acts as the contractile force during periods of lactation that express milk from a cell into the lumen. The myoepithelium encompasses the inner luminal epithelial cells, blue, that form the hollow lumen, grey, at the center of the duct. Within the ductal epithelial cells we can observe the nuclei, shown as punctate black dots. (B) In this figure the luminal cells replicate aberrantly, this is ductal hyperplasia. (C) In atypical hyperplasias the cells start to occlude the lumen. (D) Ductal carcinoma in situ (DCIS) has the lumen filled with cells but an intact basement membrane still surrounds the mammary gland duct constraining the malignancy. We can also see there is a shift in the nuclear/cytoplasmic ratio in the epithelial cells; this means the nucleus size is increasing while the cytoplasmic fraction is decreasing in some cells. (E) When the integrity of the basement membrane is lost, the malignant cells are not confined to the duct and can invade into the local stroma; this may lead to distal metastasis. Also, we still see the persistence of the altered nuclear/cytoplasmic ratio in the malignant cells.
The Axl cell surface ligand is a transmembrane receptor, the ligand-binding domain of Axl is on the extracellular surface and the kinase domain, yellow box, is intracellularly located. (A) Gas6, green, binding the N-terminus of Axl in a 1:1 ratio, causes Axl homo-dimerization. (B) Two monomers of Axl on adjacent cells can bind and interact (C) Radical oxygen species (ROS) can activate Axl homo-dimers (D) Proteases are able to cleave the extra cellular domain of Axl proximal to the cell membrane and this liberates soluble Axl (sAxl). sAxl can bind Gas6, green, in the extracellular environment, stroma, and/or plasma. (E) Axl is able to hetero-dimerize with cell surface receptors including epidermal growth factor (EGF). Structurally, we see the N-terminus of Axl two immunoglobulin (Ig) like domains followed by two fibronectin type III domains and a cleavage site proximal to the cell membrane (E). The intracellular C-terminus domain demonstrates the kinase domain as containing the tyrosine kinase residues that are phosphorylated, signaling Axl activation.

*Adapted with permission from “Axl-dependent signaling: a clinical update”. Korshunov, Vyacheslav et al.*33
Axl activation has been implicated to initiate many pathways including: cell survival, adhesion, cell migration, proliferation, inflammatory and cytokines production for natural killer (NK) cell differentiation and innate immune cell development. Axl binds its extracellular ligand Gas6 (green) in a 1:1 ratio, causing homodimerization of Axl and stimulating intracellular signaling. Ligand binding causes the phosphorylation of tyrosine residues and initiates kinase activity of Axl, which phosphorylates secondary proteins.

*Adapted with permission from Machado, Heather*
CHAPTER 2: ROLE OF GAS6 IN MAMMARY GLAND DEVELOPMENT

Specific Aim 1 is to determine the influence of Gas6 on mammary gland development. Here, we compare Gas6<sup>-/-</sup> to Gas6<sup>+/+</sup> mice in order to directly study the role for Gas6 in mammary gland development.

INTRODUCTION

The study of Gas6 signaling via TAMRs in the mammary gland and how this may effect development is unknown. We believe because of Gas6’s roles with the innate immune system and because TAMRs have been found to play a role in clearing of apoptotic cells and debris, that Gas6 will be important during remodeling that occurs during mammary gland development and pregnancy.

During puberty, the ovaries secrete estrogens and progesterone hormones in the endocrine system. Estrogen’s role during puberty is to stimulate ductal elongation and bifurcation, the splitting of a single branch into two, of the mammary gland<sup>51</sup>. One contributing factor of this process is the proliferation of mammary gland epithelium. This proliferation accompanies epithelial invasion of the TEBs and degradation of the fatty stroma of the mammary gland<sup>61</sup>. Progesterone secreted by the ovary during the estrous cycles induces side-branching<sup>51</sup>. The combination of these circulating factors during puberty causes the rudimentary ductal tree present at birth to expand into a functional mammary gland. Formation of a functional mammary gland also requires apoptosis for the creation of the lumen comprising the hollow continuous duct. We believe that the
Gas6/Axl signaling pathway will alter this process due to its potential effects on proliferation, invasion, apoptosis, and cell clearance \textit{in vivo}. In a paper by Cooke et al, they identified that the Gas6 MerTK plays a role in clearing of apoptotic debris\textsuperscript{24}.

As previously discussed in \textbf{Chapter 1}, the mammary gland develops post-natally, \textbf{Figure 1}, beginning with the onset of puberty, when the mouse is approximately 3 weeks old and endocrine hormones begin to circulate. This circulation of progesterone and estrogen also stimulates ductal elongation for mammary gland development. Estrus is a cyclical pattern of 4 phases: proestrus, estrus, metestrus, and diestrus\textsuperscript{52,53}. Each phase is identified by a distinct combination of epithelial cell types present in the vaginal smears\textsuperscript{52,53}. The longest phase, diestrus, is also correlated with the most secondary and tertiary branching within the mammary gland; this is followed by a phase of regression of the side-branches and the cycle begins again with proestrus\textsuperscript{52,53}. Thus, the ability to identify murine estrus cycle phases is essential when harvesting mammary glands as side-branching can change with the estrus phase.

The effect of Gas6\textsuperscript{-/-} on mammary gland development currently remains unknown. Since Gas6 is a secreted protein that has been shown to be produced by a variety of different cell types and it has a wide range of roles in the body, including things like innate immune cell development, by breeding and using the Gas6\textsuperscript{-/-} animal we can determine the role of Gas6 in mammary gland development. While the mouse model has been employed by other investigators to examine the function of receptor tyrosine kinases (RTKs) Tyro3/Axl/Mer
(TAMR) in vivo, no one has utilized the TAMR knock out mouse in mammary gland studies of mammary gland development. When Axl receptor is ablated, animals display hyperactive antigen presenting cells (APC), autoantibody production, defective apoptotic cell clearance, demyelination leading to multiple sclerosis, and a decrease in blood vessel integrity. The consequences of TAMR receptors knockout in an animal lead to a state of: hyper-inflammation, increased lymphoproliferation leading to autoimmunity, organ defects, spermatogenesis defects and male infertility, chronic hepatitis, impaired NK cell function, and impaired haemostasis. However, when the TAMR RTK ligand Gas6 is specifically knocked out, mice are protected against thrombosis. When both Gas6 and its homolog Protein S are knocked out, the mice show defective clearance of apoptotic cells.

Here, we begin to investigate how Gas6−/− will alter the stages of mammary gland development using the Gas6−/− animals. Mammary gland development in mice is a dynamic process involving proliferation, invasion, apoptosis and clearing of cells and all of these processes are necessary for the development of a functional mammary gland. By using the Gas6−/− mice, we can ablate paracrine, autocrine, and endocrine functions of this complex signaling system.

The mammary gland arises from progenitor cell populations and these correlate these with mammary gland developmental stages. As the mammary gland expands post-natally, the mammary stem cell populations differentiate into the progenitor populations that will eventually make all of the cells that are necessary for a functional mammary gland. Research has shown that alveolar
cells will differentiate to produce milk proteins that is excreted into the lumen of the mammary gland duct\textsuperscript{54}, while luminal cells will comprise the lumen that lines the hollow duct of the mammary gland\textsuperscript{54}. Myoepithelial cells arise from a progenitor and surround the mammary gland duct in order to provide contractile force for the excretion of milk into the lumen\textsuperscript{54}. Limiting dilution experiments \textit{in vivo}, where transplanted mammary epithelial cells can recapitulate an entire mammary gland when transplanted into a cleared fat pad of a mouse, support this observation\textsuperscript{55}. To further support this evidence, we collected and visualized mammary glands from age matched WT and Gas6\textsuperscript{-/-} animals during time points selected to be representative of each phase of mammary gland development.
MATERIALS AND METHODS

Mouse colony establishment

We acquired Gas6−/− mice from Peter Carmeliet and established a colony at the Tulane-Downtown Vivarium, in accordance with proper animal handling and maintenance conditions in compliance with federal laws, and institutional animal care and use committee (IACUC). We received prior approval for all experiments and kept homozygous animals (wild type Gas6+/+ and knockout Gas6−/−) and heterozygous animals (Gas6+/−) in static cages. Animals were provided sunflower seeds and breeding huts for enrichment. Gas6−/− breeding pairs were given LoveMash™ (BioServ #S3823P) as a food supplement in order to support reproductive performance by aiding in male and female fertility, increase litter size, decrease newborn pup cannibalism, increase pup survival, and increase maternal milk production. For these studies, we also used C57BL/6 Gas6+/+ mice (Jackson Laboratories) to establish a WT breeding colony.

Mouse genotyping and colony maintenance

PCR was used to verify pup genotype. Mice 21 days old were tagged, and the tip of the tail was biopsied. Each tail tip was cut steriley and placed in a thin wall PCR tube (USA Scientific #1402-8120) with 50μL of tail lysis buffer supplemented with 1.5μL of Proteinase K (LifeTech #AM2548) administered to each tube immediately prior to digestion. The lysis buffer (50mM tris (Fisher BP1521) pH 8.8, 1mM EDTA (Fisher BP119500) pH 8.0, 0.5% Tween 20 (Sigma #P7949-500ml) in deionized water) was stored at room temperature. The tail, tail lysis buffer, proteinase K mixture was maintained at 55°C in a PCR
machine (Applied Biosystems 2720 Thermal). The tail digestion process was run overnight. After the overnight digestion (16-18 hours), the PCR machine cycles to 99°C for 10 minutes, and then to 4°C until tube removal. Next, the sample is centrifuged for 30 seconds using a bench-top QuickSpin to pellet large debris. The supernatant, containing the genomic DNA, is then transferred to a 1ml tube, diluted 1:3 by volume with deionized water, and mixed by flicking. The genomic DNA in suspension can then be genotyped or may be stored at 4°C. AmpliTaq Gold®360 Master (Invitrogen #4398881) is used to provide amplification during genotyping. Primers were acquired from IDT Integrated DNA Technologies as follows: mGas6 1s 5'- GAG TGC CGT GAT TCT GGT -3' (reference number 120733394), mGas6 2A 5’-CCA CTA AGG AAA CAA TAA CTG-3' (reference number #120733395), NEW 3 Gas6 5’-ATC TCT CGT GGG ATC ATT-3' (reference number #120733396). Primers were mixed with 12.5µL Amplitaq Gold and 11µL of deionized water and run on the PCR machine. PCR program for amplification: step 1 94°C for 3 minutes, step 2 94°C for 30 seconds, step 3 55°C for 1 minute, step 4 72°C for 2 minutes, repeat steps 2 through 4 for a total of 35 cycles, step 5 72°C for 7 minutes, and step 6 4°C for storage. The amplified DNA was then run on a 1% agarose gel made with 1X tris base Fisher #BP1521), acetic acid (Fisher A38500), EDTA (Fisher BP119500) (TAE), and 5µL of ethidium bromide. Samples were then loaded alongside a 1KB ladder (Invitrogen #10787-018) and the gel was run at 100V for 30-45 minutes. Gel was analyzed for visualization of two bands: one at 350 basepairs (Gas6+/−), and a second at 500 basepairs (Gas6+/+). Heterozygous animals would have both the 350
basepairs band and a band at 500 basepairs.

**Mouse euthanasia**

Animals were then euthanized by exposure via liquid inhalation of Fluriso™ (isoflurane from VetOne #NDC13985-046-40) or carbon dioxide and secondarily cervical dislocation was performed once respiration ceased. The animals’ mammary glands were harvested 2 hours post-injection as described below.

**Gland harvesting and staining**

The number four inguinal mammary gland was dissected from the animal and spread on paper portion of Parafilm (Fisher S37440). The gland was allowed to air dry for one minute and then placed in a cassette labeled with pencil that is Xylene-resistant. The now encased mammary gland was then submerged in a glass jar of 4% Paraformaldehyde (PFA) (Fisher Scientific AC416785000) for one hour on ice. For 4% PFA preparation, 450mL deionized water was covered and heated to 60°C, to which 20g of PFA was added. Sodium hydroxide (Fisher Scientific ss255-1) solution was added drop-wise until the PFA particles dissolved, while maintaining the solution at 60°C to prevent PFA breakdown. The solution was removed from the heat, 50mL of 10 X PBS was added, and adjusted to pH 7.2 with HCl. The solution was filtered through a 0.22µm pore filter and sterilized PFA was aliquoted and stored at -20°C until needed. Following one hour in PFA fixation, the mammary glands were transferred to Carmine Alum staining solution (1g carmine (sigma C1022), 2.5g aluminum potassium sulfate (Sigma A-717) in 500 ml of deionized water) overnight at room
temperature. After the overnight incubation, the gland was destained and dehydrated in sequential washes of 75%, 95% and 100% HistoPrep Ethanol (Fisher Scientific #HC8001gal). Next, the glands were placed in Xylene (Fisher Scientific #X4-4) for three total washes, 15 minutes each, and stored overnight. See Table 2 for a list of all mammary glands that were harvested and stained.

_Gland Imaging_

In order to visualize the structure of the mammary glands, whole mount images of mammary glands were taken using Leica M165FC stereoscope with Nikon DS-Fi2-U3 color camera. We captured multiple images at each magnification, ranging from 0.73x to 12x with a 10x objective eyepiece. Whole mammary glands were mounted in Permount™ (Fisher Scientific #SP15-100) mounting media and imaged within 24 hours of mounting. Permount was dissolved by submersion in Xylene and mammary glands were submerged in Xylene for long-term storage.

_Analysis of whole mount mammary glands_

Nikon NIS Elements Basic Research 4.30.1 imaging software was used with its corresponding analytical tools for measurements and examination of murine mammary glands. The number 4 inguinal mammary gland was used due to its structure (Figure 8); it has a centrally located lymph node that can be used as a reference point for ductal elongation. Table 2 demonstrates the tag numbers and counts for the Gas6^{-/-} and WT mammary glands we analyzed here in. NIS Elements has both tools for measuring distance and for conducting counts of
things like TEBs or side-branches. It is important that the relay was set to 0.6 in the NIS software and the correct magnification was selected so that accurate measurements could be taken. Images were saved as *.TIFF or *.ND2 files to ensure both high quality images and metadata associated with images were collected.

**Embedding and sectioning of mammary glands**

The Histology Core Facilities at Tulane University embedded mammary glands in paraffin wax blocks. The Histology Cores made tissue sections of animal’s mammary gland in each group of *Table 2* and because of lack of experience the majority of the mammary glands were destroyed or lost due to improper sectioning. So we purchased and can now use our lab microtome (Leica) to cut 0.5µm sections of tissue that were placed on glass slides (Thermo Scientific Super Frost Plus #US4951plus) and air-dried overnight at room temperature.

**Hematoxylin & eosin staining of sectioned tissue**

Mammary glands were stained by hematoxyline & eosin (H&E). Tissue sections went through the following process: step 1 was 3 consecutive washes in fresh xylene (Fisher X4-4) for 3 minutes each, step 2 was 3 consecutive washes in 100% HistoPrep ethanol (Fisher HC-800-1gal) for 3 minutes, step 4 was a wash in 95% HistoPrep ethanol in ethanol and deionized water for 3 minutes, step 5 was 80% HistoPrep ethanol in ethanol and deionized water for 3 minutes, step 6 was 75% HistoPrep ethanol in ethanol and deionized water for 3 minutes, step 7 was a deionized water wash for 3 minutes, step 8 was 5-30 dips in freshly
filtered hematoxylin (Poly Scientific S212A-1GL), step 9 was a quick rinse in deionized water, step 10 was a 5 minute wash in tap water, blotting, step 11 was acid ethanol (70% ethanol and water + 0.25% hydrochloric acid (Fisher A144500)) dip for 10 seconds, step 12 was tap water for 2 minutes, step 13 was distilled bottled water for 2 minutes, step 14 was a rinse in double deionized water for 2 minutes, step 15 was eosin (Poly Scientific S176-1GL) counterstain for 45 seconds, step 16 was a brief dipped into deionized water, step 17 was 95% HistoPrep ethanol for 10 dips in three separate containers for a total of 30 dips, step 18 was 10 dips in 100% Histoprep ethanol divided in 2 separate containers for a total of 20 dips, step 19 was a 2 minute incubation in 100% Histoprep ethanol, step 20 was when the slide undergoes two 5 minute incubations in xylene, and then incubated in fresh xylene (15 minutes or overnight). The tissue section was then mounted in Permount, a coverslip was added, and the slide was allowed to cure overnight at room temperature before imaging. For observations, hematoxylin stained the nuclei of cells blue, while eosin was the counterstain, staining eosinophilic structures red/pink.

**Real-time PCR for determining Gas6 and Axl expression**

The axillary (numbers 2 and 3) and inguinal (numbers 4 and 5) mammary glands were harvested. Only the number 4 inguinal mammary gland has a visible central lymph node that was removed prior to harvesting. Tissues were placed in phosphate-buffered saline (PBS) and kept on ice until processing. The tissue was manually macerated, resuspended in digestion media, 0.5ml antibiotic-antimycotic (Invitrogen #15240-062), 100mg collagenase (2mg/ml) (Sigma
#1108879300 in 50 ml DMEM/F12), and rocked at 200-250 rpm for 1 hour at 37°C to aid in tissue digestion. Following the 1 hour digestion, we were able to wash and pellet organoids by centrifugation at 600x g, and mammary epithelial cells (MECs) by resuspending in wash buffer (5% fetal bovine serum (FBS), 1% antibiotic-antimycotic (Invitrogen #15240-062), in DMEM/F12. The tissue was incubated at room temperature for 5 minutes and then chloroform (Sigma C2432-500ml) was added at a 1:5 chloroform:lysate ratio, shaken for 15 seconds, and then incubated for 3 minutes at room temperature. Next we spun samples at 12,000x g for 15 minutes at 4°C to cause a phase separation with the upper aqueous phase containing the cellular RNA. This was collected and transferred to a fresh tube. Isopropanol in an equal volume to chloroform added previously and 1 µL GlycoBlue™ (ThermoFisher AM9515) were added, the components were mixed by inversion, and incubated at room temperature for 10 minutes. At this time, the RNA pellet could be visualized due to the presence of glycolblue, so the supernatant was aspirated off and the pellet was washed with 75% Ethanol. The pellet was then resuspended by vortexing followed by centrifugation at 7,500x g for 5 minutes at 4°C. The supernatant was again removed and the pellet was allowed to air dry. The pellet was resuspended in RNase-free water (VWR 47743-720) and warmed to 55°C for 10 minutes. Finally, the RNA suspension was stored at -80°C. We examined the human cell lines for Gas6 and Axl expression as well, but that data is not included here. **Table 1** contains the primer set information for the real-time PCR conducted within this work. However, the mouse Gas6 primers information could not be located in the
lab we believe it was order by A. Daniels at Baylor University so IDT would not release the number or sequence for this primer set.

<table>
<thead>
<tr>
<th>Table 1 Primer Sets for Real-time PCR (qPCR)</th>
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<tr>
<td><strong>MOUSE</strong></td>
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<tr>
<td>Name Primer</td>
</tr>
<tr>
<td>18s rRNA For</td>
</tr>
<tr>
<td>18s rRNA Rev</td>
</tr>
<tr>
<td>mAxl For</td>
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<tr>
<td>mAxl Rev</td>
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<tr>
<td>mGADPH For</td>
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<tr>
<td>mGADPH Rev</td>
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</table>

**FACS analysis for mammary gland progenitor cell populations**

MECs were isolated as outlined above, from the number 2, 3, 4 and 5 mammary glands of age matched mice from the following animal groups: C57BL/6 Gas-/ and C57BL/6 Gas+/+. Pelleted organoids and MECs were washed with 15ml of PBS without metals to remove all serum. Cells were digested further with dilute trypsin-EDTA (0.05% Trypsin-EDTA (Life technologies #25200114) in DMEM/F12). Trypsin activity was then be neutralized with wash buffer, and cells passed through a 70μm filter into a 50ml conical tube with the total volume increased to 15ml. Cells were spun at 450 x g for 5 minutes to pellet the MECs, which were resuspended in HBSS. For these studies, we used a Mouse Epithelial Cell Enrichment Kit (Stem Cell Technology v1.1.0 #19758) and manufacturer’s instructions were followed for the enrichment of MECs. Primary antibodies BV711 rat IgG, e450 hm IgG, FITC rat IgG, PE rat IgG, CD24-BV711, CD29-e450, CD14-FITC, and c-Kit-PE) were added to
enriched cells for a 30 minute incubation, followed by a wash with HBSS+ (Invitrogen #14025-34) and spin at 3000rpm for 5 minutes. Pellets were then resuspended in 500µL HBSS+ and filtered using a 40µm filter with 1µL of Sytox Red into flow tubes. Facs Aria, operated by Dorota Wyczechowska at LSUHSC Cancer Center Immunology Core, was used to perform the analyses. Data were received in Diva6 software, and were later analyzed by Heather Machado using FlowJo software.

**Statistical data analysis**

Data sets were compared using student t-test method for determination of statistically significant differences. For each data set, mean and standard error of the mean (SEM) were reported. P-values of <0.05(*), p<0.01(**), or p<0.001 (***) indicate statistically significant differences between data sets and were represented as such. For this study, N=1 represents the average of the 2 mammary glands extracted from a single animal.
RESULTS

Mouse colony maintenance and collection

We encountered problems while breeding, cannibalism of the newborn pups, sudden unexpected death, or the loss of adult animals, resulting in low animal numbers for use in these studies. Table 2 outlines the animals that we were able to collect and were used in this analysis.

<table>
<thead>
<tr>
<th>Table 2 Tag Numbers of Animals Collected</th>
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<tr>
<td>At Development Time Points Not in Diestrus</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>C57BL/6 Gas6**/+</td>
</tr>
<tr>
<td>5 weeks</td>
</tr>
<tr>
<td>8 weeks</td>
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<tr>
<td>12 weeks</td>
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Gas6/Axl expression in mammary gland

In Figure 5 we utilized genomic data analyzed in the form of a microarray, compiled by Chad Chritton, that was from isolated whole mammary gland lysate. This lysate was collected at different points during development, pregnancy and involution. Figure 5 demonstrates that Gas6 expression changes during mammary gland development. Gas6 can be produced by a variety of cells, including mammary gland epithelium and macrophages that infiltrate the mammary gland during mammopoeisis. From the microarray (Figure 5) we can see that Gas6 expression peaks during early pregnancy and late involution.

We verified the expression of Axl and Gas6 in whole tissue of C57BL/6 mice by real-time PCR, and Figure 6 demonstrates that the WT animals do have endogenous Gas6, while the Gas6**/- mice do not. The results (Figure 6) also
demonstrate that the WT and the Gas6$^{-/-}$ both have endogenous Axl expression, while Gas6$^{-/-}$ has less Axl than WT mice.

**Gas6 alters mammary gland stem/progenitor cells by FACS**

Next, we investigated if there was a difference in the stem/progenitor cell population within the mouse mammary gland when comparing the WT mice to Gas6$^{-/-}$. The mammary gland progenitor cells give rise to all the cells needed for a functional mammary gland$^{57}$. We gated for live cells that were epithelial in origin and then we see that the basal cell population, that are CD24$^+$ and Cd29$^H$, are enriched for populations of mammary stem cells (MaSCs). Gas6$^{-/-}$ and WT animals had relatively the same number of MaSCs, we observed 25.1% in the WT and 21.3% in Gas6$^{-/-}$ mice (**Figure 7**). Then we selected the luminal cell population (CD24$^H$ CD29$^L$), whose population analysis revealed apical (luminal alveolar) progenitors (CD14$^+$ cKit$^+$) as well as luminal (luminal ductal) progenitors are CD14$^+$ cKit$^H$, and myeloid progenitor lineage is CD14$^-$ cKit$^-$. We observed a shift from luminal progenitors to alveolar progenitor cell that make-up the mammary gland in the Gas6$^{-/-}$ compared to WT, see **Figure 7**. We perceived a shift in Gas6$^{-/-}$ mice an increased alveolar progenitor cells from 6.32% in WT to 17.5% in Gas6$^{-/-}$. We also see a decreased luminal progenitor cell population, see **Figure 7**, from 23.2% in WT to 12.9% in Gas6$^{-/-}$.

**Gland imaging**

In **Figure 8**, we have pointed out the landmarks of interest in a 5 week old female mouse as seen with carmine staining: the nipple and primary duct,
mammary epithelium that stains a dark red, the centrally located lymph node, terminal end buds (TEBs) are the bulbs structures at the distal ends of the epithelium furthest from the nipple, the empty fat pad is the remaining area where the mammary gland will expand as the mouse ages.

**Gas6 alters ductal elongation**

In **Figure 9A** we demonstrate positive and negative ductal elongation, also called progression during ductal elongations. In **Figure 9B** we see the comparison of Gas6\(^{+/+}\) to Gas6\(^{-/-}\) at each of the time points, and how ductal elongation differs with the presence or absence of Gas6. **Figure 9C** graphically demonstrates that Gas6\(^{+/+}\) have more ductal elongation at 8 weeks of age when compared to Gas6\(^{-/-}\) mice. This difference in the progression of the epithelium is statistically significant at 8 weeks. We observed by 12 weeks of age the Gas6\(^{-/-}\) elongation catches up to the Gas6\(^{+/+}\) and there is no longer a significant difference between the two.

**Gas6 alters TEBs and side branching**

**Figure 10A** exemplifies how we were able to count TEBs in a gland. The number of TEBs was also different between the two groups. The Gas6\(^{+/+}\) had more TEBs at 5 weeks of age compared to Gas6\(^{-/-}\) mice (**Figure 10B, 10C**). The structure of the epithelium as it pertains to side-branching is examined, **Figure 11**. The amount of side branches was less in the 5 week old Gas6\(^{-/-}\) animals compared to the Gas6\(^{+/+}\) animals (**Figure 11**).

**Gas6 alters the amount of mammary gland epithelium**
Determining the amount of epithelium was challenging as our software did not facilitate exact quantification of the epithelial tissues with in mammary gland. We were able to super-imposed a grid 1000µm x 1000µm on top of the mammary gland seen in Figure 12A and then we manually counted the boxes that had epithelium (Figure 12C). In this way, we are able to compare Gas6−/− to Gas6 WT and observed that there was overall less epithelium in Gas6−/− mice. The 5 week old animals had similar amounts of epithelium in Gas6−/− compared to Gas6 WT, but the 8 week old Gas6 WT mice had more epithelium than the age-matched Gas6−/− animals, see Figure 12. We observed that both side branching and the amount of epithelium may be different between Gas6+/+ and Gas6−/−, but these results were in virgin female mice whose stage of estrus was unknown.

**Gas6 alters mammary gland histology**

In addition to examining the mammary gland as a whole mount, we sectioned tissue to look at the structural changes that may occur due to knockout of Gas6 that are visible only by cross-sectioning. The first thing we did with our limited amount of tissue collected was an H&E examination of the tissue structure. In Figure 13, we can see the Gas6+/+ mice had hollow lumens and the mammary tissue appears to have similar organization when compared to the WT, and nothing seemed to be remarkably different. However, we observed in our limited samples that the Gas6−/− animals seem to have a thicker adjacent stroma surrounding the epithelium (Figure 13).
**Gas6 alters mammary gland involution**

During this research period we harvested animal number 453, a Gas6-/- multiparous female that was approximately 11 months old, with a date of birth 09/27/2013 and a sacrifice date of 08/11/2014. We compared the Gas6-/- female WT multiparous mammary gland from animal number 244 a females that was 5 months old, a date of birth of 02/15/2014 and sacrificed on 07/16/2014, see Figure 14. We can observed differences in duct dilatation, that may be due to the 6 month discrepancy in age between the animals. It appears from the records the females were into the process of involution. It is also unclear if the females were able to fully lactating prior to the start of involution. Both of these issues are important if we want to speculate that Gas6 alters involution. In Figure 14 we can observe other differences such as less alveologenesis which would be produce milk for suckling pups, but because of the above mentioned lack of information we can not assume the differences are solely due to Gas6.
Figure 5 Gas6 expression fluctuates during development and pregnancy

Our lab uses this microarray, courtesy of Chad Chritton, of whole mammary gland lysate shows that genomic expression of Gas6 expression temporally over the course of development and following pregnancy, with the lowest expression occurring during lactation.
Figure 6 Gas6$^{-/-}$ mammary gland tissue has decreased Axl and Gas6 expression

C57BL/6 Gas6$^{-/-}$ mice have lower expression of Axl and Gas6 in whole mammary gland lysate following isolation of Mammary Epithelial Cells (MECs) as compared to C57BL/6 Gas6 WT mice, determined by real-time PCR. Results are presented as a fold change when the WT is set to 1.
C57BL/6 Gas6−/− mice had decreased the luminal progenitor populations as compared to WT mice by FACS analysis. The C57BL/6 Gas6−/− mice show an increase in alveolar progenitor and a decrease in luminal progenitor cells compared to WT.
Landmarks within the female mouse inguinal number four mammary gland at 5 weeks of age show the nipple and primary duct present at the origin, where the mammary gland expansion begins. The branching mammary gland duct's epithelium grows through the bulbous terminal end buds (TEBs), through the empty fat pad away from the nipple, and past the centrally-located lymph node in a sprawling branched, yet directional, tree-like morphology. All scale bars represent 1000µm.
Figure 9 Gas6−/− have less ductal elongation

(A) Demonstrates how we measure ductal elongation in the number 4 inguinal mammary gland using the lymph node as a point-of-reference, using 5wk Gas6−/− mice (B) C57BL/6 Gas6−/− mice show delayed elongation at 5 weeks and 8 weeks of age compared to WT mice. At 12 weeks, this difference is no longer present. (C) Average micrometers of progression relative to the lymph node shows C57BL/6 Gas6−/− mice have significantly less ductal elongation at 8 weeks compared to WT animals. For the 5 week WT we had N=5, 8 week WT N=5, 5 week Gas6−/− N=2 and 8 week Gas6−/− N=3. *p<0.05 by Standard Student t-test. All scale bars represent 1000µm.
Figure 10 Gas6⁻/⁻ TEB numbers is different from Gas6⁺/+ 

(A) This demonstrates how we identify TEBs in the number four inguinal mammary gland using the lymph node, using 5 week Gas6⁻/⁻ mice as an example. (B) Whole mounts of C57BL/6 Gas6⁺/+ compared to C57BL/6 Gas6⁻/⁻ at 5, 8 and 12 weeks. The encircled area contains TEBs and is investigated at higher magnifications to confirm (C) C57BL/6 Gas6⁺/+ mice have fewer TEBs at 5 weeks of age compared to C57BL/6 Gas6 WT. At 8 weeks C57BL/6 Gas6 WT animals TEBs decrease, compared to 5 week old Gas6 WT, while C57BL/6 Gas6⁻/⁻ have more TEBs at 8 weeks similar to the numbers of TEBs present at 5 weeks in C57BL/6 Gas6⁻/⁻. For the 5 week WT we had N=5, 8 week WT N=5, 5 week Gas6⁻/⁻ N=2 and 8 week Gas6⁻/⁻ N=3. *p<0.05 by Standard Student t-test All scale bars represent 1000µm.
Figure 11 Decrease in Gas6−/− side-branching at 8 weeks

(A) Whole mounted mammary gland viewed on at Stereoscope at 8X magnification shows side-branching at node, branch points, which were all counted manually. (B) Whole mount comparisons of Gas6+/+ versus Gas6−/− at 5 weeks, 8 weeks, and 12 weeks show WT mice exhibit greater average side branching 8 weeks as compared to Gas6−/− mice. (C) The average number of side branches was fewer in the Gas6−/− mice at 5 and 8 weeks. For the 5 week WT we had N=5, 8 week WT N=5, 5 week Gas6−/− N=2 and 8 week Gas6−/− N=3. *p<0.05 by Standard Student t-test. All scale bars represent 1000µm.
Figure 12 Gas6^{+/+} has more mammary epithelium

(A) Demonstrates how a 1000µm x 1000µm grid was superimposed over a whole mounted mammary gland, centered at the radius of the lymph node, and grids with epithelium were manually counted (B) Whole mount comparison of Gas6^{+/+} versus Gas6^{-/-} at 5 weeks, 8 weeks, and 12 weeks with increasing amounts of epithelium as time progresses. (C) Gas6 WT animals have more epithelium than the Gas6^{-/-} at 5 weeks and 8 weeks of age. For the 5 week WT we had N=5, 8 week WT N=5, 5 week Gas6^{+/+} N=2 and 8 week Gas6^{+/+} N=3. All scale bars represent 1000µm.
Figure 13 Gas6$^{-/-}$ have thicker adjacent stroma

Hematoxylin and eosin staining (H&E staining) of mammary gland cross-sections of 5 week old and 8 week old Gas6$^{+/-}$ and Gas6$^{-/-}$ female mice shows the dark blue/purple epithelium and the pink/red stroma. The hollow lumen is evident along with ducts made of a layer of single cells, the luminal cells. The structure of the lumen appears to be unremarkable by H&E, however, the appearance of the thick stroma adjacent to the ducts in the Gas6$^{-/-}$ sections compared to the WT animals. Image scale bars represent 100µm.
Figure 14 Gas6 alters involuting mammary glands

Image of carmine stained whole mount at 4x, with an inset of the whole mammary gland. Here, we have a comparison of involuting, meaning females were sacrificed within days of a litter being born or weaned, mammary gland from C57BL/6 Gas6^{+/+} versus C57BL/6 Gas6^{−/−} mice. These are not aged match, but there appears to be less alveologenesis in the C57BL/6 Gas6^{−/−} and the ducts appeared to be dilated. All scale bars represent 1000µm.
DISCUSSION

Our first specific aim was to establish how this signaling pathway alters the developmental processes in normal mammary glands. Here we study Gas6 in mammopoeisis and how it influences proliferation, invasion, apoptosis, and the clearing of apoptotic cells during epithelial regression during the estrus cycle. During puberty, the ovaries secrete estrogen and progesterone hormones into the endocrine system. Estrogen’s role during puberty is to stimulate ductal elongation and bifurcation of the mammary gland. One contributing factor of this process of elongation is the proliferation of the mammary gland epithelium. This proliferation is necessary for epithelial invasion of the TEBs and degradation of the fatty stroma of the mammary gland. Meanwhile, the progesterone secreted by the ovaries during estrous cycles induces sidebranching. The combination of these circulating factors during puberty causes the rudimentary ductal tree present at birth to expand into a functional mammary gland. Formation of a functional mammary gland also requires apoptosis for the creation of the hollow continuous lumen and the clearing of cells during TEB invasion.

Our first mayor hurdle was to establish a successful Gas6-/- mouse colony. We found the mating pairs to be poor breeder that produced limited amounts of offspring that we could use in our studies. The animals we did collect had their genotype verified by PCR using allele specific primer set on genomic DNA from tail biopsy’s. Using real-time PCR, we were able to confirm that the WT mice expressed the receptor, AXL, and the ligand, Gas6, whereas Gas6-/- animals did
not express Gas6. This is important because we are able to confirm the affect of Gas6 in the mammary gland. However, the Gas6<sup>−/−</sup> was a troublesome animal colony, so it took over 2 years to collect enough females to start our assessments. We encountered problems with breeding, cannibalism of the newborn pups, sudden unexpected death, or the loss of adult animals. **Table 2** outlines the animals that were collected and used in this analysis. Because it took so long to acquire these animals and we had so much trouble breeding, we have a limited number of specimens. Initially, we were unaware that side-branching may be a phenotype of Gas6<sup>−/−</sup> so we did not originally estrus stage the animals and none of the animals in Table 2 were staged.

Once we learned of the effect of the estrus stage, we collected vaginal smears to visualize the stage of estrus in all subsequently collected female mice. Without estrus staging for the animals in **Table 2**, we cannot currently state that side-branching or the amount of epithelium seen are resulting as a phenomena of Gas6 expression. Also, these images for side-branching were not of independent fields of view. Instead, some of the images that were examined for side-branching were slightly overlapping and this could have caused the double counting of some branching nodes. In future studies, the appraisal should be undertaken when there are a greater number of samples and more independent fields of view can be counted.

We believe that the Gas signaling pathway will alter the process of mammary development due to its potential effects on epithelial proliferation and invasion, and apoptotic cellular clearance in vivo. To further this aim, we
compared normal mammary gland developmental stages in Gas6−/− to WT mice by observing changes occurring during typical murine maturation at: 5 week, 8 weeks, and 12 week old mice. By harvesting the inguinal mammary glands and determining the correlated vaginal epithelial composition from Gas6−/− mice at each of these time points, we may compare to age-matched WT mice and determine the role of Gas6 in early mammary development.

Utilizing this mouse model, we were able to observe limited epithelial progression, also known as ductal elongation, in Gas6−/− animals early in development as compared to WT animals (Figure 9). While this may be attributed to Gas6 absence leading to decreased proliferation of epithelial cells, this observation may also represent the shift from luminal ductal progenitor cells to epithelial ductal and alveolar populations, as seen by FACS in Figure 7. The FACS (Figure 7) was performed using 6 week old animals, which may explain a delay in ductal elongations that is later seen in the whole mounted mammary gland samples. Due to the limited number of Gas6−/− female pups, this particular experiment was not repeated to verify significance. When the animal colony is more productive in pup production, FACS should be repeated in order to determine the influence Gas6−/− has on MaSC populations to be luminal or alveolar progenitors (Figure 7). MaSCs in the mammary gland that are able to recapitulate a new mammary gland upon serial transplantation57,58. Conducting limiting dilution transplants of Gas6−/− mammary epithelial cells may be necessary to see the resulting effects of the shift in stem/progenitors cell populations. An increase in luminal progenitors has been associated with preneoplastic tissue
and tumors compared to normal mammary gland tissue\textsuperscript{59}. While alveolar progenitors are responsible for milk production during pregnancy\textsuperscript{57,60}.

Ductal morphogenesis, the progression of the terminal end buds driving through the stroma away from the nipple, was delayed in Gas6\textsuperscript{-/-} mammary glands (Figure 10). Similarly, during puberty, when the gland is expanding to fill the stroma, we observed Gas6\textsuperscript{-/-} has fewer total numbers of TEBs compared to WT mice. However, the Gas6\textsuperscript{-/-} has more TEBs that persisted into maturity, at a time when they should be undergoing apoptosis and reverting to terminal ducts. TEBs are essential for proper mammary gland formation and without a functional TEB, the cap cells and zone of apoptosis would not function appropriately and the subsequent hollow lumen would not form. The combination of delayed ductal elongation and TEB number in Gas6\textsuperscript{-/-} animals could account for the reduced amount of epithelium (Figure 12) we observed in these mice. It remains unknown if Gas6 is altering the ability of the TEBs to bifurcate, or if it is limiting the ability to TEBs to break down stroma of the mammary gland. TEBs typically persist until the mammary gland empty fat pad is filled, which could account for the increased number of TEBs at the 8 week time point in the Gas6\textsuperscript{-/-} mice. We would expect the number of TEBs to be declining by 8 weeks of age so this persistence in the Gas6\textsuperscript{-/-} may be due to the delay in ductal elongation. It appears that Gas6 expression may be dependent on the stage of development.

We can speculate from this data that Gas6 may slow the capacity of epithelial cells to proliferate \textit{in vivo} and cause the delay in ductal morphogenesis. To determine if proliferation is altered by Gas6 deficiency in mice, Ki67 can be
visualized by immunohistochemistry (IHC) or immunofluorescence (IF) when we have more embedded tissue samples. Ki67-positive cells are proliferating while Ki67-negative cells are not proliferating, and we would anticipate that Ki67 would be diminished in Gas6\(^{-/-}\) animals indicating a discrepancy in proliferation. We could also stain for matrix metalloproteinase in tissue sections to see if the stroma is being broken down, indicating whether Gas6\(^{-/-}\) TEBs are able to efficiently degrade the stroma and limit the forward progression of the mammary gland ducts in vivo.

Using the sectioned and embedded mammary glands, we were able to observe the normal hollow lumen and single cell ductal structures in the cross-sections, but the adjacent stroma proximal to the epithelium was abnormally thicker in Gas6\(^{-/-}\) animals. After loosing valuable tissue sections due to technicians unskilled in sectioning mammary glands, the Machado lab acquired our own microtome to allow for better sectioning of the fragile and limited tissue. We have the ability to stain sections of tissue with trichrome staining kit (Sigma: HT15-1kit, HT1079-1set, HT10132-1L) to test the adjacent stroma for collagen in Gas6\(^{-/-}\) animals, which may represent a new phenotype. Similarly, Gas6\(^{-/-}\) 8 week old animals had fewer side-branches (Figure 11). With knowledge that each stage of estrus changes the amount of secondary and tertiary branching, we chose the longest phase of estrus, Diestrus, to collect all subsequent animals following this study. Without knowing the estrus staging for the animals in Table 2, we cannot accurately state that side-branching or the proliferation of epithelium occurs as a phenomenon of Gas6 expression and future experiments
should contain estrus staged mice.

Upon assessment of involuting mammary glands from both Gas6$^{-/-}$ and Gas6$^{+/+}$, we found that the mammary glands may have a different phenotype in Gas6$^{-/-}$ animals versus those in WT (Figure 14). However, while these particular samples were not age matched so this observation could lead to a more in-depth assessment of pregnancy, lactation, and involution. These may be phenotypes of Gas6$^{-/-}$, but until we are able to collect age-matched cohort this remains speculation. The generalized observations that Gas6$^{-/-}$ may have a involution phenotype compared to WT animals (Figure 14) could be correlated to Gas6 since we see in Figure 5 the dynamic changes in the level of Gas6 during involution is apparent.

Breeding, harvesting, and backcrossing this colony, C57BL/6 Gas6$^{-/-}$, into Balb/c was a time-intensive task that was critical for future examination of Gas6/AXL signaling axis studies at Tulane. The Balb/c Gas6$^{-/-}$ animals will also be relevant for transplant studies to examine DCIS in vivo, because the two transplantable premalignant lesion-forming lineages, PN1a and PN1b, are both Balb/c derived. Transplant experiments would be able to determine DCIS-like lesion progression in vivo, which will be important in determining whether stromal Gas6 may be the trigger that spurs microinvasion during DCIS.

In summary, differences in Gas6 expression may correlate to phases in ductal morphogenesis during puberty. As the mammary gland develops postnatally, we expect to see a fluctuation in the amount of Axl and Gas6 during the different stages. Predictably, Gas6 will be lowest while the mammary gland is
quiescent but, upon onset of puberty and the initiation of ductal elongation, we expect to see a sharp increase in Gas6 and Gas6 signaling by TAMRs. We would expect that during ductal elongation, Gas6 would remain elevated as compared to levels in the newborn pup or pre-pubertal mammary gland. We may also observe oscillation in either Gas6 and or TAMR signaling during estrus cycling due to the expansion and regression of side-branches. Finally, we would hypothesize that Gas6 levels would decrease slightly and plateau upon reaching adulthood, because mammary gland remodeling is not as dynamic at this time-point as it is during puberty. We do not expect Gas6 to be maintained at equal levels because Gas6 is an exogenous protein that can be secreted by epithelium and infiltrating cells, such as macrophages, that enter the stroma during development and ductal morphogenesis to aid in the clearance of apoptotic cells.
CHAPTER 3: GAS6 REGULATION OF PREMALIGNANT BREAST CANCER CELLS

**Specific Aim 2** Examines the roles of Gas6 as a regulator of various biological functions in vitro including: proliferation, migration, motility, 3D morphogenesis, invasion, and survival

**INTRODUCTION**

Here, the Gas6/Axl pathway will be studied in vitro in a group of breast cancer cell lines that range from normal to malignant. We will determine whether the Gas6/Axl pathway is a regulator of cellular biological functions including proliferation, migration, motility, 3D morphogenesis, invasion and survival. The cell lines we use to investigate the subsequent signaling pathway include MCF10A, MCF10AT, MCF10DCIS.com human mammary epithelial cell lines that express Axl. We selected this cell line series because if was human cell lines that were progressively generated and have different phenotypes when transplanted in vivo that can be associated with the progression breast cancer. MCF10A cells are normal epithelial cells, MCF10AT cells are premalignant due to a H-Ras mutation, and MCF10DCIS.com have a malignant phenotype. The signaling pathway is also studied through the addition of exogenous recombinant Gas6. Additionally, we knock down Axl expression by stable transduction of MCF10AT
cells line with pLKO.1-shAXL or the control –shLUC that were selected for with puromycin and Axl knock down con confirmed by Dr Zhang via western blot. To determine if the cellular functions of proliferation, migration, motility, 3D morphogenesis, invasion and survival are altered when Axl cannot be stimulated by Gas6.

Our lab has attained preliminary data, by using an in vivo transplantable premalignant breast cancer lesion mouse model, that suggests that Gas6/Axl plays a role in breast cancer premalignant progression. A comparison of lesions with high tumor-forming potential demonstrated an increase in expression of Gas6 compared to lesions with low tumor-forming potential (data not shown). This would indicate that Gas6 might play a role in the tumor formation. Other upregulated factors in high tumor-forming transplanted cells were pro-inflammatory cytokines.

Upregulation and activation of Gas6/Axl can have tissue specific effects; in breast cancer Axl is: associated with poor prognosis, has been implicated as a regulator of metastasis and plays a role in resistance to chemotherapy. Axl is upregulated in a transplantable premalignant lesion mouse model that we use in lab. Axl expression has also been correlated to poor patient prognosis and can be correlated to tumor stage. However, the role of Axl in pre-invasive cancer, Stage 0 cancer, is unknown.

Similarly, supporting current literature revealed Axl expression in prostate cancer was correlated with reduced invasion and proliferation due to increased cell survival. Confirming this inverse relationship with DCIS progression, this
same study demonstrated that Gas6 production by a second subset of cells within the same niche decreased prostate cancer cell proliferation and was anti-apoptotic\textsuperscript{65}. In contrast, a publication by Loges \textit{et al.} shows macrophages to be a source of Gas6 that was tumorigenic\textsuperscript{66}. Overexpression of Gas6 has been investigated in osteosarcoma, glioblastoma, ovarian cancer, and prostate cancer and is associated with poor patient outcome\textsuperscript{65}.

The previously discussed studies of Gas6 function \textit{in vivo}, seen in Chapter 2, combined with our preliminary studies, provided the support that Gas6 is present along with its ligand and may be signaling to influence breast cancer progression. Because of the role of Axl in metastasis, invasion and progression, we considered that Gas6 may be providing a protective role and altering the cells ability to proliferate, migrate, invade, or survive. Here, we examine these functional pathways with respect to Gas6 expression. \textbf{We hypothesize that Gas6 regulates biological processes including: proliferation, invasion, migration, and cell survival \textit{in vitro} of human mammary epithelial cells.} We expect that these same cellular functions will be deregulated via the pressure and influence of Gas6/Axl signaling to promote breast cancer progression.

We start our examination of these biologically significant pathways by looking at proliferation and saw Gas6 had no effect \textit{in vitro}. Downstream effects via Gas6/Axl signaling pathway showed AKT phosphorylation increased, FAK phosphorylation increased, STAT3 phosphorylation decreased, NFkB phosphorylation was unchanged, and both MMP-9 and Snail decreased with
Gas6 treatment. Gas6 did not alter cell migration or 3D morphogenesis in vitro. Following these initial experiments, we determine that Gas6 does influence invasion. Finally, it appears that Gas6 does not alter survival of cells but the presence of the Axl receptor does increase cell survival.

There is existing evidence that Gas6 can be derived from not only epithelial cells but also the stroma and infiltrating innate immune cells like macrophages\textsuperscript{67}, so we wanted to vary the amount of exogenous Gas6 we used in vitro. Optimizing the amount of Gas6 we used for each cell line was critical, in addition to the consideration of other growth factors present in the media. We worked with both human and mouse cell lines in order to find a reproducible effect while conducting in vitro experiments. We focused our efforts using human cell lines that included MCF10A, which are normal epithelial cells isolated from a patient, MCF10AT cells, that are premalignant, and MCF10DCIS.com cells, which are a malignant cell line\textsuperscript{4}. We used these cell lines to represent a range of phenotypes from normal to malignant cell types. These allowed us to determine the role of Gas6 in proliferation, migration, motility, invasion, and survival to establish if these functions are altered in the presence or absence of Gas6.
METHODS

Cells and growth conditions

Human cell lines used in this research include: MCF10A, MCF10AT, MCF10AT-shLUC, MCF10AT-shAXL, MCF10DCIS.com. Human breast epithelial cell line MCF10A (benign) are spontaneously immortalized breast epithelial cells acquired from a patient with fibrocystic breast disease\(^68\). The premalignant MCF10AT cell line was generated by transfecting MCF10A cells with mutated T\(_{24}\) Ha-Ras gene that controls cell division\(^69\). Both MCF10A and MCF10AT complete media conditions include: 5% horse serum (Invitrogen #15630081), epidermal growth factor EGF 20ng/ml (Chemicon #EA140), Cholera Toxin at 100ng/ml (Sigma #C-8052), hydrocortisone at 0.5ug/ml (Sigma#H-4001), antibiotic-antimycotic 1% (Invitrogen #15240-062) in 500mL DMEM/F12 (Invitrogen #11330-057). Passage of cells was done using 0.25% trypsin/EDTA (life tech #25200114). MCF10DCIS.com cell line was derived from MCF10AT premalignant xenograft\(^70\). The complete media used to maintain MCF10DCIS.com media was 5% horse serum (Invitrogen #15630081), antibiotic-antimycotic 1% (Invitrogen #15240-062) in DMEM/F12 (Invitrogen #11330-057). MCF10DCIS.com cells were passaged using trypsin-EDTA (0.25%) (Life Technologies #25200114) to release adherent cells. The trypsin was neutralized by adding an equal volume of media with 5% serum and the cells were pelleted using a Eppendorf centrifuge 5810 R at 1000rpm. Serum starvation media is the corresponding cell line’s complete media minus any serum additives. Basal
media is DMEM/F12 for all cell lines. All cells were incubated at 37°C and 5% CO₂.

**Recombinant Gas6**

Human cells were treated with recombinant Human Gas6 (R&D Systems #885-GSB-050). The Gas6 was resuspended in sterile tissue culture water (Sigma W3500) then aliquoted and frozen at -80°C. The stock solution was stored at a concentration of 100µg/ml or 500µg/ml and diluted further prior to experimentation.

**Axl knockout cell line**

Stable cell lines were derived by Sheng Zhang in the Machado Lab. These stable transduced cell lines included MCF10AT-shLUC (control) and an Axl deletion line, MCF10AT-shAXL. Both cell lines were grown in complete media that includes: 5% horse serum (Invitrogen #15630081), EGF 20ng/ml (Chemicon #EA140), Cholera Toxin at 100ng/ml (Sigma #C-8052), hydrocortisone at 0.5µg/ml (Sigma#H-4001), antibiotic-antimycotic 1% (Invitrogen #15240-062) in DMEM/F12 (Invitrogen #11330-057) with 1:1000 Puromycin (Sigma P883). Passage of cells was done using 0.25% trypsin/EDTA (life tech #25200114). All cells were incubated at 37°C and 5% CO₂

**MCF10DCIS.com Gas6 treatment and Western blot**

MCF10DCIS.com cells were seeded 1.5 million in a 6cm tissue culture dish in 3ml of complete media. After 24 hours, the cell density was assessed. Once the cell density was found to be 60-70% confluent, the cells were washed
with sterile Dulbecco’s Phosphate-Buffered Saline (PBS) (ThermoFisher #14190250) and 3ml of serum starvation media (DMEM/F12 and 1% antibiotic-antimycotic) was added for a further 24 hour incubation. The next day, cells were stimulated with 500ng/ml of Gas6 in serum starvation media. Cell lysate was collected at delineated time points using ice cold RIPA. The 1X RIPA buffer was made in 10ml batches, aliquoted in 1ml vials, and frozen at -20°C. The RIPA buffer is 8.1ml deionized water, 0.05g sodium deoxycholate (DOC Sigma #D6750-25g) 300µL of 5M sodium chloride (Fisher #AC424290010) 1ml of 10% NP-40 (igapel Sigma #8896-100ml), 500µL 1M Tris (Fisher #BP1521) pH 7.5, one PhosSTOP inhibitor cocktail tablet (Roche #4906837001), and one Complete Ultra tablet (Roche #5892970001). After the dish was washed twice with ice cold PBS, RIPA was added. Dishes were manually scraped with a cell lifter and the mixture was passed through a 26G needle to mechanically sheer the cells. The collected supernatant was rocked at 4°C for 10 minutes and the cellular debris was pelleted by centrifugation (14,000 rpm, Eppendorf centrifuge 5418 R); the resulting supernatant was transferred to a fresh tube. 10µL was used to determine cell protein concentration using Pierce™ BCA Protein Assay Kit (ThermoFisher #23225) according to kit instructions. We loaded 60µg of protein lysate on a 4-12% gel (ThermoFisher #EC6038BOX). The volume that was equivalent to 60µg of protein was mixed with deionized water, 4X dye (100mM Tris pH 6.8, 400nM DL-dithiothreitol (DTT) Sigma#43815-5G, 8% sodium dodecyl sulfate (SDS) (Sigma #L3771-100G), 0.04% Bromophenol Blue BPB (Sigma #B0126-25G), 40% glycerol (Sigma #G5516-1L), and DTT (1.5g of
DTT in 10ml of deionized water). 4xDye and DTT are mixed so that the final concentration of DTT is 0.4M and water so that the total volume was 64µL. This was heated at 100°C for 5 minutes, then cooled on ice before 55µL was loaded into each well of the gel. We also loaded Precision Plus Protein WesternC™ ladder (BioRad #161-0376) in a single well of each gel. The gel was run in invitrogen Novex Mini-Cell XCell SureLock™ Electrophresis Cell apparatus running buffer (14.0g glycine (Sigma G8898-1kg), 3.0g Tris-Base (Fisher BP1521), 1.0g SDS (Sigma #L3771-100G)) and deionized water was added to increase the total volume to 1 liter. The gel was run at 140V for 15 minutes, and then the voltage was lowered to 110V for 1 hour 35 minutes at room temperature using invitrogen Novex Mini-Cell XCell SureLock™ Electrophresis Cell. While the gel ran, we made transfer buffer: 2.8g glycine (Fisher #BP3811), 6.06g Tris-Base (Fisher BP1521), 400ml methanol (Fisher #AC423240010) and the remaining volume to bring the mixture to 2L was deionized water. We used Invitrolon PVDF filter paper sandwich membrane (0.45µm pore size, ThermoFisher LC2005) and activated them for 30 seconds in methanol, followed by a 5 minute soak in transfer buffer. The gel was removed from its cassette and we used BioRad Mini Trans-Blot® Cell PROTEAN® Tetra System apparatus according to manufacturer's instructions to set up wet transfer. The transfer was run at 100V for 1 hour 20 minutes. We used Ponceau S (Sigma P3504-10G) for 5 minutes on a rocker and took an image to verify protein transfer by visualization. This was followed by submersion in deionized water to destain the gel. Next, we blocked the membrane with 5% milk (Nestle Carnation non-fat dry
milk from Amazon.com) in Tris buffered saline with 0.1% Tween (TBST). We made a 10x solution of Tris-buffered saline (TBS) 24.2g tris-base (Fisher BP1521), 80.0g Sodium Chloride NaCl  (Fisher 42429-0050) and then used deionized water to make the volume 1 liter adjusted the pH to 7.6 with hydrochloric acid using the Han lab Mettler Toledo SevenEasy pH meter. We took 100ml of 10X TBS and added 900ml of deionized water and 1ml of Tween 20 (Sigma #P7949-500ml) and mixed aggressively. The 5% milk in TBST rocked for 1 hour at room temperature. The membrane was washed for 3 quick rinses with TBST, followed by one 15 minute TBST incubation on a rocker, then 2 separate 5 minute incubations on at rocker at room temperature. The membrane was then ready to be probed with 5ml of primary antibodies (see TABLE 3) rocking overnight at 4°C.

After incubation with the primary antibody, the membrane was washed following the above scheme and 5ml of secondary antibody with 1:10,000 Precision Protein StrepTactin-HRP (Horseradish peroxidase) conjugate BioRad#1610380 was added to visualize the ladder (see TABLE 3). The HRP conjugated secondary antibodies were visualized using the kit Pierce ECL Western Blot Substrate (ThermoFisher #32106). If the signal was too weak, we used GE Healthcare Amersham ECL Select Western Blot detection Reagent kit (Sigma #RPN2235) as directed. The chemiluminescent signal was developed in a dark room and membranes were exposed to film (CL-X Posure Film 8x10 Thermo#34093) for a time that correlated to the strength of the signal. When necessary, we probed the membrane with another primary antibody and we
stripped the membrane using LifeTech #21059 Western Blot Stripper kit as directed, washed the stripped membrane with the same scheme as above, and began again at the blocking step above.

**Table 3 Antibodies and Conditions Used in Western Blots**

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<td>In with Secondary</td>
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**CST= Cell Signaling Technology; BSA=bovine serum albumin; Ab= Antibody**

**Cell culture conditions for Axl inhibitor, R428**

MCF10DCIS.com cells were grown in 6cm tissue culture dishes until they were 70% confluent, about 24 hours for 1.5 million cells. Day 2, the media was aspirated and cells were washed with PBS. We then added 3ml of serum starvation media was added for a 24 hour incubation. The cells were washed and a dose’s of R428 form 0.1µM to 30µM (Apexbio A8329) in serum starvation media was added to cells, which were pre-incubated for 1 hour. R428 is a
peptide that binds to the intracellular portion of Axl and inhibit autophosphorylation of the Axl dimer, diminishing kinase activity and this should prevent signaling. Next, MCF10DCIS.com cells were stimulated with Gas6 and cell lysate was collected by RIPA, as described above, for western blotting. Cell lysate was collected at 20 minutes and 60 minutes after treatment with Gas6.

**Isolation of RNA**

Cellular RNA was isolated and collected with TRizol ® (Ambion™ #15596026). RNA was purified using DNase I buffer (Ambion #8169G2). rDNase I (Ambion #2224G) was added to the RNA, mixed gently, and incubated at 37°C for 20-30 minutes. Next, DNase inactivation reagent (Ambion #8174G) was added and mixed by vortex, then incubated at room temperature for 2 minutes. Finally the solution was centrifuged at 10,000 x g for 1 minute, and the supernatant, which contained the RNA, was transferred to a fresh tube. The RNA’s purity was examined by Thermo Scientific Nanodrop2000 spectrophotometer (software NanoDrop 8000 Version 2.2.0 by Thermo Scientific). Isolated RNA concentration is determined using the Thermo Scientific NanoDrop 2000. Then a known amount of RNA is converted to cDNA using iScript cDNA Synthesis Kit (Bio-Rad # 170-8891). The settings for the PCR machine are as follows: 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C and then hold at 4°C for infinity. The resulting cDNA is stored at -80°C.
Real time PCR

cDNA and primer sets from **Table 1** were used to make a master mix with kit iQ SYBR GRN (BioRad 1708891) and nuclease free water. This is added to wells of a 96 well plate (BioRad plate 2239441) then 1µL of sample cDNA is added, and the plate is sealed (MSB1001). The plate is then read on a BioRad CFx96™ Real-Time System with C1000Touch™ Thermal Cycler using PCR program 95°C for 3minutes | 96°C for 1.15 seconds then 60°C for 1 minute and image| repeated fro 39 cycles then 60°C for 0.05 seconds and 95°C image and then 4°C for infinity.

Proliferation Assay using MTS

Cells were seeded in a 96-well plate at an optimized cell density so that cells are 50 percent confluent at 24 hours post-seeding in 100µL of media. Cells were serum starved for 24 hours. Depending on the cell line and dose treatment, media with Gas6 (R&D systems #885-GSB-050) was added. After confirming the cell confluence by microscopy, we added 20µL of CellTiter96®Aqueous One Solution (Promega # G3581) to each well. The plate was incubated at 37°C 5% CO₂ for 2 hours. The number of live viable cells per well can be correlated to the amount of color change or absorbance, as read using a plate reader in the lab of Dr. Hua Lu (Molecular Devices SpectraMax M5° running SOFTMAX® PRO SOFTWARE Version 5 for Windows®). The absorbance is read at 490nm

Proliferation Assay by MTT

Cells are seeded in at 96-well plate at an optimized density so that at 24 hours cells are 50 percent confluent. Cells were serum starved for 24 hours.
Then, depending on the cell line and dose treatment, media with Gas6 was added. MTT Reagent (Sigma# M2128 or Invitrogen M6494) was diluted down to 1mg/ml in DMEM without phenol red (ThermoFisher #11039-021). Next, we aspirated, added 100µL DMEM/MTT mixture, incubated with shaking for 5 minutes, and incubated for 3 hours at 30°C with 5% CO₂. Following the incubation, we aspirated off the DMEM/MTT, added 100µL of Dimethyl Sulfoxide DMSO (Fisher #BP231-100), mixed by pipetting, incubated for 5 minutes, then read absorbance at 562nm on a plate reader (FLUOstar OPTIMA with BMG Labtech Software 2.20R2, Firmware 1-26, Serial 413-0890).

**Migration scratch assay**

A confluent monolayer of MCF10A, MCF10AT or MCF10DCIS.com cells were grown in a well of a 24 well plate. Depending on the experiment, the cells were serum starved. Serum starvation required the aspiration of the media, washing with PBS, and addition of media without serum for 24 hours at 37°C. A wound, or scratch, was made using the tip of a p-200 pipet. The media was aspirated and the well was washed with PBS to remove any cell debris. Media with or without treatment was added and the wound was gauged over a time-course with a minimum of 3 images taken per well. We imaged the scratch assay using several types of microscopes, such as the ThermoFisher EVOS FL Cell Imaging System (borrowed with permission for Dr. Hua Lu lab) or the inverted Nikon Eclipse Ti microscope. The captured images were then opened in ImageJ software 1.48v [http://imagej.nih.gov/ij](http://imagej.nih.gov/ij) and the area of the wound was measured and compared across treatment groups over time. The “change in
area” was determined by subtracting the wound or scratch area at each timepoint subtracted from the area measured at the Time 0. The “change in area” divided by the starting area, multiplied by one hundred equals the percent change in area.

We also used the Olympus VivaView™ FL LCV110U incubator with incorporated microscope and associated software to obtain live cell imaging of the exact same point within a well. This time-lapse microscope captured a specific spot over the course of wound healing. Cells are seeded in 35mm poly-d-lysine coated glass bottom dish (MatTek #P35GC-0-10-C) and the scratch assay protocol as described above was followed. For the scratch assay, we imaged three separate sites on each dish and then acquired an image every 30 minutes over a 24-hour period. Next, all the images were saved and combined using VivaView™ FL software to make a movie. This movie was then analyzed in ImageJ 1.48v (http://imagej.nih.gov/ij) and the void made by the scratch was manually traced to yield an area that could be observed over the time period.

**Migrations assay in a transwell**

MCF10DCIS.com cells were seeded in complete media for 24 hours, and then harvested with trypsin. After the cell pellet was acquired as previously described, the cells were washed and pelleted twice with basal media (DMEM/F12) to remove any residual serum. A transwell system was set up using a 24 well plate (USA Scientific #cc7682-7524). Cells were counted and resuspended in basal media as to seed a 150,000 cells in the top of the 8.0µm pore transwell insert (Fisher #08-771-21). The bottom of the transwell had the
attractant media. Depending on the experiment, the attractant media was: basal media (DMEM/F12), basal media + Gas6, serum starvation media (DMEM/F12 + antibiotic-antimycotic), serum starvation media + Gas6, complete media (DMEM/F12 + 1% antibiotic-antimycotic +5% horse serum), or complete media + Gas6. The transwell was incubated for 24 hours at 37°C 5% CO₂ to allow migration.

Media was aspirated off and the transwell insert was washed twice with PBS, then permeabilized by incubating the transwell in ice cold 100% methanol (Fisher #A412-4) and incubated at -20°C for 20 minutes. Next, we aspirated methanol and washed the insert twice with PBS. We stained the transwell insert with 0.5% crystal violet (Sigma #C3886-25G) solution supplemented with 25% methanol in deionized water and incubated it at 4°C for 15 minutes. The media was aspirated and we washed the insert with deionized water. We aggressively swabbed the surface of the transwell where the cells were originally seeded, cut out the membrane with a razor, and mounted it on a slide in glycerol to image. We captured independent 10X fields of view using the color camera and Nikon Eclipse Ci microscope and, finally, counted the migrating cells with Nikon NIS Elements Basic Research 4.30.1 is the imaging software or ImageJ.

**Motility assay / Motility time lapse**

Time-lapse experiments, including the scratch assay and motility assays, used VivaView™FL software to manage image collection. Images of cells were acquired by using HAMAMATSU Camera controller C10600 ORCA-R² with digital CCD Camera C10600-10B. Cells were seeded on a poly-D lysine coated
MatTek glass bottom 35mm dishes (MatTek #P35GC-0-10-C). For CO₂ incubation with the integrated microscope used to view cells, we used the MetaMorph® for Olympus® LCV110U Version 7.7.0.0. The motility assay began with seeding 100,000 cells per dish and beginning the experiment when the cells were sub-confluent (about 20-30% confluence). Time-lapse images of the motility assay were captured on the same microscope setup and cells were seeded using the same dishes. We had 1 dish per treatment group and selected three sites per dish and imaged every 10 minutes for a 24-hour period. Next, all the images were saved and opened in SlideBook™ 5.0 program to allow for individual cells to be tracked in each site. A total of 6 cells per site, and 18 cells per dish, were tracked manually during a 24-hour period using the images captured previously. SlideBook™ 5.0 then took the manually-tracked cells and accumulated the total displacement of the tracked cell. The sum total of all movement, or point-by-point displacement from the centroid, of each cell accumulated over 24 hours yielded the total displacement per cell. The software also had a visual labeling of cells to show their movements from start (blue) to the end of the tracking (red) (see Figure 21).

3D Morphogenesis Assay

We used the 3D model system to assess the ability of epithelial cells to invade the synthetic basement membrane, or matrigel. We seeded mammary epithelial cells (10,000 MCF10A, or MCF10AT cells) in 45µL of matrigel-basement membrane growth factor reduced (VWR 47743-720 CB40230) within a chamber of an 8 well chamber slide. The cell/matrigel mixture was put at 37°C for
20 minutes to allow the substrate to gel, and then 400µL of media with 25 % matrigel was added. The culture was incubated at 37°C 5% CO₂ for 16 days and we changed the media every 3 days, continuing to supplement with 2% matrigel each time. Cells seeded in the chamber slide and pairs of wells received the same media treatment: untreated media, 50ng/ml Gas6 with 2% matrigel, 100ng/ml gas6 with 2% matrigel, or 250ng/ml Gas6 with 2% matrigel. Gas6 treatment media was added 24 hours after the 3D culture began (Day+1). We used the inverted Nikon Eclipse Ti microscope to image the 3D masses, referred to as acini, via bright field microscopy during the 3D culture.

**Immunostaining of 3D culture.**

The 3D cell culture was done using an 8 well chamber slide. The slide was fixed with 2% Paraformaldehyde PFA (Fisher AC41678-5000)in PBS for 20 minutes at room temperature and then permeabilized with 0.5% Triton X-100 (Fisher BP151100) in PBS (Fisher BP29404) for 10 minutes at room temperature, followed by three washes with 100mM glycine (Fisher BP3811) in PBS. The slide was blocked for 1 hour with 10% goat serum (Invitrogen 01-6201) or BSA (Sigma A7906), depending on the primary antibody, in fresh IF buffer (0.1% BSA, 0.2% Triton X-100 (Fisher BP151-000 100ml), 0.05% Tween-20 (Sigma #P7949-500ml) in PBS). Primary antibody was added to 10% serum in IF buffer for overnight incubation at room temperature while tented in aluminum foil. The slide was then washed with IF buffer three times and the secondary antibody diluted 1:500 in 10% serum in IF buffer and incubated for 1 hour at room temperature. After three washes with IF buffer, the slide was
mounted with vectashield (Vector Labs H-1200) and a coverslip, which was allowed to set overnight at room temperature in the dark humidified chamber. Finally, the slide was sealed with fingernail polish and imaged by confocal. Note the antibody conditions we selected did not work but our conclusions are based on observations with DAPI staining. To image DAPI we used the Louisiana State University-Health Science Center (LSUHSC) confocal microscope, Olympus IX81 using Olympus Fluoview FV10-ASW Version 04.00.03.04 software.

**Invasion assay transwell**

This protocol is similar to that of the transwell migration assay described above, with the exception that the transwell membrane was coated with 50µL of 1:10 dilution of reduced growth factor Matrigel (VWR #47743-720-CB40230) in basal media atop the transwell. The transwell was incubated at 37°C for 1 hour to allow the matrigel to coat the membrane. Prior to seeding, after the incubation period, the unbound media with matrigel was aspirated.

For MCF10AT-shLUC and MCF10AT–shAxl cell lines, we seeded 150,000 cells per transwell in 200µl of basal media (DMEM/F12). The attractant was 700µL of basal media for the negative control, or complete media (DMEM/F12, 1% antibiotic-antimycotic, 20ng/ml EGF, 5% hours serum), 100ng/ml cholera toxin, 0.5ug/ml hydrocortisone, or 1:1000 puromycin). The number of MCF10AT–shLUC cells with invasive capabilities was compared to invasive MCF10AT–shAXL cells.
For MFC10DCIS.com, cells were seeded 100,000 cells per transwell in basal media (DMEM/F12). The attractant was 700µl of basal media (negative control) and it was compared to invasion in complete media (DME/F12+1% antibiotic-antimycotic, + 5% horse serum), or complete media with 500ng/ml Gas6. The incubation, fixation, permeabilizing, and staining was the same as in the transwell migration assay above.

**Paclitaxel**

Cells were seeded and allowed to grow to 60% confluence in the wells of a 96 well plate. The complete media was aspirated and the cells were washed with PBS. The treatment media was then added. We chose to use a drug to treat human cells in culture. Paclitaxel (Sigma #Y000698) was rehydrated in DMSO (Fisher #BP231-100) to a stock concentration of 10mg/ml (this is 11.7mM), then it was aliquoted and stored away from light at -20°C. We optimized the amount of paclitaxel added as treatment and the timeframe of drug exposure in a dose response experiment. At the end of the time course, we added 20µl to each well of CellTiter96 solution (Promega #G3580) and incubated at 37°C for 2 hours. The reagent causes a colorimetric change and we could determine the amount of viable cells present by reading the absorbance at 490nM on Dr. Hua Lu’s Molecular Devices SpectraMax M5® running SOFTMAX® PRO SOFTWARE Version 5 for Windows®.
RESULTS

*Characterization of downstream protein expression following Gas6/Axl activation*

We used 500ng/ml of recombinant Gas6 (rGas6) to stimulate MCF10DCIS.com, recall these are malignant human epithelial cell line, that can be stimulated by and cause phosphorylation of Axl. We observe by Western blot that pAxl increased with Gas6 treatment (*Figure 15*). We collected cell lysate a 5 minutes, 10 minutes, 20 minutes, 30 minutes, 60 minutes, 120 minutes and 24 hours post treatment with rGas6. It appears that after 60 minutes with rGas6 treatment the level of pAxl is highest (*Figure 15*). Inversely, as pAxl increases, total Axl decreases, until 1 hour post-Gas6 stimulation, when both p-Axl and total Axl peak together (*Figure 15*).

We also observed that pAKT increases with Gas6 stimulation, pAKT increased continuously at 5 minutes, 10 minutes, 20 minutes, 30 minutes, 60 minutes, 120 minutes and 24 hours post treatment with rGas6 compared to time zero (T0) (*Figure 15*). However over this time course, from 5 minutes to 24 hours, total AKT does not change, to the increase in pAKT is due to Gas6/Axl signaling (*Figure 15*).

The amount of pFAK and total FAK appears to increase comparing time 0 (TO) to each or any time-point 5 minutes, 10 minutes, 20 minutes, 30 minutes, 60 minutes, 120 minutes and 24 hours post treatment with rGas6 (*Figure 15*). We did observed that the loading control, β-actin, did not change at any time-point.
with rGas6 treatment (Figure 15). We also looked at pNFkB and total NFkB but we did not see a change in protein expression with rGas6 treatment (Figure 15). We compared protein expression at 5 minutes, 10 minutes, 20 minutes, 30 minutes, 60 minutes, 120 minutes and 24 hours post treatment with rGas6 compared to time zero (T0) and did not observe changes (Figure 15).

While pSTAT3 (Figure 16) appears to be inhibited by Gas6 treatment, when we compare untreated (time zero (T0)) to 5 minutes, 10 minutes, 20 minutes, 30 minutes or 60 minutes post treatment we see a decrease in the expression of pSTAT3 (Figure 16). However over this same time course we observe while the levels of total STAT3 are unchanged (Figure 16). In summary our result demonstrate that AKT, FAK, STAT3 and are downstream in the signaling pathway of Axl once it has been stimulated by Gas6.

We also conducted a shorter incubation with the Gas6 treatment for 5 minutes, 10 minutes, 20 minutes, 30 minutes or 60 minutes and observed MMP-9 and Snail decreased continuously over time (Figure 16).

**R428 inhibitor decreases Axl phosphorylation**

To verify the changes in protein expression seen in the Western blots of Figures 15 and 16 are resulting from Axl signaling, we intend to utilize R428, an Axl inhibitor. We continue to use MCF10DCIS.com cell line that expresses Axl and is able to show increases in phosphorylated Axl with Gas6 treatment at 500ng/ml. Figure 17 indicates that R428 is able to inhibit pAxl, but the conditions need to be optimized for use with MCF10DCIS.com cell line. Because the R428 treatment conditions are not yet optimized to reduce the amount of pAxl but not
cause changes in total Axl when comparing time zero and Gas6 treatment. Following a one hours pre-treatment with R428 followed by 20 minutes with vehicle or rGas6 the MCF10DCIS.com cells we compared expression levels of pAxl. We observed pAxl, the activation of Axl by Gas6, in the R428 vehicle as compared to time zero (T0). Next we looked at how R428 would inhibit the phosphorylation of Axl and it appears that 0.1-1µM R428 is able to inhibit pAxl, when compared to vehicle that is cells are stimulated with rGas6, to an expression level comparable to the untreated cells that did not get rGas6 treatment (Figure 17).

### Gas6 does not alter proliferation

MTS assays were performed to determine if Gas6 induced proliferation in vitro. Using a progressive model human cell line MCF10A, normal, and MCF10AT cells, that are premalignant, treated with Gas6 concentrations ranging from 10ng/ml to 1000ng/ml, proliferation was not altered after 24 hours (Figure 18A) or 48 hours (Figure 18C) in the Gas6 treated MCF10A cells compared to control. Gas6 did not alter proliferation in MCF10AT after 24 hours (Figure 18B) or 48 hours (Figure 18D) compared to the control. Thus, Gas6 treatment does not alter proliferation in the normal MCF10A or premalignant MCF10AT cell lines after 24 or 48hour incubations. We did not use the malignant MCF10DCIS.com or Axl knock down cell lines because they were not available.

### Gas6 addition does not change cellular migration by transwell

Scratch assays performed with normal MCF10A and premalignant MCF10AT cells treated with 100ng/ml Gas6 showed no statistically significant
changes in wound closure (Figure 19). We used a second method, a transwell insert where cells migrate through a membrane towards a chemo-attractant to examine how Gas6 would change cell movement (Figure 20). We determined that MCF10DCIS.com cells are not influenced to migrate by Gas6 treatment (Figure 20).

**Gas6 does not alter motility**

Using VivaView, which allows for live cell imaging of 2D culture over time, we show cell tracking in an untreated group (Figure 21 A,C) and a Gas6 treated group (Figure 21 B,D) in either basal media (Figure 21 A,B) or in serum free media with growth factors (Figure 21 C,D). The cells tracked in the Gas6 treated group (Figure 21 B,D) did not have altered motility, measured by total displacement compared to untreated cells (Figure 21 A,C). Figure 21E is the average of cells tracked within each group, showing Gas6 does not alter cell motility in 2D culture over the course of 24 hours.

**Gas6 does not alter 3D morphogenesis**

Following 2D *in vitro* assessment, we established a 3D model culture system as outlined in paper by Dr. Alvin Lo *et al.* to examine how Gas6 may alter morphogenesis. We observed no changes in acini morphology by bright-filed microscopy in dose response to Gas6 (Figure 22).

We saw MCF10A (Figure 22) in 3D culture undergo clonal expansion that allows acini to form, this has also been observed by other labs. On Day 16, the 3D culture endpoint, we could observe acini that appeared to form hollow polarized structures. The premalignant MCF10AT (Figure 22) 3D culture had
some branching and acini bunches form, and the surface of these acini was not as smooth or round as seen in MCF10A acini, indicative of the premalignant phenotype\textsuperscript{71}. In Figure 23, we see acini of MCF10A and MCF10AT that were treated with different concentrations of Gas6 does not appear to alter 3D morphogenesis.

**Observations of 3D Culture by immunofluorescence**

MCF10A and MCF10AT cells in 3D culture were allowed to grow until Day 16 post-embedding in a synthetic basement membrane. The observations made by confocal were with the aid of DAPI, as our antibody staining technique did not work. It has been reported that on day 10-14 of 3D culture the seeded cells that have undergone clonal expansion and polarize also undergoing apoptosis to form a hollow lumen\textsuperscript{71}. For MCF10A cells, we observed acini but we can not state that Gas6 altered 3D morphogenesis, as representative images were not captured. In the MCF10AT cells, we observed branching acini but we are unable to make statements about the effect of Gas6, as representative images were not captured. (Data not shown)

**Gas6/Axl increases invasion**

When no attractant was present, neither MCF10AT-shAxl (Figure 23A) nor MCF10AT-shLUC cells (Figure 23B) showed invasion. MCF10AT-shAXL have less invasive tendencies (Figure 23D) compared to control MCF10AT-shLUC cells (Figure 23C) and we observed a decrease in the crystal violet stained cells that invaded through the transwell (Figure 23E). The average number of invading cells was similar for the negative controls, but when complete
media was the attractant, we observed that MCF10AT-shAxl were not able to invade as well as the MCF10AT-shLUC cell line. In Figure 24, we see that MCF10ADCIS.com cells, with the capacity to be stimulated by Gas6, do not have altered invasion following Gas6 treatment. MCF10DCIS.com Gas6-treated cells’ ability to invade is not different from that of vehicle treated cells.

**Axl presence does alter survival in cells treated with Paclitaxel**

We undertook the challenge of determining a dose of Paclitaxel (Taxol) that would decrease the viable cells by around 50%, the lethal dose that would inhibit 50% of the cells LD50. We believed that once we established this dose of the chemotherapeutic drug, we could treat the cells with Gas6, and that we could observe an increase in cells that would survive Paclitaxel treatment via the Gas6/Axl signaling pathway. We tested doses of paclitaxel on MCF10A and MCF10AT cells (ranging from 5nM to 500nM). We found that MCF10A did not demonstrate a dose response, but MCF10AT did have a significant reduction in viable cells after 48hours with 10nM of paclitaxel (Figure 26). We next tested how AXL knockdown cell line at doses 5-500nM of paclitaxel would influence cell survival on MCF10AT-shAXL cells and their control MCF10AT-shLUC cell line. We found that the control, MCF10AT-shLUC, did have a statistically significant reduction in the number of viable cells, as measured by absorbance, at 5nM paclitaxel compared to the control. When we compared MCF10AT-shAxl to MCF10AT–shLUC, we observed that cells without Axl were more sensitive to Paclitaxel treatment (Figure 27).
**Figure 15 Characterization of downstream protein expression following Axl activation with Gas6**

**(A)** Protein expression by western blot. **(B)** Corresponding β-actin loading controls. MCF 10DCIS.com cell line stimulated with 500ng/ml rGas6 leads to an increase in pAKT, pFAK, and total FAK over time. While pAxl appears to briefly increase at early time points, expression decreases by T120’ and 24 hour timepoints **(A)** in response to Gas6 treatment.
Figure 16 Gas6/Axl signaling decreases MMP-9 and Snail expression

MCF10DCIS.com treatment with 500ng/ml rGas6 causes MMP-9, Snail and pSTAT3 to decrease over time.  *Western blot run by Jill Rosenberg and the image is used with her permission.*
MCF10DCIS.com cell lines were pre-incubated with R428, an Axl inhibitor that inhibits auto-phosphorylation of multiple sites on the intracellular C-terminus of the Axl protein, prior to Gas6 treatment. Addition of 0.1µM and 1µM R428 appear to decrease p-Axl to levels comparable to untreated cells at T0, while 0.1µM R428 does not greatly diminish total Axl expression. Higher concentrations, 1µM and greater, of R428 inhibit both p-Axl and total Axl.
Figure 18 Gas6 does not alter proliferation in MCF10A or MCF10AT cells

After 24 hours with rGas6, MCF10A (A) or MCF10AT (B) do not have a change in proliferation when we compare the different dose of rGas6 to the control vehicle. After 48 hours with rGas6 treatment, MCF10A (C) and MCF10AT (D) do not show a change in proliferation when we compare the different dose of rGas6 to the control vehicle.
Figure 19: Treatment with Gas6 did not change scratch closure

MCF10A and MCF10AT cells treated with vehicle or 100ng/ml Gas6 did not show statistically significant differences in wound closure in Gas6 treated groups compared to vehicle treated cells. N=3
Gas6 does not increase MCF10DCIS.com cell migration in a transwell assay. (A-C) Cells stained with crystal violet have migrated through the transwell membrane. When the negative control (A) is compared to vehicle-treated cells (B) there is an increase in migration. But adding rGas6 as a chemoattractant (C) does not significantly alter migration through the transwell membrane when compared to (B) vehicle. Exogenous Gas6 does not increase cell migration (D) when we compare the average number of migrating cells per treatment group. Significant tested by Standard Student t-Test and an N=3.

Figure 20 Gas6 does not increase migration of MCF10DCIS.com cells
Figure 21 Gas6 does not alter motility of MCF10A cells

The tracking of a single cell’s total path over a 24 hour period is overlayed for an image of the final culture. The progression of time is apparent as the colorimetric change from blue (time 0) to red (endpoint) of the experiment (A-D). Imaging of untreated cells in basal media (A) compared to Gas6 treated cells in basal media (B) show cell movement as detected by the cell tracking over time. When growth factors are present but serum is absent, untreated cells (C) have a similar increase in movement as Gas6 treated cells (D), but there is not a significant difference when Gas6 is added. (E) Average total displacement of cells in each treatment group. Gas6 does not affect the motility or total displacement of MCF10A cells.
Figure 22 Gas6 does not alter 3D acini formation in MCF10A or MCF10AT cells

MCF10A and MCF10AT do not have changes in 3D morphogenesis with increasing doses of rGas6, as apparent in these 4X bright field images on Day 13 of the 3D culture, compared to untreated cells.
Figure 23 The ablation of Axl causes MCF10AT cells to decrease invasion

MCF10AT-shLUC (A and C) and MCF10AT-shAXL (B and D) invasion is compared when cells were seeded on a synthetic basement membrane and allowed to migrate towards complete media as the attractant. We compare the negative control (basal media) to the attractant (complete media) and see complete media stimulates MCF10AT-shLUC cells to invade better than MCF10AT-shAXL cells. (E) The average number of invading cells is statistically significantly higher in MCF10AT-shLUC cells when the attractant is complete media. ***p<0.0001 by Standard Student t-Test and an N=3.
Figure 24 Gas6 in complete media does not alter invasion of MCF10DCIS.com cells

(A) MCF10DCIS.com cells do not invade in negative control, but invasion increases with complete media (untreated) when complete media has Gas6. However, there was no difference in invasion between untreated cells or those treated with Gas6. (B) The average number and SEM of invading MCF10DCIS.com cell per 10x field of view is not different between untreated and Gas6 treated cells.
**Figure 25 Gas6 in serum starvation media increases invasion**

(A) Invading cells, stained with crystal violet, were significantly increased when Gas6 acted as an attractant in serum starvation media (200,000 cells were seeded). (B) Average number of invading cells in independent 10x fields increases with Gas6 as attractant. **p<0.01 by Standard Student t-Test**
Figure 26 Gas6 does not alter viable cells in MCF10AT cells treated with Paclitaxel

After 48 (A) or 72 hours (B) of treatment with 10nM Paclitaxel (Taxol), there is no significant difference in cell survival between MCF10AT treated with Gas6. When comparing the vehicle to either of the Gas6 treated we did observe a statistically significant difference. ***p>0.001 by Standard Student t-Test
Figure 27 Axl presence does alter viable cells in cells treated with Paclitaxel

MCF10AT-shAXL treated with 5nM paclitaxel had significantly fewer viable cells detected compared to the control MCF10AT–shLUC cells at (A) 48 hours and (B) 72 hours post-treatment. ***p>0.001 , ****p<0.0001 by Standard Student T test
DISCUSSION

For these studies, we used MCF10A, MCF10AT, MCF10DCIS.com cell lines in order to stimulate Axl with the addition of exogenous Gas6 to examine the effects over time. Our initial results indicated that 100ng/ml of Gas6 stimulated MCF10A and MCF10AT cells, but found that these results were not reproducible. In fact, we later discovered that MCF10A and MCF10AT cell lines had constitutively active Axl. In our original studies, the MCF10DCIS.com cells did not express Axl by western blot, nor by real-time PCR. However, we learned from other researchers that the MCF10DCIS.com cell line did, in fact, express Axl and we were provided a new stock of MCF10DCIS.com cells from a collaborator, Dr. Fariba Behbod at Kansas University Medical Center. Dr. Sheng Zhang in our lab confirmed by real-time PCR that this new stock of MCF10DCIS.com expresses Axl and responds to exogenous Gas6. Here, only results obtained using the newer MCF10DCIS.com cells that confirmed expression of Axl are presented. Due to the difficulty with human cell lines and stimulation, we also worked with mouse cell lines. We had similar issues when trying to use mouse cell lines Ph1B-cl and PN2-cl when trying to elicit Gas6 stimulation of Axl, so the data for these cell lines is not presented here and was not insightful. It was not until late 2015 that Sheng Zhang was able to make the Axl knock down in the MCF10AT cell line. Dr. Sheng Zhang transduced the MCF10AT cell line with pLKO.1 MCF10AT-shAXL or the control MCF10AT-shLUC, selected with puromycin, and knockdown confirmed by Western blot by
Dr. Zhang. The knockout of Axl in MCF10AT allowed for studies to determine how this cell line would function without Axl.

Human cell line MCF10DCIS.com can be activated by exogenous Gas6 and stimulate Axl (Figure 15). The flux of pAxl expression (Figure 15) may be attributed to negative regulation of the Gas6/Axl pathway. Once the Axl receptor is activated through Gas6 binding, it is targeted for degradation. This negative regulation of RTK has been described as “exquisitely regulated in both time and space” and “to avoid signaling errors the ultimately lead to aberrant cellular behavior and disease, cellular mechanisms have evolved to ensure appropriate signaling thresholds are achieved and maintained.” Axl stimulation causes downstream events, including an increase in pAKT, pFAK, and total FAK, and decreases in pSTAT3, MMP-9, and Snail (Figure 15 and Figure 16).

AKT has a variety of roles including: cell proliferation, cell survival, angiogenesis and among human cancers it is commonly activated. Schwertfeger et al., presented that mice show a delay in involution in AKT−/− mouse. Since total AKT is unchanged but pAKT increase, we can state that AKT is being activated. This could alter cellular proliferation or survival.

While FAK can alter morphology, cellular locomotion, proliferation, differentiation and apoptosis. The phosphorylated form of FAK is associated in increased proliferation. It has been seen that a FAK−/− will lead to ductal dilation and aberrant branching within the mammary gland. Total FAK appears to be unchanged but pFAK increase with Gas6 treatment indicating alterations in cell motility, proliferation of apoptosis.
STAT3 is also required for involution of the mammary gland and has been implicated in promoting tumor formation\textsuperscript{73,75}. STAT3\textsuperscript{-/-} mice have been observed to have a delay in involution as well as suppressed apoptosis within the mammary gland, and it is embryonic lethal\textsuperscript{76}. So STAT3 would be pro-apoptotic during involution\textsuperscript{77}. STAT3 decreases with Gas6 treatment and this could be an indication of anti-apoptotic effect of Gas6/Axl signaling.

MMP-9 does work in mammary gland degradation of the extracellular matrix, but MMP2 is the regulator of TEB invasion while MMP3 is regarded to be responsible for secondary and tertiary branching, this was observed knockout animal models\textsuperscript{78}. A decrease in MMP-9 with Gas6 treatment would suggest a limiting of invasion and secondary branching.

Snail is a transcription factor that has been linked to tumor progression and invasion\textsuperscript{79}. A decrease in Snail as a result of Gas6/Axl may limit tumor progression because Snail would not produce cytokines as a product of its transcription activity.

We can use R428 at 0.1µM to inhibit Axl phosphorylation (pAxl), via the binding of the inhibitor to the intracellular portion of the receptor (Figure 17). We see in Figure 17 that pAxl is highest in the vehicle group where the cells were treated with Gas6, pAxl decreases in the cells treated with 0.1µM R428 and Gas6 and the lowest level of Axl phosphorylation is observed in the untreated Time 0 (T0) group. R428 application to cells treated with Gas6 should impact the downstream events that are Gas6/Axl dependent. It is currently unknown why the level of total Axl is also decreasing with the application of R428.
Gas6 does not alter proliferation of MCF10A or MCF10AT cells. Gas6 does not significantly alter migration but its effect is a decrease in the speed with which wounds close (Figure 19). We were able to confirm with MCF10DCIS.com cells that migration in a transwell in the presence of Gas6, was not significantly different from the vehicle (Figure 20). Using live cell imaging, we studied cell motility and saw that Gas6 does not change the distance cells traveled over a 24-hour period compared to untreated cells (Figure 21). In a 3D morphogenesis assay, we can report findings that Gas6 does not appear to significantly alter MCF10A or MCF10AT acini formation (Figure 22) but this should be repeated as we can not confirm that representative images were captured. However, we were unable to qualitate acini phenotype for size, morphology or polarization because we did not capture independent fields of view of each treatment group and immunofluorescence staining did not work. Thus, observations of any abnormalities may not be an accurate representation of the entire group.

We report here that Axl knockdown in MCF10AT cells decreases invasion compared to the control (Figure 23). This is confirmed by MCF10DCIS.com cells increase invasion when complete media contains Gas6 as attractant (Figure 24). However when we remove complete media and thus any factors in the serum that may enhance invasion we see that MCF10DCIS.com cells have a varying ability to invade, which depends on the cell seeding density (Figure 25). This would indicated that factors in the serum may stimulate Axl and alter invasion (Figure 3).
We attempted to determine the role of Gas6 in survival by treating cells with Paclitaxel. In Figure 26, we observed that Gas6 did not significantly affect the ability of MCF10AT cells to survive treatment with Paclitaxel. But when we compared the MCF10AT-shAxl to its control, we found that cells that lacked Axl had significantly less survival at both time points (48 and 72 hours) when treated with Paclitaxel (Figure 26).

Thus, the roles exerted by Gas6/Axl signaling in vitro may be on invasion, but this is complicated by the observation that MMP-9 decreases with Gas6 treatment as seen by Western blot (Figure 16). With pSTAT3 decreased with Gas6 stimulation (Figure 15), and the observations that MCF10-shAXL cells are more susceptible to cell death induced by Paclitaxel (Figure 27), the role of Gas6/Axl may also impact the survival pathway. So Gas/Axl does play a role in regulating biological functions of mammary epithelial cells, and data here illustrates its role in invasion and survival.
CHAPTER 4: CONCLUSION

Here, we investigated the influence of Gas6 on mammary gland development in vivo by using a Gas6\(^{-/-}\) animal model. Our findings indicate that Gas6 may have an important role in development. Gas6\(^{-/-}\) animals had a decrease in ductal elongation (Figure 9), abnormal numbers of TEBs (Figure 10), less side branching (Figure 11), and less epithelium (Figure 12) compared to the WT. These effects could be due to a change in innate immune cells in the mammary gland of Gas6\(^{-/-}\) animals, because these cells that include macrophages are important for mammary gland development. These Gas6\(^{-/-}\) animals also showed a shift in the progenitor populations of the mammary epithelial cells that corresponded to a decrease in luminal progenitor cells and an increase in alveolar progenitors (Figure 7). How a shift in the mammary stem/progenitors, that may be orchestrated by Gas6, implicates and changes normal mammary gland development during pregnancy and involution is unknown. But the majority of breast cancers are thought to arise from luminal progenitors\(^{59,80}\), if Gas6\(^{-/-}\) limits luminal progenitors then it facilitates an anti-tumor environment.

Along with some histological changes in adjacent stroma proximal to the mammary gland duct in the Gas6\(^{-/-}\), animals appeared different from the WT (Figure 13). Due to limited sections Gas6\(^{-/-}\), we were unable to test the adjacent stroma for collagen, but we have the resources “on-hand”. If the thick adjacent
stroma is collagen then that may contribute to changes in side-branching\textsuperscript{81}. With the limited number of animals collected (Table 2), we have indications that the germline knock out of Gas6, and consequently the lack of intracellular signaling via TAMR, may alter mammary gland morphogenesis during development.

Using the information gained through these experiments, we propose a model of Gas6 function in which expression will be elevated and influence mammary gland development decreasing MMP-9 at points of remodeling and regression of the mammary epithelium. It has been found that ductal morphogenesis was disrupted in mutant mice with A-ZIP/F-1, a dominant negative protein that inhibits DNA binding and function of B-ZIP proteins that include C/EBP\textsubscript{β}\textsuperscript{82,83}. Our lab is also investigating the relationship between C/EBP\textsubscript{β} and Axl. We do not currently have the necessary estrus-staged animals to determine if Gas6 plays a role in mammary gland side-branching. However, this experiment will be conducted in the future.

TAMRs were not examined within our limited mammary samples, and while it was discovered that apoptosis occurring in various tissues require MerTK for phagocytosis, the precise role of Axl is unknown\textsuperscript{41,84}. Lemke \textit{et al.} found MerTK and Axl function as immune modulators during homeostasis and inflammation, respectively\textsuperscript{41}. This data is encouraging, as during mammopoeisis the gland undergoes proliferation followed by apoptosis, which would require phagocytosis and innate immune system modulation, which we believe is influenced by Axl signaling. We hope to further elucidate the potential roles of Axl in mammopoeisis once we have obtained the proper number of animals to
compare knockouts to WT. To combat the husbandry issues we routinely encountered, and to progress the work on Gas6 and mammary gland development, we undertook the backcrossing of the Gas6 germline knockout from C57BL/6 into the Balb/c animal.

During involution Gas6 is expressed but the role of Gas6/Axl in the process of pregnancy, lactation and involution (Figure 5) is unknown. We will need to have age-matched animals that have staged pregnancies and forced involution post weaning in order to begin to assess the role of Gas6. Thus to conclude specific aim 1, we were not able to determine the influence of Gas6 on mammary gland development due to limited sample size. We do have results that suggest that Gas6\textsuperscript{−/−} alters: ductal elongation, TEBs, the amount of epithelium, the adjacent stroma and the stem/progenitor cells in the mammary gland.

For the \textit{in vitro} work contained herein, we were able to work with a human cell line series, originating from MCF10A, that was stimulated by Gas6. Gas6 stimulation caused Axl activation and we were able to observe downstream signaling. Gas6 treatment lead to increased pAKT, pFAK, and total FAK over time (Figure 15), while pAxl briefly increased but decreased at later time points. In Figure 15, we saw that pSTAT3, MMP-9 and Snail both decrease over a shorter time course (Figure 16). It appears that Gas6/Axl signaling is tissue-specific and among its variety of roles it can: differentiate natural killer cells, regulate the immune response of macrophages, influence proliferation of cells, alter clearance of apoptotic cells, and lead to invasion and chemotherapy
resistance in carcinomas\textsuperscript{43}. Here, in the breast cancer cell lines, we see that Gas6/Axl stimulation acts via the increases in pAKT, and pFAK expression, indicating that Axl signaling is pro-proliferation. However, this was not supported by observations in MCF10A or MCF10AT cells \textit{in vitro}. While a decrease in MMP-9 protein expression indicates migration activity should decrease with rGas6. We observed an invasion increased, in a transwell using MCF10DCIS.com cells, and this action could be contributed to by the increase in pFAK with rGas6 treatment. We have also begun to optimize the Axl inhibitor R428, and initially observed a reduction in Axl phosphorylation when R428 was added to culture at a concentration of 0.1µM (\textit{Figure 17}). Use of this inhibitor will allow confirmation of downstream signaling effects via Gas6/Axl. Gas6 treatment did not alter proliferation (\textit{Figure 18}), migration (\textit{Figure 20}), motility (\textit{Figure 21}), or morphogenesis in our 3D assay (\textit{Figure 22}). However, these experiments were conducted in cell lines (MCF10A and MCF10AT) that we later discovered to have constitutively active Axl. Once this was brought to light, we changed our experimental design to include the use of the MCF10DCIS.com cell line, which is susceptible to Gas6 activation of Axl. Gas6 treatment in MCF10DCIS.com caused Axl stimulation (\textit{Figure 15}) but showed no changes in migration (\textit{Figure 20}) or invasion (\textit{Figure 24}) compared to untreated cells, unless the serum is removed and Gas6 is present then we observed an increase in invasion (\textit{Figure 25}). Also, Gas6 did not alter cellular survival when cells were challenged with Paclitaxel. When we used an Axl knockout cell line made in our lab, we saw that the lack of Axl decreased the ability of cells to invade (\textit{Figure 23}). In an
assay where these same cells were treated with Paclitaxel, the cell line that lacked Axl had fewer viable cells remaining after Paclitaxel treatment compared to the control cell line (Figure 27). Suggesting that the presence of Axl increase the ability to MCF10AT cells to survive treatment with Paclitaxel. To conclude aim 2 we found that Gas6 did regulate multiple cellular functions including invasion and survival.

We propose a model (Figure 28) by which Gas6 is made by epithelial or infiltrating innate immune cells, macrophages, within the mammary gland. This Gas6 can then bind and activated TAMRs and the subsequent signaling may: alter stem/progenitor cell population, increase survival, inhibit motility and inhibit transcription factors limiting cytokine production. Our findings, and this model (Figure 28), that Gas6 alters mammary gland development and may signal via proliferative and anti-apoptotic pathways are important to breast cancer research, and the changes seen in cellular function in these experiments are hallmarks of cancer progression85. Axl has been shown to be required for invasive breast cancer and expression is correlated to poor patient prognosis44,86. We do not know yet the exact force Axl signaling exerts in DCIS progression or if it facilitates cross-talk between the stroma and tumors within the microenvironment of the mammary gland. The Axl signaling pathway is being targeted not only for it’s role in breast cancer, but by researchers working on inhibitors that are now in both pre-clinical and clinical trials43,45,87–89. Since Gas6 is soluble, it can be assayed in a patient’s serum or by staining a human biopsy. If we could correlate Gas6 expression with prognosis in DCIS patients, clinicians could use Gas6 as a
prognostic indicator. Alternatively, Axl activation by Gas6 binding causes the cleavage of the extracellular domain and release of soluble Axl, which could also be a biomarker of disease\textsuperscript{41}. Additionally, this data suggests that Gas6 mediates mammary gland development \textit{in vivo} and the \textit{in vitro} data presented here supports that Gas6 alterations cellular functions such as invasion, survival, and their subsequent pathways, which are hallmarks of cancer. So Gas6 may influence premalignant breast cancer progression.
Figure 28 Model for the role of Gas6 in mammary gland development and breast cancer

In the mammary gland both epithelial cells and infiltrating innate immune cells like macrophages secrete Gas6. This Gas6 can then bind and activate TAMRs, including Axl. Gas6/Axl may: alter MaSCs stem/progenitor population, inhibit cytokine and immune response by decreasing Snail and fluctuating NFκB, work via Axl ablation decrease survival, while invasion is increase in breast cancer cell line that have Axl compared to Axl knockout and Gas6 stimulation of Axl in serum free media also increases invasion. While proliferation, motility, migration and 3D morphogenesis do not appear to be activated nor inhibited by Gas6/Axl signaling. All of these alterations of cellular functions may work in conjunction to explain the delay we observed in pubertal mammary gland development.
APPENDICES

Departmental Change 2013

It is noteworthy to state that I originally began my Ph.D. research in the laboratory of Dr. Thomas Voss, a virologist in the Department of Microbiology and Immunology. I was a member of the Voss lab, performing virus and influenza research. Dr. Voss elected to leave Tulane University for an alternative position in June 2013 and was unable to maintain his students after this time. Fortunately, Dr. Heather Machado accepted me into her lab in December 2013. Here, I will describe the research undertaken while in the Voss laboratory because it is significant and representative of my time at Tulane.

While the viral molecular determinants of transmission among hosts vs. dissemination among tissues within a single host for influenza are currently not well characterized, the Voss laboratory worked to understand the role of intracellular trafficking during virion release and the virus-host interactions that may affect the release of viral particles. During influenza infection, viruses replicate within mammalian cells and the localization of viral RNA and associated proteins to the nuclear compartment is required for efficient production of progeny virions. Egress from the nuclear compartment occurs via interaction of the viral nuclear export protein (NEP) and a component of the host cell nuclear export protein (CRM-1), which dictate apical or baso-lateral release of the virus particles from the respiratory epithelium. The resulting host-cell trafficking of
infected cells further complicates this system, which establishes transmission or dissemination. Infected cells may either enter into the airway, allowing for aerosolized transmission, or into further host tissues allowing for dissemination of the virus. My personal research focused on understanding the influenza viral components involved in establishing transmission vs. dissemination, and, specifically, the role of the NEP during transmission. Since the pathogenic potential of human and avian influenza viruses in humans is related to their ability to transmit efficiently, we hypothesized that during transmission the associated viral phenotype is determined by the intracellular trafficking of the viral proteins themselves after nuclear egress.

Using a number of approaches, we showed that NEP from virus particles with a transmissible phenotype interacted with host cells differently than viruses with a non-transmissible (dissemination) phenotype. This work supported a critical role for the NEP-CRM-1 interaction in establishing transmission or dissemination of influenza virus. Further, we assessed the influenza virus ribonucleoprotein (vRNP) export and interaction with CRM-1. We examined the intracellular trafficking and localization of viral proteins in polarized human respiratory epithelial cells. Our aim was to demonstrate phenotypically distinct egress of virus based on the transmission phenotype of viral NEP. Finally, in the ferret animal model we aimed to determine the transmission vs. pathogenic potential of NEP from seasonal, pandemic, and HPAI viruses. Taken together, these studies allow for more accurate surveillance of emerging influenza virus strains with transmissible and pathogenic potential, possible identification of new
antiviral therapy targets, and a clearer understanding of the fundamental processes of influenza replication and transmission.

Related to these studies, I was also involved in the examination of embryonated eggs from multiple different species of birds to determine their ability to amplify and make viable infectious influenza. This was of critical importance at that time because influenza was spreading rapidly among the avian species and the poultry industry was required to sacrifice large numbers of animals. Our aim was to identify alternate species of birds whose embryonated eggs were capable of amplifying influenza sufficiently for vaccine development. This is important to Public Health in the instance that chicken eggs are not available, we could still make virus for vaccine formulation and distribution during an epidemic.

During my time in the Voss lab, I also performed work in conjunction with Dr. Robert Garry’s laboratory on a novel peptide inhibitor of influenza. This peptide currently remains under development and included collaborations with Dr. William Wimley’s lab and Autoimmune Technologies LLC.
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BIOGRAPHY

Courtney Rose Bock Standlee born in New York City, but relocated and was raised in Willis, Texas. She is the eldest of four children and grew up on a working farm north of Houston, Texas. She attended Willis High School where she was the captain of the girl’s golf team. While enjoying college life, she worked to acquire her bachelor’s degree in Microbiology from the University of Houston-Downtown (UHD) where she was a member of the UHD Scholars Academy. Following graduation, she was employed at the City of Houston Health and Human Services Department where she earned her license as a Registered Sanitarian (R.S.). Following hurricane Katrina she was caravanned across the United States and was asked to present her experiences as a health inspector regulating the largest Mega-Shelter in Houston’s history. With support and encouragement from both state and federal agencies, she pursued and earned her Master’s in Public Health from the University of Texas School of Public Health (UTSPH), and shortly after married her husband, David Wayne Standlee. After 7 years of experience in public health, she pursued a Ph.D. in order to make an even greater impact in the field of public health. She began the Ph.D. program in Biomedical Science (BMS) at Tulane School of Medicine in New Orleans, LA in 2011, only ten day after she had her first child, Rex Wayne Standlee. In 2016 she earned her Doctorate of Philosophy, a mere five months after she welcome her second child a daughter Stella Rose Standlee into her life.