REGULATION OF MAMMARY LACTOGENIC DIFFERENTIATION BY SINGLEMINDED-2S

A Dissertation

by

ELIZABETH ANN WELLBERG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2009

Major Subject: Toxicology

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Approved by:

Chair of Committee,	Weston Porter
Committee Members,	Stephen Safe
	Robert Burghardt
	Thomas Spencer
Chair of Intercollegiate Faculty,	Robert Burghardt

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ABSTRACT

Regulation of Mammary Lactogenic Differentiation by Singleminded-2s. (May 2009)

Elizabeth Ann Wellberg, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Weston W. Porter

Sim2s is a basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) transcription factor. In Drosophila, the Sim2 homolog, sim, is necessary for cell fate determination during central nervous system (CNS) development. In mammals, both Sim2 isoforms are involved in development of various tissues, including muscle, cartilage, and mammary gland. Loss-of-function studies revealed a role for Sim2s in specifying epithelial cell fate during mammary development and inhibiting growth and invasion of aggressive breast cancer cells. This study determined the role of Sim2s in mammary epithelial cell differentiation. Our hypothesis is that Sim2s is sufficient to promote lactogenic differentiation *in vivo*, characterized by expression of lactation-specific genes. Two models were used to test this hypothesis: (1) a transgenic mouse, expressing *Sim2s* under control of the MMTV-LTR, and (2) the mouse mammary epithelial cell line HC11. Together, these models allow analysis of the effect of Sim2s on global mammary gland differentiation and the mechanism through which it accomplishes this in a relatively homogenous population of cells.

We determined that precocious expression of *Sim2s in vivo* is associated with upregulation of a subset of milk protein genes in nulliparous females. During early

pregnancy, Sim2s regulation of lactogenic differentiation extended to a larger group of genes. Following pup removal, Sim2s appears to promote survival of alveolar epithelial cells. *In vitro*, *Sim2s* expression is necessary for maximal *Csn2* expression, as determined by loss-of-function studies. Overexpression of *Sim2s* is sufficient to enhance prolactin-mediated *Csn2* expression. Chromatin immunoprecipitation assays performed in HC11 cells revealed enhanced recruitment of Stat5a and RNA Polymerase II (RNAPII) to the regulatory region of *Csn2* in the presence of Sim2s. In addition, Sim2s and RNAPII were found in a complex that was localized to both the promoter and coding region of the *Csn2* gene.

These studies support the idea that *Sim2s* is upregulated in a developmental stage-specific manner in the mouse mammary gland to promote the survival and differentiation of alveolar epithelial cells expressing high levels of milk protein genes. Further, Sim2s may regulate the function of a specific subset of alveolar cells by targeting the RNAPII holoenzyme complex to genes expressed during lactogenic differentiation.

DEDICATION

To my mother, Jane Wellberg, my sister, Jennifer Wellberg, and my husband Patrick Dougherty. Thank you for the constant support, patience, and love.

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NOMENCLATURE

ALDH	Aldehyde Dehydrogenase
AR	Androgen Receptor
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
BRCA1	Breast Cancer 1
C/EBP	CCAAT/Enhancer Binding Protein
CLD	Cytoplasmic Lipid Droplet
DSCR	Down Syndrome Critical Region
E2	Estrogen
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
ESR1	Estrogen Receptor
GH	Growth Hormone
GR	Glucocorticoid Receptor
HSA	Human Chromosome (Homo sapiens Autosome)
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
IHC	Immunohistochemistry
MMA	Mouse Chromosome (Mus musculus Autosome)
MMTV	Mouse Mammary Tumor Virus
MMP	Matrix Metalloproteinase

P4	Progesterone
PAS	PER-ARNT-SIM
Pgr	Progesterone Receptor
Rb1	Retinoblastoma
Q-PCR	Quantitative PCR
RT-PCR	Reverse Transcriptase PCR
TEB	Terminal End Bud
Sim	Singleminded
WT	Wild Type

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CHAPTER I

INTRODUCTION: MAMMARY GLAND DEVELOPMENT

The mammary gland is an excellent model for the identification of factors that are involved in the development and differentiation of an organ. It is composed of a network of ducts, made of inner luminal epithelial cells and outer myoepithelial cells, which are embedded in a matrix of fibroblasts, adipocytes, vasculature, immune cells, and connective tissue. Some rudimentary development takes place *in utero*, but most of the ductal branching and cellular differentiation happens after birth. In young females, mammary development is linear, and during reproduction, the gland undergoes cycles of differentiation and regression. Please refer to Figure 1 for a diagram of the phases of mammary gland development and associated structures. The mammary epithelium is susceptible to oncogenic transformation; however to understand how various factors are involved in mammary cancer formation or suppression, it is imperative to elucidate the normal developmental and differentiation processes of the tissue.

Embryonic Mammary Gland Development

In mice, mammary gland development begins during mid-gestation, at approximately embryonic day 10.5 (E10.5) (Hens and Wysolmerski 2005). Formation of a mammary or milk line is the first indication of the mammary structures.

This dissertation follows the style of *Genes & Development.*

Five pairs of condensed, rounded placodes then form on the surface of the epithelium at E11.5, in the order 3, 4, 1, 5, and 2. Placodes 1-3, 4 and 5 become the thoracic and inguinal glands, respectively. Invagination of the mammary epithelium into the condensed mesenchyme takes place between E11.5 and E12.5. In females, the invading epithelial bulb begins to elongate into a primary duct that, at birth, has approximately 15-20 branches.

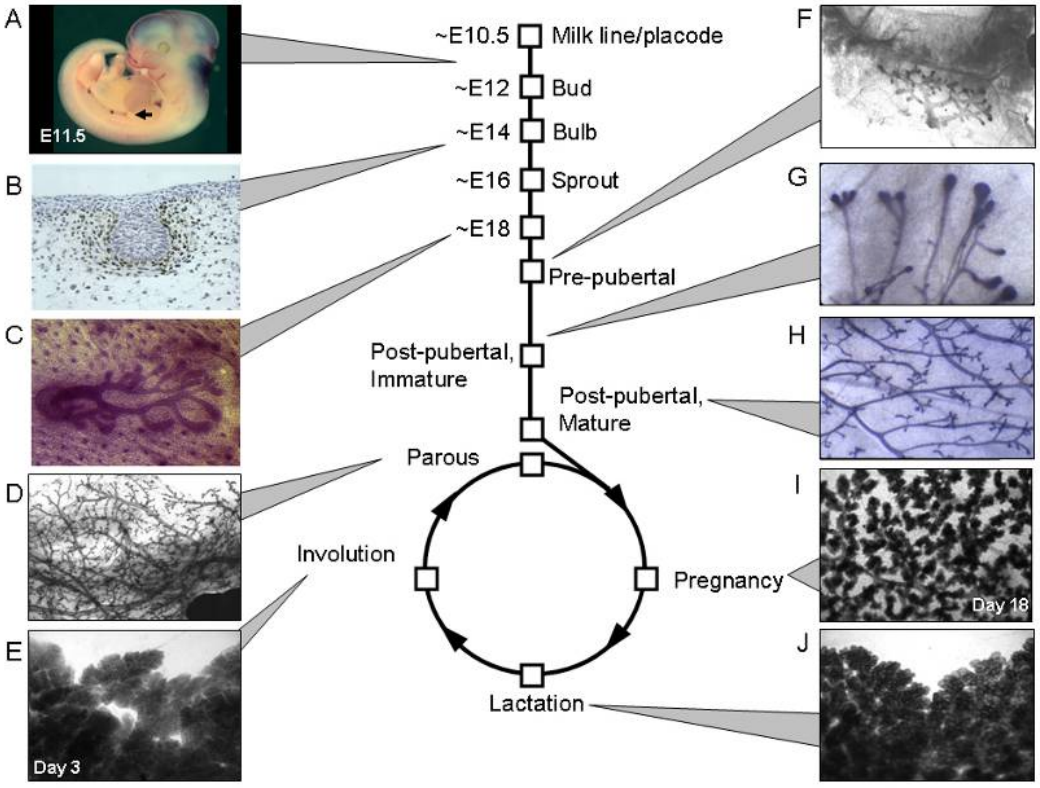

Figure 1. Phases of Mouse Mammary Gland Development. Courtesy of Mike Lewis, Ph.D., Baylor College of Medicine. A, Mammary line at E11.5. B, Mammary bud. C, Mammary Sprout. D. Whole mounted (WM) mammary gland from parous female. E. WM mammary gland at involution day 3. F. WM prepubertal mammary gland. G. WM juvenile gland showing TEB structures. H. WM mature mammary gland. I, WM mammary gland at pregnancy day 10. J, WM mammary gland during lactation.

Studies performed in mice lacking ESR1, ESR2, Pgr, GHR, or PrlR revealed that embryonic mammary development is hormone-independent (Hens and Wysolmerski 2005) . Several investigations have been done to determine the gene expression requirements for the formation of placodes and mammary buds, and it has been found that members of the Wnt, FGF, Tbx, and PTHrP family play important roles in embryonic mammary development (Hens and Wysolmerski 2005). The ability to determine the stage of gestation in mice and accessibility of the embryonic tissue throughout development makes the mouse an excellent model to use for the study of mammary gland formation; however, the rapid development of the embryo makes it difficult to pinpoint specific stages at which some structures develop. The mammary line, for example, was described as an anatomical structure in developing rabbit embryos more than 30 years ago (Veltmaat et al. 2003). The existence of such a structure in mice was controversial until 2004, when it was detected in mice, at E11.75 (Veltmaat et al. 2004).

For my research, I have used the mouse as a model; therefore, the following sections, will describe the development of the mouse mammary gland beginning in the embryo. The cycles of growth and regression that occur during pregnancy and involution, respectively will also be discussed. Throughout the introduction, I will discuss the development of the human mammary gland if it has been described, in as much detail as is known today, and also discuss some highlights of experiments performed in other species such as cows, goats, rabbits, and rats. The structural characteristics of human mammary gland development have been described, yet it

remains to be determined if the same gene families are involved in both mouse and human development. In addition, some of the differences in the mouse and human mammary gland will be discussed to illustrate the care that should be taken when extrapolating findings from mouse models to human development.

Formation of the Mammary Line and Placodes

The onset of mouse mammary gland development begins with the formation of the mammary or milk line. Until 2004, the description of this line as an anatomical structure was only revealed from studies performed in rabbit embryos (Veltmaat et al. 2004). The first indication that such a structure existed in the mouse embryo was discussed in a review by Veltmaat and colleagues (Veltmaat et al. 2003). A mouse engineered to express TOPGAL, a beta-galactosidase gene under control of a promoter that was inducible by Lymphoid Enhancer Factor/T Cell Factor (LEF/TCF) family members and beta-catenin, was described in 1999 in a study examining hair follicle formation (DasGupta and Fuchs 1999). This mouse was used to investigate the role of canonical Wnt signaling in mammary gland development and was generated following publication of a study that cited a lack of teeth, hair follicles, and mammary glands in *Lef1*$^{-/-}$ mice (van Genderen et al. 1994). Additionally, mice engineered to express the Wnt inhibitor, Dkk1, under control of a keratin 14 promoter ubiquitously expressed throughout the dermis, did not develop mammary buds. The review in 2003 cited personal communication from Dr. Wysolmerzski in the description of TOPGAL expression forming two lines extending from forelimbs to hindlimbs of male and female

mouse embryos at E10.5. This suggested that Wnt expression and signaling was the first step in formation of the mammary line, and subsequent mammary glands (Veltmaat et al. 2003). In 2006, data from the TOPGAL reporter mouse was published, revealing that initial Wnt signaling was seen in the mammary mesenchyme, but shifted to the epithelium at E12.5. In addition, expression of Patched-1, which is a target of the Hedgehog (Hh) signaling pathway, was reduced in the mesenchyme surrounding the mammary placodes in *Lef1*$^{-/-}$ mice. Together, these studies reveal a requirement for Wnt signaling in the induction of mammary growth, and for proper integration of other signaling pathways, such as that mediated by Hh ligands, during embryonic mammary formation (Boras-Granic et al. 2006).

Early in 2004, *in situ* hybridization experiments were published that revealed *Wnt10b* and *Wnt6* to be the earliest expressed Wnts along the presumptive mammary line. Further, Veltmaat, and colleagues described the appearance of a cuboidal, pseudostratified epithelial layer of cells adjacent to squamous dermal epithelial cells from the forelimb to hindlimb at E11.75. This slightly raised anatomical structure appeared 0.5 days after the appearance of a continuous line of Wnt10b expressing cells in the same region. This was the first description of a mouse mammary line analogous to that seen in rabbit embryos. Later in 2004, Chu and colleagues published the data collected from the TOPGAL reporter mouse (Chu et al. 2004). Since the TOPGAL reporter was activated at E10.5 and expression of Wnt10b, as determined by *in situ* hybridization, was not detected until E11.25 (Veltmaat et al. 2004), it seemed as though another molecule may act in parallel or upstream of Wnt signaling to activate Wnt

downstream targets, and initiate the process of mammary development. Interestingly, expression of Wnt10b and Wnt6 in E11.25-E11.75 embryos is fragmented and not continuous along the mammary line, and corresponds with the underlying somites (Veltmaat et al. 2004). It has been proposed that a signal from the dermamyotome, or the dorsal region of the somite, specificies the mammary epithelial cells and initiates formation of the line and placode (Veltmaat et al. 2006).

The fibroblast growth factor (FGF) family members are required for mammary gland development in the mouse embryo (Mailleux et al. 2002). *Fgf10* is expressed in the dermamyotome at E10.5, and mammary glands 1, 2, 3, and 5 fail to develop in mice lacking *Fgf10*, making it an excellent candidate for the molecule responsible for specification of the mammary epithelium. Loss of *Fgfr2IIIb* results in the same phenotype as that of *Fgf10*$^{-/-}$ mice. *Fgfr2IIIb*, which encodes the receptor for Fgf10, is expressed in the epithelium from E10.5 to E11.5, and its ligand in the underlying condensed mesenchyme. Communication between the compartments is thought to be necessary for cell fate determination. Wnt and Fgf signaling and Tbx3 are thought to have a reciprocal relationship. *Tbx3*$^{-/-}$ mice have no mammary placodes and do not show regional expression of *Wnt10b* or *Fgf10*, placing this gene upstream of Wnt and FGF signaling pathways. Alternatively, *Tbx3* expression is induced by Fgf in the mammary line of cultured embryos (Eblaghie et al. 2004) also placing it downstream of this pathway. During development, *Tbx3* is expressed along the mammary line at E10.25, so it too could be involved in specification of this structure (Eblaghie et al. 2004).

Shortly after specification of the mammary line, condensed, raised placodes form on the epidermis. Cells in these placodes express *Wnts*, *Fgfr2IIIb*, and other genes involved in cell migration and proliferation. Ectodysplasin (*Eda*), a tumor necrosis factor (TNF) ligand superfamily member, and its receptor, *EdaR*, are expressed in the mesenchyme and epithelial placode cells, respectively (Pispa et al. 2003). Overexpression of *Eda* in mice results in supernumerary and enlarged mammary placodes along the mammary line (Mustonen et al. 2003). EdaR signaling is thought to promote placode formation and could be involved in directing the positions of the placodes along the line. As supernumerary placodes develop exclusively along the mammary line in the embryo with *Eda* overexpression, it is likely that the Eda/EdaR pathway lies downstream of specification of the mammary epithelium.

Bud and Sprout Formation

The mesenchyme plays a critical role in the specification and growth of the mammary epithelium, and this is not limited to embryonic development. After E12.5, there is a resting phase in development, characterized by an absence of DNA synthesis for 24 hours. Until this point, male and female embryos develop identically. At E13.5, gonadal gene expression leads to production of androgens in male embryos. This is one important difference between mouse and human mammary development. In human males, the mammary bud does not regress as it does in mice; instead, it remains underdeveloped but is still susceptible to oncogenic transformation, although this is a relatively rare occurrence (Fentiman et al. 2006). In male mouse embryos, PTHrP

signaling in the mesenchyme induces expression of the AR (Dunbar et al. 1999). Activation of mesenchymal AR by circulating androgens causes condensation and eventual disconnection of the developing sprout from the nipple region, and ultimately the male bud degenerates. Interestingly, this does not take place in Testicular Feminization Syndrome mice, lacking androgen production. Further, tissue recombination studies, performed on embryos of both sexes, reveal a mesenchyme-dependent effect of male mammary degeneration (Drews and Drews 1977).

Once the fate of the epithelial cells has been established as 'mammary', the function of the tissue depends on the epithelium. The structure of the gland, however, is still dependent on the mesenchyme. The fat pad begins differentiating around E15 to E16. This corresponds to the renewed growth of the mammary epithelium after the resting phase. In females, with the onset of adipogenesis in the fat pad comes proliferation of the epithelial cells and elongation of the primary duct. A requirement of the differentiating fat pad was revealed from experiments combining E17 mammary epithelium and E14 mesenchyme, and transplanting the tissue under the kidney capsule in mice. Short hyperplastic branches were formed in the presence of condensed, undifferentiated mesenchyme, in contrast to the longer, less proliferative branches formed in adipocyte-rich mesenchyme (Sakakura et al. 1982).

Tissue recombination and transplant techniques have been widely used in the study of mammary gland development. A role for mammary-specific mesenchyme was established in 1976 with the classic study by Sakakura and colleagues (Sakakura et al. 1976). Salivary gland rudiments from E14 mice and mammary epithelial buds from E16

mice were recombined and transplanted under the kidney capsule of 4 week old females. The morphology of the outgrowths resembled salivary gland structure, showing the importance of mesenchymal instruction in ductal patterning. When the outgrowths were harvested from females that were 5-10 days post-partum, it was determined that lobuloalveolar development had taken place in the recombined tissue, and that the ducts were distended with milk (Sakakura et al. 1976). By the time the female mouse is born, her mammary bud contains 10-15 branches and occupies a small fraction of her adipose-rich fat pad. The growth of the juvenile mammary gland is isometric until acted on by circulating hormones, released when the female begins sexual maturation.

Adolescent Development

Once the female enters puberty, hormones, in cooperation with locally acting factors, induce formation of enlarged, club-like structures at the ends of the rudimentary branch points. These terminal end buds (TEBs) are the motile units of the adolescent mammary gland. They are responsible for generating the entire ductal structure, correctly spaced and distributed throughout the mammary fat pad. TEBs are composed of an outer layer of cap cells that eventually give rise to the basal myoepithelial cell layer, and several layers of inner, body cells, which are the luminal cell precursors. Pubertal hormones and stromal-epithelial interactions are largely responsible for the correct formation of the adult mammary gland (Figure 2).

Transplant studies showed that the stromal environment ultimately patterns the adult epithelial structure and morphology. When epithelium from a strain of mouse with

highly branched mammary ducts is grafted into stroma from a lesser-branched strain, the resulting outgrowth resembles that of the recipient strain rather then the donor (Naylor and Ormandy 2002).

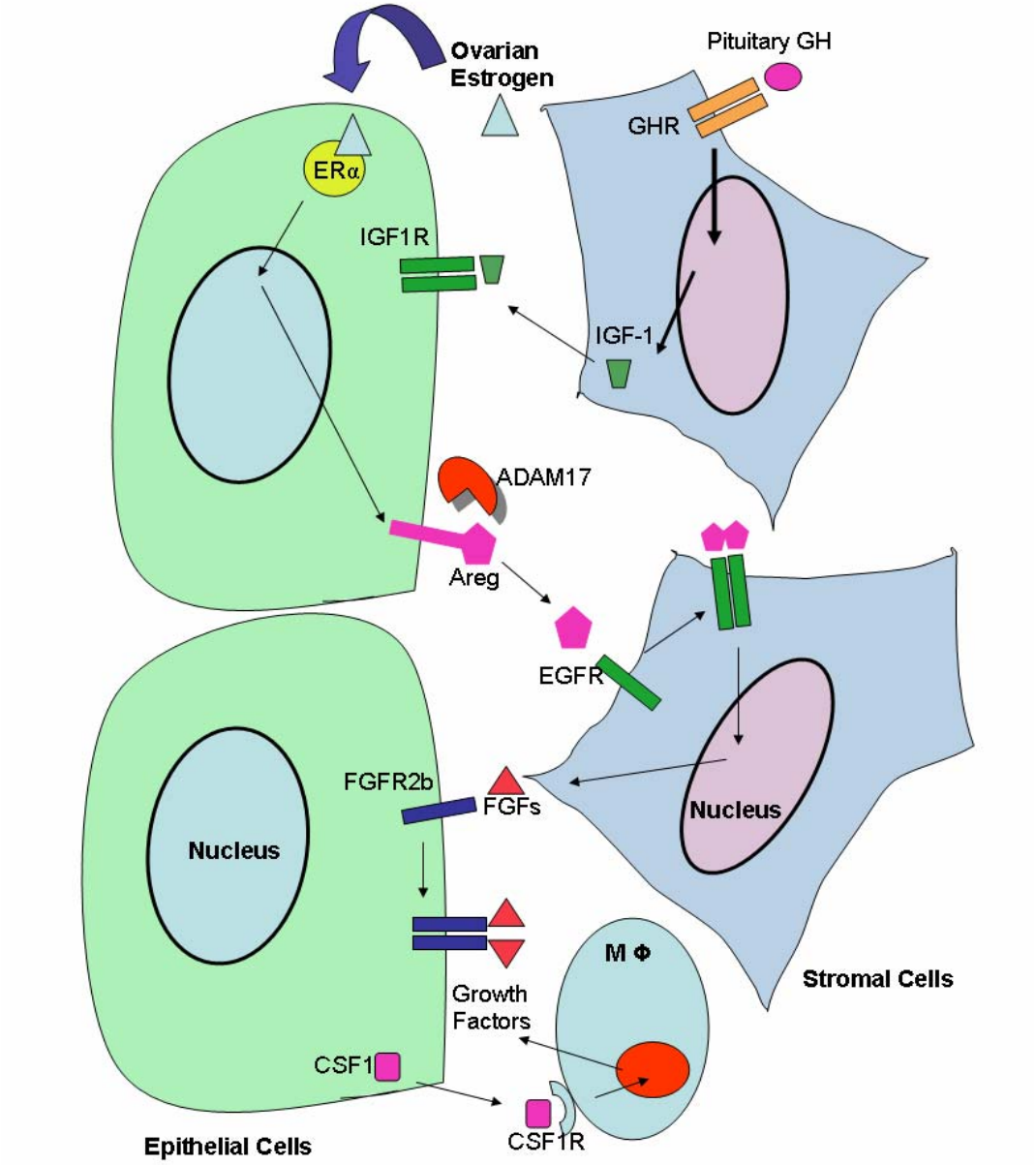

Figure 2. Stromal-Epithelial Interactions Governing Adolescent Mammary Development. Adapted from Sternlicht, et. al., 2000.

The mouse and human mammary glands differ greatly in their stromal compositions and branching morphology, but human cells will not form their characteristic, lobular pattern when transplanted into a mouse mammary fat pad (Kuperwasser et al. 2004). In mice, the stroma is composed mostly of adipocytes that are found uniformly engorged regardless of their proximity to the ducts. In humans, however, the stroma varies. Large adipocytes can be found between the lobules, but the stroma supporting an individual lobule is dense and composed of fibroblasts. Since we have already begun to realize the importance of the mesenchyme and stroma to the growth of the epithelium, it will be interesting to determine if the stromal-epithelial interactions during human development were correctly modeled by the mouse.

The extracellular matrix (ECM) composition is thought to play an important role in guiding the movement of TEBs. Cell proliferation within the TEBs, combined with Type I Collagen deposition along the neck of the structure, are responsible for pushing the growing duct forward into the fat pad. This condensation of the ECM is also believed to play a role in bifurcation and turning of the invading TEB.

Other stromal components known to participate in regulation of ductal outgrowth include macrophages and eosinophils. Using IHC, Gouon-Evans and colleagues showed that both macrophages and eosinophils are found scattered throughout the developing adolescent mammary gland, interspersed in the TEBs, and highly concentrated around the neck of the advancing TEBs (Gouon-Evans et al. 2000). Macrophages are differentiated cells from the mononuclear phagocytic lineage, and require colony stimulating factor 1 (Csf1) from tissues for their survival, differentiation, growth, and

recruitment. After irradiating mice, and thus eliminating macrophages, it was revealed that the ductal tree was stunted and had reduced branching. Bone marrow transplants performed two hours after irradiation were able to rescue the growth defect. Using a *Csf1*$^{-/-}$ mouse, which has numerous abnormalities including reduced fertility, dense bones, malformed teeth, and low immune cell numbers, the same group showed that TEB formation was delayed, and overall TEB number was reduced, leading to decreased ductal branching. Additionally, ducts that formed were often disoriented (away from the end of the fat pad) and the fat pad itself was atrophied. Treatment with recombinant human Csf1 rescued the developmental defects and implantation of estrogen pellets, used to ensure the outgrowth abnormalities were not due to ovarian dysfunction, did nothing to change the effect of Csf1 loss (Gouon-Evans et al. 2000).

Eotaxin is a chemoattractant for eosinophils, which also play a role in immune response. Using eotaxin$^{-/-}$ mice, Gouon-Evans and colleagues also evaluated ductal outgrowth and TEB formation (Gouon-Evans et al. 2000). While ductal length was not affected by loss of eosinophil recruitment, TEB formation and ductal branching were both reduced. These studies reveal a role for differentiated immune cells in proper mammary tree formation and branching. It is likely that the macrophages and eosinophils secrete factors responsible for changes in ECM composition or that instruct epithelial cells to proliferate. The macrophages located within the advancing TEB are thought to be responsible for clearing apoptotic epithelial cells and for proper lumen formation (Gouon-Evans et al. 2000).

While the stroma and its components play crucial roles in the proper development and morphogenesis of the adolescent mammary gland, it is the interaction between it and the epithelium that is instrumental for accurate patterning and to maintain the balance between proliferation and apoptosis during ductal elongation. Using ovarectomy, hypophysectomy, and conditional knockout approaches, researchers have uncovered important interactions between circulating pubertal hormones, stromal components, and epithelial cells. Circulating growth hormone (GH), released from the pituitary gland during puberty, binds receptors in the mammary stroma, and causes production and release of IGF-1, which binds its receptor on adjacent epithelial cells. IGF1R activation then promotes ductal elongation. Treatment with either estrogen (E2) or progesterone (P4) can enhance the effect of IGF1 on elongating ducts, but neither hormone alone produces the same results (Sternlicht 2006). The requirement for ESR1 is somewhat controversial and has been shown to be stromal (Cunha et al. 1997), epithelial (Mallepell et al. 2006), and both (Mueller et al. 2002). E2 can activate EGFR and EGFR ligands can rescue ductal elongation defects in ovarectomized and $ESR1^{-/-}$ mice. The only endogenous EGFR ligand produced in the mammary gland is Amphiregulin (Areg). At puberty, estrogen induces Areg expression and production in epithelial cells. The membrane-bound ligand is then cleaved by ADAM17 and freed to bind stromal EGFR. Activation of EGFR in the stroma can stimulate MMP2 production, which promotes cell survival in the advancing TEB. While ablation of the Pgr does not alter ductal elongation, it does attenuate tertiary side branching. P4, acting through its receptor, is thought to promote tertiary branching through Wnt4 production.

It has been shown that FGFR activation is critical for the proper formation of the mammary placodes during embryogenesis (Veltmaat et al. 2003). Unfortunately, genomic deletion of the *Fgfr2IIIb* results in embryonic lethality, making it difficult to examine any adolescent or adult mammary gland defects as a result of receptor loss. Recently, Dr. Zena Werb's laboratory used the Cre/LoxP system to generate a conditional *Fgfr2IIIb*$^{-/-}$ mouse (Lu et al. 2008). Cre-mediated excision, driven by the MMTV promoter expressed in the mammary epithelium, occurred by 3 weeks of age in this model. By 5 weeks of age, the mammary ducts in the mutant animals showed reduced branching, and this phenotype persisted throughout adolescent development. Closer examination of *Fgfr2IIIb* mutants revealed defects in TEB cell proliferation, and this same defect was observed during pregnancy-associated alveolar expansion. It is likely that Fgf ligands, produced by the mammary stroma, influence proliferation of cells in the TEB to promote ductal outgrowth.

In the human, mammary gland development is quite poorly characterized and appears similar to that in the mouse, but some distinct differences have been reported. By 13 weeks of gestation, human fetuses have well defined mammary buds with few branches that are surrounded by dense mesenchyme (Tobon and Salazar 1974). During the second trimester, the ductal structures have extended and branched further, but have not penetrated the subcutaneous adipose tissue. By the third trimester, the epithelial ducts are distended with secretions and the cells contain cytoplasmic lipid droplets, a phenomenon that has not been reported in rodent models. The structure of the mammary gland in humans can be characterized by morphological type and functional stage.

There are 3 morphological types and 5 functional stages. The early morphological type is characterized by simple structural formations and ducts, with advanced lobular structures and branched ducts described by later categories, and all three are present in the human mammary gland at birth. Using criteria of the functional stages, it has been postulated that the human mammary gland develops advanced lobular structures during embryogenesis and that these structures acquire secretory activity, then undergo involution, similar to that observed following lactation. Interestingly, in the human mammary gland, no differences are seen between males and females in morphological type or functional stage from birth until 2 years of age, and the ductal structures can vary greatly between individuals (Tobon and Salazar 1974; Anbazhagan et al. 1991).

In adolescent humans, mammary structural development is, again, highly variable, but in most subjects, the outer edges of the breast have developed lobular structures with multiple layers of epithelium. One study investigated the molecular characteristics of the mammary cells in the fetus, infant, and adolescent (Naccarato et al. 2000). The authors found diffuse Bcl2 staining throughout development, indicating few apoptotic cells, and low ESR1/Pgr reactivity until puberty, when the level of nuclear receptor staining increased to about 50% of the epithelial cells. In this study, cell proliferation was also examined and was found to be low during fetal and infant development, similar to what is seen in the mouse. There was no mention of an association between Pgr staining and proliferating cells, which has been previously described in mouse mammary epithelium (Seagroves et al. 2000).

The previous sections have described the importance of mesenchymal- or stromal-epithelial interactions in the specification, patterning, and growth of the mouse mammary gland, and these interactions are likely important for human development as well. Factors present in circulation or produced locally by the mammary stroma act on the developing epithelial structures to promote growth, invasion, or differentiation. The next sections will focus more on the epithelial cells, themselves, and how the functions they acquire make them unique to the mammalian species.

Acquisition of Epithelial Cell Fate

Identification of Mammary Stem Cells

Mammary gland differentiation is biphasic. The first phase of differentiation occurs during embryonic development when, hypothetically, one mammary epithelial stem cell gives rise to progenitor cells that proliferate and acquire characteristics that make up the different components of the adult gland, including ductal, alveolar, and myoepithelial cells. Later, during pregnancy, the epithelium expands to fill the mammary fat pad and specialized alveolar epithelial cells acquire the ability to produce milk. This phase of differentiation will be discussed later. The two levels of differentiation are not exclusive, as stem cells are required to participate in expanding the adult mammary gland during each pregnancy.

The existence of mammary stem cells was first determined in 1959(Deome et al. 1959). Epithelium from adult donor mice was harvested and transplanted into the fat pad of juvenile recipient mice once cleared of its epithelium (Deome et al. 1959). These

studies showed that a small amount of epithelium from an adult could reconstitute a functional mammary gland and also could be serially transplanted several times. Since then, many mammary gland biologists have used the technique to analyze the potential of epithelial cells to reconstitute a mammary gland under varying conditions. This technique has been especially useful to elucidate the role of some genes in mammary gland development when deletion of both alleles causes mid- to late-embryonic lethality, or to determine if the effect of gene loss originates in the stromal or epithelial compartments. In 1988, Drs. Gil Smith and Dan Medina performed similar studies to evaluate the outgrowth potential of different portions of the mammary gland from mice at varying stages of development. They showed that any portion, i.e. end bud, tertiary duct, primary duct, or alveolar structure, could efficiently reconstitute a mammary gland in 6 weeks unless the donor female was lactating (Smith and Medina 1988). In that case, the fat pad did not completely fill with epithelium unless the animal became pregnant. These results suggested that mammary stem cells exist in all portions of the mammary tree at all stages of development, and that cell proliferation is slower if the cells are harvested from a lactating female. They went on to identify a morphologically distinct cell located throughout the mammary gland that was hypothesized to be a mammary stem cell. This large pale-staining cell was thought to have low oxidative metabolism and mitotic potential *in vivo*. The number of these cells was reduced in the lactating mammary gland.

Isolation of Mammary Stem Cells According to Marker Expression

For the past several decades, mammary gland biologists have tried to isolate the elusive mammary stem cell using cell sorting techniques followed by limiting dilution and serial transplantation, which are the functional assays of mammary stem cell activity. In 2006, experiments were describes in which a fully functional mammary gland was produced from a single stem cell in a cleared mammary fat pad (Shackleton et al. 2006). The authors evaluated these cells using markers previously identified in other tissue types. CD24, or heat stable antigen, is a cell surface-bound protein first identified in B cells. Later, it was found to be highly expressed in tumors of various origins, including those found in the mammary gland. In the adult mouse mammary epithelium, CD24 expression can be divided into 3 groups: absent, low, and high. Of these groups, cells with low CD24 expression ($CD24^{low}$) were most likely to reconstitute a mammary gland when transplanted to a cleared fat pad (Regan and Smalley 2007). Sorting cells using CD24 and CD29, or beta1-integrin, Dr. Visvader's lab showed that one cell from the $CD29^{high}/CD24^{+}$ (corresponding to $CD24^{low}$ described elsewhere) was capable of complete mammary gland formation in 6 of 102 cases. The authors further showed that the $CD29^{high}/CD24^{+}$ population of cells also showed high expression of CD49f, or alpha6-integrin. Both CD29 and CD49f are basal epithelial cell markers and it has been hypothesized that stem cells exist in the basal compartment of the mammary gland (Taddei et al. 2008).

Earlier work focused on the Side Population or SP cells that were revealed after staining with Hoescht 33342 dye and sorting (Alvi et al. 2003). These cells expressed

high levels of *Abcg2*, also known as the Breast Cancer Resistance Protein-1 (BCRP-1). Members of the ABC transporter family participate in efflux of compounds from cells, and of the Hoescht dye; however, the work published in Nature in 2006 did not find a correlation between mammary stem cells and SP cells and lately, there have not been many studies reported that focus on this population. Figure 3 depicts a diagram of cellular differentiation in the mammary gland.

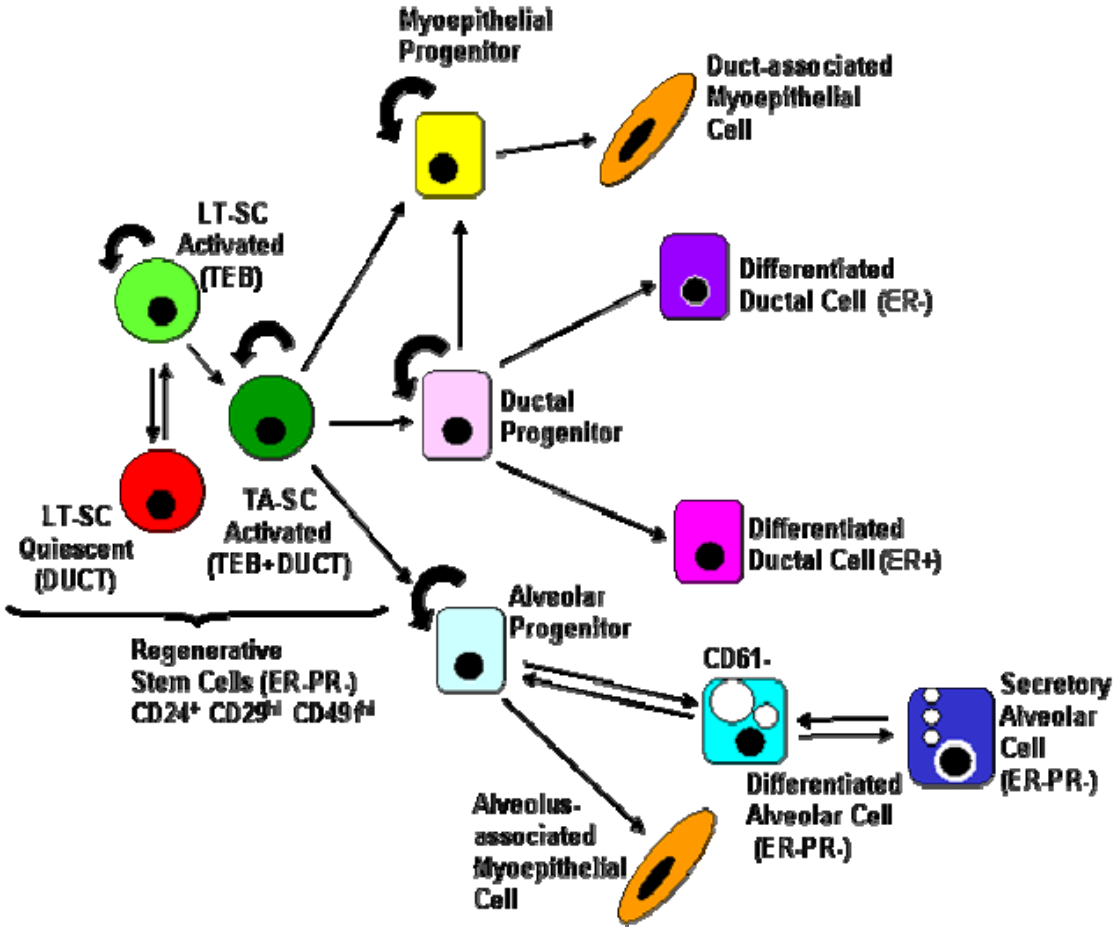

Figure 3. Cellular Differentiation in the Mouse Mammary Gland. Schemiatic drawing of the progression of mammary stem cell differentiation and associated marker expression. Long-term stem cell (LT-SC) and transit amplifying stem cell (TA-SC). Courtesy of Mike Lewis, Ph.D., Baylor College of Medicine

Although sorting primary mammary epithelial cells based on these markers can provide enrichment for certain populations, the identifiers are just markers. Some groups have begun to elucidate genes associated with marker variation or with stem cells in other tissue types, providing more information on the inner workings of the mammary stem cell. In 2007, Ginestier and colleagues evaluated aldehyde dehydrogenase 1 (ALDH1) activity and expression as an indicator of multipotency in the mammary gland (Ginestier et al. 2007). ALDH1 is a detoxifying enzyme that catalyzes the oxidation of aldehydes. The enzyme is proposed to play a role in stem cell differentiation by converting retinol to retinoic acid. Additionally, ALDH1 activity is high in hematopoietic and neural stem cells from mice and humans. Ginestier et. al. showed that, in humans, ALDH1 positive cells make up approximately 8% of the total epithelial population and are located in the terminal ductal lobular units (TDLUs), similar to mouse TEBs, where mammary stem cells are proposed to reside. Using a humanized fat pad transplantation model, the investigators showed that only ALDH1 positive cells were capable of mammary outgrowth formation.

In the mature adult female mouse, approximately 30-40% of mammary luminal epithelial cells express ESR1 and Pgr. These nuclear receptor positive cells are located throughout the ductal network and are hypothesized to stimulate proliferation of adjacent, receptor negative cells, by a paracrine mechanism. In young nulliparous animals, however, most of the luminal epithelial cells express ERα and PR. Recently, Dr. Seagroves and colleagues evaluated the effect of C/ebpb loss on mammary development (Seagroves et al. 2000). In that study, they showed that in the absence of

C/ebpb, the distribution of Pgr positive cells resembles that found in an immature mammary gland. They postulated that uniform expression of hormone receptors in immature nulliparous females prevents hormone-dependent alveolar development prior to the completion of ductal elongation. Once the ductal network is established, alveolar expansion is appropriate and hormone receptor expression adopts a more non-uniform pattern. In mature *C/ebpb*$^{-/-}$ glands, steroid receptor expression remained uniform and did not adopt the chimeric pattern found with increasing age. The authors hypothesized that, when the entire duct was analyzed, uniform steroid receptor expression reflected globally, a less differentiated gland. Their data suggested that *C/ebpb* expression and activity is required for proper maturation of the mammary luminal epithelial cells during development.

Some genes associated with cell cycle progression or DNA damage responses have been shown to be involved in mammary epithelial cell differentiation. BRCA1, mutated in some cases of breast cancer, is required for the differentiation of mouse (Kubista et al. 2002) and human (Furuta et al. 2005) mammary epithelial cells. In addition, human mammary tissue from individuals with germline BRCA1 mutations has a higher proportion of ALDH1 positive cells, presumed to be undifferentiated progenitors (Liu et al. 2008). Retinoblastoma1 (Rb1), a well-characterized tumor suppressor, is induced during mid-pregnancy in the mammary gland, and has an interesting role in mammary epithelial cell differentiation. Embryos lacking Rb1 die in utero, but mammary buds can be transplanted and their growth evaluated (Robinson et al. 2001). While there are no defects seen in ductal outgrowth or secretory

differentiation, which takes place during mid-pregnancy, the possibility remains that Rb1 participates in regulating the function of the gland during lactation. This step of development cannot be evaluated in a transplant model and a study published on a conditional Rb1 mutant model only discussed tumor formation. When a constitutively active form of Rb1 is expressed in the mammary epithelium, the gland displayed reduced ductal growth, precocious lactogenic differentiation, and interestingly, tumor formation after a long latency, likely due to sustained epithelial cell survival (Jiang and Zacksenhaus 2002). From these studies, it is clear that some genes involved in the commitment of mammary stem cells to a luminal fate are also involved in the differentiation that takes place beginning in mid-pregnancy. The next sections will describe that stage of mammary development in detail.

Pregnancy and Lactation

Secretory Differentiation

The open architecture of the nulliparous mammary gland allows space for the expansion of the epithelium that takes place during pregnancy. With each estrus cycle, small tertiary branches form along the ducts that will eventually give rise to functional alveolar structures. Hormones, such as P4 and Prolactin (Prl), are released into circulation in early pregnancy and promote proliferation of luminal epithelial cells and lobule formation. Functional differentiation of the mammary gland is divided into stages. Lactogenesis I, also known as secretory differentiation, takes place during the later part of pregnancy in most mammals and is characterized by elevated expression of

milk protein genes and formation of lipid droplets in the alveolar epithelium. These effects were documented in cows and rabbits in 1933 and 1950 respectively (Folley 1956). Lactogenesis II, or secretory activation, occurs at or near parturition and is associated with abundant milk production and secretion, and other changes in alveolar epithelial cells that are discussed below. Various circulating and local factors work together to stimulate the synthesis and expulsion of milk throughout lactation.

Progesterone and Prolactin are two of the most important hormones involved in proper pregnancy-associated development and function of the mammary gland. Two isoforms of Pgr are expressed in the mammary epithelium (Aupperlee et al. 2005). PgrA and PgrB, both encoded by the same gene, have different expression profiles during ductal morphogenesis. PgrA is most highly expressed in luminal cells during virgin development, but PgrB expression increases at midpregnancy, while neither isoform is detectable during lactation. Complete ablation of both Pgr isoforms results in severe reproductive and hormonal abnormalities, making it difficult to assess the effect of Pgr loss in the mammary epithelium specifically. Isoform specific knockout mouse studies have revealed that PgrB is required for proper alveolar expansion in the pregnant animal (Mulac-Jericevic et al. 2003). Females deficient in PgrB expression were able to lactate; however, the pups they nursed were malnourished and smaller than those fed by control animals. Gross examination of mammary glands from PgrB null mice revealed fewer, but properly developed alveoli. It was also found that expression of receptor-activator of NFκB signaling ligand (RANKL), thought to play a role in pregnancy-associated epithelial cell proliferation, was decreased in PgrB null mice. The authors concluded

that P4 exerts its effects in the mammary epithelium during pregnancy by activating PgrB, which promotes production and secretion of RANKL. By activating RANK on adjacent cells, RANKL stimulates activation of Cyclin D1 and proliferation, leading to the formation of numerous alveoli. Recently, a study was published that evaluated, in detail, the pattern of PgrA and PgrB expression throughout mouse mammary development (Aupperlee et al. 2005). It was determined that PgrB was not expressed until day 14 of pregnancy, well after alveoli have formed. It is possible, then, that PgrA mediates the initial burst of proliferation seen during the first week of pregnancy, as that is the predominant isoform expressed in the epithelium, and that PgrB can compensate for the loss of PgrA in isoform-specific knockout mice.

Prolactin plays an important role in alveolar formation and secretory differentiation, in addition to its necessity for sustained lactation and alveolar cell survival. The effect of anterior pituitary hormones on the mammary gland was investigated in 1928 and 1930 by Stricker, Grouter, and Strausburg. Extract from this tissue was shown to have lactogenic effects in rats (Folley 1956). In addition, A. T. Cowie performed hypophysectomies on intact, lactating rats at four days post-partum (Folley 1956). He showed that, without the pituitary gland, only 4% of pups survived to weaning age. Several separate studies, done in the mid- to late 1940s, showed that purified Prl injected into the rabbit mammary gland induced localized lactation, indicating the hormone's direct effect on the mammary epithelium (Folley 1956). Prolactin, which we now know is secreted from the anterior pituitary gland, acts by binding to its receptor (Prlr) that exists in a short and long form. Activation, and

subsequent dimerization, of both isoforms of Prlr leads to Jak2 phosphorylation, but only activation of the long form can result in phosphorylation and nuclear translocation of Stat5a. Activated Stat5a binds γ-interferon activation sites (GAS) with the sequence TTCNNNGAA, in the promoters of target genes including *Wap* and *Csn2*. Complete ablation of the *Prlr* leads to infertility, among other problems (Ormandy et al. 1997). Analysis of $Prlr^{+/-}$females, though, revealed a defect in pregnancy-associated lobulo-alveolar formation and reduced pup survival. This effect was attenuated with increasing age of the female at first pregnancy, and was almost completely reversed by the second lactation cycle.

Further characterization of Prlr function in the mammary gland took place two years later. Cathrin Brisken and colleagues closely examined the mammary glands of $Prlr^{+/-}$ females and also found that those with difficulty nursing pups had poorly developed alveolar structures (Brisken et al. 1999). When mammary rudiments from $Prlr^{-/-}$ females were transplanted into cleared recipient fat pads, pregnancy-associated outgrowths were minimal as was milk production. These studies confirmed that Prl, acting through its receptor in the mammary epithelium, is required for alveolar outgrowth and function of the mammary gland during pregnancy and lactation.

Confirmation of the Prl signaling cascade was achieved by performing loss-of-function studies on Jak2 and Stat5a. Deletion of *Jak2* in the mammary epithelium resulted in attenuated alveolar formation and Wap production during lactation. Additionally, using a LacZ reporter transgenic mouse model, investigators showed that Jak2 was required for survival of differentiated alveolar epithelial cells (Wagner et al.

2004). Genomic ablation of *Jak2* resulted in embryonic lethality; however, transplanted mammary rudiments from these animals failed to respond to treatment with E2 and P4, which mimicks pregnancy effects, by forming alveoli. Two markers routinely used to assess differentiation of mammary epithelial cells are Nkcc1 and Npt2b. Nkcc1 is expressed in the developing virgin and declines during pregnancy. Conversely, Npt2b is upregulated during late pregnancy and lactation. Analysis of marker expression in *Jak2*$^{-/-}$ transplanted glands revealed sustained Nkcc1 expression and a lack of Npt2b upregulation at the end of pregnancy (Shillingford et al. 2002). In the absence of Stat5a, similar defects were observed (Liu et al. 1997). *Stat5a*$^{-/-}$ mothers could not nurse young and their mammary glands did not expand during the later stages of pregnancy. Examination of the few alveolar cells that formed revealed lipid droplet accumulation, indicating an increase in metabolic activity normally associated with secretory differentiation; however, the cells never acquired secretory activity. Gene expression analysis showed a defect in Wap production, similar to that identified with *Jak2* deletion. Together, these studies highlight the importance of the Prlr/Jak2/Stat5a signaling cascade in alveolar formation and secretory activation in the mammary epithelium during late pregnancy and lactation.

Secretory Activation

Secretory activation takes place at parturition as a result of P4 withdrawal. In mice, P4 is produced by the ovaries throughout pregnancy and declines shortly before the birth of the pups. In humans, the placenta is responsible for the circulating P4 during

much of pregnancy, and placenta removal is necessary for onset of abundant milk production. At parturition, Prl levels surge leading to augumented production of milk proteins and other components. In addition to its role in alveolar formation, Prl, released from the brain in response to suckling, can act by signaling through its receptor to promote cell survival via induction of IGF1 and subsequent inhibition of IGFBP5, which is involved in involution after pup removal (Flint et al. 2006).

In addition to protein, lipids, lactose, ions, and water are secreted from alveolar epithelial cells, and the composition of these constituitents varies across mammalian species. In humans, for example, only 5% of breast milk is composed of lipid, while mice produce milk that contains 25-30% fat. Depending on the diet, the fatty acid composition may vary, but the percent of lipid remains consistent (Rudolph et al. 2007b). Secretory activation is characterized by copious milk production, upregulation of lipogenic enzymes, down regulation of fatty acid beta-oxidation enzymes, closure of epithelial tight junctions, and polarization of cellular organelles. Proteins, water, lactose, and calcium are released from alveolar epithelial cells through an exocytotic pathway. Lipids are synthesized in the epithelial cell and aggregate into a unique membrane-encased structure, called a milk fat globule, which is secreted by a budding process. Other transport pathways exist for the movement of some proteins, ions, and small molecules, such as glucose and amino acids. Closure of the tight junctions during lactation prevents paracellular transport, normally used by low molecular weight substances and large solutes to travel between cells, and back and forth from the interstitial space to the lumen of the duct.

The proteins produced during secretory differentiation and lactation include members of the Casein family, whey acidic protein (Wap), WDNM1, and alpha-lactalbumin. Of these, the milk protein, beta-Casein, is the most widely studied and its regulation the best characterized. Both *in vivo* and *in vitro*, hormones such as insulin, glucocorticoids, and Prl are known to induce transcription of the *Csn2* gene. In 1993, Happ and Groner showed that mammary gland factor (MGF now known as Stat5a) was the nuclear factor that responded to hormones to induce *Csn2* transcription (Happ and Groner 1993). The HC11 cell line is an excellent model to use for studying the regulation of *Csn2*, as the gene is one of the only physiologically relevant milk protein genes expressed upon Prolactin treatment. In addition to Stat5a, C/ebpb, GR and YY1 have all been shown to bind the *Csn2* promoter and play positive or negative regulatory roles in transcription (Kabotyanski et al. 2006). Mutational analyses performed on the rat *Csn2* promoter identified C/ebp binding sites that were critical for both basal and lactogenic hormone-induced activation of *Csn2* (Doppler et al. 1995). Conversely, the nuclear factor, YY1, was shown to be important in the negative regulation of *Csn2* expression and was dissociated from the *Csn2* proximal promoter upon addition of Stat5a (Meier and Groner 1994). ChIP assays, performed on samples isolated at specific timepoints during HC11 cell differentiation, have showed that within 15 minutes of Prl stimulation, Stat5a is recruited to GAS sites in the *Csn2* promoter (Kabotyanski et al. 2006). In addition to various milk proteins, the alveolar epithelial cells synthesize and secrete large amounts of lipid throughout lactation (Rudolph et al. 2007a). Milk lipid, secreted in the form of triglycerides (TG), generally comes from three sources: dietary

fat, mammary stromal adipocytes that regress beginning in pregnancy, and *de novo* lipogenesis that relies glucose and other macromolecular resources. The genes responsible for TG synthesis begin to increase at mid-pregnancy, explaining the appearance of cytoplasmic lipid droplets (CLDs) in the mammary epithelium at this stage. At parturition, there is a drop in expression of fatty acid beta-Oxidation genes and an increase in lipogenic enzyme mRNA expression. This shift in mRNA expression was proposed to be regulated by a balance in PPARγ and LXR activity and subsequent induction of SREBP1c. This bHLH family member also plays a role in the activation of lipogenesis in the 3T3-L1 mouse preadipocyte line. Interestingly, in the mammary glands of mice fed high fat chow, SREBP1c induction was minimal, as was expression of its target genes. This indicates that SREBP1c and its downstream targets are responsible for de novo lipogenesis when dietary fat sources are unavailable. Additionally, in mice on a high fat diet, the adipose compartment of the mammary gland undergoes minimal regression, while in mice fed normal (i.e. 4% fat) chow, adipocytes are completely de-lipidated by parturition.

Involution

Natural Loss of Function

A normal mouse lactation period lasts 21 days. According to the lactation curve, the maximum secretory ability of the gland is reached nearly 11 days before the pups are old enough to be weaned (Hanrahan and Eisen 1970). The decline in secretory ability of the mammary gland is referred to as secretory diminution (Hadsell et al. 2006), and has

been hypothesized to be due to several things. Secretory cell aging, proposed in 1989, is thought to play a role in the failure of lactation, although this theory has not been confirmed (Hadsell et al. 2007). In mice, activity of the mitochondrial cytochrome C-oxidase, or Complex IV of the electron transport chain, increases until day 10 of lactation and then begins to decline (Hadsell et al. 2007). Forced involution in rats has been associated with increased oxidative damage to mitochondrial DNA (Esteve et al. 1999), while in mice, oxidative DNA damage begins to increase during the gain-of-function phase of lactation (Hadsell et al. 2006). Although these studies suggest that alveolar epithelial mitochondria experience a loss of function associated with oxidative damage, there are no reported associations between this stress and changes in mitochondrial function during prolonged lactation or involution. Additionally, it is unclear if secretory diminution is centrally or peripherally mediated, but likely involves the interplay of the stromal and epithelial compartments in the mammary gland.

Cross-fostering experiments in both rats and mice have been shown to prolong the secretory ability of the alveolar epithelial cells by ensuring complete and frequent milk removal from the gland (Hadsell et al. 2006). It is likely that pup behavior, such as eating solid food, and leaving the nest to explore the cage, causes them to suckle less than if they were younger and contributes to the natural loss of function. Additionally, the mother may encourage less nursing after the pups grow teeth.

To avoid the sporadic involution of different lobules as a variable in studying involution, many investigators use a forced-weaning model. This involves normalizing the litter sizes to approximately 8 pups and allowing the mother to lactate for 10 days

before the pups are removed and the glands are harvested. This model, combined with several studies reporting genes associated with failed lactation, has led to the discovery of important regulators of involution. There is an overwhelming abundance of studies that report either accelerated or delayed involution when various genes are manipulated, but only a few factors have central roles. Those factors will be discussed in the next section.

Regulation of Mammary Involution

Using the forced-weaning model, it was revealed that involution occurs in two phases: an immediate, reversible phase, induced by the build up of milk in the alveoli, and characterized by a small amount of apoptotic epithelial cells that have been shed into the lumens, and a later, irreversible phase that involves complete gland remodeling by MMPs, and lipid accumulation in the stromal adipocytes (Lund et al. 1996). The first phase lasts 24-48 hours, and during this time, circulating prolactin levels begin to decrease. It has been shown that Prl-mediated Stat5a activation is important for the function and survival of the alveolar epithelial cells (Flint et al. 2006). Several mouse models have revealed that LIF production by epithelial cells, acting through its receptor and Jak activation, causes phosphorylation of Stat3. Following Stat3 activation, C/ebpd and IGFBP5 are induced, and lead to upregulation of members of the pro-apoptotic Bcl2 family including *Bak* and *Bax*. Phosphorylation and activation of Stat5a ceases, and expression of anti-apoptotic genes, such as *Bcl2a1* and *Bcl2l2*, is reduced. Mice with deficiencies in LIF, Stat3, and C/ebpd all share similar phenotypes, experiencing delayed

involution characterized by sustained distention of alveoli with milk, low levels of active Caspase-3, and maintenance of alveolar structures (Chapman et al. 2000; Humphreys et al. 2002; Kritikou et al. 2003; Thangaraju et al. 2005). IGFBP5 has been shown, in wild type glands, to bind and sequester IGF1, which promotes epithelial cell survival, in addition to its role in adolescent development. In the absence of LIF and Stat3, involution does occur, although it is delayed. Under these circumstances, cell death is mediated largely by p53 activity.

Three to four days after pup removal, expression of MMP3 and C/ebpb increases in the mammary gland. Currently, there is very little literature on the role of C/ebpb during mammary involution. It is worth noting that each of the studies mentioning the association of C/ebpb with mammary involution did so by evaluating whole mammary gland lysate by either western blot, northern blot, or PCR analysis (Raught et al. 1995; Gigliotti and DeWille 1998; Gigliotti and DeWille 1999; Thangaraju et al. 2004). Because C/ebpb is important for adipogenesis, and mammary epithelial regression during involution happens concurrently with differentiation of adipocytes in the stroma, it is possible that the upregulation of C/ebpb during involution actually takes place in the mesenchymal comparment and that it does not have a critical role in alveolar disintegration.

MMP3, or stromelysin 1, participates in degradation of the basement membrane and has been found to be primarily produced by stromal cells immediately adjacent to alveoli (Lund et al. 1996). Several studies have revealed a requirement for basement membrane integrity, and association with epithelial cells in lactogenic differentiation and

function (Barcellos-Hoff et al. 1989; Chen and Bissell 1989; Streuli et al. 1991). Using teat-sealing experiments, which allow the elucidation of pathways involved in mammary involution in the presence of circulation lactogenic hormones, Talhouk and colleagues determined that TIMP production prevents MMP expression during the initial, reversible stage of involution (Talhouk et al. 1992). In addition, transgenic mice expressing MMP3 in the mammary epithelium, under the control of a *Wap* promoter expressed during lactation, had smaller alveoli and experienced early involution (Talhouk et al. 1992). Together, these studies highlight the mammary gland as an excellent model to evaluate normal processes associated with cell death and tissue remodeling.

A Short History of Sim2

Singleminded-2 (Sim2) is a member of the bHLH-PAS family of transcription factors. PAS proteins influence transcription of target genes as heterodimers and recognize consensus response elements in gene regulatory regions. *Sim2* is one of two mammalian homologs of Drosophila Singleminded (*dsim*), which is required for proper CNS development in fly embryos. Located in the Down Syndrome Critical Region (DSCR) of HSA21, *Sim2* is thought to influence some of the developmental abnormalities associated with Down Syndrome (DS). Our research has focused on SIM2 as a potential mammary tumor suppressor based on a study published in 2000 examining the solid tumor occurrence in individuals with DS (Hasle et al. 2000). Of the 2800 people surveyed in the study, the investigators expected 7 cases of breast cancer and observed none. Thus, previous studies performed in our lab have evaluated the

relationship between SIM2 and cancer cell growth, invasion, and metastasis. Until we began our investigation, it was not determined if Sim2 had a role in normal development and differentiation of the mammary gland.

Drosophila Singleminded

Loss-of-function studies performed in Drosophila have revealed a role for *dsim* in establishing the fate of the central midline cells, which are the first to be specified during embryonic neurogenesis. In the absence of *dsim*, the midline cells degenerate, and other precursors lack expression of ventral epidermal, and lateral neuronal markers (Chang et al. 2001). Conversely, ectopic expression of *dsim* in mutant embryos rescues CNS defects and causes non-midline cells to adopt a midline fate (Menne et al. 1997). Additionally, the factors produced by the midline cells, and thus by *dsim* activity, are required for the specification and differentiation of lateral neural cells and for expression of dorsoventral patterning genes during embryonic neurogenesis. Later in embryogenesis, instructive signals from midline cells are thought to influence differentiation of mesodermal cells, which eventually adopt a dorsal median fate. While *dsim* expression is restricted to the single row of midline cells during early development, its role in activating transcription of spitz class genes is necessary to achieve the precise gradient of secreted, instructive factors that ultimately influence the patterning and differentiation of multiple cell types in the Drosophila CNS.

In Drosophila, dsim forms a heterodimer with tango (tgo), homologous to mammalian ARNT, and this association has been shown to require the PAS domain in

the N-terminal region of the dsim protein, while the basic domain is required for association of the dsim/tgo heterodimer with DNA response elements. A consequence of inhibiting dsim/tgo heterodimerization was observed in dsim mutant flies. Using a temperature-sensitive allele, which substitutes a phenylalanine for serine in the HLH domain of dsim, investigators showed that the mutant flies could only walk in circles and had reduced fertility. They found that the dsim/tgo heterodimer is required for proper formation of the portion of the CNS responsible for walking behavior, and to correctly pattern genital discs and the anal pad anlagen (Pielage et al. 2002). A separate study, evaluating mutations of dsim binding sites in target genes, revealed that dsim acts as a transcriptional activator, and that association of dsim with DNA was not required for repression of transcription, indicating that it inhibits gene expression by activating repressors (Estes et al. 2001).

Outside of the CNS, dsim is necessary for the migration and eventual fusion of mesectodermal cells with ventral muscle fibers (Zhou et al. 1997). In addition, left-right asymmetry was not established in the Drosophila gut in the absence of dsim expression, which is reminiscent of the CNS phenotype observed under the same circumstances. Based on data showing a requirement of dsim expression and transcriptional activity for the formation of midline cells, and on the necessity of secreted factors produced by midline cells for the proper patterning and differentiation of the entire Drosophila CNS, dsim has been called the “master” regulator of neurogenesis in this organism (Nambu et al. 1990). This title is somewhat misleading, as it is clear that the requirements for dsim activity extend beyond the Drosophila CNS cell types, and in the next sections, I will

describe the role singleminded proteins play in the development and function of multiple structures in higher species.

The singleminded (sim) gene has been identified and cloned in xenopus and zebrafish. In these organisms, sim is expressed in the developing CNS during embryogenesis. In adult zebrafish, as in mammals, there are two homologs of dsim, Sim1 and Sim2. A requirement for SIM1 in the development of isotocin cells, part of the preoptic-neurohypophysial system, has been established (Eaton and Glasgow 2006). *Sim2* is expressed in muscle, heart, liver, eyes, gills, and intestines, but whether or not Sim2 is required for development of these structures and for differentiation of the CNS, as it is in Drosophila, is yet to be determined (Coumailleau et al. 2000; Wen et al. 2002).

Mammalian Singleminded Proteins

In 1995, investigators published the identification of one human homolog of *dsim* in the DSCR of Chromosome 21 (Chen et al. 1995). Two years later, two homologs of *dsim* were cloned using exon trapping. In addition to *Sim2*, this group identified *Sim1*, located on HSA6 (Chrast et al. 1997). In this same study, the authors reported the production of a short isoform of *Sim2*, called *Sim2s*, due to alternative splicing. The two isoforms of Sim2 are identical except for the lack of a proline/alanine domain in the C-terminal region of Sim2s (Chrast et al. 1997). In mice, screening of a cDNA library from E11.5 embryos revealed a mouse homolog of dsim located on MMA16, now known to be mouse *Sim2* (Yamaki et al. 1996) (Figure 4). Our lab first identified the short isoform of mouse *Sim2*, called *Sim2s*, which is produced by a similar mechanism

as the human isoform (Metz et al. 2006) (Figure 5). The mouse and human Sim2 proteins act as transcriptional repressors, contrary to dsim, but both short isoforms have variable activity depending on the promoter and cell type (Metz et al. 2006).

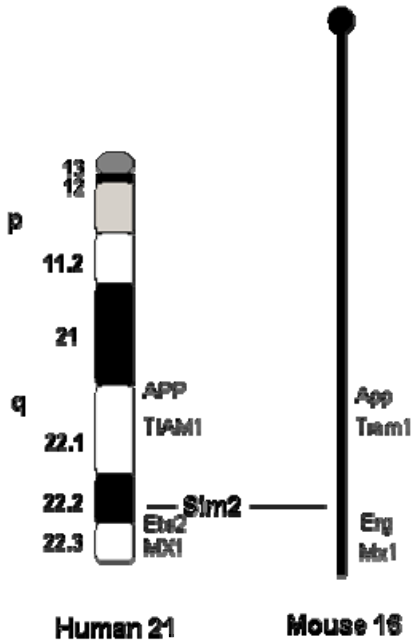

Figure 4. Location of *Sim2* in Humans and Mice**.** Schematic drawing of human chromosome 21 and mouse chromosome 16 showing location of Sim2 genes. Adapted from Chrast et. al., 2000.

Several studies have been performed, using mouse models, to elucidate the involvement of Sim2 in CNS development due to its critical chromosomal location. The initial identification and characterization of mammalian *Singleminded* genes was done in 1995 and early 1996 by several groups. In the May 1995 issue of Nature Genetics, Chen and colleagues first reported the discovery of 6 exons with sequence homology to *dsim* located on HSA21, in an attempt to isolate genes associated with the DSCR (Chen et al.

1995). In the summer of 1995, Muenke and colleagues published a study on holoprosencephaly. While they did not find an association between HSA21 and holoprosencephaly, they did mention the existence of 2 human homologs of dsim, located on HSA6 and 21 (Muenke et al. 1995). At the same time, Dahmane and colleagues reported the use of exon trapping to identify a portion of the DSCR with sequence similarity to dsim and detected 2 transcripts by northern blot analysis in the human fetal kidney (Dahmane et al. 1995). In this study, the authors evaluated expression of *Sim2* by ISH in developing rat embryos and in 16 week old human fetuses. The strongest expression of *Sim2* was found in the skull and facial primordia, and also in the vertebral body and ribs of E13-E15 rats, while the CNS was strongly labeled in the region of the basal hypothalamus. In human fetuses, similar regions as seen in the rat embryos expressed *Sim2*. In 1996, the same group that originally reported the identification of a dsim homolog on HSA21 cloned a singleminded gene from a mouse E11.5 cDNA library, to begin to analyze the structure of the protein (Yamaki et al. 1996).

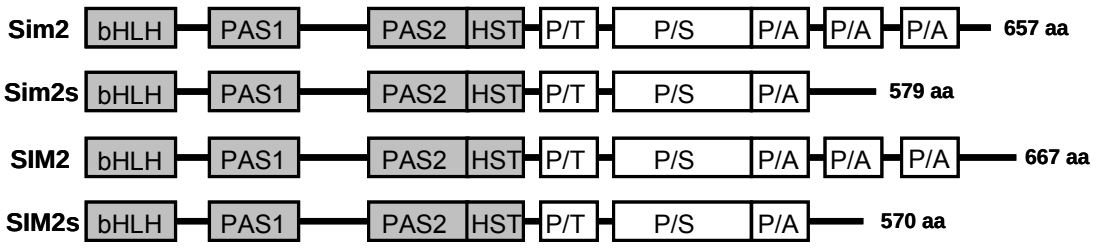

Figure 5. The Mammalian Singleminded Proteins. Schematic drawing of human and mouse Sim2 proteins with conserved and variable domains. Adapted from Chrast, et. al., 1997.

They determined that the amino terminal region of the mammalian singleminded protein showed similarity to dsim, the AHR, PER, and ARNT, but the carboxy-terminus did not show any homology to the Drosophila isoform. Based on analysis of the protein structure, it was concluded that the Sim homolog identified in this study was identical to the *Sim2* isoform that was reported previously. Finally, in 1997, both human homologs of dsim, *Sim1* and *Sim2* were cloned (Chrast et al. 1997).

With the discovery of two mammalian homologs of dsim, *Sim1* and *Sim2*, and the localization of *Sim2* to HSA21 and a syntenic region in MMA16, came several studies evaluating the possibility that SIM2 was involved in the developmental abnormalities and mental retardation associated with the DS population. The Fan Laboratory initially generated a construct to use for disruption of *Sim2* expression (Goshu et al. 2002), but Shamblott and colleagues published the first study examining the consequence of *Sim2* ablation on mouse embryonic development (Shamblott et al. 2002). Although *Sim2*$^{-/-}$ pups were born in the proper Mendelian ratio, none survived to weaning age. Close examination of *Sim2*$^{-/-}$ pups revealed stomachs devoid of milk, and instead, full of air. Most had severely cleft palates, characterized by decreased cell density and a high composition of hyaluronan (HA) in the ECM. Analysis of HA Synthetase expression revealed a significant increase with the loss of both *Sim2* alleles, as compared to heterozygote and wild type animals. On the contrary, the study published by Goshu and colleagues described a slightly different phenotype of the *Sim2*$^{-/-}$ mice, originally generated by another group (Goshu et al. 2002). As reported

previously, *Sim2*$^{-/-}$ mice died shortly after birth. In disagreement with the previous study, reporting cleft palates and aerophagia associated with *Sim2* loss, these investigators discovered abnormally developed ribs, intercostal muscles, and diaphragms in *Sim2* mutants. In fact, they reported that in both wild type and mutant pups, the palate was properly formed and joined at the midline. The defects in the muscle and rib development found in mutant animals were presumably responsible for the respiratory distress and cyanosis observed at birth and shortly before death. Regardless of discrepancies in the literature, the early lethality of *Sim2*$^{-/-}$ mice eliminates this model for the use of studying behavioral changes resulting from *Sim2* disruption. Generation of trisomy 16 and Sim2-overexpressing mice, however, helped overcome this obstacle.

Evaluation of mice with complete trisomy of MMA16 was reported in 1985, but these animals die in utero, making it difficult to study behavior and full CNS development (Epstein et al. 1985). Two mouse models, each with partial trisomy of MMA16 that includes the region homologous to the DSCR on HSA21, have been generated and their behavior evaluated, in an attempt to establish models of Down Syndrome. The Ts65DN mouse was created first, and was found to have reduced memory and learning behavior as judged by performance in a 12-arm radial maze, to have spontaneous locomoter hyperactivity, and low performance in a Morris Water Maze (Reeves et al. 1995; Demas et al. 1998). It has been suggested that locomoter hyperactivity is a non-specific indicator of abnormal CNS development, and the reduction in performance on various learning tasks suggests partial trisomy of Chromosome 16 somehow influences communication between regions of the CNS. In

1998, quite by accident, Sago and colleagues derived another partial trisomy 16 mouse while attempting to generate a *Sod*$^{-/-}$ allele. This mouse carries an extra copy of a slightly smaller DSCR than the one described previously. Called Ts1Cje, this mutant also showed impaired performance in the Morris water maze (Sago et al. 1998). Neither model is described as having abnormally developed facial or limb structures. It can be presumed that both mouse models described above have three copies of *Sim2* as a result of partial trisomy 16; however, in neither study did the investigators examine expression of the gene or production of the protein. In 1999, Ema et. al. created a transgenic mouse overexpressing *Sim2* under control of the chicken Actb promoter (Ema et al. 1999). This study revealed mild deficits in contextual fear conditioning and, interestingly, in the Morris water maze task, associated with *Sim2* overexpression. Aside from the reduced performance on various behavioral tasks, the authors also reported the expression of *Sim2* in wild type adult mouse skeletal muscle, kidney, lung, stomach, thalamus, hippocampus, and amygdala. Two other *Sim2*-overexpressing mouse models were generated in 2000, bearing one or two additional copies of the gene, contrary to the model described in 1999 that used a chicken Actb promoter to drive transgene expresison (Chrast et al. 2000). While the investigators of the former model reported reduced fear responses and learning impairments, those results could not be reproduced in this study. The authors did report a higher tolerance of pain in the transgenic mice and noted reduced social behavior in female mice, but attributed the learning deficits of the mice in the first study to the artificial levels of *Sim2* in that model. Taken together,

these data indicate that overexpression of *Sim2*, resulting from trisomy 21, may play a role in learning impairments associated with the DS population.

Singleminded-2 and Cancer

In addition to its potential role in the etiology of DS, *Sim2* has been studied as a putative oncogene in several tissue types. *Sim2s* expression has been reported in tumor-derived cell lines, and tissue samples from carcinomas of the colon, prostate, and pancreas, but not in breast, lung, or ovarian cancers (DeYoung et al. 2003b). Using antisense oligonucleotides, this group has reduced *Sim2s* expression in cell lines derived from both colon and pancreatic carcinomas (DeYoung et al. 2003b; DeYoung et al. 2003a; Aleman et al. 2005). In cells with low levels of *Sim2s*, they reported reduced proliferation, increased caspase-dependent apoptosis, and slower growth in nude mouse xenograft models when compared to control cells. Surprisingly, in the discussion of their work, the authors describe the possible association of *Sim2s* with Down Syndrome, but Hasle and colleagues did not find an increase in colon tumors associated with such individuals (Hasle et al. 2000). There is no mention, thus far, of pancreatic or prostate tumor occurrence in the DS population by any group, although several investigators report a decrease in all solid tumors associated with trisomy 21 (Hasle et al. 2000; Hasle 2001; Dixon et al. 2006; Sullivan et al. 2007).

Our lab has found a very different relationship between *Sim2s* and cancer. Most of the work published on *Sim2* as a tumor suppressor has been done in breast cancer cells, and it is worth noting that De Young et. al. did not detect any expression of *Sim2s* in mammary carcinomas (DeYoung et al. 2003b). The expression of *Sim2s* has been

shown to be high in normal mammary epithelial cells, and to decrease with transformation to cancer cells (Kwak et al. 2007). When *Sim2s* is re-introduced to MD-MBA-435 cancer cells, proliferation, invasion, and anchorage-independent growth are all reduced. Additionally, in this cell type SIM2s reduced mRNA levels and activity of MMP3, known to be involved in cancer metastasis (Kwak et al. 2007). *Sim2s* is the primary isoform expressed in relatively non-aggressive MCF-7 breast cancer cells, albeit at a low level. Elimination of SIM2s, using shRNA, led to an increase in proliferation, invasion, and growth of cells in nude mouse xenografts (Laffin et al. 2008). In this model, SIM2s was found to inhibit expression of *Slug* and MMP2, both known contributors to cancer invasion and metastasis, and reduction of SIM2s led to a loss of epithelial characteristics, such as E-Cadherin and Keratin-18 expression, and acquisition of the mesenchymal markers, N-Cadherin and Vimentin.

Recently, it was shown that stem cells and cells undergoing EMT were very similar (Morel et al. 2008). By using cell sorting and other techniques, the authors were able to conclude that the EMT process requires a de-differentiation of epithelial cell types to a progenitor-like cell population under normal developmental circumstances. Our data suggest that SIM2s is necessary for the maintenance of the differentiated epithelial phenotype, characterized by expression of epithelial-specific keratins and cell contact proteins; therefore, our hypothesis is that Sim2s promotes differentiation of epithelial cells in the mammary gland, possibly playing a role in the formation and function of alveolar epithelial cells during lactation.

CHAPTER II

MATERIALS AND METHODS

Cell Culture

HC11 cells were maintained in growth media consisting of RPMI 1640 (GIBCO 22400-089), 10% calf serum (Atlanta Biologicals S11450), 5 µg/mL insulin (Sigma I6634), 50 µg/mL gentamicin (GIBCO 15710-064), and 50 ng/mL rhEGF (QED Biosciences Inc., 36001P). Cells were passaged approximately every other day, based on confluence. For differentiation, cells were allowed to remain confluent for 3 days, with fresh growth media added daily. Then, cells were washed 2 times with PBS, followed by addition of priming media (PM). Basal PM included RPMI 1640 (GIBCO), 10% charcoal-stripped donor horse serum (Atlanta Biologicals S12150), 50 µg/mL gentamicin (GIBCO), and 5 µg/mL insulin (Sigma). Hydrocortisone (HC Sigma H0888) was added to a final concentration of 1 µg/mL for 24 h. Then both HC and ovine Prolactin (Prl NIDDK oPRL 21) were added at 1 µg/mL for the duration of the differentiation protocol (ranging from 4 h to 4 days), adding fresh PM + HC and Prl every 24 h. Differentiation was assessed by formation of cytoplasmic lipid droplets in the majority of the cells, and also by expression of *Csn2*.

Chromatin Immunoprecipitation Assay (ChIP)

Chromatin Harvest

Formaldehyde (Sigma F1635) was added to fresh culture media (final concentration 1%) and cells were incubated at room temperature (RT) for 10 min with gentle rocking. Glycine (Sigma G8898) was added to a final concentration of 125 mM and allowed to quench formaldeyhde for 5 additional min at RT. Cells were washed 2 times with ice-cold PBS and scraped in cold PBS containing 25x Complete protease inhibitors (CPI Roche 11-697-498-001). Cells were pelleted by spinning at 2000 rpm (805 x g) for 4 min using an Eppendorf 5810R centrifuge chilled to 4 C. SDS lysis buffer (50mM Tris pH 8.1, 10 mM EDTA, 1% SDS, 25x CPI) was added, and resuspended cells were incubated on ice for 10 min. Using a sonicator (Heat Systems Ultrasonics Inc., Model W-380), DNA was sheared in 10 second pulses, 10 times, allowing the lysate to cool on wet ice for 1 min after every 2 pulses. Debris was pelleted by spinning at 13,200 rpm (16.1K x g) in an Eppendorf 5415D centrifuge for 10 min at 4 C. Chromatin was stored at -80 C in 100 µL aliquots.

Standard ChIP Assay

One aliquot of chromatin was used for each assay. ChIP dilution buffer (0.01% SDS, 1.1 % Triton X-100, 1.2 mM EDTA, 16.7 mM Tris pH 8.1, 167 mM NaCl, 25x CPI) was added to 5-fold dilution. Normal rabbit serum (5 µL Santa Cruz sc2338) and 60 µL salmon sperm DNA/Protein A or Protein G Agarose (Upstate Protein A 16-157; Protein G 16-201) was added for 30 min and chromatin was precleared at 4 C with

agitation. Beads were pelleted by spinning in an Eppendorf centrifuge at 200 x g for 1 min. Lysate was precleared once more with 60 µL of agarose beads (no serum). Antibodies were added and chromatin was agitated at 4 C overnight (see Table 2 for antibody sources and conditions). The following day, 60 µL of agarose beads were added for 1 h at 4 C with rocking. Beads were pelleted and washed consecutively for 10 min in each solution: low salt wash (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.1, and 150 mM NaCl), high salt wash (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.1, and 500 mM NaCl), lithium chloride wash (0.25 M LiCl, 1 % NP-40, 1 % sodium deoxycholate, 1 mM EDTA, and 10 mM Tris pH 8.0), and twice in TE buffer (10 mM Tris pH 8.0 and 1 mM EDTA). TE washes took place at RT and others at 4 C. Immune complexes were eluted from beads in 1 % SDS and 0.1 M $NaHCO_3$, adding 250 µL to each aliquot and rocking for 15 min at RT, then repeating for a total of 500 µL eluate. NaCl was added for a final concentration of 0.3 M with 1 µL of 10 mg/mL RNAse-A. Eluate was incubated at 65 C for 5 h to reverse formaldehyde crosslinks. Two and one half volumes of 100 % EtOH were added to each sample and they were placed at -20 C overnight. On the third day, chromatin was pelleted by spinning in an Eppendorf centrifuge at 16.1K x g for 10 min. Supernatant was removed and the pellet resuspended in 100 µL H_2O, with 2 µL 0.5 M EDTA, 4 µL 1 M Tris pH 6.5, and 1 µL of 20 mg/mL proteinase K (Sigma 93161722). Samples were incubated at 45 C for 2 h, and then purified using a Qiagen PCR purification kit (Qiagen 28106). DNA was eluted in 50 µL elution buffer (supplied with kit). PCR was performed according to conditions listed in Table 1.

Table 1. Primer Sequences Used in RT-PCR and Q-PCR Reactions.

Target	Strand	Sequence	Anneal C
mCsn2 Proximal Promoter	Sense	CAC-TTG-GCT-GGA-GGA-ACA-TGT-AGT-T	59
	Antisense	ACA-TCT-GAA-GTT-CTT-ACC-TTT-AGT-GG	
mCsn2 Exon VII	Sense	CAT-ATG-CTC-AGG-CTC-AAA-CCA-TCT-CT	60
	Antisense	GTA-CTG-CAG-AAG-GTC-TTG-GAC-AGA-C	
mActin Exon	Sense	TAC-AGC-TTC-ACC-ACC-ACA-GC	57
	Antisense	AAG-GAA-GGC-TGG-AAA-AGA-GC	
HA Sequence	Sense	AAG-AAC-CAA-CCC-ATA-TCC-CC	54
	Antisense	GGC-ATA-ATG-CGG-CAC-ATC-ATA-AGG	
GAPDH	Sense	CTA-ACA-TCA-AAT-GGG-GTG-AGG	54
	Antisense	TCA-TAC-TTG-GCA-GGT-TTC-TCC	
Keratin-18	Sense	CAT-CGT-CTT-GCA-GAT-CGA-CAA	Q-PCR
	Antisense	GAC-TGG-CGC-ATG-GCT-AGT-TC	
Claudin-7	Sense	TCC-CTG-GTG-TTG-GGC-TTC-T	Q-PCR
	Antisense	ACA-GCG-TGT-GCA-CTT-CAT-G	
Wap	Sense	TCA-GTC-CAT-GTT-CCC-AAA-AGC	Q-PCR
	Antisense	CTC-GTT-GGT-TTG-GCA-GAT-GA	
WDNM1	Sense	CGC-CCC-CAC-GCA-GTT	Q-PCR
	Antisense	GCC-AGA-GCA-CGA-TGG-ATC-TG	
Lalba	Sense	GAA-TGG-GCC-TGT-GTT-TTA-TTT-CA	Q-PCR
	Antisense	TGT-GCT-GCC-GTT-GTC-GTT	
pan Sim2	Sense	TCA-CGT-CTT-CAG-CAG-CAA-GAA	Q-PCR
	Antisense	AGA-AGC-GTG-CCA-CCT-CAC-A	
Sim2s	Sense	AAC-CAG-CTC-CCG-TGT-TTG-AC	Q-PCR
	Antisense	ACT-CTG-AGG-AAC-GGC-GAA-AA	

Re-ChIP Assay

The standard ChIP protocol was followed until beads were washed with TE. Following TE washes, beads were incubated with 1 volume of 20 mM DTT at 37 C for 30 min with agitation. Beads were pelleted by centrifugation at 16.1K x g and supernatant was diluted 15-fold in re-ChIP dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, and 20 mM Tris pH 8.1) and 25x CPI. Ten percent was reserved for input samples, and the remainder was divided in half, for primary antibody incubation and IgG control. After addition of antibodies, samples were processed

according to standard ChIP protocol, except for the final step, in which DNA was eluted after clean-up in 30 µL elution buffer instead of 50 µL.

Western Blot Assay

Protein Isolation

Cells were washed once with PBS and scraped in PBS containing 25x CPI (Roche). Cells were pelleted by spinning in a pre-cooled Eppendorf centrifuge at 2000 rpm for 4 min. Lysis buffer [20 mM Tris-Cl pH 8.0, 137 mM NaCl, 10 % glycerol, 1 % NP-40, 2 mM EDTA + 25x CPI + phosphatase inhibitors (0.5 mM NaMolybdate, 0.1 mM Na Orthovanadate, and 1 mM NaF)] was added and resuspended cells were agitated at 4 C for 30 min. Debris was pelleted by spinning in a cooled Eppendorf centrifuge at 16.1K X g for 10 min. Aliquots of 200 µL were stored at -20 C. If used immediately, they were stored at 4 C. Protein content was estimated using the RCDC Protein Assay (BioRad 500-0120).

Standard Analysis

Protein samples were diluted in 30 µL of H_2O per sample. Six µL of 6x SDS loading buffer (60 % glycerol, 0.3 M Tris pH 6.8, 12 mM EDTA, 12 % SDS, 6 % beta-mercaptoethanol, 0.5 % bromophenol blue) were added and samples were boiled for 5 min, followed by 5 min of cooling on ice. Acrylamide gels ranging from 8%-12% were used for analysis. Depending on the size of the target protein, gels were run at 110mV (constant V) for 1-2 h and transferred to PVDF membranes for 1.5 to 3 h at 110mA

(constant mA). After a 5 min wash in PBS + 0.05 % Tween 20 (PBST), membranes were blocked for 1 h or overnight in PBST + 5 % milk (BioRad 170-6404). See Table 2 for antibody sources and incubation conditions. Proteins were visualized using the Amersham ECL Plus western blotting detection reagent (GE Healthcare RPN 2132) on Amersham Hyperfilm (GE Healthcare RPN1678K). All films were scanned using a Dell All-in-one scanner.

Table 2. Antibodies Used for ChIP Analyses.

Antigen	Source	Catalog #	ChIP (µg)
RNAPII	Abcam	ab-26721	5
S5RNAPII	Abcam	ab-24759	5
Stat5a	Chemicon	AB3163	8
AcH3	Upstate	06-599	5
Cdk9	Santa Cruz	sc7331	5
Sim2	Chemicon	AB4145	5

PCR Analysis

RNA Isolation from Cells

Cells were washed with PBS and RNA was isolated using the RNEasy Mini Kit (Qiagen 74106) following the protocol for spin isolation. Qia-Shredder columns were used to homogenize cells prior to RNA isolation (Qiagen 79656). RNA was eluted in 30 to 50 µL RNAse-free H_2O and stored at -80 C. To remove contaminating genomic DNA, 8 µL aliquots of RNA were treated with 10 U of DNAse (Roche 10776785001)

and 1 µL 10x DNAse buffer (200mM Tris pH 8.4, 20mM $MgCl_2$, 500mM KCl) for 15 min at room temperature. One µL of stop solution (50 mM EDTA) was added to inhibit the DNAse reaction. RNA concentration was determined using a BioRad Spec (BioRad Smartspec Plus).

RNA Isolation from Tissue

To isolate total RNA from tissue, sections of approximately 0.5 cm by 0.5 cm were homogenized in Trizol reagent (Invitrogen) for 30 seconds. Samples were then centrifuged for 5 min at 16.1K x g to pellet debris. Supernatant was mixed with 200 µL of chloroform and allowed to incubate at RT for 5 min. Following centrifugation at 12K x g for 15 min, aqueous layer was mixed with 1 mL of 75 % EtOH and inverted several times. Samples were centrifuged for 10 min at 12K x g. Supernatant was aspirated and pellets were washed in 75 % EtOH, then centrifuged for 5 min at 7.5K x g. Supernatant was aspirated again, and pellets were resuspended in 100 µL of RNAse-free H_2O. Rehydrated RNA was subjected to purification according to that described in the previous section for RNA isolation from mammalian cells.

Reverse Transcription

Depending on the RNA concentration, 1-2 µg of RNA was used for reverse transcription reactions. One µL of each 10 mM dNTPs (Invitrogen) and Oligo dT (Invitrogen 18418-012) or random primers (Invitrogen 48190-011) was added to RNA in H_2O for a total volume of 12 µL. The sample was incubated at 70 C for 10 min, then 4

µL 5X first strand buffer, 2 uL 0.1 M DTT, 1 µL RNAse Out (Invitrogen 10777-019), and 1 µL Superscript III reverse transcriptase (Invitrogen 18080-044) were added for a new total volume of 20 µL per sample. The sample was incubated at 42 C for 50 min, followed by 70 C for 15 min. cDNA was diluted to 20 to 25 ng/µL with H_2O and stored at -20 C.

Q-PCR (Real Time)

Two µL of each cDNA sample were mixed with 12.5 µL 2x SyberGreen master mix (Applied Biosystems 4309155), 8 µL H_2O, and 2.5 µL of both sense and antisense primers and added to a 96 well plate (Applied Biosystems MicroAmp N801-0560). Reactions were run according to the following cycle conditions: 95 C for 10 min, and 40 cycles of 95 C for 10 seconds followed by 60 C for 1 min. Analysis was performed using the ddCT method. For mouse mammary tissue samples, expression of *Claudin 7* was used to normalize mRNA levels of assayed genes (Blackman et al. 2005).

RT-PCR

The following master mix was used for RT-PCR analysis: 10x PCR buffer, 0.4 µM dNTPs, 2 mM $MgCl_2$, 1 µM each sense and antisense primers, 1.25 units of Taq polymerase (Invitrogen 18038-042), and 2 µL cDNA sample in a total volume of 25 µL per reaction. For all PCR reactions, the initial denaturation step was performed at 94 C for 5 min and the final elongation at 72 C for 5 min. The first denaturation round of

each cycle was performed at 94 C for 30 seconds in all reactions. Other varying conditions for each primer set are listed in Table 1.

Immunostaining

IHC

Tissue was harvested and immediately placed in chilled 4% paraformaldehyde for 12-24 hours, depending on the thickness of the tissue. The Veterinary Integrative Biosciences Histology Core Facility paraffin-embedded and sectioned all tissue, and also provided all H&E stained sections. For immunohistochemical or immunofluorescent analysis, slides were heated in a 60 C oven for 30 min to melt the paraffin, followed by deparaffinization in graded alcohols according to the following protocol: 2 washes in Xylenes for 5 min each, 2 washes in 100 % EtOH for 3 min each, 1 wash for 3 min in each 95 % EtOH, 70 % EtOH, and PBS. Antigen retrieval conditions varied depending on the antibody and target tissue and can be found in Table 3. All antigen retrieval was performed in 10 mM sodium citrate buffer in the microwave. For IHC, endogenous peroxidases were blocked by incubating the slides in 3 % H_2O_2 for 6 min. The Avidin/Biotin blocking kit was used for all IHC stained slides (Vector Labs SP-2001). Sections were blocked in PBST containing 10 % normal horse serum (NHS), and incubated in primary antibody diluted in PBST + 1 % NHS, or IgG (Mouse IgG Upstate 12-371; Rabbit IgG Santa Cruz sc2027) overnight at 4 C. After incubation in secondary antibody, diluted 1:250 in PBST + 1 % NHS, the ABC Kit (Vector Labs PK-6200) was used for signal amplification and the DAB Kit (Vector Labs SK-4100) was used to

visualize proteins. Slides were counterstained with methyl green [0.5 g methyl green in 100 mL 0.1 M sodium acetate buffer (1.36 g sodium acetate trihydrate, 100 mL H_20, pH 4.2 with glacial acetic acid)] and mounted using Permount (Fisher). The M.O.M. Kit (Vector Labs BMK-2202) was used for mouse primary antibodies.

Table 3. Antibodies Used for Immnohistochemical Analyses.

Target	Source	Catalog #	Dilution	Ag Retrieval
Npt2b	Alpha Diagnostics	NPT2B11-A	1.400	1' high 9' med
Aqp5	Alpha Diagnostics	AQP51A	1.250	None
β-Casein	Santa Cruz	FL-231	1.100	None
PR	Neomarkers	RB9017	1.600	1' high 9' med
Cleaved Caspase-3	Cell Signaling	9611S	1.200	1' high 9' med
Ki67	Neomarkers	RM-9106	1.500	1' high 9' med
HA	Sigma	H6908	1.250	1' high 9' med
Sim2	Millipore	AB4145	1.250	15 min high

Animal Handling/Genotyping

All mice were housed in the Comparative Medicine Program main facility with ad libitum access to water and a standard rodent diet containing 4 % fat. To genotype mice, the tip of the tail was treated with lidocaine gel for approximately 2 min, then less than 0.5 cm was cut for analysis. Styptic powder was used to control bleeding. All tail tissue to be used for genotyping harvested from live mice was done so between 2 and 4 weeks of age. Tails were kept at 4 C until digestion. To isolate genomic DNA, the Promega Wizard Kit was used following manufacturers protocol for mouse tail tissue.

Rehydrated DNA was quantitated using a BioRad Spectrophotometer and diluted to a final concentration of 25 ng/μL. Two μL of diluted DNA were used for PCR. To genotype MMTV-mSim2sHA mice, the following primers were used: MMTV Sense and mSim2 Seq2 Antisense, expected product size 850 bp. To genotype MMTV-Neu mice, the following primers were used: rNeu Sense and rNeu Antisense, expected product size 600 bp. Alternatively, the RED-Xtract-N-Amp Kit (Sigma XNAT) was used beginning in June 2008 for genomic DNA isolation and PCR analysis. All steps performed were according to the manufacturers instructions for extraction and PCR conditions, except that less than 0.5 cm of tail tissue was used rather than the recommended 1.0 cm.

Retroviral Transduction

Retroviral plasmids were transfected into 293T Viral Packaging Cells, which stably express Amphotrophic envelope proteins and are referred to as 293-Ampho. Up to 10μg of plasmid was transfected using Gene Juice (Novagen 70967), in a 3:1 ratio of Gene Juice (μL) to DNA (μg). Media was changed 24 h later, and collected for infection 48 h and 72 h later. Viral media was filtered through 0.45 μM syringe filters and polybrene was added to a final concentration of 4 μg/mL. Viral media was added to target cells, followed by centrifugation at 1200 rpm for 1 h. Target cells were incubated at 32 C for the duration of the infection protocol to promote viral stability. Selection was carried out using puromycin at varying concentrations, depending on the target cell

type. Selection was considered complete when all cells in a mock-infected plate were dead.

CHAPTER III

THE ROLE OF SIM2S IN MAMMARY GLAND DEVELOPMENT

Sim2s Expression is Regulated by Developmental Stage

Previously, we have shown that *Sim2* is highly expressed in normal mammary epithelial cells, and that expression is lost in aggressive breast cancer cells (Kwak et al. 2007). In addition, we have determined that SIM2s is the predominant isoform expressed in the human MCF7 breast cancer cell line (Laffin et al. 2008). To evaluate the expression pattern of *Sim2s* in the mouse mammary gland, RNA was isolated from whole mammary gland lysates harvested during virgin development, pregnancy, lactation, and involution. Q-PCR analysis was used to determine the relative amounts of *Sim2s* expressed in the mammary glands during each developmental stage (Fig. 6A). *Sim2s* expression is detectable in mammary glands from nulliparous, or virgin, females, and it declines during pregnancy, when the epithelial cells are rapidly proliferating. It then begins to increase in late pregnancy and reaches a peak during the first week of lactation, when the gland is terminally differentiated and secreting large amounts of milk. *Sim2s* expression declines again during involution, when most of the milk-producing alveolar epithelial cells are dying and the gland is remodeling. *In situ* hybridization was used to evaluate *Sim2* mRNA localization in the mammary glands of both nulliparous and lactating mice (Fig. 6B). High levels of *Sim2* mRNA were found to be localized in the epithelial cells of the lactating gland, as compared to those of a nulliparous female.

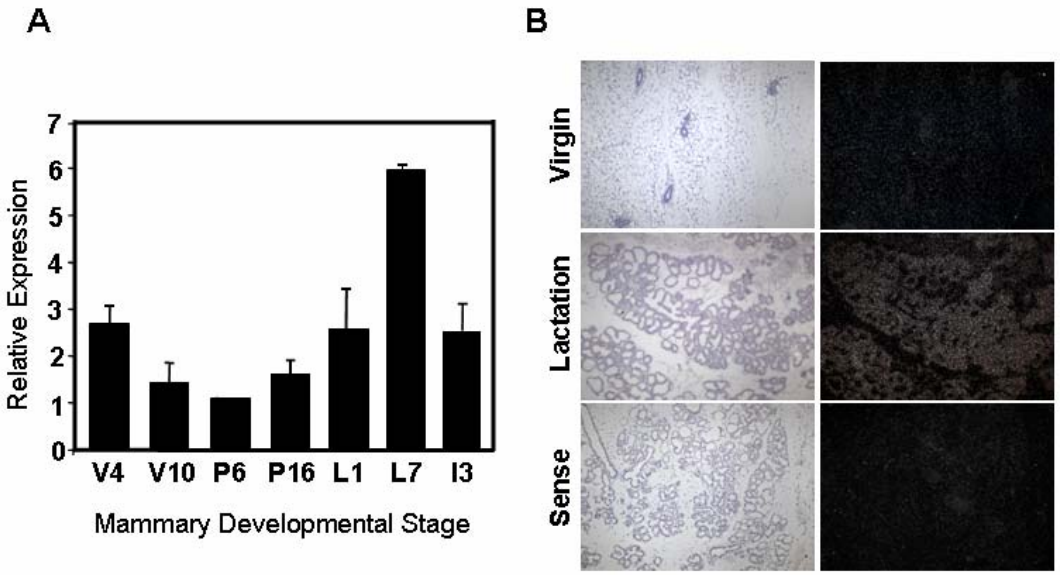

Figure 6. *Sim2s* Expression during Mammary Gland Development. *(A)* Expression levels of *Sim2s* in weeks 4 and 10 of virgin development (V4 and V10, respectively), days 6 and 16 of pregnancy (P6 and P16, respectively), days 1 and 7 of lactation (L1 and L7, respectively), and day 3 of involution (I3) as determined by Q-PCR analysis. N=3. *(B)* Localization of Sim2 mRNA to epithelial cells during lactation, as determined by *in situ* hybridization.

The mammary gland experiences a gain of function phase during the first week of lactation, and the synthetic activity of alveolar epithelial cells is maximal at approximately day 10 of lactation. To more closely evaluate the expression of *Sim2s* during the first week of lactation, RNA was isolated from whole mammary glands harvested 1, 4 or 7 days after parturition (L1, L4, and L7, respectively), and subject to Q-PCR analysis. In addition, expression of four major milk protein genes was evaluated, including *Csn2*, *Wap, Lalba*, and *Expi*, which is known as WDNM1. *Csn2* encodes the β-Casein protein, *Wap* and *Expi* encode secreted protease inhibitors, and *Lalba* encodes α-Lactalbumin, a subunit of the lactose synthetase enzyme. *Sim2*, *Csn2*, and *Wap* showed a progressive increase in expression from day 1 through day 7 of lactation (Fig. 7A-C). Interestingly, expression of *Lalba* and *Expi* was highest on day 1 of lactation, and did not increase further during the first week of lactation, suggesting that expression of these genes is regulated by a different mechanism than *Csn2* and *Wap* (Fig. 7D-E).

Generation of the MMTV-Sim2sHA Mouse

In Drosophila, *dsim* is required for proper formation of the central midline of the developing CNS, and to establish midline cell fate (Chang et al. 2001). Expression of *dsim* in lateral neural cells causes them to adopt a midline fate, characterized by expression of midline-specific genes (Menne et al. 1997). Based on the role of *dsim* in cell fate determination and the high expression of *Sim2s* in differentiated mammary epithelium, we hypothesized that Sim2s plays a role in maintaining lactogenic differentiation and function of the alveolar epithelial cells. To test our hypothesis, we

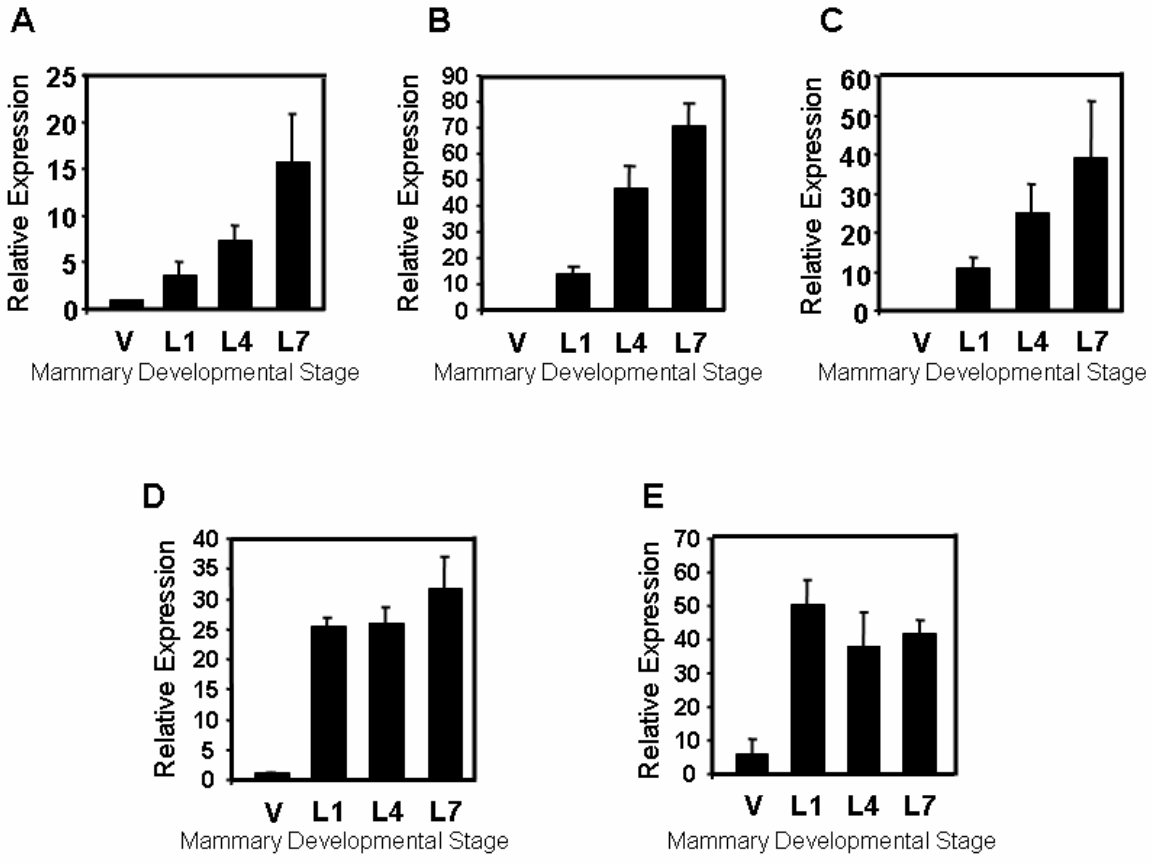

Figure 7. Mammary Gene Expression during the First Week of Lactation. Q-PCR analysis was used to determine relative expression of milk protein genes. N=3 for all timepoints. (*A*) *Sim2s* expression. (*B*) *Wap* expression. (*C*) *Csn2* expression. (*D*) *Lalba* expression. (*E*) *Expi* expression.

created a transgenic mouse model, which expresses HA-tagged Sim2s under control of the mouse mammary tumor virus long terminal repeat (MMTV LTR) promoter (Fig. 8A), which confers expression to the mammary epithelium. It has been reported that MMTV can drive expression in several tissues in addition to the mammary epithelium (Robinson et al. 2001). RNA, isolated from various organs of three adult transgenic females, was pooled and evaluated for transgene expression. Using primers that specifically recognize the transgene, we determined that the MMTV LTR drives expression of the *Sim2s* transgene exclusively in the mammary gland (Fig. 8B). Immunohistochemistry was used to detect the HA tag in mammary glands from eight-week virgin (V8) WT or transgenic females. Using IHC, we observed HA positive cells in the mammary epithelium of transgenic females, but not in non-transgenic littermates (Fig. 8C). In addition, HA positive staining in transgenic glands showed a chimeric pattern, which is consistent with other reported MMTV-driven transgenes (Robinson et al. 2001).

Mice have three pairs of thoracic mammary glands, and two pairs of inguinal glands for a total of 10 mammary glands. Due to the accessibility, the number 4, or first inguinal, glands are routinely utilized for mammary gland biology studies. Mammary tissues were harvested from female mice at various developmental timepoints including nulliparous, pregnant, lactating, and involuting glands. Mammary glands were harvested during the estrus phase, due to the possibility of ovarian hormone influence on expression of some mammary specific genes (Robinson et al. 1995). Unless otherwise indicated, all females were nulliparous or primiparous. For involution samples, females

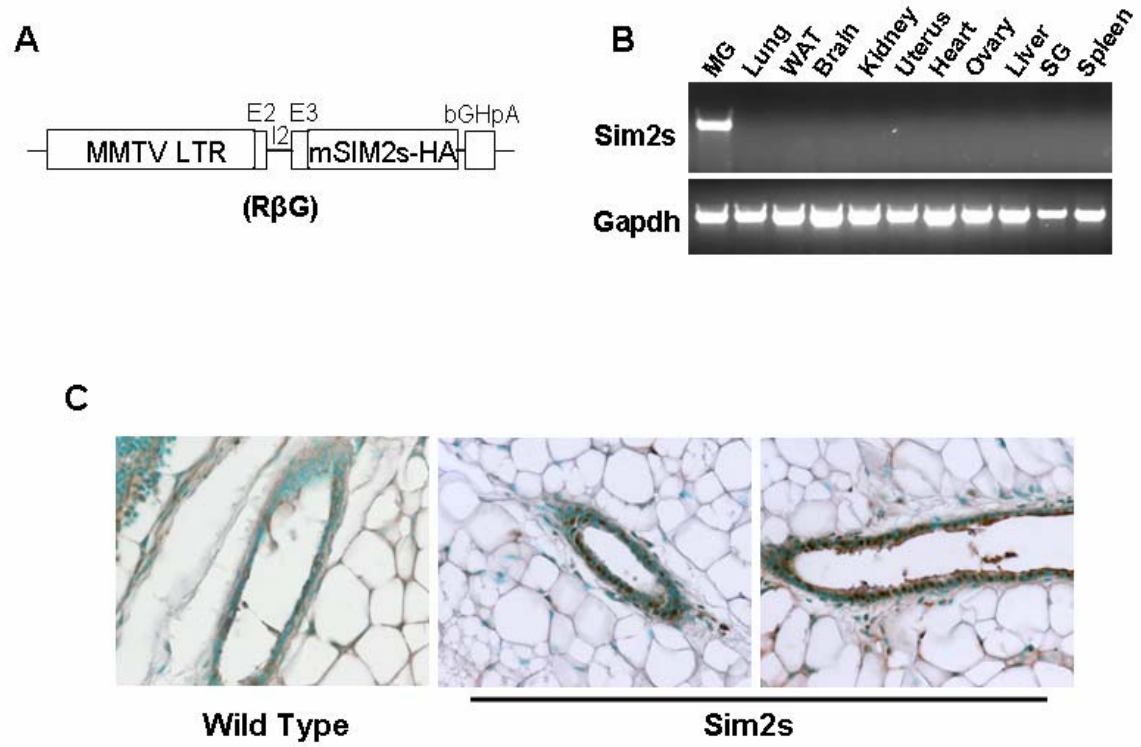

Figure 8. Generation of the MMTV-Sim2s HA Transgenic Mouse. (*A*) Schematic diagram of the construct used to generate the mouse. The MMTV-LTR sequence construct was provided by Dr. Jeffery Rosen at Baylor College of Medicine. Exons 2 and 3 (E2, E3) and Intron 2 (I2) are derived from the rabbit β-globin gene (RβG). The poly-A signal is derived from the bovine growth hormone gene (bGHpA). (*B*) RT-PCR analysis of three pooled samples from trasngenic females showing expression of the Sim2s-HA transgene in the mammary gland. MG; mammary gland, WAT; white adipose tissue, SG; salivary gland. *Gapdh* was used as a control for cDNA integrity. (*C*) IHC analysis of HA localization in WT and transgenic mammary glands from mature nulliparous females.

were allowed to nurse pups for 10 days, and the glands were harvested at given time intervals after forced weaning.

During virgin development, no gross morphological defects were observed in TEBs or mature ductal structures in transgenic females as compared to WT controls (Fig. 9A-B). In addition, no abnormalities were detected at pregnancy day 6 or 16 (P6 and P16, respectively; Fig. 9C-D). Two weeks after pup removal, transgenic glands were indistinguishable from WT glands (Fig. 9E).

Mammary glands were harvested from both WT and transgenic females at L1, L4, and L10. Evaluation of epithelial structures in H&E stained sections did not reveal any morphological differences between the two groups at any stage (Fig. 10A-C). At L1, the alveoli occupied a substantial portion of the mammary gland, and the relative amount of epithelium increased by L4. At L10, visibly distended alveoli could be observed. Upon closer examination (inset) it was revealed that the cells within the alveoli were metabolically active and many contained CLDs. Overall, examination of the mammary glands of transgenic mice at various stages of development did not reveal any gross morphological differences, when compared to glands from WT mice.

Accelerated Lactogenic Differentiation in Transgenic Females

It has been reported that early expression of genes associated with cell fate determination can promote the formation of differentiated alveolar epithelial cells in nulliparous females (Jiang and Zacksenhaus 2002; Kouros-Mehr et al. 2006; Oakes et al. 2008). These reports, taken together with the pattern of *Sim2s* expression in WT

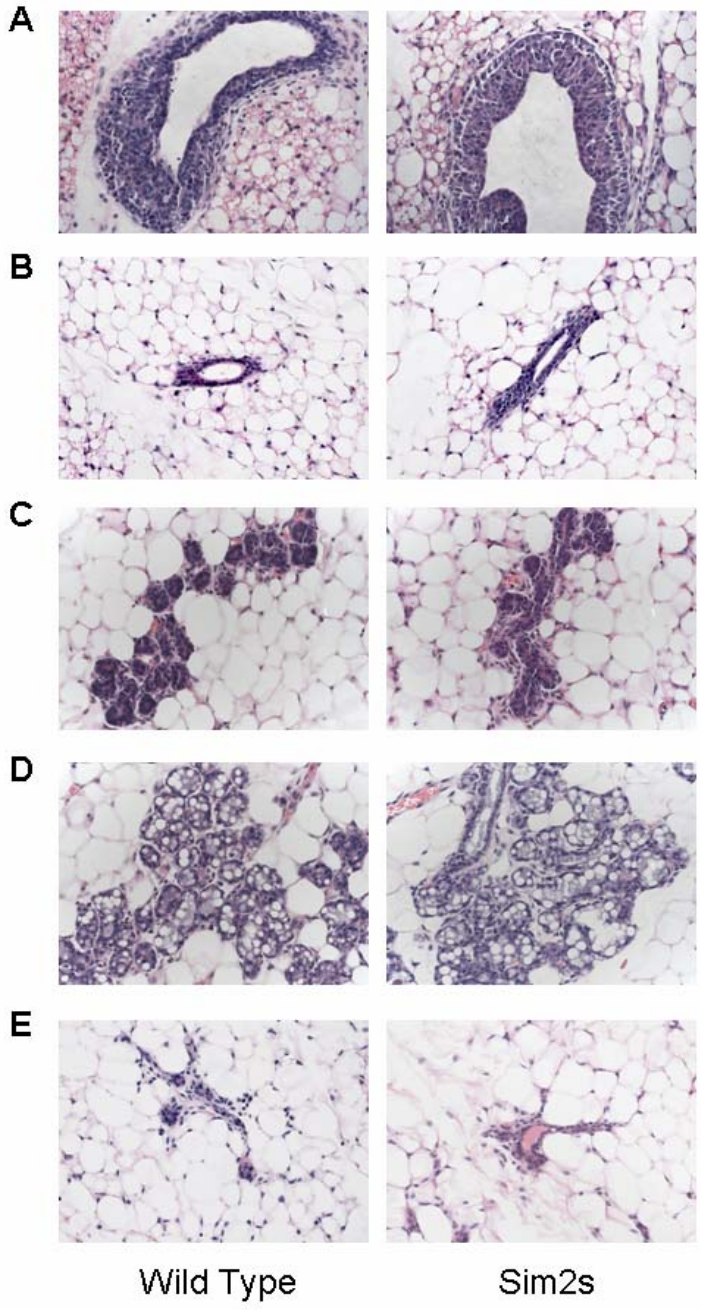

Figure 9. Evaluation of Mammary Morphology in Female Mice. Left is Wild Type and right is Sim2s. (*A*) and (*B*) TEB and mature ductal structures in 5- and 8-week (V5 and V8) old nulliparous females, respectively. (*C*) and (*D*) Lobulo-alveolar development at pregnancy day 6 (P6) and day 16 (P16). (*E*) Alveolar regression 2 weeks following pup removal (I2w).

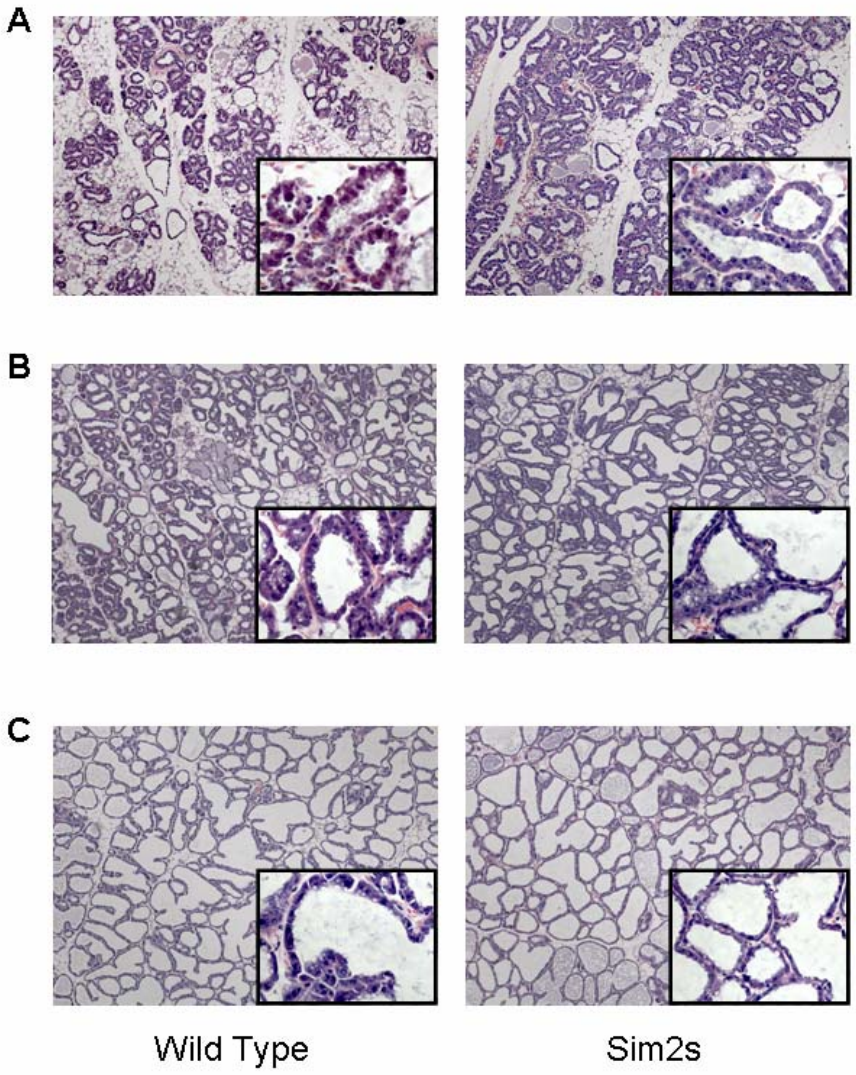

Figure 10. Mammary Gland Morphology in WT and Transgenic Females during Lactation. Left is Wild Type and right is Sim2s. (*A*) Comparison of H&E stained sections of mammary glands at lactation day 1 (L1). (*B*) Comparison of H&E stained sections of mammary glands at lactation day 4 (L4). (*C*) Comparison of H&E stained sections of mammary glands at lactation day 10 (L10).

mammary glands, led us to hypothesize that precocious expression of *Sim2s* in the mammary epithelium would promote lactogenic differentiation. To determine if precocious *Sim2s* expression leads to upregulation of milk protein genes, Q-PCR analysis was used on RNA isolated from whole mammary glands of V8 females. When compared to WT glands, mRNA levels of *Csn2* and *Wap* in transgenic glands were significantly elevated (Fig. 11A-B). Surprisingly, differences were not detected in the expression of *Lalba* or *Expi* in transgenic glands compared to WT (Fig. 11C-D). The differential regulation of Csn2 and Wap by Sim2s was reminiscent of the patterns of gene expression observed during the first week of lactation in WT glands (Fig. 7). Together, these data suggest that Sim2s plays a role in regulating the transcription of a subset of milk protein genes in the mammary epithelium.

Various cell surface proteins have been determined to be developmentally regulated in the mammary gland. Aquaporin-5 (Aqp5) is localized to the luminal surface of ductal epithelial cells, and its expression is lost during lactation. Solute carrier 34a2 (*Slc34a2)*, which encodes the sodium-inorganic phosphate cotransport protein referred to as Npt2b, is upregulated during lactation and is localized to the luminal surface of the epithelium. Immunohistochemistry was used to evaluate Aqp5 and Npt2b in mature mammary glands from nulliparous WT or transgenic females. Differences in Aqp5 were not detected between the groups at this developmental stage (Fig. 12A). In approximately 70% of transgenic glands examined, Npt2b was found on the luminal surface of the ductal epithelial cells (Fig. 12B). In addition, we were also able to detect β-Casein in the cells of mature nulliparous transgenic mice (Fig. 12C).

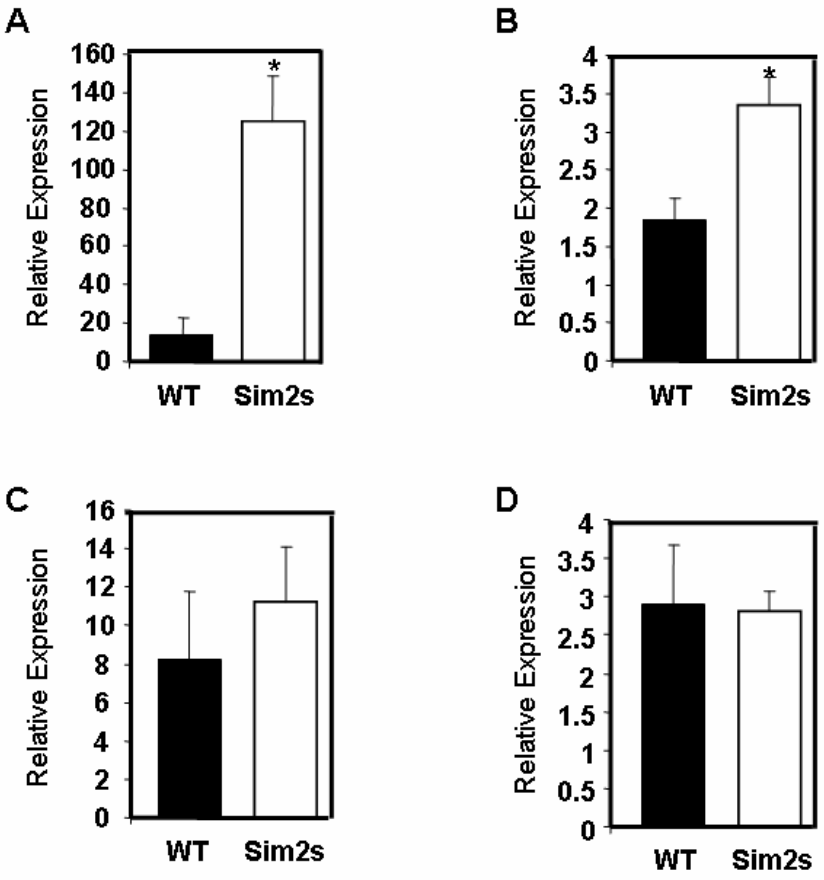

Figure 11. Analysis of Milk Protein Gene Expression in Mature Mammary Glands from WT and Transgenic Females. Q-PCR was used to evaluate gene expression in samples from nulliparous wild type (WT) and transgenic (Sim2s) females. N=5 for all timepoints. (*A*) *Csn2* expression. (*B*) *Wap* expression. (*C*) *Lalba* expression. (*D*) *Expi* expression. * $p < 0.05$

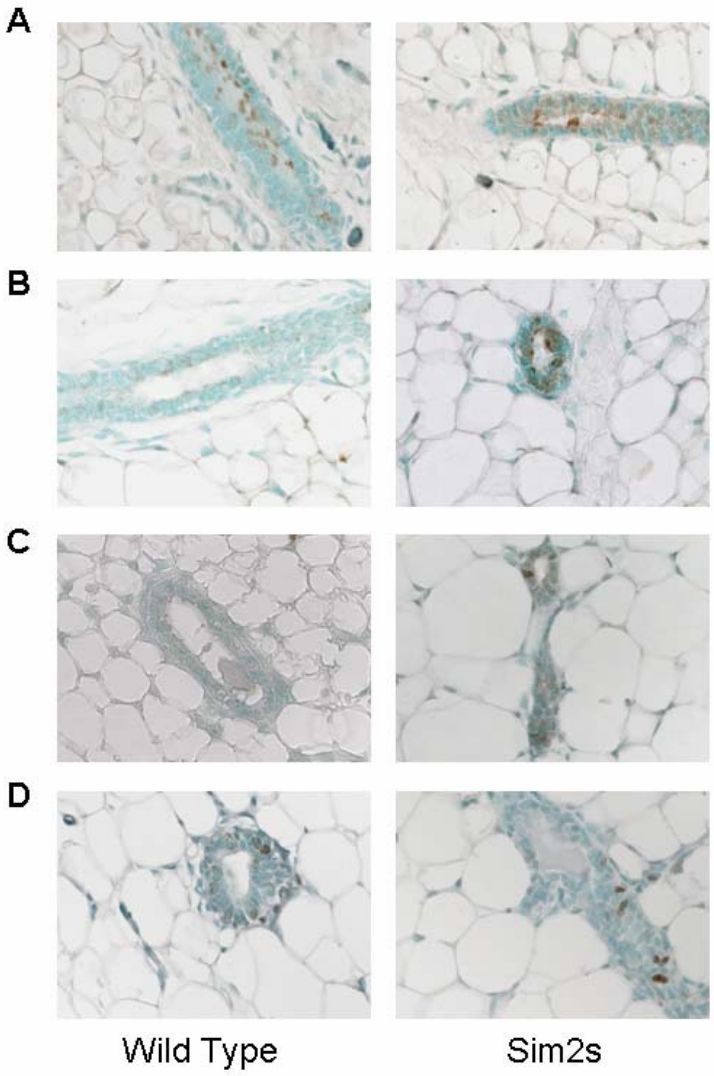

Figure 12. Analysis of Differentiation Markers and Proliferation in Mature Mammary Glands from Nulliparous WT and Transgenic Females. Left is Wild Type and right is Sim2s. (*A*) IHC analysis of Aqp5 localization in nulliparous mammary glands. (*B*) IHC analysis of Npt2b localization in nulliparous mammary glands. (*C*) IHC analysis of β-Casein production in nulliparous mammary glands. (*D*) IHC analysis of Ki67 localization in nulliparous mammary glands.

We have previously shown a relationship between *Sim2* expression and cell proliferation (Kwak et al. 2007). To determine if proliferation is reduced in transgenic glands, IHC was used to detect Ki67 expression. No differences were seen between WT and transgenic glands with regards to proliferation, which was not surprising given the normal ductal morphology (Fig. 12D).

PR has been shown to be required for proper alveolar expansion during pregnancy. Further, the pattern of mammary PR expression has been proposed to reflect global gland maturity (Seagroves et al. 2000). In immature virgins, PR is found in nearly all ductal epithelial cells. This has been hypothesized to prevent premature side branching and alveolar formation prior to completion of branching morphogenesis. Once the female reaches maturity, PR expression adopts a more chimeric pattern, and it is not found in every cell. During pregnancy, overall amounts of PR decrease in the gland, and PR is undetectable during lactation (Aupperlee et al. 2005). We have shown that Sim2s can promote lactogenic differentiation, characterized by increased expression of milk protein genes, and upregulation of Npt2b in luminal epithelial cells. Immunohistochemical analysis was used to determine if Sim2s altered the expression of PR in mammary glands from WT and transgenic females. Differences in PR expression were not detected in glands from mature nulliparous females (Fig. 13A & D). At P6, mammary glands of transgenic mice expressed significantly less PR than glands from WT mice at the same timepoint, consistent with accelerated lactogenic differentiation (Fig. 13B & E). Although this observation was not true for every sample, the reduction

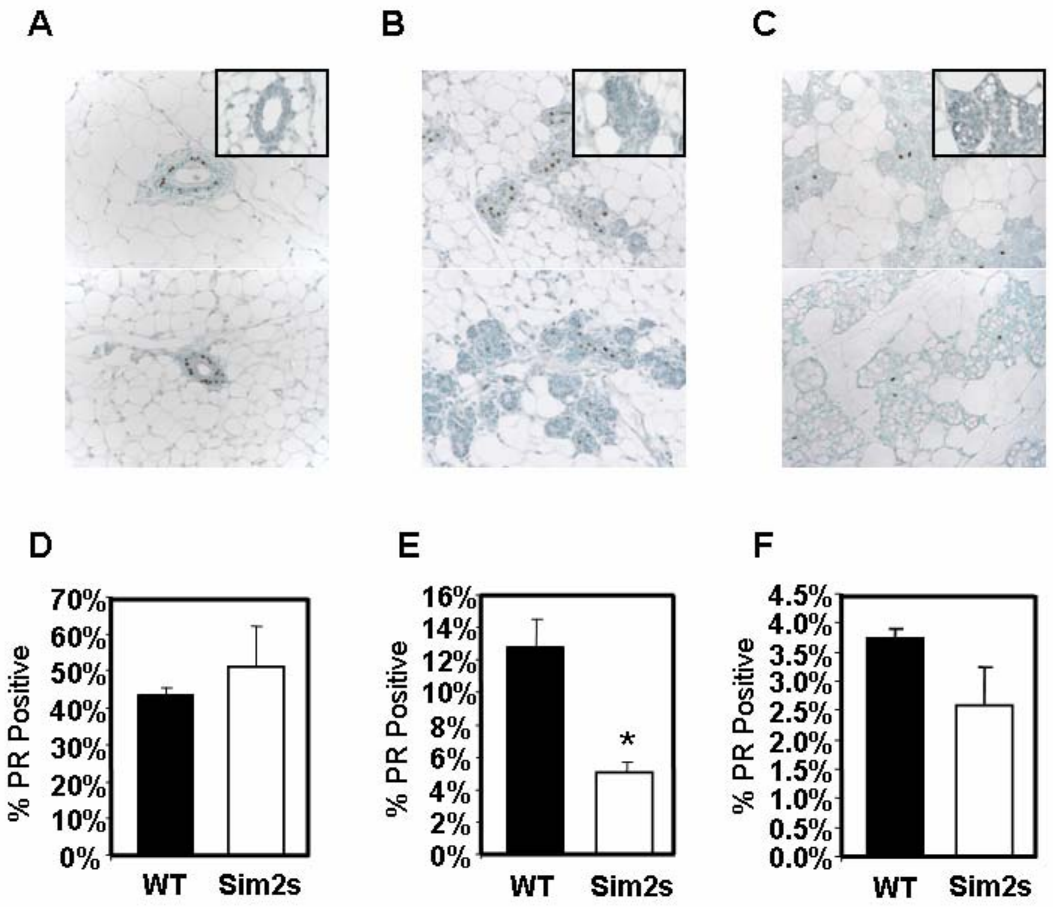

Figure 13. Immunohistochemical Analysis and Quantitation of PR Expression and Localization in Mammary Glands from WT and Transgenic Mice. For PR IHC, wild type samples are top and Sim2s samples are bottom. N=4 for all samples used in quantitation. (A) IHC analysis of PR in mammary glands from 8-week nulliparous females (V8). (B) IHC analysis of PR in mammary glands from day 6 pregnant females (P6). (C) IHC analysis of PR in mammary glands of day 16 pregnant females (P16). (D), (E), and (F) Quantitation of PR positive nuclei out of total luminal epithelial nuclei in V8, P6, and P16 mammary glands, respectively. Insets represent negative controls. * $p<0.05$.

in PR expression associated with transgenic females was significant in the majority (70%) of P6 glands. Significant differences in PR expression were not detected at P16 (Fig. 13C & F).

Mammary Differentiation during Pregnancy and Lactation

Milk protein genes are expressed asynchronously during pregnancy (Robinson et al. 1995). *Expi* and *Csn2* are detectable in early pregnancy, but show high variability in the amount and localization of expression. *Wap* is expressed at low levels several days after *Expi* and *Csn2*; however, it is restricted to cells that express *Csn2*, and the two show the same pattern of mRNA localization (Robinson et al. 1995). Expression of *Lalba* is not detectable until very late in pregnancy. In mammary glands of transgenic females, we have shown an acceleration of lactogenic differentiation during virgin development compared to WT littermates. To determine if transgenic females experience enhanced alveolar cell differentiation during pregnancy, RNA was isolated from whole mammary glands and used in Q-PCR analysis for *Expi*, *Lalba*, *Csn2* and *Wap.* At P6, mRNA levels of all four of the genes analyzed were significantly higher in glands of transgenic females compared to WT (Fig. 14A-D). By P16, however, there were no detectable differences between the two groups (Fig. 14A-D). These data suggest that the epithelial cells experience accelerated differentiation during early pregnancy in transgenic females.

Upon parturition, milk protein gene expression is dramatically enhanced, but does not yet show uniform expression throughout the alveolar and ductal epithelium

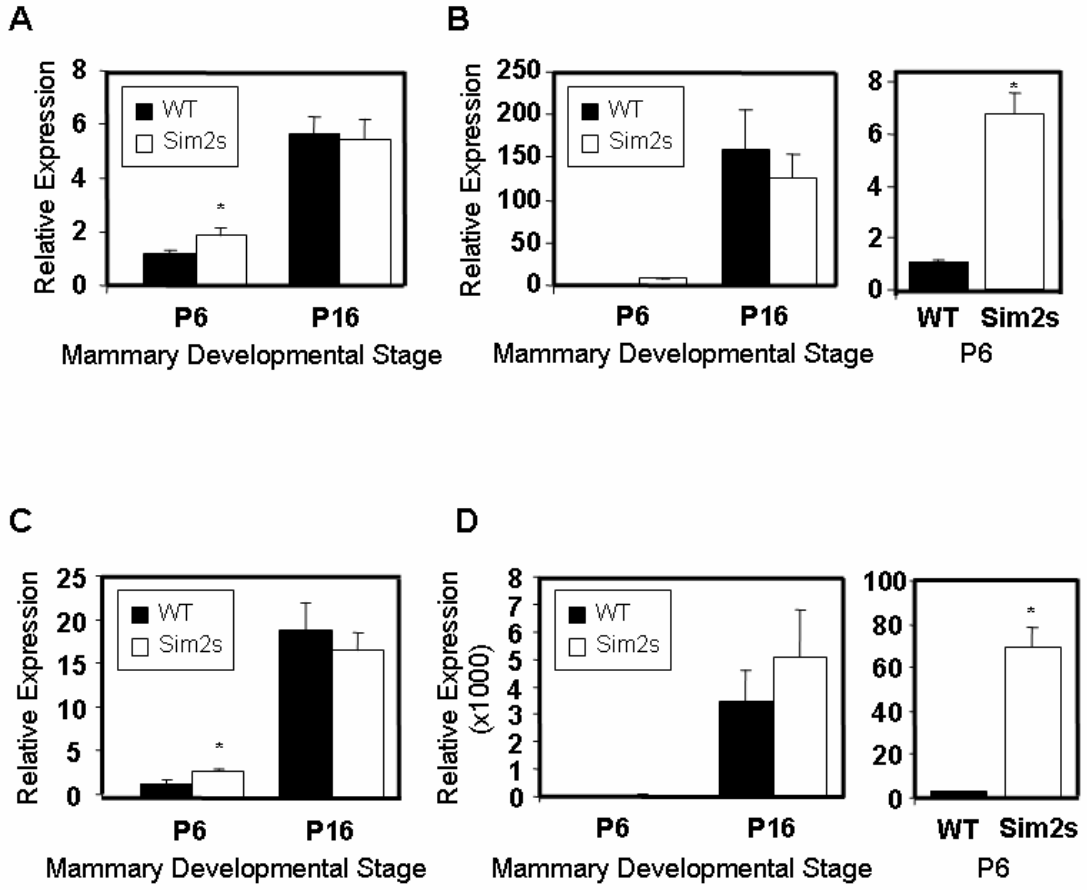

Figure 14. Expression of Milk Protein Genes in Mammary Glands from Early- and Late-Pregnant WT and Transgenic Mice. Q-PCR was used to evaluate gene expression in mammary glands from day 6 (P6) and day 16 (P16) pregnant WT and transgenic (Sim2s) females. N=4 for all timepoints. (*A*) *Expi* expression. (*B*) Left, *Lalba* expression and right, *Lalba* expression at P6. (*C*) *Csn2* expression. (*D*) Left, *Wap* expression and right, *Wap* expression at P6. *p< 0.05

(Robinson et al. 1995). To determine if MMTV-Sim2s females express higher levels of milk protein genes during the first week of lactation, Q-PCR was used to analyze *Expi, Lalba, Csn2* and *Wap*. Differenced were not detected in the expression of *Expi* or *Lalba* at L2 or L4 between WT and transgenic glands (Fig. 15A & B). Both *Wap* and *Csn2* were upregulated from L2 to L4 in WT glands, but only *Csn2* expression was significantly enhanced in transgenic glands at L4 (Fig. 15C & D). It is possible that the levels of expression of *Wap*, *Expi,* and *Lalba* were too high to detect significant differences between WT and transgenic glands.

Delayed involution following pup removal has been reported in transgenic mice expressing genes associated with precocious lactogenic differentiation, such as *Stat5a*, MTA-1, and a constitutively active form of pRb (Iavnilovitch et al. 2002; Jiang and Zacksenhaus 2002; Bagheri-Yarmand et al. 2004). To characterize mammary glands of transgenic mice following involution, females were allowed to nurse pups for 10 days, and then weaned. Mammary glands were harvested 24, 48, or 72 hours after pup removal and stained using H&E (Fig. 16A-C). Examination of H&E stained sections from females harvested at the indicated timepoints revealed a noticeable delay in postlactational epithelial regression by 48 hours after pup removal, and a more dramatic delay 72 hours after weaning (Fig. 16B-C). IHC analysis was performed on sections to detect the active Caspase-3 protein, which is involved in apoptosis of alveolar epithelial cells (Fig. 16D-F). There appeared to be more cleaved Caspase 3-positive cells in the mammary glands of WT females at all three timepoints as compared to glands from

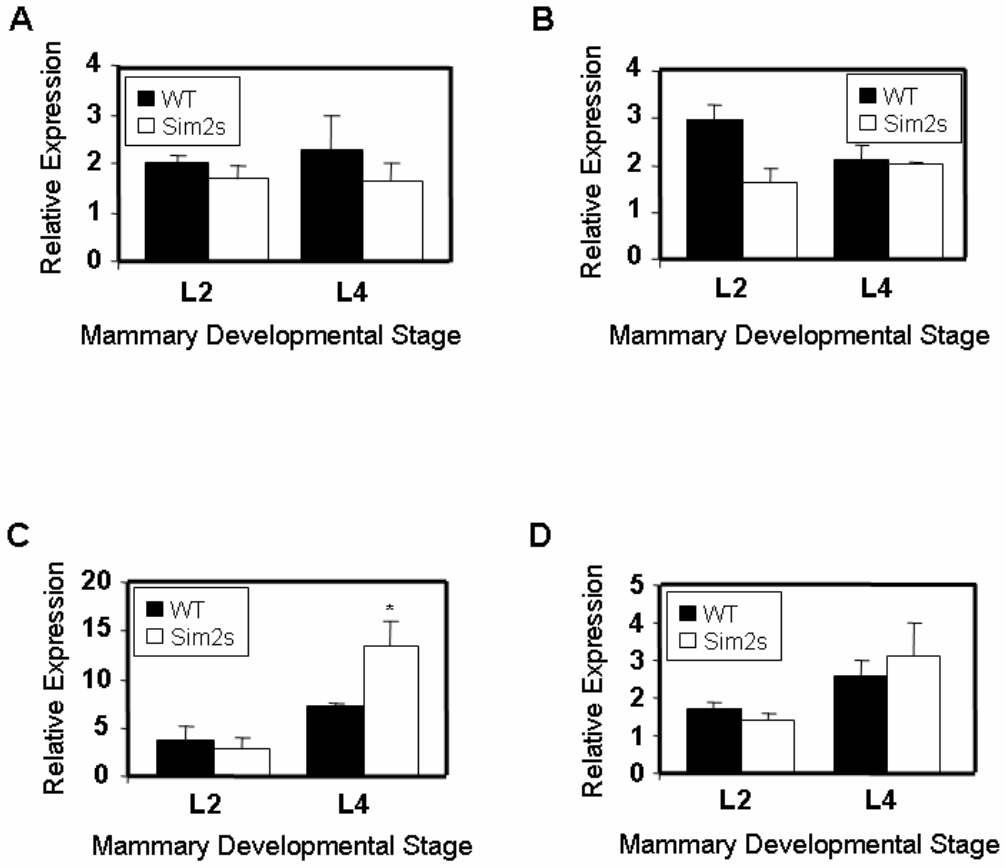

Figure 15. Gene Expression Analysis of Mammary Glands during Lactation from WT and Transgenic Females. Q-PCR was used to evaluate gene expression in mammary glands from day 2 (L2) and day 4 (L4) lactating WT and transgenic (Sim2s) females. N=4 for all timepoints. (*A*) *Expi* expression. (*B*) *Lalba* expression. (*C*) *Csn2* expression. (*D*) *Wap* expression. * $p < 0.05$

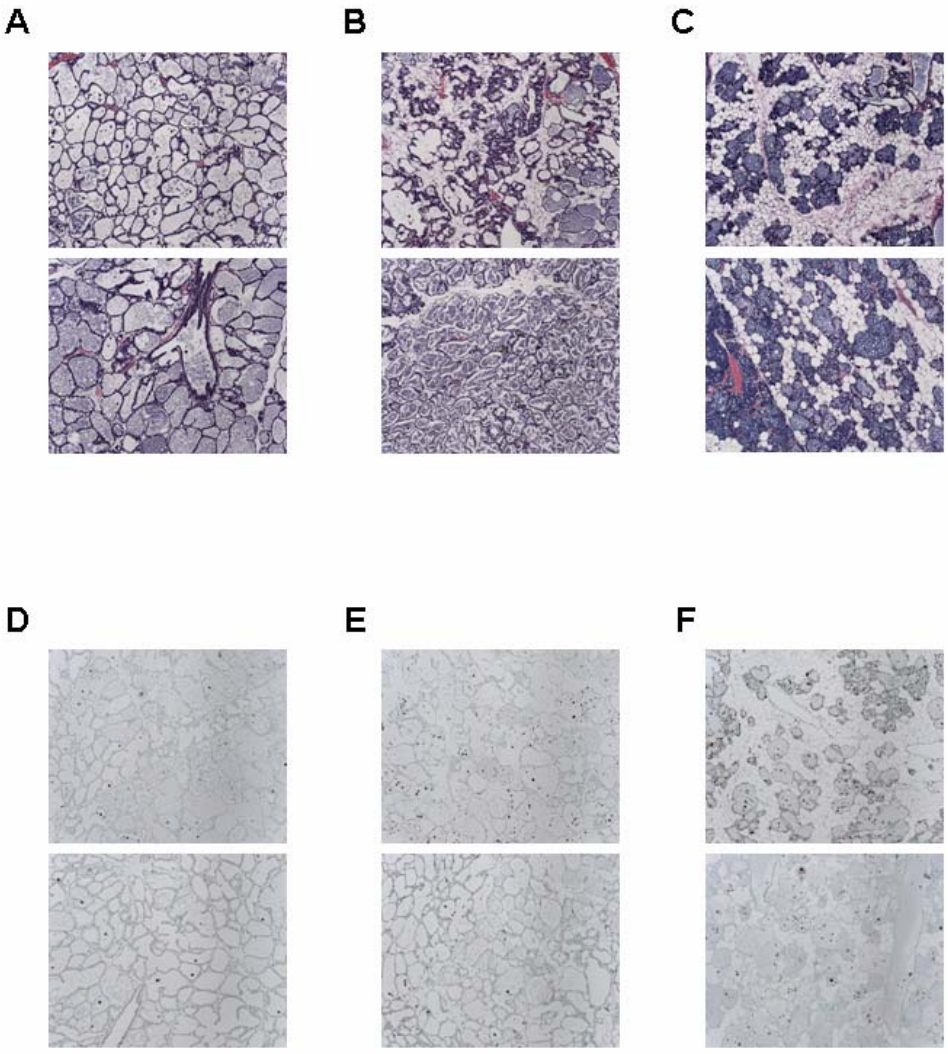

Figure 16. Mammary Gland Morphology and Immunohistochemical Analysis of Active Caspase 3 During Involution in WT and Transgenic Females. All top images in each group are from WT females and all bottom images are from transgenic females. (*A*), (*B*), and (*C*) H&E stained sections of mammary glands from WT and transgenic females harvested 24, 48, or 72 hours, respectively, after pup removal. (*D*), (*E*), and (*F*) IHC analysis of active Caspase 3 in mammary glands from WT and transgenic females harvested as indicated in (*A*), (*B*), and (*C*).

transgenic females, indicating a reduction in apoptosis that is consistent with a delayed epithelial regression following pup removal.

IHC was also used to detect Sim2 in mammary glands harvested from WT females 72 hours after forced weaning. Using this technique, we found Sim2 localized to the epithelial cells lining the alveoar lumens, but did not detect Sim2 in the cells that had been extruded from the epithelial layer (Fig. 17A arrows). Nuclear staining was not observed using IgG as a negative control (Fig. 17B). Together, these data suggest that Sim2 promotes cell survival following lactation, and loss of *Sim2* expression is a prerequisite for apoptosis of alveolar epithelial cells.

In mice, we have shown that *Sim2s* is developmentally regulated and is highly expressed in terminally differentiated mammary epithelium. In addition, precocious expression of *Sim2s*, driven by the MMTV LTR, leads to upregulation of *Csn2* and *Wap* in mammary glands from mature nulliparous females. This is accompanied by moderate production and appropriate localization of Npt2b. Reduced levels of PR in early pregnancy, associated with enhanced expression of four major milk protein genes suggests that mammary epithelial cells from transgenic females experience accelerated lactogenic differentiation. By late pregnancy, no differences in gene expression were detected between WT and transgenic glands. During the first week of lactation, *Csn2* was significantly upregulated in glands from transgenic females compared to those from WT controls. In addition to early lactogenic differentiation seen during mammary development in transgenic females, a delay in involution, characterized by reduced levels of active Caspase 3, was observed. Cells that had been extruded from alveolar

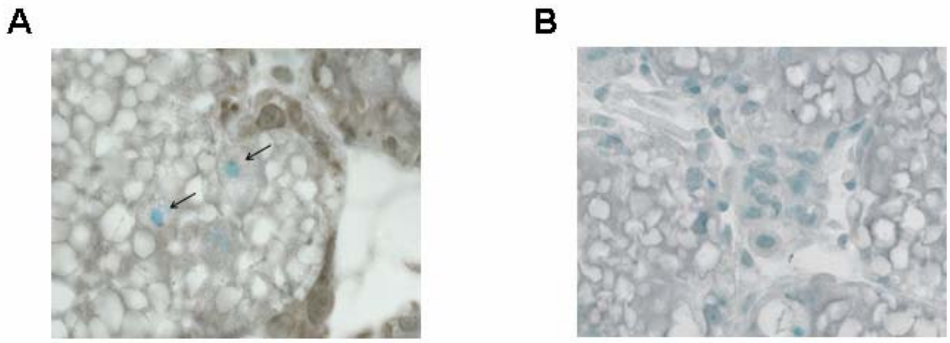

Figure 17. Immunohistochemical Analysis of Sim2 Localization during Involution in Mammary Glands from WT Females. (*A*) IHC analysis of Sim2 localization in a mammary gland from a WT female 72 hours after pup removal. Arrows indicate extruded, Sim2-negative cells. (*B*) IgG was used as a negative control.

walls in WT females were found to be negative for Sim2 expression, as determined by IHC analysis. The HC11 cell line, described in Chapter I and discussed in detail in the next chapter, is an excellent model to use for elucidation of the factors that regulate transcription of the *Csn2* gene.

CHAPTER IV

SIM2S AND LACTOGENIC DIFFERENTIATION *IN VITRO*

Sim2s **Expression in HC11 Cells**

HC11 cells are derived from the COMMA-D line, isolated from a mid-pregnant BALB/c mouse (Ball et al. 1988). After they reach confluence, treatment with hydrocortisone (HC) and prolactin (Prl) results in a robust induction of *Csn2*. This cell line provides an excellent model to study the transcriptional regulation of one major milk protein gene in response to hormonal stimulation. We have shown, *in vivo*, that *Sim2s* expression increases during the first week of lactation, when circulating Prl levels are high. To determine if *Sim2s* is regulated in a similar manner *in vitro*, Q-PCR analysis was used to evaluate expression pattern of *Sim2s* in proliferating, undifferentiated (UN) HC11 cells, and in cells treated with hydrocortisone and Prl for 1 or 4 days (Prl1 and Prl4, respectively). *Sim2* expression was induced after 1 day of Prl treatment, and the expression increased further 4 days after Prl treatment (Fig. 18A). Western blot analysis, performed on undifferentiated HC11 cells or cells treated for 4 days with Prl, revealed that Sim2s was the predominant isoform expressed in this cell type (Fig. 18B). Detection of beta-Casein by western blot analysis confirmed that the cells responded appropriately to hormonal stimulation, and beta-Actin was used as a control for protein loading (Fig. 18B).

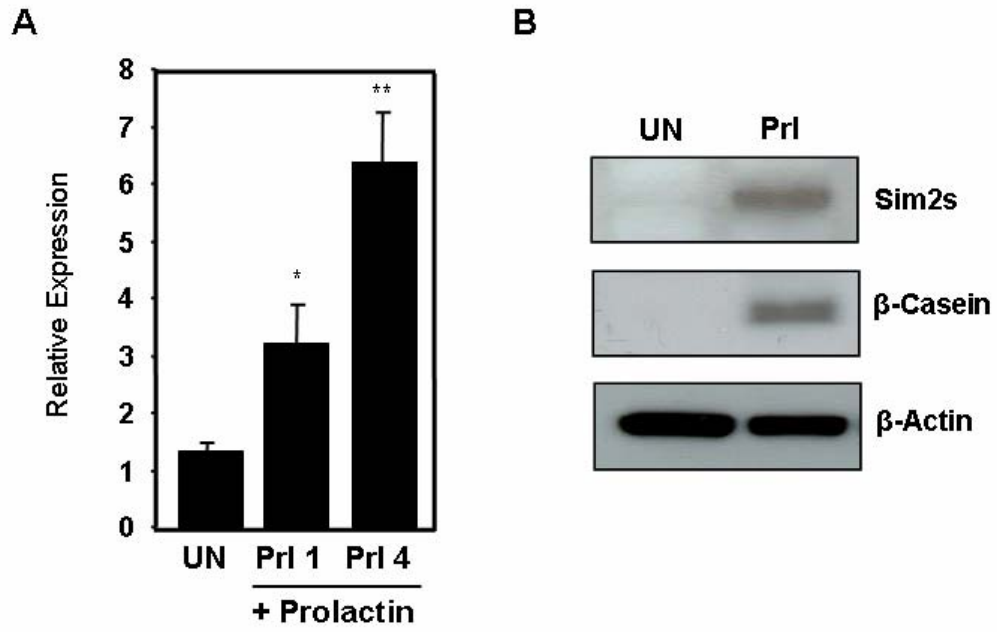

Figure 18. *Sim2s* Expression in HC11 Cells. N=3 samples for each timepoint analyzed by Q-PCR. *(A)* Expression of *Sim2s* in undifferentiated HC11 cells (UN), and in cells treated for 1 (Prl1) or 4 (Prl4) days with HC + Prl. *(B)* Western blot analysis of Sim2s and β-Casein protein levels in undifferentiated HC11 cells (UN) and cells treated with HC + Prl for 4 days (Prl). β-Actin was used as a loading control.* $p<0.05$; **$p<0.01$.

Csn2 Expression Is Directly Related to *Sim2s* Expression

To recapitulate the model described in the previous chapter, retroviral transduction was used to express *Sim2s* (Sim2s), or empty vector (Control) in HC11 cells. RNA was isolated from undifferentiated (UN) Control and Sim2s HC11 cells, or from cells treated for 4, 8, and 24 hours with HC and Prl (Prl). Q-PCR analyis revealed a significant increase in expression of *Csn2* in Sim2s cells 4 hours after hormonal stimulation, and the enhanced expression was maintained throughout the timecourse (Fig. 19A). In Control cells, the pattern of *Csn2* induction in response to hormonal stimulation was similar to that seen in Sim2s cells (Fig. 19A inset). *Sim2s* overexpression, however, was not sufficient to induce *Csn2* in the absence of Prl (UN). A higher amount of β-Casein protein was also detected in Sim2s cells compared to Control cells following Prl treatment, using Western blot analysis (Fig. 19B).

To determine if Sim2s is necessary for *Csn2* expression in differentiated HC11 cells, shRNA was used to reduce protein levels. Two constructs, targeting either Exon 6 or Exon 10 of mouse *Sim2* were expressed in HC11 cells, using retroviral transduction. A scrambled (Scr) sequence, not know to target any mammalian gene, was used as a negative control. *Sim2* expression was reduced approximately 50% using this technique. Q-PCR analysis revealed a significant decrease in the amount of *Csn2* in cells with reduced Sim2 using the construct targeting Exon 6 (siSim2), when compared to Scr controls (Fig. 20A). Similar results were obtained using the shRNA construct targeting Exon 10 (data not shown). A decrease in β-Casein protein was seen, as well, in siSim2

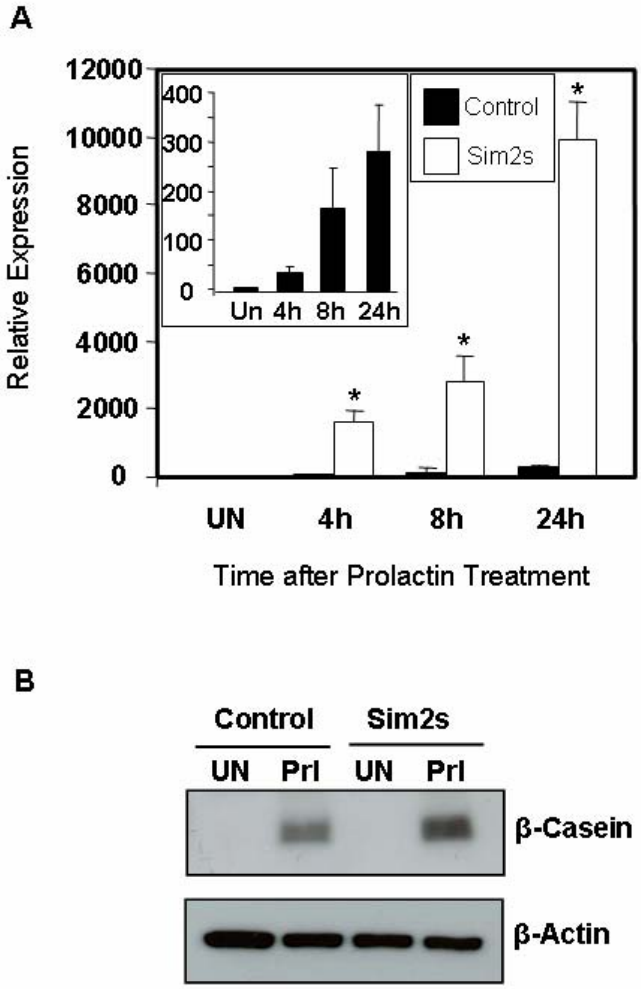

Figure 19. *Sim2s* Overexpression Enhances *Csn2* Production in HC11 Cells. *(A)* Q-PCR was used to detect *Csn2* in undifferentiated cells (UN) and cells treated with HC + PRL for 4, 8, and 24 hours. Inset represents Control samples. N=3 for each timepoint. *(B)* Western Blot analysis of β-Casein in Control and Sim2s undifferentiated HC11 cells (UN) and cells treated with HC + Prl for 4 days (Prl). β-Actin was used as a loading control. * $p<0.05$

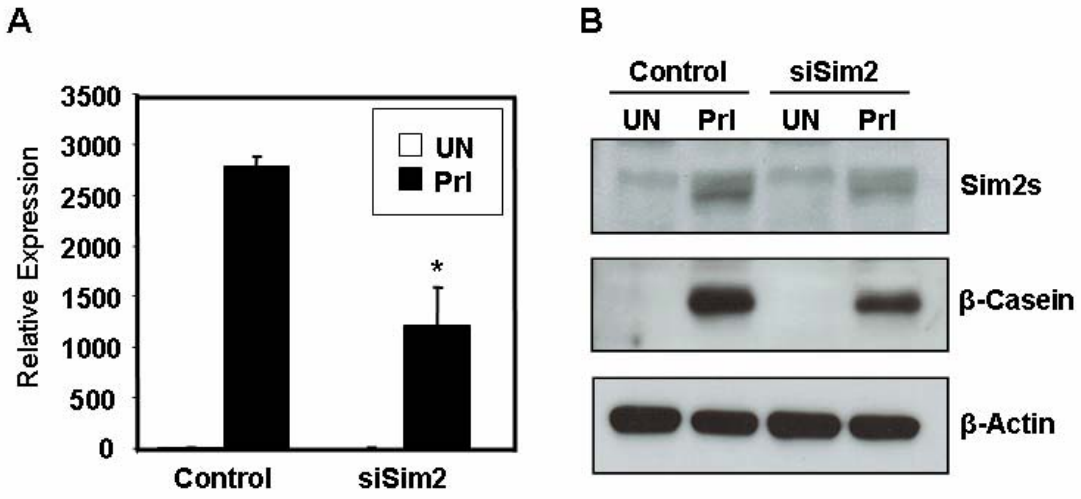

Figure 20. Reduction of Sim2s Levels Leads to Attenuated *Csn2* Production in HC11 Cells. (*A*) shRNA targeting exon 6 of *Sim2* (siSim2) or encoding a Scrambled sequence (Control) was expressed in HC11 cells. Q-PCR was used to analyze *Csn2* expression in undifferentiated cells (UN) or cells treated for 4 days with HC + Prl (Prl). N=3 for each timepoint. (*B*) Western Blot analysis of Sim2s and β-Casein in Control and siSim2 cells treated with HC + Prl for 4 days (Prl). β-Actin was used as a loading control. * $p < 0.05$

cells compared to Scr cells (Fig. 20B). These data suggest that upregulation of *Sim2s* during HC11 cell differentiation is required for maximal *Csn2* expression.

Analysis of the *Csn2* Proximal Promoter

The proximal region of the *Csn2* promoter has been well characterized, and many of the factors that regulate expression of the gene have been identified (Kabotyanski et al. 2006) Figure 21A depicts a diagram of the *Csn2* gene. Within 1 hour of Prl treatment, an increase in association of Stat5a, GR, C/EBPβ, and RNA Polymerase II (RNAPII) can be detected at the *Csn2* proximal promoter. Using ChIP analysis, performed on chromatin harvested from undifferentiated HC11 cells or cells treated for 4, 8, or 24 hours with Prl, we detected a transient increase in the amount of Stat5a associated with the *Csn2* promoter in Control cells, and a greater, sustained association of Stat5a in the Sim2s cells (Fig. 21B). Western blot analysis revealed no increase in the amount of total Stat5a in Sim2s HC11 cells compared to Control cells (Fig. 22), so the difference in Stat5a found at the Csn2 promoter between the two cell types is likely due to enhanced recruitment in the Sim2s cells.

The pattern of RNAPII association with the *Csn2* promoter followed that of Stat5a for both the Control and Sim2s cells, and higher amounts of RNAPII were found to be recruited to the *Csn2* promoter in Sim2s cells (Fig. 21B). The C-terminal domain (CTD) of RNAPII is composed of heptad repeats with the amino acid sequence YSPTSPS. Phosphorylation on Serines 2 and 5 are required for promoter escape and

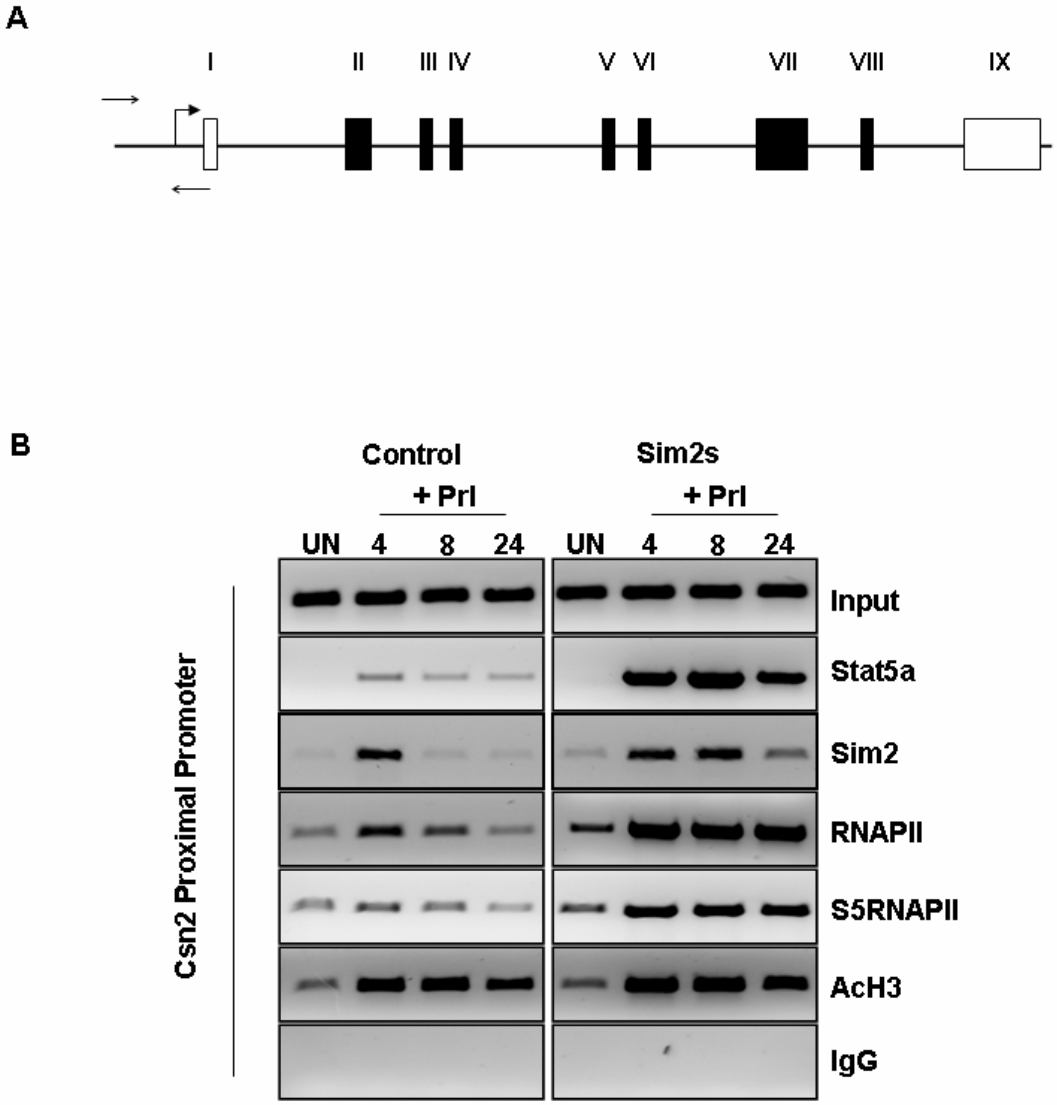

Figure 21. ChIP Analysis of the *Csn2* Proximal Promoter Region. (*A*) Diagram of the *Csn2* sequence. Open boxes represent non-coding exons and closed boxes represent coding exons. Filled arrowhead represents the transcriptional start site and open arrowheads represent primer annealing sites. (*B*) ChIP assays performed on chromatin harvested from undifferentiated HC11 cells (UN), or cells treated for 4, 8, and 24 hours with HC + Prl.

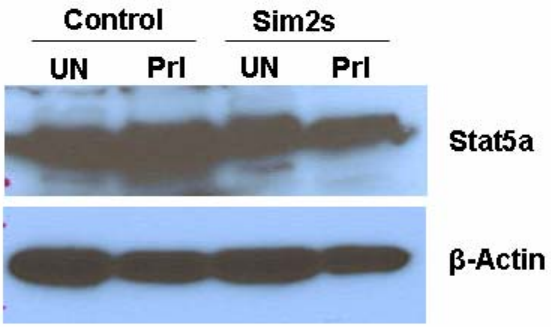

Figure 22. Western Blot Analysis of Stat5a in HC11 Cells. Protein isolated from undifferentiated HC11 cells (UN) or cells treated with HC + Prl for 12 hours was used for western blot analysis. Stat5a is approximately 97kDA. β-Actin was used as a loading control.

elongation of the RNAPII complex. In Sim2s cells, more active, or Serine 2-phosphorylated, RNAPII was found to be associated with the *Csn2* promoter, when compared to Control cells, and this association was sustained throughout the timecourse, similar to that of Stat5a. Sim2 was found to be transiently associated to the *Csn2* promoter in both the Control and Sim2s cells, although the *Csn2* regulatory region does not contain a consensus binding site for Sim2 or other PAS proteins. Interestingly, association of acetylated-Histone H3 (AcH3) with the *Csn2* promoter was relatively constant in both Control and Sim2s cells, after hormonal stimulation. This suggests that overexpression of *Sim2s* does not alter the accessibility of chromatin surrounding the *Csn2* regulatory region; rather, it appears to enhance the recruitment of factors to the promoter after PRL stimulation.

ChIP analysis was also used to evaluate the factors associated with the coding region of the *Csn2* gene (Figure 23A). Analysis of Stat5a occupancy was used as a negative control, as the protein has been shown to bind the proximal promoter region only (Fig. 23B). Both total and active RNAPII showed a similar pattern of association to that found at the promoter region, in both Control and Sim2s cells. Greater amounts of both forms of RNAPII were recruited in the Sim2s cells as compared to Control cells (Fig. 23B). Cdk9 is part of the P-TEFb complex that participates in promoting elongation of RNAPII by phosphorylating Serine 2 in the CTD. A transient association of Cdk9 with the *Csn2* coding region was detected in Control cells, but this recruitment was sustained in Sim2s cells (Fig. 23B). Surprisingly, Sim2 was found to be associated with the *Csn2* coding region in both Control and Sim2s cells, and the pattern of

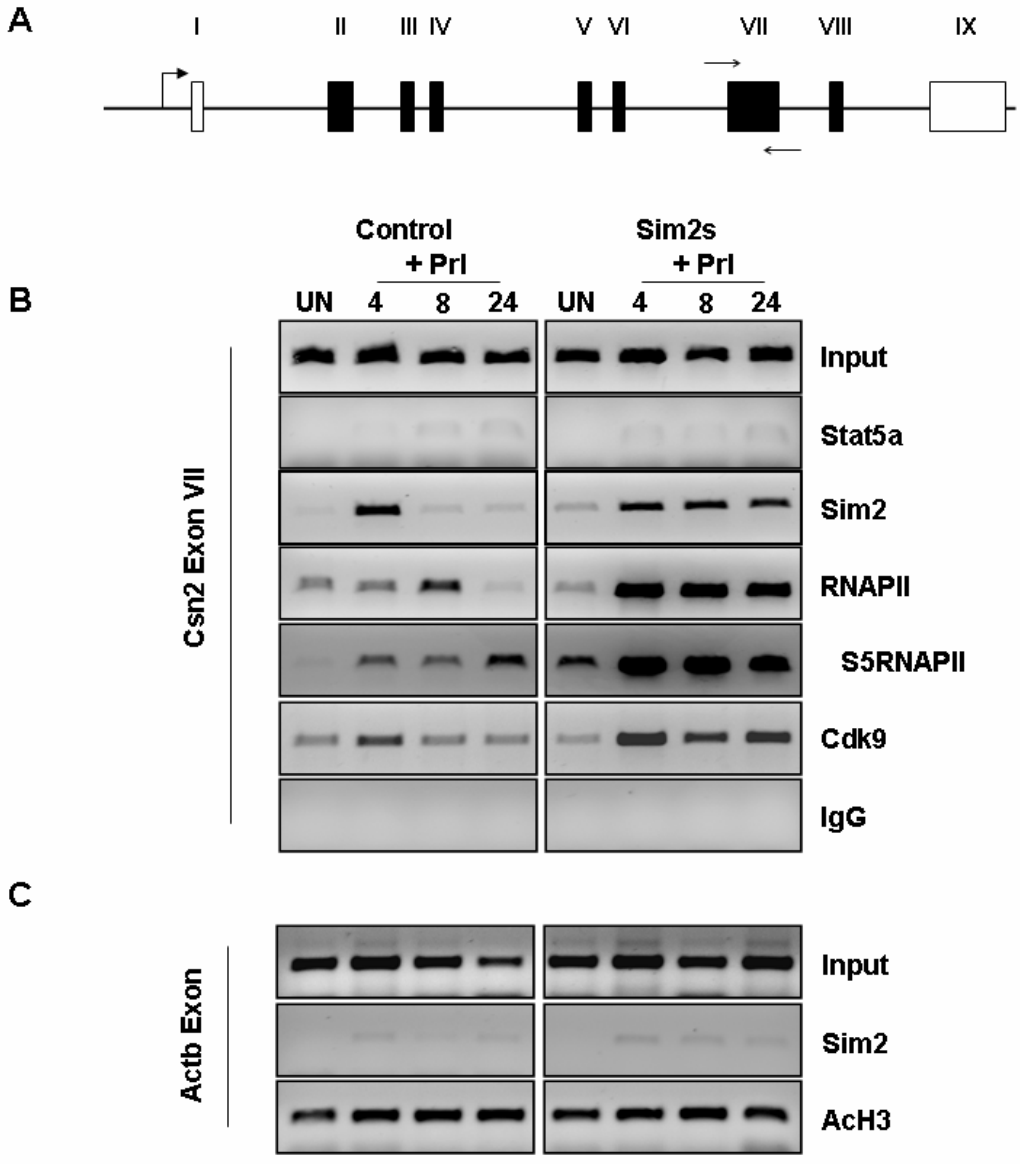

Figure 23. ChIP Analysis of *Csn2* Exon VII. (*A*) Diagram of the *Csn2* sequence described in detail in Figure 22. (*B*) ChIP assays performed on chromatin harvested from undifferentiated HC11 cells (UN), or cells treated for 4, 8, and 24 hours with HC + Prl. (*C*) ChIP analysis was performed on indicated HC11 chromatin samples to detect AcH3 and Sim2 association with a coding exon of *Actb*.

recruitment was similar to that of RNAPII (Fig. 23B). Sim2 was not detected in association with a coding exon of the *Actb* gene, and AcH3 was used as a positive control (Fig. 23C). The detection of Sim2 in the coding region of *Csn2* suggests that it interacts with the general transcriptional machinery that remains associated with DNA throughout the gene.

To determine if Sim2 and RNAPII associate with one another at specific sites in the *Csn2* gene, Re-ChIP analysis was performed on chromatin harvested from Control cells treated for 4 hours with HC + Prl (Fig. 24). This experiment revealed that Sim2 and RNAPII were both found in a complex associated with the *Csn2* proximal promoter and coding regions after Prl stimulation of HC11 cells.

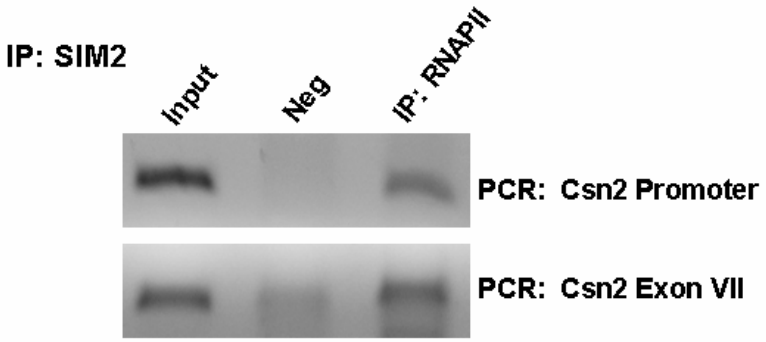

Figure 24. Re-ChIP Analysis in HC11 Cells. Chromatin from Control HC11 cells treated for 4 hours with HC + Prl was used for Re-ChIP analysis. First IP was performed with Sim2 antibody, input was reserved and remaining chromatin used for IP with RNAPII antibody or no antibody as a control. PCR was performed using primers for the *Csn2* proximal promoter and Exon VII.

CHAPTER V

CONCLUSIONS

Sim2s Expression and Cellular Differentiation

The *Singleminded* gene was initially identified in Drosophila (*dsim*), where it plays a role in cell fate determination in the CNS during embryogenesis (Menne et al. 1997; Chang et al. 2001). Our laboratory was the first to identify a role for mammalian *Sim2*, a *dsim* homolog, in normal development of the mammary epithelium, and to uncover a requirement for SIM2s in the maintenance of epithelial cell fate in breast cancer cells (Laffin et al. 2008). It was determined that the short *Sim2* isoform, *Sim2s*, was found to be highly expressed in the terminally differentiated epithelial cells of the mouse mammary gland (Fig. 6A). Based on the role of dsim in cell fate determination and of SIM2s in maintenance of mammary epithelial cell fate, Sim2s likely participates in promoting and maintaining an alveolar cell fate in the mammary epithelium.

To test our hypothesis, we created a transgenic mouse expressing *Sim2s* driven by the MMTV-LTR in the mammary epithelium (Fig. 8), and altered levels of *Sim2s* in the mouse mammary epithelial cell line, HC11 (Figs. 19 & 20). The HC11 cell line provides a valuable resource to evaluate the regulation of milk protein gene transcription in response to hormonal stimulation (Ball et al. 1988). Analysis of various tissues from transgenic female mice revealed transgene expression in the mammary epithelium. The pattern of expression, as determined by IHC analysis of HA localization (Fig. 8C), was

consistent with that of previously reported MMTV-driven transgenes (Robinson et al. 2001).

Morphologically, mammary gland development in transgenic female mice was not altered when compared to WT controls (Figs. 9 & 10). TEB and mature ductal structures were normal in nulliparous females, and lobulo-alveolar formation took place during pregnancy. Following pup removal after 10 days of lactation, mammary glands from transgenic females appeared to regress more slowly than those from WT females, and had fewer cleaved caspase 3-positive cells, as determined by IHC analysis (Fig. 16). Ultimately, the glands involuted and were able to expand again during the next pregnancy.

Enhanced Differentiation *in Vitro* and *in Vivo*

During virgin development, expression of *Csn2* and *Wap* were significantly upregulated in mammary glands from transgenic females compared to those from WT animals (Fig. 11A & B). In addition, Npt2b was detected at the luminal surface of the ductal epithelial cells in approximately 70% of transgenic females (Fig. 12B). In WT females, Npt2b is not detectable during virgin development, but becomes localized to the apical surface of the ducts during lactation (Shillingford et al. 2002). Interestingly, there were no differences in Aqp5 expression or localization between WT and transgenic mammary glands (Fig. 12A). *Aqp5* is highly expressed and located on the luminal surface of ductal epithelial cells during virgin development, but is lost during pregnancy and lactation (Shillingford et al. 2003). The detection of both Aqp5 and Npt2b in

epithelial cells of transgenic nulliparous females suggests precocious, but incomplete, lactogenic differentiation. Using IHC, we detected beta-Casein sporadically produced in epithelial cells of transgenic females (Fig. 12C). Moderate upregulation of *Csn2* in the mammary epithelium is not uncommon during estrus in nulliparous females (Robinson et al. 1995). With each cycle, some epithelial cells acquire the ability to transcribe small amounts of both *Csn2* and *Wap*, but neither is detectable during diestrus. Females used for these studies were sacrificed during estrus, so the differences between the two groups can be attributed to the transgene expression; however, the fate of these partially differentiated cells after estrus is not known. The data, revealing a reduction in postlactational apoptosis following forced weaning, and showing the extrusion of Sim2-negative epithelial cells into the alveolar lumen (Fig. 17A), suggest that differentiated epithelial cells in transgenic nulliparous females may accumulate with each estrus cycle due to enhanced cell survival. During pregnancy, the patterns of *Csn2* and *Wap* expression are identical, although *Csn2* is detectable prior to *Wap* (Robinson et al. 1995). Neither shows uniform expression, and both are localized to the same alveolar cells in late pregnancy. During lactation, expression of milk protein genes is high, but none show a uniform pattern throughout the mammary gland (Robinson et al. 1995). Currently, the tools are not available to distinguish between cells that do and do not produce *Csn2* and *Wap* in an alveolar structure. It is possible that the Sim2s transgene leads to the differentiation and survival of this cell type in the mammary epithelium after estrus, and endogenous Sim2s maintains this cell fate during lactation.

The level of expression and pattern of PR localization in the mammary epithelium was reported to be developmentally regulated (Seagroves et al. 2000; Aupperlee et al. 2005). In immature nulliparous femals, PR expression is high, and localized to nearly all luminal epithelial cells. As the female progresses through puberty and ductal elongation is complete, the pattern of PR expression becomes less uniform. It has been proposed that PR controls cell proliferation in the mammary gland in a juxtacrine manner, as PR is rarely associated with a proliferating cell in mature mammary glands. Hypothetically, PR activation stimulates production of mitogenic factors, which are secreted and promote growth of adjacent, PR-negative, cells (Seagroves et al. 2000). The uniform pattern of expression displayed by PR in immature virgins is likely a mechanism to prevent precocious side branching and cell proliferation prior to the completion of branching morphogenesis. During pregnancy, the relative amount of PR positive cells decreases, and no PR is detectable during lactation (Aupperlee et al. 2005).

In mammary glands of mature nulliparous transgenic females, differences were not detected in distribution or expression levels of PR, when compared to WT glands (Fig. 13A). In early pregnancy, however, there were significantly fewer PR positive cells in epithelial cells of transgenic glands compared to WT glands (Fig. 13B). This decrease in PR expression could reflect a globally more differentiated gland in the transgenic females. During late pregnancy, relatively few cells expressed PR, and differences were not observed between WT and transgenic glands (Fig. 13C). It is

possible that, during late pregnancy, expression of PR is too low to detect any significant differences between the groups.

In addition to evaluating expression of PR during pregnancy, we analyzed the expression of milk protein genes in glands from WT and transgenic females at P6 and P16 (Fig. 14). Significant increases were observed in expression of *Expi*, *Lalba*, *Csn2* and *Wap* in early pregnancy in glands from transgenic females. In the WT mammary gland, early pregnancy is associated with rapid cell proliferation and alveolar formation, but alveolar epithelial cells have not yet undergone secretory differentiation. At this stage, endogenous Sim2s expression was relatively low (Fig. 6). By P16, significant differences were not detected in expression of the four listed milk protein genes between WT and transgenic glands. In late pregnancy, the alveolar epithelial cells continue to proliferate, but are beginning to undergo secretory differentiation, characterized by accumulation of CLDs and moderate expression of milk protein genes. Our data indicate that precocious *Sim2s* expression, during early pregnancy, promotes moderate upregulation of milk protein gene expression, but by late pregnancy, expression is high enough to render undetectable any changes due to transgene expression.

Sim2s and Lactation

At parturition, several changes take place in the mammary gland that promote abundant milk production for the newborn offspring; Circulating levels of P4 decrease and Prl levels surge, which ensures high transcription rates of essential genes. Milk protein gene expression is dramatically upregulated and select mRNAs become

stabilized. Although expression of *Expi, Lalba*, *Csn2* and *Wap* is greatly enhanced, they continue to show a non-uniform pattern of expression throughout an alveolar unit (Robinson, 1995). In addition, we have shown that *Exp*i and *Lalba* were most highly expressed immediately following parturition, and did not increase further during the first week of lactation (Fig. 7D & E). On the contrary, expression of *Csn2* and *Wap* continued to increase during that week, peaking at L7 (Fig. 7B & C). Interestingly, *Sim2s* expression followed that exact pattern (Fig. 7A). These results, taken with the non-uniform expression pattern, suggest that all milk protein genes are not regulated by the same mechanisms, and that Sim2s may be involved in fine-tuning the expression of certain genes during lactation.

Loss-of-function studies, performed on some of these genes, reflect different requirements of their expression for pup survival. It has been reported that loss of *Csn2* (Kumar et al. 1994) or *Wap* (Triplett et al. 2005) does not result in pup mortality. *Csn2*$^{-/-}$ mammary glands produce milk with a slightly altered protein composition, and pups nursed by *Csn2*-deficient mothers are runted, but they survive. Loss of *Wap* does not result in pup growth defects during the first 8 days of lactation. It is only during the last half of lactation that pup growth becomes slower and the offspring are significantly smaller. This indicates that maximal expression of *Wap* and *Csn2* immediately after parturition is not required for the survival of the offspring. It is likely that the mammary tissue has evolved mechanisms to prevent the unnecessary use of valuable resources during lactation, and these mechanisms involve precise control of gene expression. Conversely, loss of *Lalba* in the mammary gland leads to pup mortality within 8 hours of

their birth (Stacey et al. 1995). Alpha-lactalbumin is a subunit of the lactose synthetase enzyme. Lactose, a major milk carbohydrate, helps move water into the milk and is responsible for its fluidity. Milk produced by *Lalba*$^{-/-}$ mothers is highly viscous and cannot be expelled from the ducts. In this case, maximal expression of the *Lalba* gene at parturition is clearly necessary for the survival of the offspring. Currently, there are no reported loss-of-function studies on *Expi*. Based on the expression of *Sim2s* during lactation and the effect of the *Sim2s* transgene on *Csn2* and *Wap* levels during virgin development, it is possible that, during lactation, Sim2s functions to participate in the precise control of the expression of a subset of milk protein genes in specific cells.

Analysis of milk protein gene expression during the first week of lactation in WT and transgenic females revealed interesting results (Fig. 15). In WT glands, the expression of *Csn2* and *Wap* increased from L2 to L4 (Fig. 15C & D). Differences were not detected in *Wap* expression between glands from transgenic and WT females; however, we did observe a statistically significant increase in *Csn2* expression in glands from transgenic females at L4, when compared to WT glands (Fig. 15C). Significant differences were not detected in either *Expi* or *Lalba* expression at L2 or L4 in WT or transgenic glands (Fig. 15A & B). Overall, precocious expression of *Sim2s* in the mammary epithelium resulted in accelerated differentiation of the mammary epithelium, characterized by developmental stage-specific upregulation of certain milk protein genes. In addition, other changes associated with lactogenic differentiation took place in the mammary glands of transgenic females, including upregulation of Npt2b and loss of PR expression. During the first week of lactation, however, only *Csn2* was significantly

upregulated. In the next section, the possible mechanism contributing to enhanced *Csn2* expression will be discussed.

Regulation of *Csn2* Transcription

As described previously, the HC11 cell model provides an excellent system to study regulation of *Csn2* transcription. Within 15 minutes of hormonal stimulation, Stat5a, GR, and RNAPII are recruited to the *Csn2* promoter, and mRNA can be detected 2 hours later (Kabotyanski et al. 2006). Using HC11 cells, we have shown that Sim2s expression increases with hormone treatment, in a similar pattern seen during the first week of lactation *in vivo* (Fig. 18A). Overexpression of *Sim2s* was not sufficient to induce *Csn2* in the absence of Prl; however, it resulted in significantly enhanced steady-state levels of *Csn2* mRNA in the presence of Prl, and this difference was detected as early as 4 hours after hormone treatment (Fig. 19A). Reduction of *Sim2s* levels, using shRNA, resulted in attenuated Csn2 expression and beta-Casein production (Fig. 20A & B). Together, these data suggest that, in HC11 cells, Sim2s is necessary for maximal Prl-induced *Csn2* expression.

ChIP analysis of the *Csn2* proximal promoter region revealed transient increases in the association of Stat5a and RNAPII in control cells (Fig. 21B). Interestingly, Sim2 was found to be associated with this region as well, although there are no consensus binding sites for the protein in the *Csn2* promoter (not shown). The relative amounts of Stat5a and RNAPII recruited to the *Csn2* promoter were markedly higher in the Sim2s cells, and they remained associated for 24 hours. As reported previously, the *Csn2*

promoter region was found to be associated with acetylated Histone H3 (AcH3), consistent with genes undergoing active transcription. The pattern and relative amounts of AcH3 at the *Csn2* promoter did not differ between Control and Sim2s cells. These data indicate that *Sim2s* overexpression may enhance recruitment of Stat5a and RNAPII to a given *Csn2* promoter, rather than stimulating accessibility of more promoters in a population of cells. In addition to enhanced stability of the *Csn2* mRNA, an increase in the transcription rate of the *Csn2* gene has been reported in HC11 cells after Prl treatment (Ball et al. 1988). Although not evaluated in this model, it is possible that Sim2s participates in altering the rate of transcription of *Csn2* in response to Prl in HC11 cells, and possibly *in vivo*.

ChIP analysis was also used to evaluate the factors associated with the *Csn2* coding region (Fig. 23B). Similar to what was detected at the proximal promoter, in Control cells RNAPII and Sim2s were both found to be transiently associated with *Csn2* Exon VII, and the association was greater in Sim2s cells. Stat5a was not detected in this region in either cell type. Cdk9, a subunit of P-TEFb, known to promote transcription elongation by phosphorylating Serine 2 of the RNAPII CTD, was found to be recruited to the *Csn2* coding region in a similar pattern to RNAPII in both cell types. In addition, RNAPII and Sim2 were found to be in a complex at both the proximal promoter and ExonVII, by Re-ChIP analysis (Fig. 24). These results indicate that Sim2s associates with RNAPII beyond the proximal promoter region of the *Csn2* gene. It is possible that Sim2s serves as a cell type-, or differentiation stage-specific subunit of the RNAPII

holoenzyme complex to promote transcription under hormonal stimulation. The precise mechanism by which Sim2 accomplishes this has not been investigated.

The Function of Sim2s in the Mammary Epithelium

In these studies, we have determined that the expression of *Sim2s* in the mouse mammary gland is developmentally regulated and peaks during the first week of lactation. In addition, precocious expression of *Sim2s in vivo* promotes an alveolar epithelial cell phenotype, characterized by upregulation of milk protein gene expression in glands from nulliparous and pregnant transgenic females, production and proper localization of Npt2b, and reduction of PR during early pregnancy. Milk protein gene expression, *in vivo*, is not uniform during pregnancy or even during lactation (Robinson et al. 1995). The increased expression of some milk protein genes in mammary glands of transgenic nulliparous females suggests that a subpopulation of differentiated alveolar cells is over-represented in this model, perhaps due to prolonged cell survival. Several studies have been performed to evaluate the signature of the mammary stem cell, both during normal development and oncogenic transformation (Vaillant et al. 2007). The identified markers allow certain cell types to be isolated, and many investigators have associated the expression of other genes, many localized to the nucleus, with these various surface markers. Unfortunately, the markers that would allow the identification of specific alveolar epithelial cells that produce *Wap* and *Csn2* during estrus are not available.

The question remains, what is the function of a protein that is identified as cell type specific, i.e. progenitor, luminal, alveolar, or myoepithelial? One striking difference between progenitor cells and their differentiated daughters, in many tissues, is the array of genes that are expressed. In many systems, specific genes are upregulated during cell commitment processes that ultimately define the function of the tissue. It is likely, then, that some proteins can alter global transcription within a cell when they are produced. The observation that Sim2s promotes the expression of genes associated with differentiation is consistent with the previously published role for Sim2 as a putative mammary tumor suppressor (Kwak et al. 2007). In the mouse PyMT mammary tumor model, for example, the progression from adenoma to adenocarcinoma involves the loss of cellular differentiation (Kouros-Mehr et al. 2008). Our data, from HC11 cells, showed recruitment of RNAPII and Stat5a to the *Csn2* promoter in the presence of Sim2s, and revealing an association of Sim2s and RNAPII at the proximal promoter and coding exon of *Csn2*, suggest that Sim2s participates in recruiting transcriptional machinery to the regulatory region of differentiation-specific genes. It is possible that the inverse relationship between Sim2s expression and cancer cell aggressiveness involves the effect of Sim2s on tumor cell differentiation.

The conformation and subunit composition of the general transcription factors can vary depending on the tissue, the cell type within a tissue, or the developmental stage of the tissue. In Drosophila spermatocyte development, it has been proposed that transcriptional complexes can vary in the composition of TBP-associated factors (TAFs) that together with TBP form $TF_{II}D$, to regulate the expression of testis-specific genes

(Hiller et al. 2004). $TF_{II}D$ associates with promoters of genes to recruit RNAPII and promote transcription initiation. In mice, it has been shown that the cellular localization of a TAF is another level of transcriptional regulation during differentiation (Pointud et al. 2003). TAF7L was identified as a germ cell-specific paralog of TAF7. In spermatogonia, TAF7L is localized to the cytoplasm, but becomes nuclear and replaces TAF7 in the $TF_{II}D$ complex during spermatogenesis. A study performed in mouse ovaries determined that loss of TAF105 affected expression of a subset of genes associated with follicle development (Freiman et al. 2001). The authors proposed that cell type specific subunits of $TF_{II}D$ in mammalian cells lead to specific regulation of gene expression programs during differentiation. Cell type specific replacements for TBP have been identified in spermatocytes and developing myotubules, as well (Zhang et al. 2001; Deato and Tjian 2007).

Based on these studies and our data, we propose a model in which *Sim2s* is expressed in a developmentally-regulated, and possibly cell type specific manner, to interact with RNAPII and its associated factors and promote expression of genes associated with lactogenic differentiation in the mammary gland (Figs. 25 & 26). In addition, we hypothesized that Sim2s plays a role in alveolar cell survival during lactation, which is likely not exclusive from its role in transcription regulation. Future studies will most certainly involve loss of Sim2s function and analysis of altered transcription programs, and also biochemical assays to evaluate the potential interactions between Sim2s and components of the general transcription machinery.

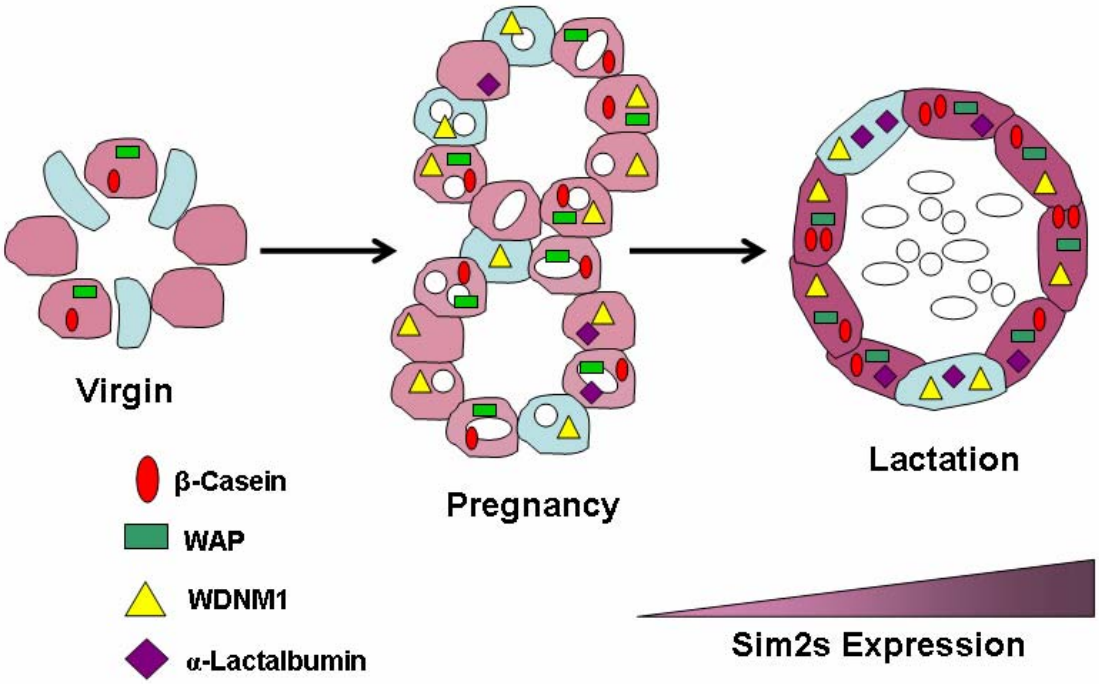

Figure 25. Proposed Model of Sim2s Expression and Association with Milk Protein Gene Expression.

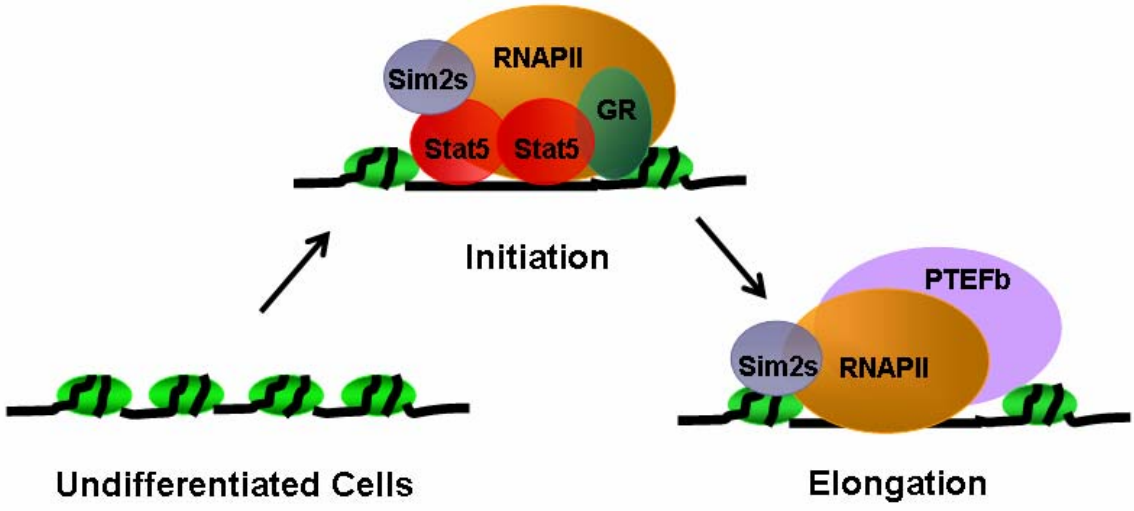

Figure 26. Proposed Model of the Interaction of Sim2s with General Transcription Factors during Transcription.

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VITA

Name:	Elizabeth Ann Wellberg
Address:	Texas A&M University College of Veterinary Medicine and Biomedical Sciences 4458 TAMU College Station, TX 77843-4458
Email Address:	ewellberg@cvm.tamu.edu
Education:	B.S. in Biomedical Sciences, Texas A&M University, 2004 Ph.D. in Toxicology, Texas A&M University, 2009