REGULATION OF MAMMARY CELL DIFFERENTIATION AND METABOLISM BY SINGLEMINDED-2S

A Dissertation

by

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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

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August 2013

Major Subject: Toxicology

ABSTRACT

Ductal carcinoma i*n situ* (DCIS) has been shown to be a precursor to invasive ductal cancer (IDC). Though the progression of DCIS to IDC is believed to be an important aspect of tumor aggressiveness, prognosis and molecular markers that predict progression are poorly understood. Therefore, determining the mechanisms by which some DCIS progress is critical for future breast cancer diagnostics and treatment.

Singleminded-2s (SIM2s) is a member of the bHLH/PAS family of transcription factors and a key regulator of differentiation. SIM2s is highly expressed in mammary epithelial cells and lost in breast cancer. Loss of *Sim2s* causes aberrant mouse mammary development with features suggestive of malignant transformation, whereas over-expression of *Sim2s* promotes precocious alveolar differentiation, suggesting that *Sim2s* is required for establishing and enhancing mammary gland differentiation. We hypothesize that *SIM2s* expression must be lost in pre-malignant lesions for breast cancer to develop.

We first analyzed *Sim2s* in the involuting mammary gland, which is a highly tumor-promoting environment. *Sim2s* is down-regulated during involution, and forced expression delays involution. We then analyzed SIM2s expression in human breast cancer samples and found that SIM2s is lost with progression from DCIS to IDC, and this loss correlates with metastasis. SIM2s expression in DCIS promoted a differentiated phenotype and suppressed genes associated with de-differentiation. Furthermore, loss of *SIM2s* expression in DCIS xenografts increased metastasis likely due to an increase in hedgehog signaling and matrix metalloproteinase expression. Interestingly, we found metabolic shifts with gain and loss of *SIM2s* in not only DCIS cells, but also MCF7 and SUM159 cells. *SIM2s* expression decreased aerobic glycolysis and promoted oxidative phosphorylation through direct upregulation of

CDKN1a and senescence. Loss of *SIM2s*, conversely, promotes mitochondrial dysfunction and induction of the Warburg effect. This is the first time *CDKN1a* and cellular senescence have been indicated as causative to metabolic shifts within cancer cells.

These studies show a new role for *SIM2s* in metabolic homeostasis, and this regulation is lost during tumorigenesis. These data indicate *SIM2s* is at the apex where aging, metabolism, and disease meet – regulating the delicate relationship between the three.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Porter, and my committee members, Dr. Dees, Dr. Dindot, Dr. Phillips, and Dr. Safe, for their guidance and support throughout the course of my research.

Thanks also go to my friends and colleagues and the toxicology department faculty and staff for making my time at Texas A&M University a rewarding experience. I also want to thank my Texas A&M collaborators: Dr. Burghardt, Dr. Mouneimne, Dr. Payne at the Image Analysis laboratory for assistance with electron microscopy and live cell imaging, Dr. Hilty and Mukundan Ragavan in the Dept. of Chemistry for NMR analysis; Dr. Chapkin and Dr. Fan in the Dept. of Nutrition for assistance in mitochondrial bioenergetics; and the Histology Core Facility. I would also like to thank Dr. Behbod at the University of Kansas Medical Center and Dr. Medina at Baylor College of Medicine for their extensive support throughout this project through providing cells, human samples, and intellectual input.

Finally, thanks to my mother and father for their unconditional encouragement and support throughout my long career as a student. I couldn't have done this without them.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Breast Cancer

Breast cancer is the most common type of cancer occurring in women in the United States, and remains one of the leading causes of mortality (Figure 1)(Group, 2013). Breast cancer occurs when cells in the breast proliferate uncontrollably, ignoring the signals from the body to stop.

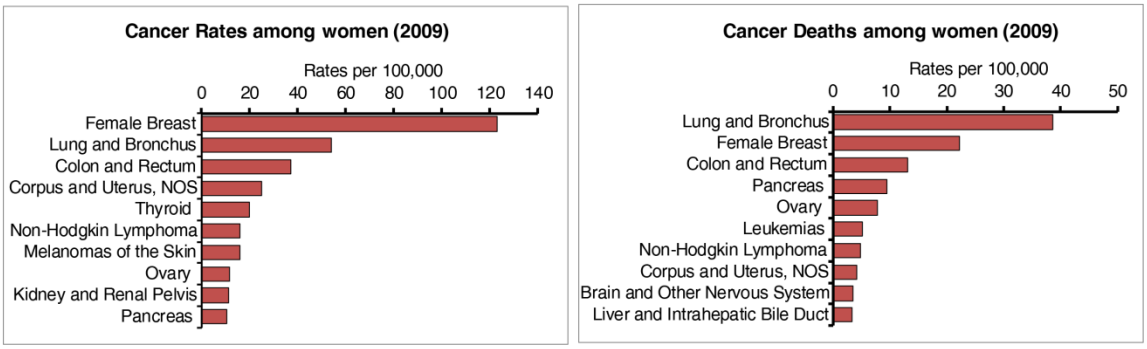

Figure 1. Cancer Rates in the United States of America. Data is courtesy of CDC cancer statistics. Breast cancer is the higher occurring cancer among women, and the second leading cause of mortality in the USA.

Approximately 50-75% of breast tumors begin in the ductal tree (ductal carcinoma), while 10-15% begin in lobular structures (Dillon, 2010). In 2013, an estimated 232,340 new cases of invasive breast cancer will be diagnosed, along with 64,640 new cases of *in situ* breast cancer. The estimated number of breast cancer related deaths in 2013 is 39,620 (American Cancer Society). In the United States, approximately 22% of women between the ages of 45-

54 are diagnosed with breast cancer. Despite drastic advances in diagnostics and therapies, there remains a need for more efficient biomarkers and treatments.

Breast cancer is a collection of diseases that have diverse histo-pathological phenotypes, genetic make-ups and prognostic indicators (LaMarca and Rosen, 2008; Vargo-Gogola and Rosen, 2007). Non-invasive breast cancer is characterized by hyperplastic cells that are proliferating within the ductal structure of the mammary gland, but have not spread to nearby tissue. This early stage of the disease is termed ductal carcinoma *in situ* (DCIS) or the less common lobular carcinoma *in situ* (LCIS). Due to improved detection methods, the diagnosis of DCIS has risen from less than 1% of diagnosed breast cancers to 15-25% (Jones, 2006; Norton et al., 2010). DCIS has been shown to be a precursor to invasive ductal cancer (IDC), with 20-30% of DCIS showing evidence of invasion upon diagnosis (Burstein et al., 2004; Maffuz et al., 2006). Invasive breast cancer occurs when rapidly growing cells from within ductal and lobular structures invade through the stroma into nearby tissues. From here tumor cells can spread to lymph nodes and to distant organs (metastasis). Metastasis to distant organs is the primary cause of mortality in breast cancer patients. Research has provided many biomarkers that aid in diagnoses and chemotherapy, as well as characterization and classification of this disease.

Breast Cancer Subtypes

Analysis of primary breast cancers for genetic mutations and unique genetic phenotypes has led to the characterization of 6 breast cancer sub-types: luminal A, luminal B, HER2 positive, and basal-like (Table 1), as well as the lesser known normal-like and claudin-low (Cancer Genome Atlas, 2012; Perou et al., 2000; Sorlie et al., 2001).

Table 1. Classification of Breast Cancer Subtypes.

Molecular Subtype	Immunohistochemical staining					
	ER	PR	HER2	CK5/6	CK8/18	EGFR
Luminal A	+	+	-	-	+	-
Luminal B	+	+	+	-	+	-
HER2	-	-	+	-		-
Basal	-	-	-	+	-	+

The use of high-throughput sequencing and protein arrays have enabled scientists to view whole-genome changes that are acquired with the onset of breast cancer, thus identifying unique profiles that divide breast cancer based on certain genotypic characteristics.

Luminal breast cancers make up the most heterogeneous subtype of breast cancers in terms of genes expression and mutation (Cancer Genome Atlas, 2012). Luminal-type tumors are mainly characterized by the expression of luminal genes such as GATA binding protein 3 (GATA3), X-box binding protein 1 (XBP1), and cytokeratins 8 and 18 (CK8/18). More commonly, luminal cancers are identified by the expression of hormone receptors: estrogen receptor (ER) and progesterone receptor (PR) (Perou et al., 2000). ER^+/PR^+ breast cancers require hormone stimulation to maintain tumor proliferation and integrity. As such, endocrine therapy that blocks this signaling is highly effective in treating these cancers. Luminal breast cancers can be further broken down into subtypes A and B by whether, in addition to ER and PR, the tumors also express human epidermal growth factor receptor 2 (HER2) (Luminal B).

HER2 overexpression occurs in approximately 30% of primary breast cancers (Slamon et al., 1989). Also called Neu, ErbB2, and CD340, HER2 expression in tumors activates mitogenic pathways such as STAT, AKT, and MAPK. HER2 expression is associated with lower survival rate and high recurrence rates and metastasis, and is often associated with

increased expression of epidermal growth factor receptor (EGFR) (Cancer Genome Atlas, 2012). Although resistant to hormone therapy, new treatments, such as trastuzumab, that directly target HER2 (and therefore leave healthy tissue alone) are very effective (Damodaran and Olson, 2012; Tinoco et al., 2013).

If a tumor is missing all three of these biomarkers, it is said to be a triple-negative, or basal, breast cancer. Triple-negative breast cancers, which comprise approximately 15% of breast cancers, are hard to classify, and as such are a heterogeneous mixture of various subtypes. Triple negative breast cancers are said to be basal-like when they are highly proliferative, poorly differentiated, and express the basal marker cytokeratin 5/6 (CK5/6) (Perou et al., 2000) and this accounts for approximately 75% of triple negative breast cancers. Triple-negative breast cancers are often characterized by expression of genes such as Ki67 and Proliferating Cell Nuclear Antigen (PCNA) regulating proliferation, as well as loss or mutation of p53 (Cancer Genome Atlas, 2012). These cancers are often harder to treat, and their inherent aggressive characteristics account for a much higher proportion of breast cancer mortality compared to luminal subtypes (Oakman et al., 2010). A relatively newly characterized triple-negative breast cancer is the claudin-low subtype (Herschkowitz et al., 2007). In addition to being a triple-negative breast cancer, claudin-low subtype tumors also are characterized by low expression of adhesion genes such as E-cadherin (CDH1) and claudins, as well as a strong mesenchymal phenotype. Additionally, claudin-low breast cancer have a unique signature of $CD44^{+}/CD24^{-}$ breast tumor-initiating cells, which are linked to chemoresistance and metastasis (Hennessy et al., 2009).

Thanks to many new treatments and biomarkers, our understanding of breast cancer progression has been greatly enhanced. However, much remains to be understood. Research has increasingly shown that the normal development of the mammary gland includes a delicate

balance that is the basis for cancer development when insults occur. In order to understand the progression of breast cancer, we must also understand the normal mammary gland and how the normal function is mis-regulated in carcinogenesis.

Normal Mammary Gland Development

The mammary gland is a unique reproductive structure of mammals that, with the exception of humans and some dairy calves, is critical for reproductive success. Mammary glands are epithelial organs that at birth are extremely rudimentary, and do not reach full development until lactation following parturition. The mammary is a complex structure of lactating lobules, ducts for milk passage, supporting tissue, muscles, fat, and lymph nodes. Throughout puberty, menstruation, pregnancy, lactation, and menopause the structure and activity of the mammary gland dynamically changes to meet the varying signals of these cycles.

The mammary gland is composed of three major epithelial cell types including ductal, alveolar and myoepithelial cells, which undergo dramatic morphological and genetic changes associated with different stages of mammary gland differentiation (Siegel and Muller, 2010; Visvader, 2009). A rudimentary ductal structure develops *in utero*, however, all subsequent development occurs at the onset of puberty. During puberty, mammary gland development is regulated through a branching morphogenic mechanism driven by terminal end buds (TEB). TEBs are highly proliferative and invasive, invading the mammary fat pad to develop the rudimentary ductal structure (Sympson et al., 1995; Sympson et al., 1994). As the TEB invades, the ductal structures remaining are formed through highly regulated apoptotic and differentiation mechanisms. The onset of estrous cycling promotes side branching and alveolar budding (Andres and Strange, 1999). Prolactin and progesterone are largely responsible for alveolar bud formation during post-pubertal growth by stimulating proliferation through paracrine signaling

with Wn4 and Rank Ligand (RankL) (Brisken et al., 2000). During pregnancy and lactation, progesterone and prolactin levels increase, causing the mammary gland to enter a cyclic transition characterized initially by growth and differentiation of lobuloalveolar structures, a switch to milk production during lactation, and regression to a state similar to the pre-pregnant virgin gland during involution. Similar to the hematopoietic system, a differentiation hierarchy of mammary stem cells has been identified in the adult mammary gland that give rise to the luminal and myoepithelial lineages (Figure 2) (Visvader, 2009). The luminal lineage can be further sub-divided into ductal cells that line the ducts and alveolar luminal cells that expand in response to lactogenic hormones to form alveolar units. There is also increasing evidence that the differentiation hierarchy in the developing mammary gland may be linked with the different sub-types of breast cancer. Gene expression profiling studies suggest that breast cancer heterogeneity is a result of the transformation of specific stem cell or progenitor cell populations responsible for normal mammary gland development (Figure 2) (Siegel and Muller, 2010; Visvader, 2009).

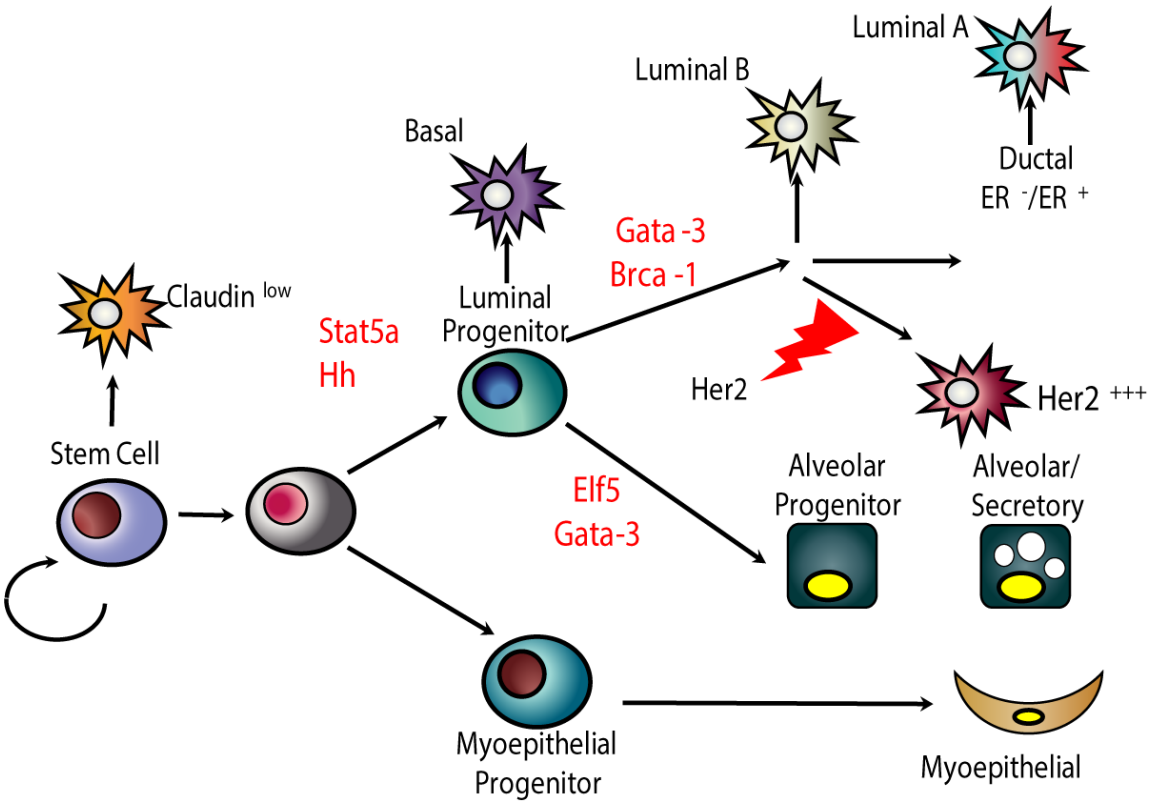

Figure 2. Mammary Gland Hierarchy and Derivation of Breast Cancer Subtypes. The mammary gland is composed of a unique hierarchy of stem, progenitor, and differentiated cells. Mutations in various cell types lead to the different subtypes of breast cancer.

Mammary gland involution is the regression of a lactating mammary gland post weaning to its quiescent state. Involution is characterized by a decrease in milk protein, collapse of alveolar structure, apoptosis of epithelial cells, and reinvasion of adipocytes (Baxter et al., 2007; Furth, 1999; Thangaraju et al., 2004; Walker et al., 1989; Wilde et al., 1999). Involution has been shown to proceed in two separate phases, first an acute response phase characterized by a decrease in milk protein synthesis, and epithelial cell apoptosis (Baxter et al., 2007; Bierie et al., 2009; Clarkson and Watson, 2003; Furth, 1999; Henson and Tarone, 1994; Jaggi et al., 1996; Lund et al., 1996; Marti et al., 1999; Quarrie et al., 1996). This initial phase is reversible and

occurs for 1-3 days post weaning (varies with species). The second phase begins at 72 hours post weaning, and is characterized by a collapse in the alveoli, extensive epithelial apoptosis, and breakdown of the basement membrane by matrix metalloproteinases (MMPs) (Stein et al., 2007). Involution is a unique process with a wound healing signature and controlled inflammation, both of which are associated with breast cancer progression, metastasis, and survival. During this process terminally differentiated luminal epithelial cells proceed through two pathways: the most prominent is cellular apoptosis, the remaining undergo a de-differentiation back to their pre-lactating state. The extracellular matrix of the involuting mammary gland has also been shown to enhance metastasis, and the gene expression signature of the involuting gland has been associated with poor prognoses in breast cancer diagnosis (Marti et al., 1999; McDaniel et al., 2006; Schedin et al., 2007; Stein et al., 2009). Extensive research has looked at gene expression, basement membrane changes, and signaling in involution and its respective correlation with breast cancer. Research has shown that metastatic breast cancer shares a gene expression profile similar to the involuting mammary gland (Clarkson and Watson, 2003; Clarkson et al., 2004; Come et al., 2004; Henson and Tarone, 1994; Jager et al., 1997; Lefebvre et al., 1992; Lyons et al., 2009; McDaniel et al., 2006; O'Brien and Schedin, 2009; Pensa et al., 2009; Radisky and Hartmann, 2009).

Pregnancy-associated breast cancer is one of the more aggressive, highly metastatic, types of breast cancer and often has a poor prognosis (Lyons et al., 2009; Newcomb, 1997; Polyak, 2006; Russo and Russo, 1998). Research has indicated that this is due to hormonal changes during pregnancy and lactation, and more recently the microenvironment and unique wound healing signature of the involuting mammary gland (Clarkson and Watson, 2003; Lund et al., 1996; McDaniel et al., 2006; Newcomb, 1997; O'Brien and Schedin, 2009; Radisky and Hartmann, 2009; Stein et al., 2009).

Stat5 in the Mammary Gland and Breast Cancer

Transcription factor cascades controlling functional gene expression are key events in regulating the differentiation potential of mammary epithelial cells. In the mammary gland, signal transducer and activator of transcription 5A (STAT5a), a well-known non-tyrosine kinase-containing cytokine receptor-activated transcription factor, is a key modulator of three different cellular outcomes: differentiation, survival, and proliferation (Liu et al., 1997; Silva, 2004). STAT5a can be activated via serine phosphorylation and de-phosphorylation, as well as interactions with other cellular proteins (Johnson et al., 2010; Wang et al., 2003). As a key regulator of mammary cell fate and lactation, STAT5a is activated by diverse and overlapping signaling pathways. These same pathways are potentially involved in the aberrant up-regulation of STAT5a in breast cancer.

Studies in normal mammary gland development have established the vital role for STAT5a in alveolar differentiation and lactation. STAT5a expression is present throughout virgin development of the mouse mammary gland, with highest levels of expression seen during pregnancy and lactation, coinciding with expression levels of Whey Acidic Protein (Liu et al., 1995). Using deletion transplant studies, loss of STAT5a in mouse mammary epithelium did not affect virgin ductal development (Miyoshi et al., 2001). However, STAT5a null mammary glands failed to differentiate and form alveoli or express milk proteins, resulting in locational failure in early pregnancies. Interestingly, after subsequent pregnancies, coupled with suckling pups, STAT5b expression and phosphorylation increased to eventually rescue the lactation defect (Liu et al., 1998). Complete knockdown of both STAT5a and STAT5b prevent this rescue, and significantly inhibited proliferation and differentiation in the mammary gland. Using conditional gene activation Cui et al (2004) examined this loss of STAT5 at different stages of mammary gland development to determine its effect. Loss of STAT5 expression prior to

pregnancy prevented epithelial proliferation and differentiation, and caused lactation failure. Loss of *STAT5* during late pregnancy using a WAP Cre gene promoter, which itself (WAP) is a milk protein induced in differentiating mammary tissue, did not completely ablate functional lactation, although alveolar structures were fewer in the $STAT5^{fl/fl}$ mice. However, further analysis showed that loss of STAT5 in lactating epithelial cells resulted in a de-differentiation, and was characterized by loss of WAP protein expression and the apical secretory marker Npt2b. Loss of STAT5 in late pregnancy also increased apoptosis in the lactating gland, indicative of an involution phenotype. Indeed, under normal circumstances, a cyclic relationship with loss of STAT5 and induction of STAT3 is a key step in the onset of involution (Philp et al., 1996).

In the mammary gland, STAT5a expression can be regulated by many different pathways. The most commonly identified pathway is gene induction through pregnancy hormones and prolactin. Alveolar epithelial cells respond to prolactin by initiating a Janus kinase 2 (Jak2)-mediated phosphorylation and dimerization with cytoplasmic STAT5a, resulting in activation and nuclear translocation of STAT5a, which then binds regulatory elements of milk protein genes, increasing their expression and subsequent lactation (Happ and Groner, 1993; Li and Rosen, 1995). However, in studies where the prolactin receptor (PrlR) was knocked down in mammary epithelium, STAT5 could still be induced by a myriad of other cytokine and hormone signals. While loss of PrlR in mammary epithelium inhibited lactation in the same manner as STAT5 loss, it was not as severe. PrlR-null mammaries were able to form small, open alveolar lumina, indicating that other cytokines were potentially activating STAT5 (Miyoshi et al., 2001). Miyoshi et al. showed that injection of epidermal growth factor (EGF) or growth hormone (GH) was sufficient to induce activation of STAT5a in PrlR-null mammary glands. However, other work done by Gallego et al. (2001) shows that neither one of these is necessary for normal mammary gland development, while PrlR is essential.

Although prolactin-mediated STAT5a activation is central to alveolar epithelial cell function, it is not the only pathway responsible for initiating mammary gland specification towards terminal differentiation. Other transcription factors, including the glucocorticoid receptor (GR), CCAAT-enhancer binding protein-beta (CEBPβ), Elf-5 and GATA-3 also play a role in lactogenic differentiation (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2008; Kouros-Mehr et al., 2006; LaMarca et al., 2010). Thus, commitment to cellular differentiation in the mammary gland requires activation and coordination of multiple signaling pathways to ensure that specific mammary lineage commitment is achieved. However, it is still not clear how these proteins control the specification of distinct cell types within the differentiation hierarchy of the developing mammary gland (Asselin-Labat et al., 2007; LaMarca and Rosen, 2008).

Ductal Carcinoma In Situ

DCIS is also a heterogenous group of diseases within itself, characterized by a neoplastic mammary lesion that is confined to the ductal-lobular system of the breast. It is hypothesized that nearly all invasive ductal carcinomas proceed from DCIS, and the ability to detect this early stage of breast cancer has allowed clinicians to target DCIS before the onset of invasive disease. However, research has yet to find reliable biomarkers that dictate the progression of DCIS to invasive ductal cancer (IDC). An estimated 14-60% of women with untreated DCIS will later develop invasive disease (Burstein et al., 2004).

Pathological markers such as nuclear grade, comedo necrosis, and size have been associated with an increased risk of DCIS progression, however these features are confounded when also addressing treatment and follow-up (Chin et al., 2004; Cocker et al., 2007; Ma et al., 2003; Porter et al., 2003; Yao et al., 2006). Serial analysis of gene expression (SAGE) showed no distinct genetic signature that differentiates DCIS from IDC. Indeed, most genetic changes

occurred at the normal to DCIS transition. In fact, extensive studies using fluorescent in situ hybridization (FiSH), microarrays, and whole genome analysis of human DCIS and IDC lesions have not consistently identified any classical markers for progression from DCIS to IDC (Hernandez et al., 2012; Muggerud et al., 2010). Molecular profiling of DCIS compared to IDC shows the same heterogeneity and sub-types previously described for IDC (Clark et al., 2011). However, the frequency of these subtypes varies between DCIS and IDC, indicating differences in subtype progression that may merit further research. Cocker, et al. (2007) studied the potential role or proteolytic enzymes in promoting the progression of DCIS to IDC, however, their results were inconsistent . Current research has also not determined the mechanisms that cause some DCIS to progress and others to lie dormant. Many confirmed oncogenes and tumor suppressors such as p53, PTEN, ERBB2, and MYC have been analyzed in DCIS and IDC samples; and consisten changes in gene expression indicating progression to invasion were not observed (Behling et al., 2011; de Biase et al., 2010; Knudsen et al., 2012; Liu et al., 2010; Lu et al., 2009; Miron et al., 2010; Rajan et al., 1997; Schmidt et al., 2010; Yao et al., 2006). ERBB2 was overexpressed in 25% of invasive tumors, and 50-60% of DCIS lesions, and must be correlated with 14-3-3ζ or its downstream regulator SIAH to better predict the risk of progression from DCIS to IDC; changes in PTEN signaling between DCIS and IDC were also not observed. MYC has been implicated as a possible genetic signature predicting DCIS and IDC, however the genetic imbalances and heterogeneity seen in these tumors did not show a definitive correlation (Heselmeyer-Haddad et al., 2012). However, FiSH studies of ERBB2, ESR1, CCDN1, and MYC showed no significant changes in their amplification in the progression from DCIS to IDC (Burkhardt et al., 2010). While a correlation has been established between hormone receptors ER and PR and DCIS, no change in p53 expression is seen during DCIS progression (Liu et al., 2010). Another study by Lee et al. showed that suppression of

genes involved in cell adhesion and signaling, and protease inhibition significantly increased DCIS progression, and pointed toward increased cathepsin activity (which is involved in autophagy) as a possible mechanism for DCIS progression (Lee et al., 2012). Currie et al. studied the potential role of tumor initiating cells (cancer stem cells) in the progression from DCIS to IDC. Tumor initiating cells are defined as de-differentiated, stem cell-like cells that are able to generate the heterogeneous cells of the tumor. In breast cancer, TICs have been identified as $CD44^{+}/CD24^{-}$ (cluster of differentiation 44/24, cell surface proteins) cells that are also positive for aldehyde dehydrogenase (ALDH) activity (Al-Hajj et al., 2003; Ginestier et al., 2007). Higher percentages of TICs have been implicated in basal breast cancers and have been associated with enhanced invasion and metastasis (Charafe-Jauffret et al., 2009; Charafe-Jauffret et al., 2008; Dontu et al., 2003; Korkaya and Wicha, 2007). Analysis of these three TIC markers in matched DCIS and IDC samples showed similar TIC populations (Currie et al., 2013). Although some possible indicators have been found between DCIS and IDC, no causal links to induced DCIS progression have been established, and no major pathways have emerged as necessary for the onset of invasive disease.

Emerging evidence indicates that response to metabolic stress and hypoxia promote progression of DCIS to IDC (Espina and Liotta, 2011; Hu et al., 2008; Lee et al., 2012; Schmidt et al., 2010). Indeed, hypoxic stress and nutrient deprivation are well established inducers of mutagenesis and genetic instability (Bindra and Glazer, 2005; Mathew et al., 2009; Mathew and White, 2011). In order for DCIS to progress, these lesions must circumvent stress-induced death and senescence, and adapt to using alternative sources of energy. HIF1α up-regulation in a hypoxic environment has been implicated as an inhibitor of p53-mediated cell death and the DNA damage response (Sendoel et al., 2010). De-phosphorylation of retinoblastoma (RB) in response to hypoxia and nutrient deprivation is responsible for cell-cycle arrest and senescence,

however, research in DCIS indicates this pathway is compromised, resulting in uncontrolled proliferation regardless of stress signals (Berman et al., 2010; Gauthier et al., 2007). A similar study further analyzed RB and the related tumor suppressor phosphatase and tensin homolog (PTEN) as potential regulators of DCIS progression (Knudsen et al., 2012). Using human DCIS samples, loss of RB protein expression was found to be significantly related to recurrent DCIS or progression to IDC. However, PTEN expression showed no correlations with DCIS progression. It was suggested that RB and PTEN expression together was a promising prognostic indicator for DCIS progression, through dual regulatory roles in cell cycle progression and an adaptive growth advantage via PTEN loss (Knudsen et al., 2012). In order to survive the stressed environment during tumorigenesis, autophagy has also been implicated in survival. Interestingly, autophagy has been to shown to have both tumorigenic and tumor-suppressive qualities, which will be further discussed. Autophagy has been shown to be activated during hypoxia and nutrient stress, and is hypothesized to be a major survival mechanism in DCIS cells (Debnath, 2011; Jin, 2006; Lozy and Karantza, 2012; Mathew et al., 2009).

MCF10DCIS.com Cells as a Novel Model for DCIS In Vivo

Until recently, mechanistic research of DCIS and the mechanism of cancer progression has been challenging due to lack of *in vivo* models. Studies were restricted to human samples, limiting real-time experimentation and modulation. Development of the MCF10DCIS.com (DCIS) cell line provided a useful and novel technique for modeling DCIS progression in vivo (Miller, 2000; Miller et al., 2000; Tait et al., 2007). Developed from the premalignant MCF10AT cells, DCIS cells were obtained from xenograft lesions of MCF10AT cells that mimicked DCIS *in vivo*. *In vitro*, DCIS cells grow in a single monolayer, and all cells express keratin and smooth muscle actin. DCIS cells, upon injection, form rapidly growing DCIS lesions, characterized by tightly packed lobular structures, many with central necrosis (comedo

DCIS). After injection and growth, these DCIS lesions will ultimately progress and become invasive, similar to a normal human DCIS lesion. It is hypothesized that *in vitro* DCIS cells are still in a bipotent progenitor state, and *in vivo* they differentiate into the myoepithelial and luminal cells necessary for DCIS development. Thanks to the development of this model, researchers are now able to actively study the genetic and environmental effects on DCIS progression.

Initial studies using this model were used to elucidate the potential role of the myoepithelial cell layer and fibroblasts in DCIS progression. Using subcutaneous injections, Hu et al. (2008) verified the bipotent activity of the DCIS cell line and development of DCIS lesions with intact basement membranes and myoepithelial cell layers, which other human breast cancer cell lines were unable to do. Using immunohistochemical analysis and flow cytometry, the bipotent progenitor capabilities of the DCIS cell line were verified. *In vitro*, DCIS cells are uniformly positive for both basal and luminal markers, and only upon injection do they differentiation into separate luminal and myoepithelial cells. Additionally, microarray analysis confirmed that genetic alterations in DCIS xenografts mimicked genetic alterations commonly found in human breast carcinomas, such as downregulation of cell cycle regulator p16 (CDKN2A). SAGE and microarray analysis showed that the myoepithelial layer in DCIS xenografts and human DCIS lesions was largely responsible for maintenance of the extracellular matrix (ECM), cytoskeleton, and basement membrane remodeling. Further analysis of matrix metalloproteinases (MMPs), which have been highly indicated in degradation of the basement membrane and promotion of invasion, found that MMP14 was highly upregulated in DCIS associated myoepithelial cells. Co-injection of DCIS cells with fibroblasts resulted in invasive ductal carcinoma rather than the previously described DCIS lesions, but this could be reversed by the addition of normal myoepithelial cells. Importantly, none of these differences induced

genetics changes in the epithelial cells of DCIS xenografts, indicating a paracrine effect of fibroblasts on the bipotent progenitor potential of DCIS cells. This study elegantly elucidated a potential role for tumor progression that explains the lack of genetic changes with the onset of invasive disease.

More recently, a new technique, the mouse intraductal (MIND) injection has been developed to better mimic the natural environment of human DCIS *in vivo* (Behbod et al., 2009). In addition to the DCIS.com cell lines, a second DCIS-like SUM225 cell line and primary human DCIS cells can be used in this model. Using immunosuppressed mice, these cells were injected into the inguinal mammary gland through the nipple. This results in a DCIS lesion within the actual ductal structure of the mouse mammary gland, enhancing the similarities to a human mammary gland DCIS lesion. Interestingly, in this study, only the DCIS.com cells became invasive, the SUM225 cell line and primary human samples did not. In the intraductal model, the previously described comedo style DCIS of the DCIS.com cells was replaced by a cribiform pattern, where the epithelial cells have not completely filled the ductal region. In this model, the myoepithelial and basement membrane layers are developed by the mouse mammary gland, whereas the epithelial cells are from the human cell lines, indicating that the bipotent potential of the DCIS.com cell lines is altered in this model. This modeling experiment has also been used with human primary DCIS to continually grow human primary DCIS samples through repeated intraductal injection. Using this model, Valdez et al. (2011) were able to grow human DCIS cells within mouse mammary ducts that maintained that characteristics of the original DCIS pathology. This allows for long-term study of DCIS lesions in an *in vivo* situation with lower risks of genetic alteration.

The establishment of these unique models for DCIS progression has significantly enhanced our ability to study early onset and progression of breast cancer. These models will be

critical for identification of markers for progression, and identification of novel and effect treatments that prevent the development of invasive disease.

Breast Cancer Differentiation

A major indicator of overall tumor aggressiveness and an important prognostic indicator is the differentiation status of the tumor. Understanding differentiation and the identification of genes regulating differentiation are major themes in cellular biology. While the discussion of differentiation permeates the study of breast cancer, understanding the definition and important of differentiation in breast cancer progression is vital.

In the normal body, differentiation is identified as the process by which a less specialized cell (ie. stem cell) becomes a more specialized cell with a specific function (ie., neuron). Thus, differentiation is vital for human growth and development. The mammary gland is unique in that it goes through repeating rounds of differentiation (specialization) and de-differentiation with menstrual cycling, and most importantly with pregnancy and lactation. We have already discussed the various levels of differentiation that occur within the mammary gland during normal development, and also how it diverges at various points of this pathway resulting in unique subtypes or breast cancer. The mammary gland reaches its most differentiated state, named terminal differentiation, shortly after parturition, at the peak of lactation. During this state the cells are highly metabolically active but do not proliferate. After weaning, most of these terminally differentiated cells are unable to de-differentiate to the mammary glands pre-pregnancy state, and therefore, they undergo apoptosis. These terminally differentiated cells are also highly prone to cell death, and are more susceptible to outside insults such as chemotherapeutics. Throughout the cycles of differentiation within the mammary gland, it must always maintain the ability to form a subsequent, functional gland for later pregnancies, which is

hypothesized to occur through the regulation of mammary stem cells (Visvader, 2009). While regulation of this process is highly controlled in the normal gland, the unique ability of this organ to cycle in its differentiation status makes tumor differentiation of the utmost importance in breast cancer.

In breast cancer, tumors are often graded according to their state of differentiation, or de-differentiation – in accordance with how closely they resemble the normal tissue of the mammary gland (NCI). The more de-differentiated a breast tumor is, or the less it resembles the mammary gland, the worse the prognosis. Poorly differentiated tumors are often highly invasive and metastatic, in addition to being highly proliferative (Bloom and Richardson, 1957; Contesso et al., 1987; Liu et al., 2007). Indeed, the parameters used for breast cancer grading are directly related to the differentiation characteristics of the mammary gland (Jogi et al., 2012). Tumor grading in breast cancer is often based on several parameters – including tubule formation and nuclear pleomorphisms, both of which are directly related to tumor differentiation. Tubule formation is indicative of the cells' ability to maintain polarity, whereas large nuclei and variations in nuclear size are markers for low differentiation. Additionally, in ductal carcinoma *in situ*, the development of hypoxic regions within lesions correlates with a high localization of de-differentiated cells and progression to invasive breast cancer, and promotes the hypothesis that hypoxia, and specifically HIF1α, plays an important role in loss of differentiation.

The importance of differentiation in breast cancer progression and aggressiveness has stimulated research on identification of critical pathways of differentiation that are altered with the onset of cancer. Several transcription factors have been implicated, including GATA3, p53, HIF1α, and the previously mentioned STAT5a (Kouros-Mehr et al., 2008; Kouros-Mehr et al., 2006).

GATA3 in Mammary Gland and Breast Cancer Differentiation

GATA3 was first identified as a key transcription factor that is highly expressed in the luminal epithelial cells of the mature mammary gland (Kouros-Mehr et al., 2006). GATA3 is expressed in all luminal cells of the mammary gland, but not in the capsule of the TEB. Loss of GATA3 in the mouse mammary gland severely disrupted normal development. While embryonic and pre-pubertal development was similar to WT littermates, at the onset of puberty, the GATA3 null glands failed to develop beyond the pre-pubertal stage, and these glands exhibited defects in side branching and structural defects. GATA3 null luminal epithelial cells exhibited a loss of cellular polarity, identified by multiple layers of epithelial cells within ductal structures. Using a doxycycline-inducible system, Kouros-Mehr et al. (2006) allowed mice to read adulthood before targeting GATA3 in the mammary gland. Even after puberty, loss of GATA3 induced a drastic loss in ductal structure, and a loss of polarity in luminal epithelial cells. Loss of GATA3 induced loss of luminal markers β-casein (CSN2) and CDH1, and also increased proliferation within the mammary gland, showing that GATA3 expression as critical for both the development and maintenance of the differentiated mammary gland (Kouros-Mehr et al., 2006).

Additional work in mammary cancer showed that loss of GATA3 in breast cancer promoted tumor growth and metastasis (Kouros-Mehr et al., 2008). Using the Mouse Mammary Tumor Virus Polyoma Middle-T (MMTV-PyMT) tumor system, GATA3 was shown to be down-regulated in breast tumors and was lost during tumor de-differentiation. Tumors that had metastasized to distant organs were consistently devoid of GATA3 expression. By examining a panel of breast cancer cell lines ranging from less aggressive, luminal to highly metastatic, basal types, Kouros et al. (2008) verified that less aggressive cell lines have detectable amounts of GATA3, which is progressively lost in more aggressive cell lines. Re-establishment of GATA3

in PyMT tumors significantly inhibited metastasis and promoted differentiation. Expression of GATA3 in PyMT tumors also induced CSN2 expression, indicative of terminal differentiation, and the tumors themselves formed ductal structures rather than disorganized tumors. Although tumor sizes were larger in GATA3 expressing tumors, this was due to the formation of cysts full of secretory material from the tumor rather than enhanced proliferation. These tumors had a 27 fold decrease in lung metastasis compared to controls. Interestingly, loss of GATA3 in early stage mammary carcinomas was not sufficient to induce tumor progression, since loss of GATA3 induced apoptosis in GATA3 positive cells, and did not completely eliminate GATA3 expression in subsequent tumors. Further analysis indicated that tumor progression occurred through the clonal expansion of the GATA3 null population of cells in the tumor, eventually overtaking the GATA3 positive population and conferring stem-like, basal characteristics on the tumor. These studies show a vital role for GATA3 expression in the promotion of differentiation, and its potential as a diagnostic indicator in breast cancer.

Singleminded

Originally discovered in *Drosophila*, d*sim* was found to be a key regulator of CNS midline differentiation and cell fate (Crews et al., 1992; Kasai et al., 1992; Lewis and Crews, 1994; Nambu et al., 1990; Nambu et al., 1991). It was found that d*sim* knockdown in embryos was fatal due to a lack of differentiation, and the d*sim* expression was sufficient and necessary for CNS development. These studies also examined the collaboration of *dsim* with tango (*dtgo*, homologous to mammalian ARNT) for association with DNA response elements and proper formation of the CNS (Pielage et al., 2002). This research concluded that *dsim* was a master regulator of neurogenesis (Nambu et al., 1990). Two mammalian homologs of *dsim* have been identified: Singleminded 1 (SIM1) and Singleminded 2 (SIM2) (Chrast et al., 1997). SIM1 is

located on chromosome 6, whereas SIM2 is located on the Down Syndrome (DS) critical region of chromosome 21 and is believed to contribute to many of the physiological abnormalities associated with trisomy 21 (Chrast et al., 2000). Sim2 plays an important role in development as *Sim2* null mice die shortly after birth due to multiple abnormalities including cleft palate, improper diaphragm development and rib defects (Goshu et al., 2002; Shamblott et al., 2002).

SIM2 is a member of the basic helix-loop-helix (bHLH)/Per-Arn-Sim (PAS) transcription factor. bHLH describes the basic structural motif of the transcription factor, and PAS describes the unique protein domain within the protein that functions as a signal sensor influencing the transcription of target genes (Ponting and Aravind, 1997). The bHLH superfamily contains transcriptional regulators that function in multiple fundamental biological processes. The basic bHLH region is involved in DNA binding and the helix-loop-helix motif is a dimerization domain. Both are necessary for the formation of functional DNA binding complexes required for transcription. bHLH proteins can be divided into three subfamilies: those with the bHLH domain only; those with a bHLH domain contiguous with a leucine zipper (Zip); and those with a bHLH domain contiguous with a Per-Arnt-Sim (PAS) domain (Kewley et al., 2004). The bHLH/PAS family of transcription factors form heterodimers that recognize sequences such as the xenobiotic response elements (XRE), the hypoxic response elements (HRE), and central midline elements (CME) (Kewley et al., 2004). The PAS domain, which consists of two adjacent repeats of approximately 130 amino acids, is important in regulating dimerization specificity (Kewley et al., 2004). Activation of signaling occurs when one bHLH/PAS protein dimerizes with another bHLH/PAS protein, namely ARNT. DNA binding and regulation of gene expression occurs through transcription machinery recruitment, elongation control, or transcriptional repression (Kewley et al., 2004).

SIM2 in Down Syndrome

DS is a genetic condition characterized by an extra chromosome 21 (also known as Trisomy 21). This extra chromosome, resulting in an overexpression of genes on this chromosome, results in complications in body and brain development. Some common physical symptoms include: a flat face with an upward slant to the eye, small hands and feet, poor muscle tone, and white spots on the iris of the eye. In addition to the classical mental and physical symptoms, people with DS are also prone to a unique subset of health problems, most notably, increased susceptibility to childhood leukemias and germ-line cancers, but a greater than 50% decreased development of solid tumors including cancers of the breast, lung, and other epithelial cancers (Boker et al., 2001; Boker and Merrick, 2002; Hasle et al., 2000a; Hasle et al., 2000b; Hasle et al., 2000c; Hill et al., 2003; Satge et al., 1998). Children with DS are 500 times more likely to develop acute leukemias, and a majority of adults with DS develop Alzheimer's or other neurodegenerative diseases (Seewald et al., 2012; Xavier et al., 2009). DS individuals often develop amyloid plaques as early as their 40s, and up to 75% of people with DS develop dementia. Research has shown that extra copies of amyloid precursor protein (APP), located on chromosome 21, are associated with increased Alzheimer's in DS individuals (Zigman and Lott, 2007; Zigman et al., 1996). Interestingly, long-term studies analyzing cancer in DS populations has shown that people with DS have a lower occurrence of solid tumors, especially breast cancer (Hasle et al., 2000c; Xavier et al., 2009). A substantial population of individuals with DS were followed through a national cancer registry to examine malignancy rates in a DS population compared to a matched normal population (Hasle et al., 2000c). In a population of approximately 2814 DS individuals, 60 cases of cancer were seen, which is significantly higher than the cases in a normal population; however, 60% of these cases were leukemia. Out of the 60 cases, only 24 were solid tumors, with 48 expected. Even more striking is the observation that

women with DS are 10-25 times less likely to develop breast cancer compared to age-matched normal populations (Benard et al., 2005; Hasle et al., 2000a; Hasle et al., 2000b; Hasle et al., 2000c; Hill et al., 2003; Satge et al., 1998). The unique relationship between DS and tumorigenesis has led to extensive research of the DS Critical Region (DSCR) of chromosome 21 for potential tumor suppressors and oncogenes that would attribute to this unique tumor phenotype. Studies have established a unique paradox where the biological features of DS are also general characteristics of cancer-prone individuals (Nizetic and Groet, 2012). Chromosome instability, increased DNA damage and defective DNA repair, immunodeficiency, and the presence of oncogenes on the DSCR are all features of DS that increase specific cancer susceptibility. However, the presence of tumor suppressor genes and anti-angiogenic genes in the DSCR lead to the conclusion by Nižetić and Groet (2012) that the DS phenotype is more susceptible to cancers that do not depend on angiogenesis, such as leukemia. These conclusions are insufficient; however, to explain epidemiological findings that some solid tumors do occur in DS populations, indicating that there are complex, tissue specific effects of the genetic makeup of DS patients.

SIM2 expression during fetal development in the CNS as well as the facial, skull, and vertebra primordial indicate that the expression of *SIM2* could be involved in the phenotypes associated with DS (Dahmane et al., 1995). Indeed, the spatial and temporal location of SIM2 expression in the brain during development corresponds to the regions affected by DS (Rachidi et al., 2005). The short isoform of SIM2 (SIM2s) was discovered in 1997 in the study of the DSCR of chromosome 21 (Chrast et al., 1997). Using exon trapping, Chrast et al (1997) and associated discovered the additional isoform of SIM2 that was 10kD shorter than the long isoform, and one repressive Proline/Alanine- rich region was deleted (Figure 3).

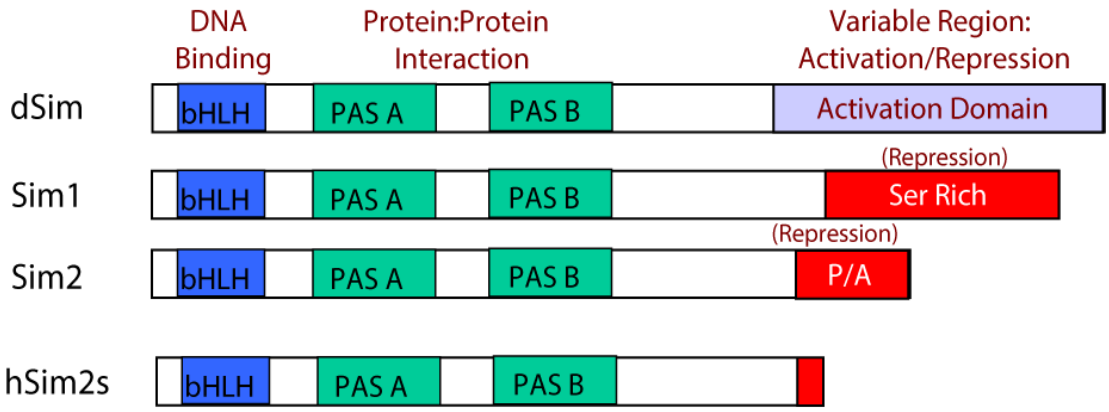

Figure 3. The Mammalian Single-minded Protein. Schematic drawing of human and mouse Sim2 proteins with conserved and variable domains. Adapted from Chrast et al. (1997).

Chrast et al (1997) also showed high similarity between *dsim*, mouse *Sim1* and *Sim2*, and human *SIM1* and *SIM2*. To further elucidate the potential role of *SIM2* in DS, Ema et al. (1999) overexpressed *mSim2* in the hippocampus and amygdala of mice. They found that while these animals were viable and fertile, they had defects in fear conditioning and spatial learning and memory, indicating that the overdosage of *Sim2* may be important for the mental retardation seen in DS (Ema et al., 1999). In a similar study, Chrast et al. (2000) developed an artificial bacterial chromosome overexpressing *Sim2* with only one or two additional copies. Similar to previous experiments, these mice developed normally with no histological abnormalities. However, *Sim2* transgenic mice exhibited reduced exploratory behavior and enhanced anxiety, as well as reduced social interactions and increased pain tolerance. These studies provide strong evidence for the role of SIM2 in the etiology of DS. More recently, it was shown that Sim2 protein levels are upregulated in the cortex of Tc1 DS mouse models (Spellman et al., 2013). Using DS-derived lymphoblastoid cell lines and cortex material from two DS transgenic mouse models, Spellman et al. (2013) analyzed protein levels for 20 genes in the DSCR of chromosome

21, including Sim2. Out of the 20 genes analyzed, *Sim2* is one of only two transcription regulators that were elevated in trisomy in both transgenic mouse models analyzed. In addition to baseline level analysis of SIM2 in DS, analysis of single nucleotide polymorphisms (SNPs) in the SIM2 gene have also been associated with DS (Chatterjee et al., 2013; Chatterjee et al., 2011). These investigators examined two coding, nonsynonymous SNPs in *SIM2* in DS populations compared to their parents, as well as ethnically matched controls. These SNPs were selected using FastSNP analysis due to their potential deleterious role in *SIM2* functioning. The first SNP called rs2073601 (C/A), showed significant upregulation in DS populations compared to parents and control groups; however, the second SNP (rs2073416 A/G) showed no significant changes. These studies established that rs2073601 SNP on the *SIM2* gene occurs at a high frequency in DS populations, and may lead to an alteration in protein function, thus contributing to the DS phenotype. Further *in silico* analysis was performed to predict pathways regulated by SIM2, including pathways involved in nervous system development, signal transduction, and induction of apoptosis. These studies provide strong evidence for a causal role of SIM2 in the DS etiology.

Characterization of SIM2

Human orthologues or *SIM2* are highly conserved with the murine and drosophila homologues, however, there are differences in the carboxyl terminus, where *dSim* acts as a transcriptional activator and murine Sims functions as a transcriptional repressor (Ema et al., 1996; Probst et al., 1997). While human *SIM2* mimics the murine *Sim2* and is thought to be a transcriptional repressor, the loss of proline/alanine rich domains in the short isoform (SIM2s) indicates the potential for SIM2s to operate either as a repressor or activator (Chrast et al., 1997; Moffett et al., 1997). Moffett et al. (1997) studied the transcriptional activity of murine Sim2 to determine its similarities to *dSim*. Using a series of structural deletions, Sim2 was determined to

heterodimerize with Arnt through its bHLH-PAS region, similar to other Arnt partners, Ahr and Hif1α. Further immunoprecipitation analysis (IP) indicated that unlike its relative genes Per and Arnt, Sim2 does not form homodimers. Further analysis of Sim2 repression domains using deletion mutations identified a trans-repression element within the carboxyl terminus. By separating the C terminus into three unique regions based on the amino acid enrichment (serine-threonine, proline-serine, and proline-alanine), Moffett et al. (1997) determined the presence of two separable repression domains within the carboxyl terminus. It is interesting to note that in the earlier study by Chrast et al. (1997) that found SIM2s, part of the C-terminus is lost in the short isoform, enhancing the potential for both repression and activation (Chrast et al., 1997). Using a mammalian two-hybrid system, Moffett et al. (1997) determined that Sim2 dominantly represses Arnt mediated transactivation, but not DNA binding. Additionally, the transactivation of Hif1α through heterodimerization with Arnt is a well-established hypoxic response in cells. By inducing a hypoxic environment with Hif1α, Arnt, and Sim2, researchers were able to show that Sim2 interferes with Hif1α/Arnt transactivation by competitively dimerizing with Arnt. Competitive interference with Hif1α indicates the potential for Sim2 to inhibit hypoxic and dioxin-induced gene expression. Further work by Moffett et al. (2000) was done to evaluate the transcriptional properties of murine Sim1 and Sim2 . Using reporter assays, Sim1 and Sim2 were found to bind to central midline elements (CME) in promoter regions, and this activity was dependent on the inclusion of Arnt. Interestingly, while Sim1/Arnt activates gene expression, Sim2/Arnt does not unless the carboxy-terminus is deleted. Additionally, Sim2 expression was shown to dominantly sequester Arnt and block Sim1 mediate transactivation. These data indicate a unique mechanism for Sim2 as a transcriptional repressor by blocking the availability of Arnt to other transcription factors.

Further analysis of SIM2 transcriptional activity has been reported by Woods et al. (2002). Based on the role of SIM2 in competing with HIF1α for ARNT, Woods further examined the potential role of SIM2 expression during hypoxia (Woods and Whitelaw, 2002). Analysis of SIM cellular localization defined SIM proteins as mammalian class I bHLH/PAS proteins, meaning they are constitutively nuclear. The key nucleotides of the CME, which bind SIM2, are also present in the hypoxic response element (HRE). Using luciferase reporters, SIM1/ARNT complexes enhanced HRE transactivation, whereas SIM2/ARNT complexes did not. However, the presence of SIM2 ablated HIF1α/ARNT mediated transcriptional activation of an HRE-regulated reporter gene. Using mutational analysis, chromatin immunoprecipitation (ChIP), Woods et al. (2002) discovered that this repression was not just from competitive dimerization with ARNT, but also with direct binding to the HRE sequence. Indeed, SIM2 can repress the hypoxic activity of HIF1α by competitively dimerizing with ARNT and binding the HRE. However, prolonged hypoxic treatment decreased SIM2 protein levels while increasing HIF1α protein, indicating an inverse relationship between HIF1α and SIM2 expression as needed in a hypoxic environment. This has led to the theory of a "hypoxic switch" in cells where SIM1/2 are coexpressed with HIF1α. These data indicate a potentially different activation and repression activity for SIM2 based on specific cis-element binding.

Additional characterization of SIM2 discovered that SIM2 is polyubiquitinated though interaction with E3 ubiquitin ligases (Okui et al., 2005). SIM2 was found to have a PEST motif, a sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T), using computer analysis. PEST motifs are typically found in short-lived proteins such as transcription factors and cell cycle regulators that are controlled by proteolysis. Initial attempts to examine SIM2 protein expression presented problems in finding the appropriate band size via western blot, which could be overcome with proteasome inhibition, thus, these problems are likely due to the

rapid ubiquitination of the protein. Using co-transfection experiment with SIM2 and various ubiquitin mutants, Okui e al. (2005) verified the ubiquitination of SIM2 protein on lysine residues. Further mutation of SIM2 showed that ubiquitination occurred on multiple lysine residues between the PAS1 and PAS2 domains. The potential SIM2 interaction with E3 ubiquitin ligases was analyzed using co-expression of a selection of ligases (Parkin, HHARI, Siah1, and AIRE) with SIM2, followed by IP analysis. Results showed that Parkin and HHARI interact with SIM2, whereas Siah-1 and AIRE did not. This binding is due to unique IBR (in-between ring) and RING2 (really interacting new gene) domains on Parkin. This interaction has many implication for brain development, as Parkin is ubiquitous in the brain at all stages of development, whereas Sim2 is not, and Parkin has been strongly implicated in the etiology of Parkinson's disease and mitochondrial function (Kitada et al., 1998; Pilsl and Winklhofer, 2012).

Previous work in our laboratory has elucidated the unique transcriptional characteristics of Sim2s compared to its long isoform, Sim2 (Metz et al., 2006). Using 3' RACE analysis in mouse kidney and liver to identify the mouse homologue of *SIM2s*, Metz et al. (2006) showed that the unique short isoform that was previously detected in human tissues is also present in the mouse, again lacking the Pro-Ala rich repressor region present in full-length Sim2. Tissue analysis of full-length and short Sim2 showed that both isoforms were expressed at high levels in kidney and skeletal muscle; however the ratios between the two differ indicating that their expression is tissue-specific. Using the two-hybrid liquid culture assay and co immunoprecipitation (CoIP), our laboratory verified that Sim2s can also heterodimerize with Arnt and Arnt2, similar to Sim2, and that Sim2s preferentially binds Arnt over Arnt2. Luciferase assays showed that Sim2s significantly repressed hypoxia-induced reporter gene expression, and that this phenotype could be potentially overcome with addition of Arnt, but not Arnt2. These assays also indicated that Sim2s mediated repression was not by competition with Hif1α for Arnt

binding, but rather direct interaction with the hypoxic response element (HRE). Using similar methods, Metz et al. (2006) also verified that *Sim2s* expression significantly represses dioxin-induced gene expression and was attenuated by increasing amounts of Arnt, indicating that this repression is caused by competition for Arnt binding. Since Sim2s is missing one of the repressor domains present in full length Sim2, analysis of the previously described Sim2 repressor activity on the central midline element (CME) need to be analyzed to determine if Sim2s had less repressive activity (Moffett and Pelletier, 2000). Luciferase studies on CME-controlled gene expression showed that Sim2s significantly increased reporter gene expression when expressed with Arnt. This transcriptional activity was mediated by the transactivation domain of Arnt, as mutation of this domain ablated CME-controlled gene expression. Through this work, it was determined that Sim2s has differential effects on CME and HRE-controlled gene expression compared to full length Sim2. Sim2s can also be both a transcriptional activator and repressor that depends on the cis-element and Arnt heterodimerization, adding a level of complexity and fine tuning to Sim2s mediated gene expression.

Sim2s in the Normal Mammary Gland

Previous work in our laboratory has examined the role of *Sim2s* in mouse mammary gland development. In normal mouse mammary gland development, *Sim2s* expression is developmentally regulated. In early mammary gland development, branching morphogenesis is a tightly regulated proliferative process, guiding the development of the basal ductal tree before the onset of puberty (Sympson et al., 1994). During development of the ductal tree, EMTs play a vital role in regulating migration and tissue development. A concert of proliferation, apoptosis, and differentiation are vital for the final structure of the ductal tree: a hollow lumen with luminal epithelial cells, mesenchymal cells, and stroma. Initial analysis by our laboratory in early development showed that Sim2s is the primary Sim2 isoform present in the early virgin

mammary gland (Laffin et al., 2008). Additionally, Sim2s expression was detectible in the ductal epithelial glands, indicating a potential role in mammary gland development. Using *Sim2*$^{-/-}$ mammary bud transplant models, the mammary buds were transplanted from embryonic lethal *Sim2*$^{-/-}$ mice and transplanted into the cleared fat pad of nude mice. At 8 weeks of age, the mammary glands from these mice were harvested and analyzed. Whole mount analysis showed an increase in alveolar budding. Significantly, H&E analysis of *Sim2*$^{-/-}$ and WT glands revealed that *Sim2*$^{-/-}$ glands failed to hollow appropriate, and the alveolar buds seen under whole mount analysis were disorganized epithelial cells reminiscent of hyperplasia. Mason's trichrome immunohistochemistry showed that *Sim2*$^{-/-}$ gland had less collagen and disrupted basement membranes. Indeed, examples of invasion through the stroma into the surrounding fat pad are seen in *Sim2*$^{-/-}$ glands and were also associated with an increase in the expression of proliferative marker Ki67 and a decrease in TUNEL apoptotic staining. Staining for cell polarity marker Aquaporin 5 showed a loss of polarity in *Sim2*$^{-/-}$ glands, which is a hallmark of tumorigenesis (Ellenbroek et al., 2012). These studies show that loss of *Sim2* has a significant impact on normal mammary epithelial organization. Further analysis of EMT markers in the *Sim2*$^{-/-}$ glands showed a decrease in CDH1 staining with an increase in Mmp2 and β-catenin protein levels. Additionally we saw a significant increase in EMT regulator Slug in the *Sim2*$^{-/-}$ mammary glands.

While detected in early development, Sim2s levels remain low in virgin and early pregnant glands, which correlate well with Sim2s' hypothesized role in differentiation. *Sim2s* expression increases during late pregnancy and peaks during mid-lactation when the gland is terminally differentiated and metabolically active (Wellberg et al., 2010). Sim2s expression in the mammary gland is specific to the nuclei of luminal alveolar mammary epithelial cells as previously mentioned. The correlation between Sim2s expression and lactation led to our

hypothesis that Sim2s regulates this process in the mammary gland, promoting the onset of lactation and terminal differentiation. Over expression of *Sim2s* using a Mouse Mammary Tumor Virus (MMTV) long terminal repeat (LTR) promoter found that virgin mice undergo precocious lactogenic differentiation. While the ductal structure of the virgin mouse mammary was indistinguishable between wild type and MMTV-*Sim2s* mice, immunohistochemical and Q-PCR analysis showed the epithelial cells of the transgenic mice expressed milk proteins such as whey acidic protein (WAP),Csn2, and solute carrier Npt2b (a polarity marker that is upregulated with the onset of lactation) – which are hallmarks of lactation. This was believed to be a partial induction of alveolar differentiation, as the polarity marks Aqp5, which is normally lost during lactation, remained expressed in MMTV-*Sim2s*HA mammary glands. Indeed, when using the normal mouse mammary cell lines HC11 and CIT3, *Sim2s* expression was upregulated in response to prolactin-induced differentiation. HC11 and CIT3 cells are unique mouse mammary epithelial cell lines derived from the COMMA-D cell line that can undergo lactogenic differentiation with exposure to hydrocortisone and prolactin (Ball et al., 1988; Kabotyanski et al., 2009). Stable lentiviral transduction inducing *Sim2s* expression in these cells enhances their response to prolactin, significantly increasing Csn2 expression. Analysis of *Sim2s* levels in undifferentiated and differentiated cells showed that *Sim2s* levels increased within 24 hours of hormone stimulation and continued to elevate through day 4. Lentiviral transduction of these cell lines was performed to overexpress *Sim2s*, to establish whether enhanced *Sim2s* expression played a role in prolactin induced differentiation. *Sim2s* enhancement in HC11 and CIT3 cells significantly upregulated *Csn2* expression following hormone induction. Additionally, shRNA transduction targeting *Sim2s* in these cells reduced Csn2 expression after prolactin exposure by 50%. While not completely abrogated, this indicated a role for *Sim2s* in the robust hormone-mediated induction of Csn2 and differentiation. ChIP analysis of confirmed transcriptional

regulators of lactogenic differentiation showed a significant enhancement of recruitment to the *Csn2* promoter in *Sim2s* overexpressing cells. Both Stat5a and RNA Polymerase II (RNAPII) binding were enhanced in *Sim2s* expressing cells, and they presented a more rapid response to hormone induction. Additionally, further ChIP analysis confirmed that Sim2s directly binds and regulates the expression of Csn2 milk protein through elongation control and recruitment of transcriptional machinery. Sim2s re-ChIP analysis showed that Sim2s is associated with RNAPII on Csn2 but not on negative control β-actin, showing this is not a general transcriptional effect but is gene specific. These studies confirmed that Sim2s expression is necessary and sufficient to induce lactogenic differentiation.

One of the most unique aspects of the mammary gland is its ability to undergo numerous rounds of differentiation, lactation, and de-differentiation in response to the hormone signals during estrous cycling, pregnancy, and parturition. These pathways are classically highjacked during breast tumorigenesis, thus understanding their normal operation will help us elucidate the mechanisms by which they are hijacked during breast cancer. We hypothesize that Sim2s plays an integral role in normally mammary gland differentiation cycling, and thus is potentially a key pathway that must be loss for tumors to occur.

SIM2s in Cancer

In addition to the role of Sim2s in normal development and mammary gland development, SIM2s has also been implicated in multiple cancers. Interestingly, SIM2s has been described as both an oncogene and tumor suppressor depending on the organ and type of cancer. SIM2s expression was observed in colon, prostate, and pancreatic cancers, but not in breast, lung or ovarian tumors (DeYoung et al., 2003b).

In the search for tumor-critical genes on the DSCR region of chromosome 21 using large scale bioinformatics, DeYoung et al (2003) first pin-pointed *SIM2s* as a potential drug target for

solid tumors. Using RT-PCR analysis, *SIM2s* expression was detected in colon, pancreas, and prostate tumors, but not in the corresponding normal tissues. In adult tissues, *SIM2s* expression was observed only in kidney and tonsil derived samples. In fetal tissues, however, *SIM2s* was detected in the heart, kidney, and skeletal muscle. In colon tumors, *SIM2s* was found to be activated at an early stage, and was associated with increased tumor progression. These studies indicated a unique tumor phenotype with tissue specific *SIM2s* expression.

SIM2s in Breast Cancer

Our laboratory has established differential expression of SIM2s in normal and human breast cancer samples (Kwak et al., 2007). *SIM2s* expression was highest in the normal-like MCF10A and 16N breast cell lines, and was significantly decreased in breast cancer cell lines. Additionally, the expression of *SIM2s* in these cell lines decreased with the relative aggressiveness of the cell line. Luminal cancer cell lines MCF7 and T47D had moderate levels of *SIM2s*, whereas *SIM2s* was not detected in the highly metastatic MDA.MB.231 cells. This was confirmed in human primary samples using immunohistochemistry. SIM2s expression was strong and nucleic in normal mammary gland epithelial cells, and lost in primary breast tumors. Preliminary studies in the highly aggressive MDA.MB.435 breast cancer cell line showed that stable lentiviral transduction re-establishing *SIM2s* expression significantly decreased proliferation and colony formation in soft agar. Additionally, *SIM2s* expression significantly inhibited the invasive potential of MDA.MB.435 cells through inhibition of MMP3, which is known to play a role in epithelial mesenchymal transition (EMT) and breast cancer progression (Ioachim et al., 1998; La Rocca et al., 2004). ChIP and luciferase analysis of SIM2s showed that SIM2s binds the promoter of MMP3 and represses transcriptional activity.

Luminal-like MCF7 breast cancer cells express moderate levels of *SIM2s*, and loss of *SIM2s* (*SIM2si*) in these cells by RNA interference (RNAi) induced an EMT (Laffin et al.,

2008). These cells underwent a transition from the cobblestone, epithelial characteristics of the parent MCF7 cell line to a spindle-like mesenchymal morphology. Analysis of key epithelial and mesenchymal markers showed a decrease in epithelial markers CDH1 and CK18 and an increase in mesenchymal markers N-cadherin (NCad), Vimentin (VIM), and β-catenin (CTNNB1). Indeed, analysis of key EMT regulators showed a significant up-regulation of SLUG with loss of *SIM2s* (Come et al., 2006; Martinez-Estrada et al., 2006; Peinado et al., 2004; Peinado et al., 2007). To determine the tumorigenicity of these cells, they were injected into the flank of athymic nude mice. *SIM2si* xenografts grew rapidly and by day 10 were 3 times the size of Scrambled controls. *SIM2si* xenografts also had increased angiogenesis, elucidated by CD31 endothelial cell staining and VEGF. BrdU analysis of xenografts showed a significant increase in proliferation in *SIM2si* tumors. Similarly to the *in vitro* data, *SIM2si* xenografts had decreased CDH1 and increased VIM, indicative of an EMT. MCF7 cells classically require estrogen to sustain growth. However, *SIM2si* xenografts grew well in the absence of estrogen, did not respond to estrogen treatment, and immunohistochemical analysis showed that ERα was undetectable in these tumors. Based on the earlier study by our lab elucidated a mechanism by which SIM2s binds and represses MMP3, analysis of MMP expression and behavior was performed to determine if a similar mechanism was occurring in MCF7 xenografts. These experiments showed a significant increase in *MMP2* expression with loss of *SIM2s* in MCF7 tumors, whereas the aforementioned *MMP3* expression was unaltered. Luciferase assays verified that increasing amounts of *SIM2s* expression inhibited MMP2 activity. It was hypothesized that the difference in MMP regulation between MCF7 and MDA.MB.435 cancer cells is likely due to intrinsic differences between the two cell lines, as it is common for primary tumors and cell lines to have unique molecular signatures. Since *in vitro* and *in vivo* analysis showed significant alterations in EMT related proteins, we hypothesized that SIM2s may regulate these factors.

Key regulators of EMT have been well characterized in the SNAIL family of transcription factors (De Craene et al., 2005a; De Craene et al., 2005b). Analysis of key transcription factors *SNAIL* and *SLUG* showed that *SLUG* expression was significantly increased with loss of *SIM2s*. By expressing a SLUG-controlled luciferase reporter in the MCF7 Scr and *SIM2si* cells, we saw a significant increase in SLUG-controlled gene expression in *SIM2si* cells when compared to controls (Gustafson et al., 2009b; Laffin et al., 2008). ChIP analysis confirmed that SIM2s binds and represses SLUG expression through a promoter associated CME/XRE element. This data, along with the earlier work in normal mammary gland development, elucidated a role for SIM2s expression in the inhibition of an EMT through repression of SLUG and MMPs (Figure 4).

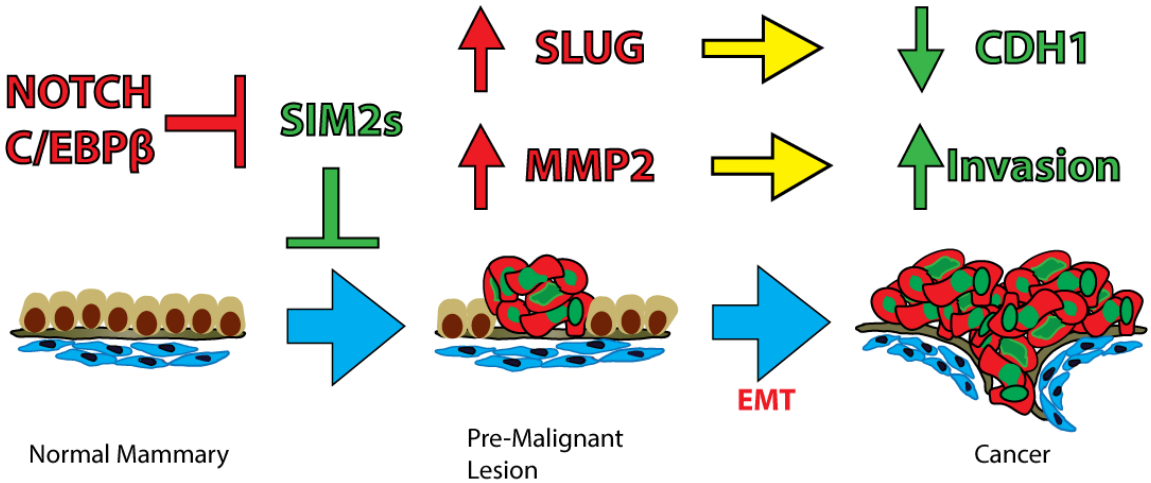

Figure 4. Singleminded-2s Induction of an Epithelial Mesenchymal Transition. During oncogenesis, upregulation of NOTCH and C/EBPbeta and NOTCH suppressive SIM2s expression, which results in upregulation of SLUG and MMP2, and concurrent EMT transition characterized by decreased CDH1 and upregulated invasion (Gustafson et al., 2009b; Laffin et al., 2008).

After characterizing the role of SIM2s in tumor inhibition, our laboratory sought to explain how SIM2s expression was lost in oncogenesis (Gustafson et al., 2009b). The *Ras* proto-oncogene is dis-proportionately activated in approximately 50% of breast cancers, and it's activity correlates with progression and poor prognosis (von Lintig et al., 2000). In breast cancer, Ras overexpression has been shown to target NOTCH signaling and the CCAT/enhance binding protein β (C/EBPβ) transcription factor (Kiaris et al., 2004; Weijzen et al., 2002). To this end, previous work in our laboratory used the Ras oncogene in the MCF10A breast cell line to determine if SIM2s expression is lost through oncogenic induction of tumorigenesis. MCF10A cells express have high levels of *SIM2s* mRNA and are considered a normal breast epithelial cell line (Kwak et al., 2007). Stable overexpression of *Ras* in MCF10A cells was achieved by transfected cells with a lentiviral Ras construct. Upon transfection, *Ras* expression resulted in MCF10A cells that exhibited characteristics of transformation and EMT, including increased invasion, anchorage independent growth, and decreased CDH1. *Ras* induced transformation decreased SIM2s protein and mRNA levels. Ras target NOTCH was activated in MCF10A-Ras cells as well as NOTCH target genes HES1, HEY1, and HEY2. Similarly, stable transduction of the MCF10A cells with a *SIM2s* shRNA (*SIM2si*) resulted in a similar transformation characterized by poor acini growth in three-dimensional culture and increased invasive potential. *SIM2si* MCF10A cells also had increased VIM and decreased CDH1 expression. This led to our hypothesis that *Ras*-mediated tumorigenesis in MCF10A operates through NOTCH signaling, which targets SIM2s. Using a SIM2s-controlled luciferase assay, cells were cotransfected with *SIM2s*-reporter construct and either NOTCH intracellular domain (NICD) or the common NOTCH target C-repeat binding factor 1 (CBF1). Analysis showed that NICD significantly inhibited SIM2s-regulated gene expression while CBF1 did not. Thus, the effect of NOTCH on SIM2s was independent of its usual CBF1 mechanism. ChIP analysis

confirmed that NICD directly bound to the Sim2s promoter in Ras-overexpressing cells, indicating that NOTCH inhibits SIM2s expression to promote tumor progression. In addition to NOTCH, C/EBPβ has also been shown to be an important mediator of *Ras*-induced tumorigenesis (Grimm and Rosen, 2003; Seagroves et al., 1998). Analysis of *Ras*-transformed MCF10A cells showed a significant increase in the truncated dominant negative isoform (LIP), with no changes in other C/EBPβ isoforms. To see if changes in these isoforms were sufficient to alter SIM2s expression, MCF10A cells were stable transduced with all 3 isoforms (LAP1, LAP2, and LIP). Western blot analysis revealed that the aforementioned LIP was the only isoform to reduced SIM2s protein levels. Previous studies of C/EBPβ in the mouse mammary gland have shown that loss of C/EBPβ impairs ductal outgrowth and differentiation (Seagroves et al., 1998). Analysis of C/*EBPβ*$^{-/-}$ glands for *Sim2s* showed a significant increase in *Sim2s* expression. These data show roles for NOTCH and C/EBPβ as inhibitors of SIM2s in both normal mammary gland development and oncogenesis. While *Ras*-mediated oncogenesis is complex and activates many pathways, it is of interest that both these pathways mediate *SIM2s*, as they have differing molecular signatures that vary with tumor sites (Gomis et al., 2006; Sundaram, 2005).

SIM2s in Other Cancers

Additional work by DeYoung et al. (2003) determined whether SIM2s would make a viable drug target in colon cancer cells using a *SIM2s* antisense RNA chimera . Treatment of colon cancer cells resulted in a rapid inhibition of proliferation and induction of apoptosis, whereas, treatment of MDA.MB.231 breast cancer cells had no effect on proliferation or invasion. This work established a unique, tissue specific role for SIM2s expression and gene regulation. In later studies, the effect of SIM2s on p53 and caspase dependent apoptosis was determined in colon cancer (Aleman et al., 2005). Treatment of SIM2s expression colon cancer

cells with a *SIM2s* antisense RNA chimera induced tumor-specific apoptosis, since colon tissue does not express SIM2s. This apoptotic phenotype was reverse by general caspases and specific caspase 9 and 10 inhibitors, but not caspase 2 or 8 inhibitors. Protection from cell death was also seen with MAPK inhibition and p53 inactivation in these tumor cells. The induction of apoptosis with *SIM2s* antisense was due to increased GADD family expression in concordant with p38 MAPK activation; however, this did not correlate with an increase in cell cycle inhibition or senescence. Antisense treated cancer cells saw an induction in colon terminal differentiation markers such as mucins and alkaline phosphatase (ALP). While the role of SIM2s in colon cancer is the inverse of breast cancer cells, it is interesting to note that the pathways are similar, specifically the modulation of differentiation and apoptosis. A potential role for SIM2s in pancreatic cancer has also been observed (DeYoung et al., 2003a). Using microarrays and RT-PCR, pancreatic tumors expressed higher levels of SIM2s than normal tissues. Targeting SIM2s in pancreatic tumor cells significantly inhibited growth in a dose dependent manner, and also induced apoptosis. Since *SIM2s* expression is absent in healthy colon and pancreatic tissues, DeYoung and colleagues showed that targeting SIM2s in these tumors allowed for a tumor specific target that did not affect surrounding healthy tissue.

More recently, using a similar microarray approach, Caldas et al. (2012) also identified SIM2s as a potential regulator of malignant pleural mesothelioma . Using a genome-wide measurement of gene expression levels, *SIM2s* was significantly under-expressed in pleural mesothelioma. *SIM2s* expression was also under-expressed compared to control pleural tissue, along with a corresponding increase in *SNAI2* (SLUG) expression, consistent with our previous work in the breast. This was a novel association of SIM2s with mesothelioma, and a second example of the role of SIM2s as a potential tumor suppressor. Recent studies have also associated *SIM2s* expression in glioblastoma and invasion (He et al., 2010). SIM2s expression

was not detected in the normal cortex, meningioma, or pituitary gland of the brain at the RNA or protein level. However, *SIM2s* was detected at significant levels in glioma samples and glioblastoma cell lines. The expression of *SIM2s* also increased with the grade of glioma. Knockdown of *SIM2s* in gliomas and glioblastomas by RNAi had no effect on cell proliferation, however, invasion was significantly decreased. *SIM2s* knockdown in these cells increased tissue inhibitor of metalloproteinase 2 (TIMP2) and decreased matrix metalloproteinase 2 (MMP2), which play a role in invasion and metastasis (Mendes et al., 2005). This MMP2 specific relationship has also been observed in our work (Laffin et al., 2008). The inverse relationship between the effect of SIM2s on MMP2 in the breast and glioblastoma is yet another example of the unique tissue specific effects of SIM2s expression.

In addition, a role for SIM2s in prostate cancer has also been observed. High levels of *SIM2s* were observed in prostate tumors compared to normal prostate tissue (DeYoung et al., 2003b). Halvorsen et al. (2007) first identified full length SIM2 in prostate tumors in cDNA microarrays, and Q-PCR confirmed that *SIM2s* was significantly upregulated in malignant samples. Immunohistochemical analysis of SIM2s protein levels in prostate tumors showed a significant correlation with adverse prognostic indicators such as preoperative serum prostate specific antigen (PSA), high histologic grade, and proliferation. Statistical analysis also showed that positive SIM2s expression significantly correlated with reduced survival and reduced time to metastasis. Similarly, Arredouani et al. (2009) used prostate tissue microarrays to identify genes significantly overexpressed in prostate cancer. 23 genes were overexpressed in prostate cancer, including SIM2. Q-PCR verified that *SIM2* had the highest frequency of expression among these genes, and elisa analysis for SIM2 immune antibodies in the sera of health and prostate cancer patients identified SIM2 antibodies in the sera of some prostate cancer patients, but not in the controls, indicating a humoral immune response to SIM2 expression. The presence of

autoantibodies against self-antigens correlates with clinical responses and immunotherapy. Based on this information, the possibility of deriving an effective vaccine to generate SIM2 specific lymphocytes that target prostate tumors was investigated. Using several unique epitopes, it was shown that SIM2 vaccines significantly increased the cytotoxic t-cell response to subsequent SIM2 stimulation, indicating a potential role for vaccination to enhance SIM2 tolerance in prostate cancer (Arredouani et al., 2009).

These studies indicate an important role for SIM2 in cancer, both as a potential oncogene and tumor suppressor. The previous work in our laboratory has demonstrated a role of SIM2s in breast cancer progression and inhibition. Promotion of differentiation, inhibition of invasion, and terminal differentiation are all important activities in the mammary gland that can be imperative to either maintain a functioning gland or prevent tumor progression. These pathways have been shown as causal pathways in SIM2 regulated gene transcription and has led to the hypothesis that SIM2s promotes differentiation in the mammary gland and loss of SIM2s is necessary for the development breast cancer.

Cancer Metabolism

Cellular metabolism is a complex and highly regulated series of pathways through which the cell generates energy, acquires the nucleotides necessary for growth and survival, and performs various functions necessary for organ function. For our purposes, we will limit the pathways discussed to the two major catabolic pathways of the cell: oxidative phosphorylation (cellular respiration) and glycolysis.

Oxidative phosphorylation (OXPHOS) is the major metabolic pathway through which normal cells produce adenosine triphosphate (ATP), the primary energy molecule of the cell. Oxidative phosphorylation is the most energy efficient pathway, generating most of the ATP in

the cell, and must have oxygen in order to function. In aerobic organisms, all oxidative steps in various metabolic pathways converge with OXPHOS as the final stage. OXPHOS occurs in the mitochondria, where enzymes in the citric acid cycle produce CO_2 from the precursor acetyl CoA, then the electron transport chain (ETC) operates through the reduction of oxygen (O_2) into water (H_2O) using electron transfer and the generation of a proton gradient (Nelson and Cox, 2005). In addition to ATP and CO_2, OXPHOS also is responsible for the production of reactive oxygen species (ROS) in the cell, which are important for cell signaling, and also be responsible for protein and DNA damage (Alfadda and Sallam, 2012; Verbon et al., 2012). Mitochondria have two membranes with different permeabilities – the outer membrane which is readily permeable and the inner membrane which is impermeable, and uses transporter proteins to transmit particles. It is this inner membrane that contains the electron transport chain for ATP generation. Within the inner mitochondrial membrane, named the mitochondrial matrix, resides the enzymes of the citric acid cycle and other pathways, with the exception of glycolysis, which occurs in the cellular cytoplasm (Figure 5).

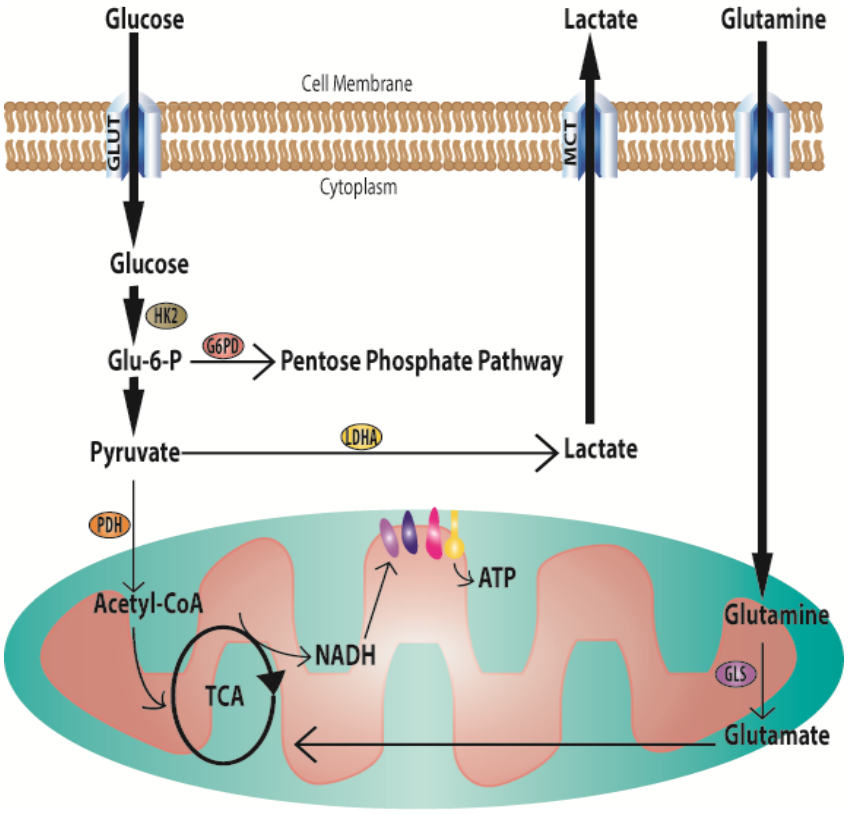

Figure 5. Metabolic Localization in the Cell. Glycolysis and lactate production or localized to the cytoplasm, while glutamate metabolism and the TCA cycle are located within the mitochondrial matrix (Cairns et al., 2011a).

Under normal circumstances, cellular respiration occurs in three distinct phases (Nelson and Cox, 2005). Cytoplasmic glycolysis oxidizes glucose in the cell through a series of 10 reactions into pyruvate (Figure 6). Under aerobic conditions, pyruvate is transported into the mitochondria, where it is decarboxylated by pyruvate dehydrogenase, resulting in acetyl-CoA (Figure 7). Under anaerobic conditions, pyruvate remains in the cytoplasm and is converted into lactate by lactate dehydrogenase (LDHA). Once converted, acetyl-CoA then enters the citric acid cycle (within the mitochondria), where it is enzymatically oxidized into CO_2, and energy is released through the production of reduced electron carriers NADH and $FADH_2$ (Figure 7).

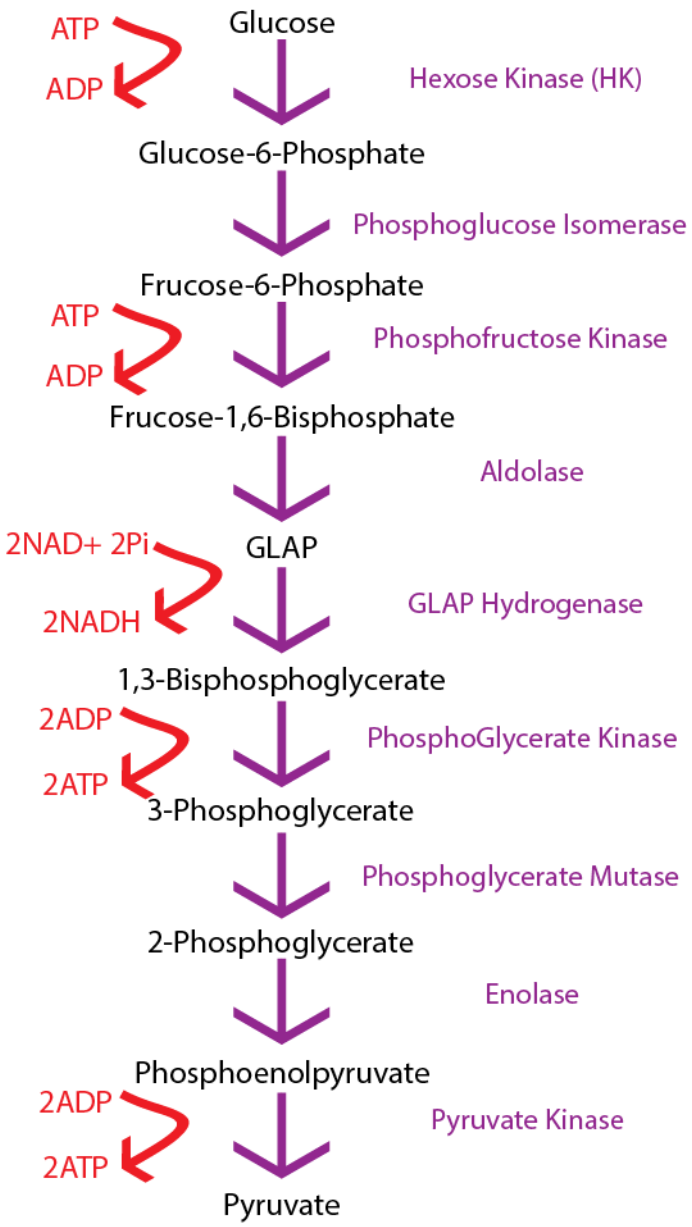

Figure 6. Normal Cellular Glycolysis. The normal consumption of glycolysis in the cytoplasm.

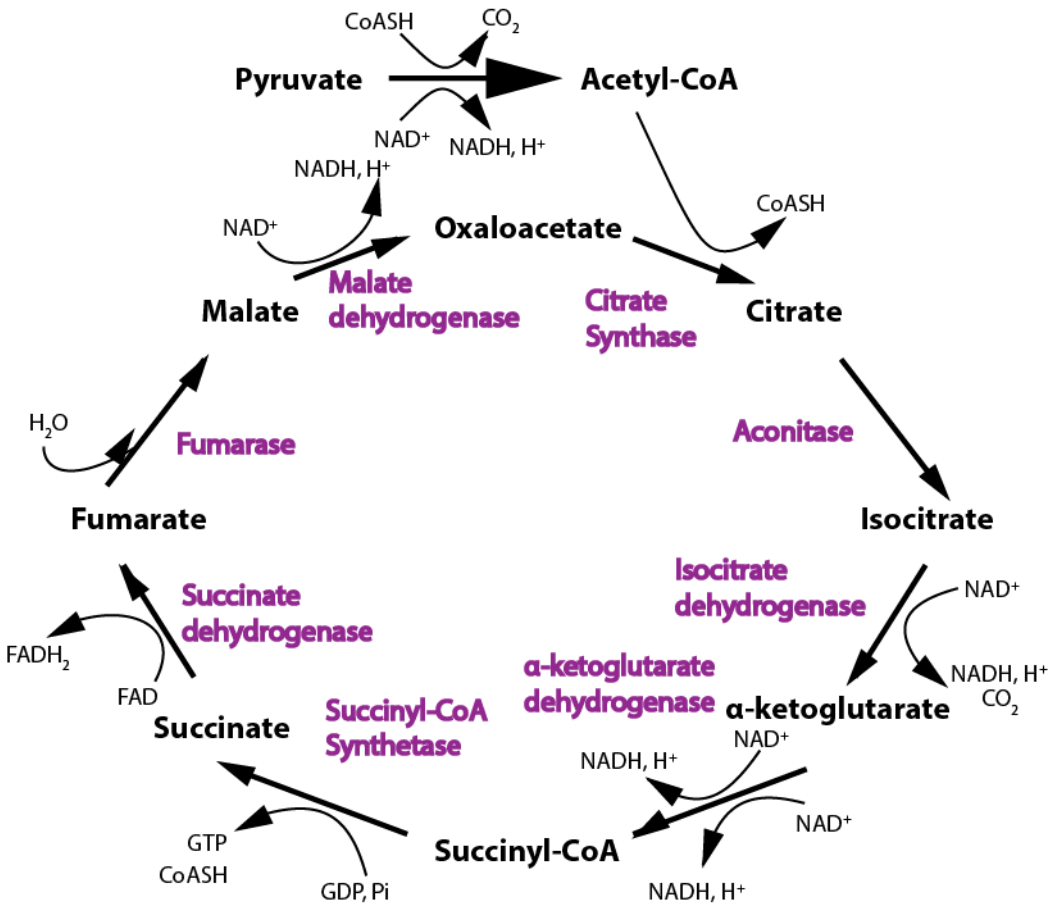

Figure 7. Tricarboxylic Acid Cycle. Pyruvate metabolism localized in the mitochondria, oxygen dependent.

Finally, in the electron transport chain (on the inner mitochondrial membrane), these electron carriers are oxidized through five complexes, generating H_2O, and ultimately ATP through the generation of a proton motive force (Figure 8).

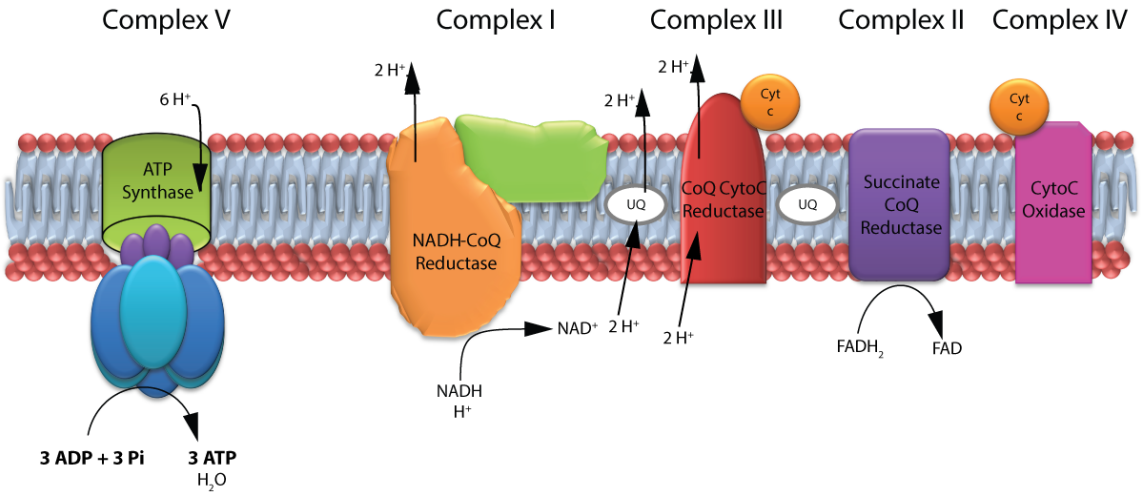

Figure 8. Electron Transport Chain (ETC). The flow of electrons across the inner mitochondrial membrane to generate ATP.

Deregulation of cell metabolism is a unique and defining feature of tumor cells. First described by Otto Warburg over eighty years ago, tumors cells preferentially utilize glycolytic pathways for energy generation while down-regulating their oxidative phosphorylation or mitochondrial energy activity, even in the presence of abundant oxygen (Warburg, 1956a; Warburg, 1956b). Warburg hypothesized that tumor cells originate from a two hit model: first the irreversible injury to mitochondrial respiration, and second the struggle for tumor cells to maintain their structure after the insult, resulting in a de-differentiation and ultimately a tumor cell. By measuring blood glucose levels in tumor veins and arteries, he established that tumors consumed significantly higher amounts of glucose out of the blood than normal tissues, and secreted more lactate. Warburg established that tumor cells are more adaptive at obtaining energy than normal cells, and in order to effectively target and kill tumor cells both glycolysis

and oxidative phosphorylation had to be thwarted. This is driven by the tumor cells' need to meet increased metabolic demands for proliferation and survive insults to mitochondrial respiration and the frequently hypoxic environment of the tumor (Bensinger and Christofk, 2012; Dang, 2012; Yeung et al., 2008). Increased glycolysis provides tumors with a selective growth advantage by supplying ATP to meet their high bioenergetics needs, including precursors required for nucleotide, amino acid and lipid biosynthesis. In fact, even though the net ATP gain for aerobic glycolysis is lower than that gained through oxidative phosphorylation, it is believed that the gain in metabolic precursors is more important for cellular survival and proliferation (Cairns et al., 2011b; Dang, 2012; DeBerardinis et al., 2007).

Normal non-proliferating cells and cancer cells use both glucose and glutamine to generate energy (Bensinger and Christofk, 2012; Dang, 2012; Kroemer and Pouyssegur, 2008; Lu et al., 2010a; Tennant et al., 2010). Differentiated cells predominantly metabolize glucose to pyruvate. Enzymes in the mitochondria facilitate the oxidation of pyruvate to produce ATP and reactive oxygen species (ROS) through electron transport-coupled oxidative phosphorylation (Figure 9).

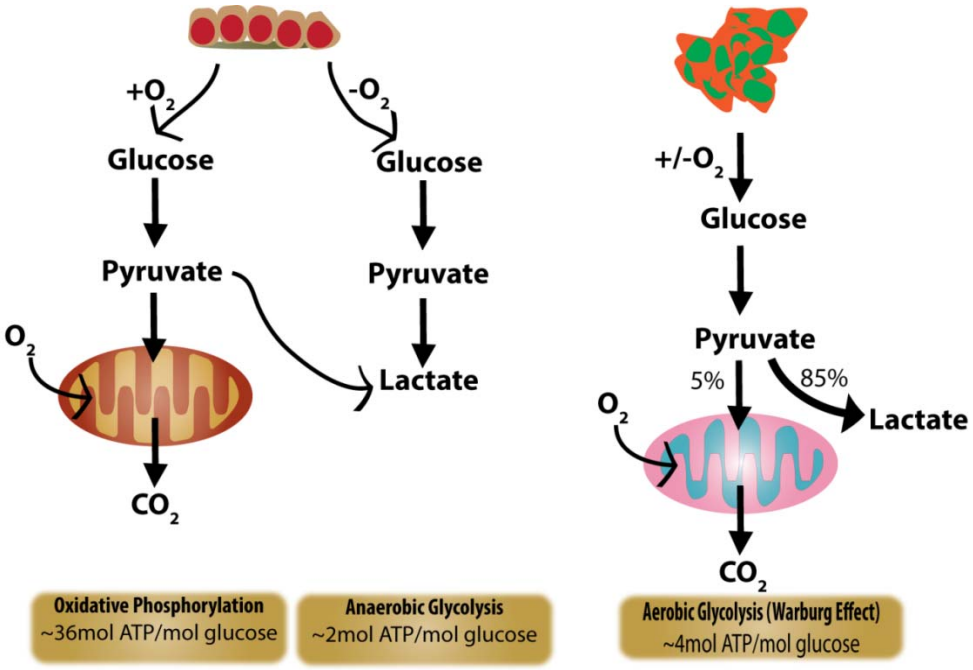

Figure 9. Normal Metabolism and the Warburg Effect. Tumor cells preferentially used glycolysis for energy production, even in the presence of abundant oxygen.

In contrast, rapidly dividing cancer cells meet metabolic demands by switching from oxidative phosphorylation to aerobic glycolysis (Figure 9). This switch has been attributed to growth factor signaling, as well as oncogene and tumor suppressor mutations (DeBerardinis et al., 2008; Ward and Thompson, 2012). Cancer cells increase their glucose uptake by up-regulating glucose transporters (GLUT 1 to 9) and hexokinase (HK) to retain glucose in the cell. The majority of the pyruvate generated from glucose in cancer cells is metabolized in the cytoplasm to lactate by lactate dehydrogenase (LDHA) and secreted into the microenvironment, lowering the pH, and increasing angiogenesis and invasion (Cardone et al., 2005; Gatenby and Gillies, 2004; Harguindey et al., 2005; Jones and Thompson, 2009; Robey and Martin, 2011). Enhanced lactate levels have been associated with metastases, tumor recurrence, and patient survival in human cancers (Hirschhaeuser et al., 2011; Semenza, 2008; Walenta et al., 2000).

Increased intracellular glucose also promotes activation of the pentose phosphate pathway through induction of glucose-6-phosphate dehydrogenase (G6PD), which generates NADPH and ribose-5-phosphate for the synthesis of nucleotides. Recent studies have also found that glutamine is an essential nutrient for cancer cells by providing intermediates for the TCA cycle and synthesis of fatty acids and to regulate redox potential (Lu et al., 2010b).

Breast Cancer Metabolism

The use of positron emission tomography (PET) in patients to detect tumors and metastatic lesions with increased glucose uptake has shown that altered metabolism in breast cancer is associated with increased tumor progression and therapeutic resistance, supporting a role for alterations in metabolism in malignant transformation (Gambhir, 2002; Gatenby and Gillies, 2004; Hama and Nakagawa, 2010; Jones and Thompson, 2009; Kroemer and Pouyssegur, 2008; Tennant et al., 2010). [^{18}F]deoxy-glucose (FDG), used in PET scans, uptake significantly correlates with glucose transporter 1 (GLUT1) expression in tumors (Robey et al., 2008). Early research in human breast cancer samples showed that GLUT1 was significantly upregulated in breast cancer when compared to healthy tissue (Brown and Wahl, 1993). Early research in MCF7 and MDA.MB.453 breast cancer cell lines examined the unique glycolytic phenotypes of these cells and their responsiveness to nutrient starvation (Mazurek et al., 1997). Their studies indicated that MCF7 cells maintained higher glycolytic flux than MDA.MB.453 cells, and were more susceptible to nutrient starvation and adenosine monophosphate levels. They also hypothesized that the unique metabolic environment of MDA.MB.453 cells would make them less prone to drugs that target electron carrier NAD^+. While the authors touched on the unique metabolic differences between the two cells lines and their responses to different stresses, they did not pursue the inherent differences and their potential role in tumor progression. A later study analyzed key, rate-limiting proteins involved in aerobic glycolysis

(Brown et al., 2002). Primary glucose transporter GLUT1, which transports glucose from the body into the cell, and hexokinase II (HK2), responsible for the conversion of hexose to hexose-6-phosphate on the outer mitochondrial membrane, were analyzed via immunohistochemistry in untreated primary human breast cancer samples. HK2 is the first enzyme of the glycolytic pathway and is a key regulator of glucose metabolism in cancer cells (Mathupala et al., 2006). While the authors found upregulation of HK2 in breast cancer, it did not always correlate with increased GLUT1 expression. However, HK2 is more consistently activated than GLUT1 in primary tumors. Analysis of primary human breast cancer cell lines showed variable rates of glucose uptake and lactate production under normal cellular conditions (Robey et al., 2008) and a linear relationship between GLUT1 expression and lactate production. Analysis of key oncogenes HIF1α and c-Myc with lactate production and glucose uptake showed that HIF1α did not correlate with glucose uptake, although it was highly expressed in all cell lines, and phosphorylated c-Myc strongly correlated with lactate production and increase glucose uptake. Expression of another oncogenic protein, AKT, did not correlate with the glycolysis in breast cancer cells (Schmidt et al., 2010). However, another study showed activation of AKT in the glycolytic phenotype of breast cancer (Schmidt et al., 2010). Analysis of primary human specimens showed significantly upregulated AKT phosphorylation and GLUT1 expression in tumors compared to normal tissues. All of these studies point to an enhanced glycolytic phenotype in breast cancer.

Further analysis of GLUT1 in MMTV-ErbB2 tumors indicated an important role for glucose uptake in tumor growth (Young et al., 2011). GLUT1 screening across a panel of breast cancer cell lines and tumors verified that *GLUT1* was the most high expressed glucose transporter in all samples. Knockdown of *GLUT1* by RNAi in ErbB2 overexpressing mammary epithelial cells significantly decreased glucose uptake and lactate production, and also

proliferation and colony formation. However, loss of *GLUT1* had no effect on ATP production. Loss of *GLUT1* did not completely ablate glucose uptake in these cells, indicating that glucose uptake may still occur by non-specific means or other glucose transporters. Cells with silenced *GLUT1* were then injected into the cleared mouse mammary glands as xenografts and there was significantly decreased tumor growth *in vivo*. While *GLUT1* knockdown inhibited proliferation, it had no effect on apoptosis, indicating the differences in tumor size are due to differences in growth. Conversely, overexpression of *Glut1* in a mouse mammary tumor cell line that has low levels of *Glut1* significantly increased glucose uptake, however had no effect on proliferation *in vitro*. When these cells were injected into mice, however, enhanced Glut1 expression significantly increased tumor growth and decreased cellular apoptosis.

In addition to GLUT1, lactate dehydrogenase A (LDHA) has also been implicated in breast cancer growth and glycolysis (Hussien and Brooks, 2011; Zhao et al., 2009). Hussein et al. (2011) analyzed location of extracellular lactate transporter MCT and LDH isoforms in MCF7, MDA.MB.231, and HMEC cells. MCT and LDH expression were expressed in both control and cancer cell lines, and cancer was associated with increased expression, but not a change in localization of these proteins. They also found that lactate transport was associated with decreased oxidative phosphorylation and increased lactate accumulation in cancer cells. Surprisingly, their studies indicated that MCT1 was down regulated in breast cancer, and when expressed it is localized to the cell membrane, indicating a potential role for the flow of lactate between tumor cells and the stroma. Research on the role of LDHA in ErbB2 expressing MCF7 and MDA.MD.453 breast cancer cells (Zhao et al., 2009) showed that there was increased glucose uptake and lactate production, and decreased oxygen consumption. Analysis of LDHA protein levels showed higher LDHA levels in ErbB2 expressing cells, and this correlated with enhanced enzyme activity. Moreover, knockdown of ErbB2 in an ErbB2 overexpressing cell

line down regulated glucose uptake and lactate production, and increase oxygen consumption. Knockdown of *LDHA* by RNAi in ErbB2 expressing tumors was sufficient to decrease the glycolytic phenotype and it was shown that ErbB2 regulation of LDHA was due to heat shock factor 1 (HSF1), a transcription factor that play an important role in stress response gene expression. ErbB2 expression in breast cancer cells also increased cell sensitivity to glycolytic inhibitors such as 2-deoxy glucose (2-DG). In contrast, ErbB2 decreased sensitivity to mitochondrial drugs such as oligomycin, which targets the ATPase complex of the electron transport chain.

Mitochondria in Cancer

In addition to glycolytic enzymes, mitochondrial dysfunction has also been implicated in cancer. Warburg's original work associated the enhanced aerobic glycolysis of tumor cells with an irreversible dysfunction of the mitochondria, where oxidative phosphorylation occurs (Warburg, 1956a). More recent research shows that while many tumors have irreversibly altered mitochondria, many continue to use cellular respiration, albeit at a significantly lower level than the use of glycolysis (Bensinger and Christofk, 2012; Crabtree, 1929; Scatena, 2012; Ward and Thompson, 2012). Mitochondria are incredibly complex organelles, and mutations to mitochondrial DNA (mtDNA), mitochondrial specific enzymes, or mitochondrial physiology can impact cellular respiration, ROS signaling, and other metabolic pathways (Wallace, 2012). Additionally, studies analyzing mitochondrial morphology and activity in cancer cells showed that mitochondrial dysfunction was due to increased mitochondrial replication, however alterations to mtDNA and the ETC prohibited increased oxygen consumption and ATP production (Shapovalov et al., 2011). This study emphasizes that mitochondrial number is not a true indicator of cellular respiration, and real-time analysis of oxygen consumption is key to understanding mitochondrial activity in tumors and tumor cells. Thanks to the development of

Seahorse Flux analysis, we are now able to measure these quantities in real-time. This multiparameter analysis of human tumor cells verified that increased levels of glycolysis correlated with decreased mitochondrial function, and that long-term, sustained glycolysis attenuated oxidative phosphorylation capacity, to the point that mitochondria are not able to adapt to compensate when glycolysis is inhibited (Wu et al., 2007). Mitochondrial length is also an important characteristic that can promote cellular survival and tumorigenesis (Gomes et al., 2011). At the induction of the self-degradation process, autophagy, mitochondria undergo fusion to elongate and avoid mitophagy (degradation of mitochondria), where the ubiquitin ligase PARKIN degrades components of mitochondrial fusion and dysfunctional organelles. The result is long, interconnected mitochondria that are spared from degradation, maintain ATP levels, and promote cell survival in low nutrient environments.

In breast cancer, mitochondrial adaptation to cellular stress has also been examined (Smolkova et al., 2010). As expected, oxygen consumption was lower in breast cancer cells compared to non-cancer cells. However, when these cells were grown in low glucose media, the rate of oxygen consumption was similar between cancer and non-cancer cells. This supports the Crabtree effect, which states that the high concentrations of glucose used for cancer cell growth inhibited oxidative phosphorylation in cancer cells (Crabtree, 1929; Rossignol et al., 2004). However, this study did not take into account other variables such as glutamine in the media. Further experiments were done by Owens et al. (2011) that examined the five complexes of the ETC in human breast cancer cells and primary tumors . Examination of a panel of breast cancer cells showed various ETC complexes were decreased in breast cancer, as well as the activities of these complexes. ETC complex defects also correlated with overall cancer aggressiveness. Complex III, the complex responsible for coenzyme Q:cytochrome c-oxidoreductase , is the most commonly reduced complex, and is sifnificantly down regulated in the highly metastatic

MDA.MB.231 cell line. Interestingly, although complex III activity was decreased in breast cancer, many of the subunits were upregulated. Knockdown of complex III subunits led to decreased mitochondrial membrane potential and increased ROS production. Inhibition of complex II proteins also decreased cellular invasion, not only in breast cancer cells but also osteosarcoma and ovarian cancer cells. A recent study by Diers et al. (2012) also found that induction of mitochondrial function in breast cancer cells through addition of pyruvate actually promotes cell growth . By substituting pyruvate for glucose in cell culture medium, proliferation of MCF7 and MDA.MB.231 cells was significantly increased compared to glucose-containing media, and the conditions did not affect cell death. Addition of pyruvate did not enhance baseline levels of oxygen consumption, but it increased reserve capacity. However, their reserve capacity was lower than cancer cells grown in a full complement including glucose and pyruvate. This study demonstrates that mitochondria play an important role in cellular proliferation and oncogenesis.

Autophagy in Metabolism and Cancer

Autophagy and mitophagy play important roles in cellular metabolism in response to nutrient stress and hypoxia. Autophagy is the process through which cells consume their own cytoplasm and organelles in autophagosomes in order to build new proteins and membranes. Autophagy can be non-selective, but is also used to selectively degrade faulty organelles, such as mitochondria (mitophagy), ribosomes (ribophagy), endoplasmic reticulum (reticulophagy), and lipids (lipophagy) (Gomes et al., 2011; Rabinowitz and White, 2010; Tanida et al., 2008). In the absence of cellular stresses, basal levels of autophagy are used in a housekeeping manner to removed damaged organelles that could become toxic. Due to the potential specificity of autophagy, the process is highly regulated, with complex pathways involved in promoting or inhibiting autophagosome development. Autophagy is integral to multiple stages in normal

mammalian development, from embryonic survival to survival immediately after parturition. Autophagy is highly active in normal embryonic cells without stress, and is a potential mechanism to ensure survival and stress repair (Salemi et al., 2012). High levels of autophagy in adult stem cells also correlate with inhibition of cancer, and are required for adult stem cell differentiation. During starvation, autophagy is activated to promote cell survival until the necessary nutrients are available. Autophagy can also be used to remove unwanted organelles to adapt to changes in the nutrient environment. These mechanisms have also presented a potential mechanism for autophagy in the prevention of aging by maintaining cellular integrity (Wirawan et al., 2012). Cellular phenotypes of aging include damage to DNA, lipids, proteins, and other organelles - thus autophagy is a key anti-aging mechanism in normal cells through organelle cleanup.

Autophagy has been implicated both as an oncogenic and a tumor suppressive process (Tsuchihara et al., 2009). The general consensus is that early stages of autophagy induction prevent malignant transformation and oncogenesis, whereas autophagy induction at later stages can promote cell survival and tumor growth (Debnath, 2011; Wirawan et al., 2012). The primary hypothesis for the promotion of tumor growth by autophagy is through cellular survival. Autophagy allows tumor cells to survive in stressed environments that would normally undergo cell death, and suppresses necrosis and inflammation responses. The molecular mechanisms that activate autophagy under these circumstances, however, are unclear (Degenhardt et al., 2006). Beclin-1 (BECN1), a critical autophagy gene, is a candidate tumor suppressor, especially in breast cancer (Liang et al., 1999). Liang et al. found that BECN1 was highly expressed in normal mammary glands, and that expression was lost in breast cancer. Subsequent studies in cell lines verified that expression of *BECN1* reduced proliferation and tumorigenesis. P53, a classical tumor suppressor integral to cell function, has also been shown to promote autophagy

through multiple mechanisms including suppression of mTOR and direct effect through transcription of the damage-regulated autophagy modulator gene (DRAM) (Crighton et al., 2006; Feng et al., 2005). Additionally research has shown that autophagy can also mediate cellular senescence, a stable cell cycle arrest during which the cells remain metabolically active (Young et al., 2009). Young et al. (2009) found *Ras* induced senescence also upregulated autophagy marker LC3II, and this upregulation could be blocked by subsequent inhibition of the senescence machinery. Their study established that induction of autophagy contributes to the establishment of cellular senescence. The primary hypothesized tumor-suppressive function of autophagy is the induction of autophagy cell death. This non-apoptotic programmed cell death pathway is upregulated in cancer cells after chemotherapy (Kondo et al., 2005). Another hypothesis regarding autophagy in tumor suppression is through regulation of genomic stability (Mathew et al., 2007) where it has been hypothesized that loss of autophagy in cancer cells increased DNA damage and gene amplification, resulting in increased genomic instability due to an accumulation of damage organelles.

In breast cancer, autophagy is also oncogenic and tumor suppressive (Gong et al., 2012; Karantza-Wadsworth et al., 2007; Oh et al., 2011). Wadsworth et al. (2007) found that inhibition of autophagy was necessary to sensitize mammary epithelial cells to insult, thus causing the DNA damage and genomic stability that are integral to cancer progression. Oh et al. (2011) studied the effect of anti-apoptotic Bcl2 on autophagy inhibition. During breast cancer progression Bcl2 is often upregulated and confers drug resistance as well as oncogenic transformation. Bcl2 was shown to directly target BECN1, and thus inhibit autophagy during oncogenesis. Using a Bcl2 mutant unable to inhibit apoptosis but still capable of suppressing autophagy, it was shown that the anti-autophagic capability of Bcl2 was integral to its oncogenic effect. Conversely, Gong et al. (2012) found that autophagy was required in breast cancer tumor

initiating cells. Autophagic flux was significantly upregulated in aldehyde dehydrogenase (ALDH) positive cells, and was integral for maintenance of TICs. While depletion of *BECN1* in a cell monolayer increased tumorigenesis, depletion of *BECN1* in the TIC specific population inhibited tumorigenesis (Gong et al., 2012). Autophagy is vital to embryonic stem cell survival and it appears that autophagy has unique cell-type specific effects in adult stem cells and differentiated cells.

The metabolic regulation in normal cells is elegantly controlled and intermingled. The derailment of this system in cancer is complex and not well understood. While it has been reviewed and studied, there remains competing theories that indicate the role of metabolism is not strictly black and white with tumorigenesis.

BNIP3, a Novel Sim2s Target Implicated in Metabolism

The identification of BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) as a target of SIM2s repression was the first indication that SIM2s plays a role in intracellular metabolism. BNIP3 is a HIF1α regulated pro-apoptotic protein that is also pro-autophagic (Bruick, 2000; Tracy et al., 2007). Farrall and Whitelaw (2009) elucidated the regulation of BNIP3 by SIM2s in prostate cancer. Microarray analysis of *SIM2s* expressing prostate cancer cells compared to controls revealed the *BNIP3* levels were down regulated with ectopic expression of *SIM2s*. Similarly to SIM2s, BNIP3 has been shown to be down regulated in pancreatic and colon cancers (Akada et al., 2005; Bacon et al., 2007). The inverse relationship between *SIM2s* and *BNIP3* expression was confirmed using addition cell lines stably transduced to overexpress *SIM2s*, indicating that BNIP3 silencing is downstream of SIM2s activity. No changes were seen in epigenetic regulation of *BNIP3* expression, thus indicating a direct regulation of BNIP3 expression by SIM2s. SIM2s competes with HIF1α to bind the HRE,

and inhibits hypoxic induced gene expression (Woods and Whitelaw, 2002). Since BNIP3 is a hypoxically regulated gene, ChIP analysis was performed to test for SIM2s binding to the HRE in the BNIP3 promoter, as well as two putative SIM2s specific response elements (S2RE) (Woods et al., 2008). SIM2s bound to the BNIP3 HRE, but not the S2REs, supporting previous studies (Woods and Whitelaw, 2002). To confirm that SIM2s binding to the BNIP3 promoter was sufficient to inhibit hypoxic induced gene expression, *SIM2s* expressing cells were grown in a hypoxic environment, resulting in enhanced *BNIP3* expression, even with the decreased levels of SIM2s that are seen after hypoxic exposure. Further analysis during hypoxic exposure verified that the binding affinity of SIM2s to the HRE during hypoxia was not altered. Additional ARNT expression during hypoxia partially alleviated SIM2s mediated repression, thus indicating that SIM2s also competes with HIF1α for ARNT heterodimerization. Further analysis showed that SIM2s mediated BNIP3 repression inhibited hypoxia induced autophagy in prostate cancer cells and therefore inhibited autophagic cell death.

BNIP3 is a protein related to the BH3-only family, which contains only a single Bcl-2 homology (BH) domain. BNIP3 induces cell death and autophagy, but upon interaction with the anti-apoptotic protein BCL2, these effects are inhibited. BNIP3 is a hypoxia regulated gene, and its expression is associated with tumor cell death. Interestingly, the molecular mechanism of BNIP3 induced cell appears to work independently of the caspase cascade and is not well understood (Vande Velde et al., 2000; Zhang and Ney, 2009). The generally accepted model of BNIP3 activity involves mitochondrial dysfunction, specifically a loss in mitochondrial membrane potential through mitochondrial pore opening, the generation of reactive oxygen species (ROS), and ultimately necrosis (Kubli et al., 2007). BNIP3 can induce mitochondrial pore opening a number of ways: through direct interaction with BAX or BAK, or indirectly via increased mitochondrial calcium uptake (Marzo et al., 1998). Indeed, knockdown of *Bax* and

Bak in mice prevented Bnip3 mediated mitochondrial cell-death, but not Bnip3 mediated autophagy (Kubli et al., 2007). BNIP3 can also form channels in the mitochondrial outer membrane, thanks to its unique transmembrane domain (Chen et al., 1997; Imazu et al., 1999). As previously mentioned, in addition to cell death, BNIP3 is as an important protein in cellular autophagy and several studies have established a role for BNIP3 in drug-induced autophagy. Interestingly, the effect of BNIP3 induced autophagy varies with the cell type. Induction of autophagy in glioma cells induced mitochondrial depolarization and autophagy cell death, however HL-1 myocytes were protected from cell death by BNIP3 induced autophagy (Daido et al., 2004; Hamacher-Brady et al., 2007; Kanzawa et al., 2005). *BNIP3* expression in skeletal muscle also induces autophagosome formation, and has been implicated in muscle wasting disorders (Mammucari et al., 2007). In a hypoxic environment, autophagy is often associated with cell survival rather than cell death, and *BNIP3* expression during hypoxia does not induce autophagic cell death. Tracy and Macleod (2007) have observed that overexpression of *BNIP3* in various tumor cell lines causes increased autophagy independently of cell death, and that knockdown of BNIP3 inhibits hypoxia-induced autophagy. Autophagy during hypoxia increases ATP production efficiency and helps combat the oxidative damage that occurs, particularly through mitophagy, in order to maintain an energy balance in hypoxic cells (Jin, 2006). *BNIP3* expression increases activation of autophagic LC3 proteins, and increases lysosomal localization to faulty mitochondria for removal (Rikka et al., 2011). Inhibition of this process using autophagy inhibitor 3-methyladenine (3-MA) resulted in extensive cell death. BNIP3 is a novel autophagy gene in that is induced by varying and sometimes competitive signals, indicating a highly controlled process that it responsive to unique cellular stimuli, as opposed to the highly conserved ATG genes. In addition to the mitochondrial autophagy and cell death mechanisms, further work by Rikka et al. (2011) established that expression of BNIP3 decrease mitochondrial

respiration proteins, thereby reducing oxidative phosphorylation and ATP levels. In addition to regulation by HIF1α and SIM2s, BNIP3 is also repressed by the classical tumor suppressor RB (Tracy et al., 2007). These competing regulators of BNIP3 expression could keep BNIP3 levels in a range that promotes autophagy without inducing cell death, as well as regulating autophagy in response to unique environmental stressors.

In breast cancer, research on BNIP3 has produced varying and conflicting data. Early work examined the expression of *BNIP3* in DCIS lesions and correlation with necrosis and tumor grade (Sowter et al., 2003). Using both *in situ* hybridization and immunohistochemistry, an array of varying DCIS lesions were analyzed for BNIP3 expression. Sowter et al. (2003) reported that BNIP3 was not expressed in normal breast tissues, but was detected in DCIS tissue. BNIP3 levels varied significantly with tumor grade, as well as necrosis score. High levels of BNIP3 were observed in high-grade, highly-necrotic DCIS lesions, and BNIP3 expression significantly correlated with invasion-associated DCIS. It is important to note that DCIS necrosis has been associated with enhance tumor progression and metastasis (Shekhar et al., 2008). However, BNIP3 expression in the invasive component did not correlate with BNIP3 staining in the adjacent DCIS lesion, nor did it correlated with grade or necrosis. Sowter et al. (2001) also established that *BNIP3* was upregulated in breast carcinoma cells lines, and hypoxia enhanced expression. In a similar study, a large number of DCIS and IDC lesions were analyzed for *BNIP3* and *HIF1α* to determine the potential use of BNIP3 as a marker of DCIS progression (Tan et al., 2007). BNIP3 was only weakly detected in normal tissues, but over 60% of DCIS samples and 70% of IDC samples expressed this protein. While there was a significantly correlation in BNIP3 expression and breast cancer compared to normal tissue, no correlation was seen between BNIP3 and progression to IDC. Nuclear expression of BNIP3 in IDC samples correlated with smaller tumor size and lower grade, as well as ER expression. Surprisingly,

BNIP3 expression did not correlate with HIF1α expression. Additionally, nuclear localization of BNIP3 was significantly related to increased disease recurrence in DCIS lesions, and shorter disease-free survival. This study indicated an enhance complexity in the regulation of BNIP3 expression, with potential HIF1α independent mechanisms and both pro and anti-tumorigenic properties. A recent study by Naushad et al. (2012) has elucidated a role for epigenetic regulation in BNIP3 expression in IDC. Combined bisulfite restriction analysis (COBRA) of the *BNIP3* gene showed that the CpG island on the *BNIP3* promoter was hypomethylated in IDC samples, and upregulated. However, the methylation profile of the *BNIP3* promoter did not correlate with hormone markers ER, PR, HER2, or mitotic index.

In contrast, the loss of *BNIP3* has also been associated with enhanced progression and metastasis. Manka et al. (2005) determined that loss of *BNIP3* in breast cancer promoted metastasis to the lung, liver, and bone, and in mouse breast cancer metastasis models, *BNIP3* was increased after exposure to hypoxia, however expression was lower in highly metastatic 4T1 cells compared to the non-metastatic 67NR cell line. Expression in 4T1 cells was also mitochondrial, compared to cytoplasmic expression in both the 67NR and 4T07 cell lines. Likewise, hypoxia also induced high levels of cleaved caspase in the non-metastatic 67NR and mildly-metastatic 4T07 cells compared to 4T1 cells. Knockdown of *BNIP3* in 67NR and 4T07 cells increased clonogenic survival, and tumor volume. Knockdown of *BNIP3* in 4T07 cells enabled tumors to form macroscopic metastatic lesions in the liver and sternum. Koop et al. (2009) also used *in situ* hybridization and immunohistochemistry to associate BNIP3 expression with IDC. Forty percent of IDC tumors were positive for *BNIP3* mRNA, and 57% were positive for BNIP3 protein, and expression was cytoplasmic and granular, as expected with mitochondrial localization. In adjacent normal tissue, immunohistochemistry showed that 8/35 samples exhibited nuclear staining of BNIP3, which is rarely seen in tumor cells. When tumor cells were

strongly positive for BNIP3, the lower levels of BNIP3 were observed in the surrounding normal tissues approximately 65% of the time, with stronger signal in normal tissue being seen 35% of the time. Again, BNIP3 expression did not correlate with HIF1α status. Clinicopathological analysis showed that BNIP3 negative tumors had a higher frequency of lymph node metastasis, as well as mitotic index. Correlations with ER, PR, necrosis, or tumor size were not observed.

The conflicting reports on BNIP3 expression in breast cancer indicated a highly regulated, complex mechanism for BNIP3 in mitochondrial function and autophagy. BNIP3 is a new link between SIM2s and HIF1α. Most importantly, BNIP3 establishes a potential role for SIM2s in the regulation of cellular metabolism and autophagy. The roles of metabolic homeostasis and autophagy in tumor onset and progression are complex, and strategies that target these pathways would provide a novel approach for chemotherapy.

P53

P53 in Differentiation

P53 is one of the most well-known tumor suppressor genes and was identified in the early 70's. P53 has been shown to have important roles in apoptosis, DNA repair, cell-cycle regulation, and genomic stability (Linzer and Levine, 1979) and because of this it has earned the name, "Guardian of the Genome" (May and May, 1999). P53 is activated by variety of stresses, such as DNA damage, UV radiation, and stress signals, and regulates various functions such as mitochondrial function, senescence and autophagy, apoptosis, and DNA damage repair (Junttila and Evan, 2009) (Figure 10).

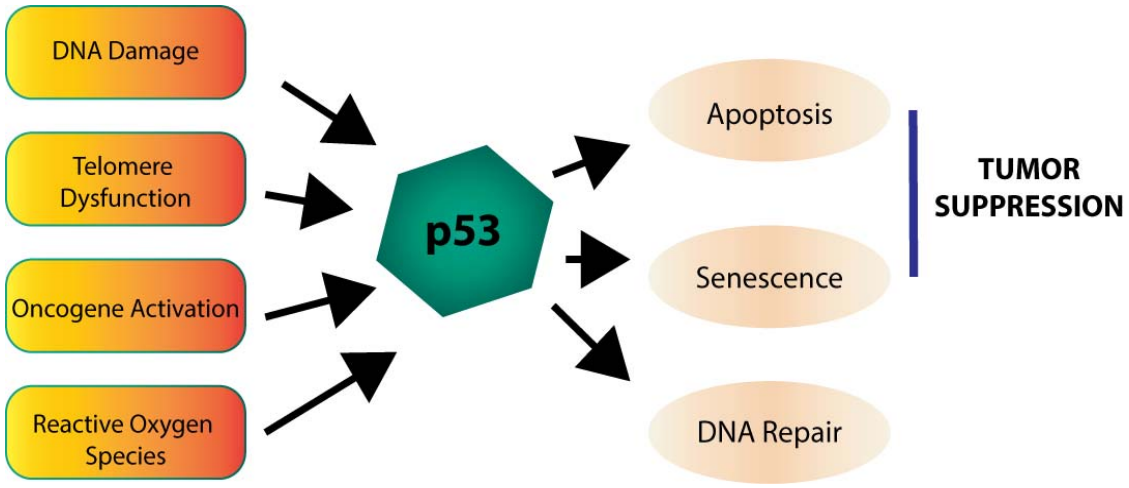

Figure 10. P53 Stress Response. Stress induction of p53 has multiple pathways, which can promote tumor suppression.

P53 is frequently inactivated or mutated in cancers, and by a wide range of mechanisms. Although extensively researched, while some methods of p53 inactivation are favored over other, it remains unclear why. One widely studied role of p53 is the promotion of cell cycle arrest through upregulation of p21. When activated, p53 transcribes cell cycle inhibitor $p21^{WIF/CIP}$ which inhibits the CDK4/6 complex, thus blocking cell cycle progression and inducing senescence (Taylor and Stark, 2001). Additionally, many p53 target genes are pro-apoptotic, thus loss of p53 contributes to the survival of tumor cells (Jerry et al., 2000).

P53 mutation in breast cancer is associated with increased tumor aggressiveness and worse prognosis (Gasco et al., 2002). While p53 mutation occurs in approximately 50% of breast cancers, and it is enriched in the inheritable BRCA1/BRCA2 mutated tumors, hypothetically due to the increased genomic instability caused by germline mutations (Greenblatt et al., 2001). While p53 has been indicated as a potential prognostic marker for breast cancer, immunohistochemical analysis of breast cancer samples for p53 are inconclusive, as they cannot

differentiate between wild-type and mutant p53. Mutant p53 expression is associated with increased tumor growth, metastasis, and chemotherapeutic resistance (Allred et al., 1994).

Aside from p53's role in cell cycle regulation and apoptosis, it has also been shown to play an important role in normal cell differentiation and tumor de-differentiation (Molchadsky et al., 2010). In normal embryonic development, *p53* expression is high during organogenesis, but is almost un detectable in terminally differentiated tissues (Schmid et al., 1991). Initial studies on *p53* knockout mice appears as though *p53* null mice were viable and physiologically normal, but later generations exhibited frequent developmental defects in extremity development and fertility (Armstrong et al., 1995). These mice were also more susceptible to spontaneous cancers (Donehower et al., 1992). Further research in p53 function has established it as a critical regulator of differentiation in multiple tissues. P53 has been shown to be a critical regulator of neuronal differentiation, slowing growth and maintaining neuronal stem cell potential (Sah et al., 1995; Zheng et al., 2008). In p53 null mice, neurons proliferate uncontrollably, resulting in exencephaly and neural tube defects. In neurons, P53 has been shown to promote differentiation through two major pathways: progression through neurite outgrowth and cell-cycle arrest. P53 in osteogenic differentiation has a slightly different role. While P53 is considered a promoter of differentiation, studies have indicated that it acts as a negative regulator in bone development (Lengner et al., 2006; Wang et al., 2006). Loss of P53 accelerated osteoblast differentiation, resulting in higher levels of bone formation and bone density. The associated increase in proliferation has also been associated with a higher risk for osteosarcoma formation. Additionally, P53 has been shown to regulate skeletal muscle differentiation through maintenance of active retinoblastoma (RB) (Porrello et al., 2000). As noted with other tissues, loss of P53 in myogenic cells is associated with increased rhabdomyosarcomas (Choi and Donehower, 1999). Initially, the role of P53 in hematopoietic cell differentiation was not

observed, however later research has shown that P53 is necessary for B-cell maturation, which promotes their tumor suppressing activity (Donehower et al., 1992; Shaulsky et al., 1991). Finally, P53 has also been implicated in adipogenic differentiation. Adipocyte differentiation is another example of where P53 acts as an inhibitor of differentiation (Hallenborg et al., 2009; Inoue et al., 2008). P53 is down regulated during normal adipocyte differentiation, and loss of *P53* results in increase proliferation of adipocytes. P53 induction is also seen in obese mice, possibly promoting lipid accumulation in adipocytes. P53 expression in adipose tissues in animals with insulin resistance showed characteristics of aging and inflammation, likely due to P53 upregulation of P21 and senescence (Ahima, 2009). It is these effects on adipocytes that has led to the study of p53 in metabolism, we will discuss below.

In the mammary gland and breast cancer, P53 is an important target for hormone-mediated tumor suppression. While P53 is stabilized with exposure to stresses such as radiation, estrogen and progesterone are also sufficient to increase P53 expression in the mammary gland (Becker et al., 2005). Thus P53 is upregulated during pregnancy, and hypothetically works to promote luminal cell differentiation and prevent mammary tumorigenesis due to the enhanced mammary epithelia proliferation that occurs during this time (Jerry et al., 2000; Medina and Kittrell, 2003). Original work attempting to analyzed breast cancer in *p53* null mice indicated that loss of *p53* had no effect on development of breast cancer. However, these mice often died young due to lymphomas, therefore, obscuring a possible mammary tumor phenotype. Thus, Jerry et al. (2000) used a mammary specific *p53* knockout mouse to study the effects of *p53* loss specifically in the mammary gland. Their studies found that loss of *p53* alone was sufficient to induce significant levels of breast cancer, and exposure to hormones just increased the tumorigenicity in *p53* null glands. Studies have shown that full-term pregnancy at a young age has a preventative effect on breast cancer, and Sivaraman et al. (2001) hypothesize that this

preventative effect is the early upregulation of P53 in the differentiating mammary gland. Interestingly, while P53 expression peaks and is necessary during pregnancy and lactation, involution can proceed independently of p53 (Li et al., 1996). Li et al. showed that the major apoptosis that occurs during mammary gland involution is P53-independent, but still uses P53 apoptotic targets Bax and Bcl-x, indicating P53-independent mechanisms for their activation. Studies by Jerry et al., however, showed that loss of p53 delayed mammary gland involution, followed by compensation by p53-independent mechanisms (Jerry et al., 1998; Jerry et al., 1999). These studies indicated that p53 promoting the first stage of rapid cellular apoptosis in the involuting mammary gland, indicated through upregulation of p21.

P53 in Metabolism

While extensively characterized in differentiation, P53 has also been heavily implicated in the regulation of normal and tumor metabolism. We have already established the importance of P53 upregulation in response to various environmental stresses. So it also makes sense that P53 is upregulated in response to nutrient stress such or low oxygen. However, enhanced aerobic glycolysis, as is common in cancers, has been shown to suppress P53, and may potentially help cancer cells evade P53 induced apoptosis and senescence (Zhao et al., 2008). Hypoxia has also been shown to induce P53, however research is conflicting whether P53 stabilization is a direct effect of low oxygen or indirect due to DNA damage and nutrient deprivation (Alarcon et al., 1999; An et al., 1998; Pan et al., 2004). Similarly, mitochondrial ROS are an important activator of P53 (Karawajew et al., 2005). These authors found that inhibition of mitochondrial function through targeting of the ETC or MMP suppressed P53 activation. However, while these studies elucidated the promotion of classical P53 stress response, including senescence and inhibition of proliferation, new studies have shown novel mechanisms for P53 in the direct regulation of metabolic pathways.

P53 has been shown to suppress glycolysis and promote oxidative phosphorylation through suppression of GLUT1 and GLUT4 (Schwartzenberg-Bar-Yoseph et al., 2004). Additionally, two key P53 repression targets: P53-induced glycolysis and apoptosis regulator (TIGAR) and synthesis of cytochrome c oxidase (SCO2) have been shown to regulate various aspects of metabolism as well (Bensaad et al., 2006; Madan et al., 2011; Won et al., 2012). These proteins have been especially studied in breast cancer, where P53 expression significantly correlates with decreased TIGAR and SCO2 expression (Won et al., 2012). However, decreased SCO2 expression wax associated with histological grade, metastasis, and poorer prognosis. This study was not able to determine the presence of mutant p53, which may provide a confounding factor. The authors hypothesize that it is the upregulation of these genes with mutation/loss of P53 that can induced the Warburg effect in breast cancer cells. TIGAR has been shown to promote the pentose phosphate pathway (PPP) in mitochondria, which lowers ROS and increases NADPH (Bensaad et al., 2006; Cheung et al., 2012). Under hypoxia, HIF1α promotes the translocation of TIGAR to the mitochondria where it increases hexokinase 2 (HK2) activity. This shunting toward the PPP in the mitochondria decreases TCA cycle activity, thus lowering ROS and protecting the cells from apoptosis. P53 has also been shown to inhibit the PPP through binding and repressing the rate-limiting enzyme, glucose-6-phosphate dehydrogenase (G6PD) (Jiang et al., 2011). This inhibition of the PPP would have the opposite effect, promoting the TCA cycle and oxidative phosphorylation. Conversely to what was seen in breast cancer cells, P53 actually has been shown to upregulate SCO2 expression, which in turn upregulates oxidative phosphorylation through the cytochrome c complex (COX) (Madan et al., 2011). SCO2 and COX are critical for the major site of oxygen utilization in the cell. P53 regulation of SCO2 has been shown to be the modulator of balance between respiratory pathways and glycolysis (Ma et al., 2007; Matoba et al., 2006). Other work has also shown that

loss of *P53* in malignancy is associated with increased mtDNA mutations, mitochondrial dysfunction, and direct regulation of COXII by P53 (Compton et al., 2011; Zhou et al., 2003). Additional studies have shown a direct regulation of monocarboxylate transporter (MCT1), the key lactate transporter, by P53 (Boidot et al., 2012). P53 directly binds the MCT1 promoter and inhibits lactate efflux under normal conditions. At the onset of hypoxia, P53 is lost and MCT1 expression increases, thus increasing lactate transport, and ultimately glycolytic flux. This was verified in human breast cancers, and MCT1 expression was associated with poorer prognosis. Further studies attempting to upregulate *P53* in tumors showed that pharmacologically activating *P53* significantly inhibited glycolysis and HIF1α and promoted cell death in tumors and breast cancer cells (Zawacka-Pankau et al., 2011).

P53 has also been shown to participate in regulation autophagy through DRAM as well as other indirect mechanisms (Abida and Gu, 2008; Crighton et al., 2006). As previously mentioned, autophagy is believed to be both oncogenic and tumor suppressive, and P53 has been shown to be both an activator and an inhibitor of autophagy (Tasdemir et al., 2008a; Tasdemir et al., 2008b). Interestingly, the pathways P53 upregulates autophagy through are different from the pathways through which it inhibits autophagy. Loss of *P53* has been shown to induced ER stress, which in turn promotes ER specific autophagy (reticulophagy) as party of the unfolded protein response (UPR) (Morselli et al., 2008; Tasdemir et al., 2008a). Since the presence of low oxygen and mitochondrial ROS have already been shown to induce the P53 stress response, it stands to reason that P53 is integral to repair oxidative stress and damage. The same pathways involved in P53-mediated autophagy and senescence are also key for the exertion of P53-dependent antioxidant mechanisms (Sablina et al., 2005; Yoon et al., 2004). The role of P53 in oxidative stress is highly complex, and examples have been shown where P53 can also be pro-oxidation through upregulation of BAX and PUMA, and thus induce senescence and

apoptosis (Macip et al., 2003). This regulation by P53 might regulate oxidative adaption between basal, low stress environments and high damage conditions.

With these major roles in oxidative phosphorylation and oxidative stress, it is expected that P53 also have important roles in mitochondrial quality and function. Initial studies examined ischemic damage in the heart, and found that loss of *P53* caused an increase in mitophagy, thereby decreasing the levels of damaged mitochondria, and increasing survival after myocardial infarction (Hoshino et al., 2012). They found that P53 and TIGAR were inhibited in response to ROS, which in turn activated BNIP3 and mitophagy. This is an example of mitophagy's ability to prevent oxidative damage in organs through degradation of faulty mitochondria, which has also been implicated as being mandatory for extended lactation in the mammary gland (Hadsell et al., 2011). Furthermore, an addition P53-inducible gene, MIEAP, has been shown to regulate mitochondrial quality and autophagy (Kitamura et al., 2011). In a similar mechanism to P53/TIGAR, ROS and BNIP3 promote mitochondrial quality control through upregulation of MIEAP induced mitophagy, and this effect can be ablated with inactivation of *P53*. These studies indicate that not only is P53 integral for genome quality and repair, but also the repair and maintenance of metabolic machinery as well.

More recent studies have elucidated another mechanism through which P53 helps to inhibit the Warburg effect and promote normal cell metabolic homeostasis: through interactions with NFκB signaling. NFκB signaling is an important regulator of immune and inflammatory responses, and its mis-regulation is known to be tumorigenic (Johnson and Perkins, 2012; Johnson et al., 2011). During tumorigenesis, NFκB subunit RELA translocates to the mitochondria where it inhibits mitochondrial gene expression, oxygen consumption, and ATP – thus promoting glycolysis. Expression of P53 however, blocks this effect by inhibiting RELA's interaction with the heat shock protein mortalin through competition and sequestration.

Interestingly, RELA has been shown to have varying effects depending on cellular context, and these effects are determined by P53. RelA expression in mouse embryo fibroblasts upregulates p53, thus enhancing OXPHOS and inhibiting glycolysis through upregulation of Sco2 (Mauro et al., 2011). Other work has shown that RELA upregulates GLUT3, increasing glucose uptake and lactate production (Kawauchi et al., 2008). In fact, this upregulation of GLUT3 is required for Ras-induced cell transformation. The cross-talk between NFκB and P53 adds an additional fine-tuning to metabolic homeostasis, and loss of this feedback can hyperactivate NFκB, and promote the Warburg effect. So far RELA is the major NFκB subunit that has been implicated in metabolic transformation, but there is potential crosstalk with other subunits as well.

P21

P21 in Cellular Senescence and Differentiation

One of the most well-known mechanisms of P53 action is through upregulation of P21 (CDKN1A), and promotion of cellular senescence. Senescence is an important mechanism for proliferation arrest in potential cancer cells, as well as tissue repair, inflammation, and aging. Originally described in the 1960's, senescence was described even then to be important for aging and tumor suppression (Hayflick, 1965). Hayflick's work found that normal diploid cells were not immortal *in vitro*, and could only undergo a set number of divisions, and this number varied with the cell type. Cellular senescence is classically described as the irreversible growth arrest that occurs when cells encounter an oncogenic insult. Almost all division-competent cells can undergo senescence when properly stimulated (Campisi and d'Adda di Fagagna, 2007). Characteristics are senescent cells are: flattened and enlarged cell size, expression of senescence-associated β-Galactosidase (SA-βGal), upregulation of *P16INK4a* and *P21* (Reddy and Li, 2011; Rodier and Campisi, 2011; Shay and Roninson, 2004). Senescent stimuli include DNA damage,

telomere erosion (aging), oncogenic insult, and epigenetic disruption (Rodier and Campisi, 2011). In addition, environmental stress can induce senescent without direct genomic insult. Serum starvation, oxidative stress, and other environmental factors have been indicted in senescence induction. These stimuli that induce senescence all have the ability to initiate or promote tumor growth, making cellular senescence one of the large tumor-suppressive pathways that must be overcome in oncogenesis (Reddy and Li, 2011; Rodier et al., 2007). Studies of human tissues and cancers have shown that cellular senescence is lost in most, if not all cancers. Also, research shows that early oncogenic transformation induces senescence (oncogene induced senescence, OIS), and this must be overcome through loss of *P53*, *CDKN1A*, and *P16* in order to tumors to develop (Figure 11).

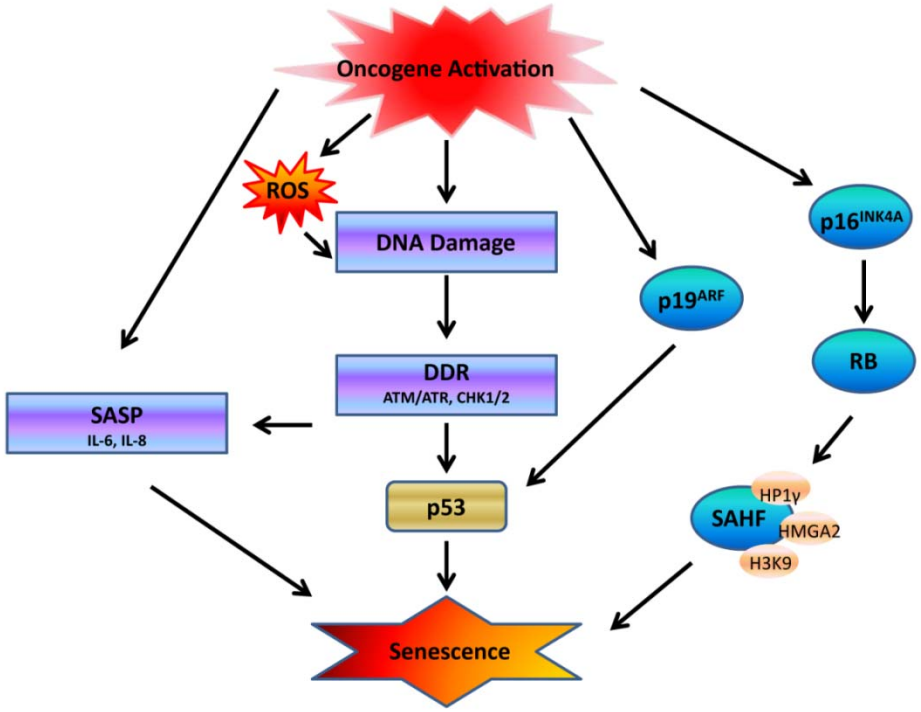

Figure 11. Oncogene Induced Senescence. Upregulation of senescence in response to initial oncogenic events occur four potential pathways.

Some tumor cells may retain the ability to senesce, and may do so in response to chemotherapeutics (Schmitt et al., 2002). As is seen in P53, metabolism, and autophagy, cellular senescence can also be tumorigenic in the appropriate context. As previously mentioned, senescence is largely induced and implicated in aging – and cancer is primary an age-related disease (Campisi et al., 2011). Not much work has been done to elucidate how senescence promotes late-life cancer, however some hypothesize that the senescence secretory phenotype induced the secretion of factors that can stimulate cancer cells (Kuilman and Peeper, 2009). For example, work has shown that factors secreted by senescent fibroblasts (amphiregulin, growth-related oncogene α, and interleukin 6) stimulated proliferation of premalignant epithelial cells (Bavik et al., 2006; Coppe et al., 2010).

While senescent cells are similar to terminally differentiated cells, they are not the same. However research has not clearly delineated the two. In fact, several studies have shown that P21 promotes differentiation in various tissues and circumstances. P21 is associated with a G1 arrest, and this arrest is frequently characterized in cellular differentiation (Steinman et al., 1994). Scientists found that P21 was induced by multiple differentiation signalers during hematopoietic differentiation, and this induction could occur independently of P53, and was coupled with other differentiation markers. P21 has also shown to be expressed in well-differentiated chondrosarcoma cells, and was associated with a better prognosis in these tumors (Hiraoka et al., 2002). In colorectal cancer cell lines, escape from P21-mediated OIS resulted in cell de-differentiation and enhanced survival (de Carne Trecesson et al., 2011). P21 and OIS have been established in many premalignant lesions as an important checkpoint to inhibit tumor progression (Reddy and Li, 2011). P21 has been indicated to promote differentiation in a myriad of other cells lines independently of P53, making it a powerful competitor for promotion of tumor differentiation.

In the mammary gland and breast cancer, P21 has already been established as a key barrier to tumor progression. Initial studies in MCF7 cells showed that oncogenic overexpression of HER2 induced senescence in a dose-dependent manner (Trost et al., 2005). A similar study actually showed that the co-expression of HER2 and senescence in breast epithelial cell lines induced a secretory phenotype that promoted metastasis (Angelini et al., 2013). In MDA.MB.231 cells, HER2 induced senescence promoted metastasis independently of cytokine attraction. Additional work in MCF7 cells showed that tumor-initiating cells survive through down-regulation of the senescence pathway (Karimi-Busheri et al., 2010). This enhanced survival allows for radiation resistance in mammosphere culture. Human pituitary tumor-transforming gene1 (hPTTG1) is an oncogene commonly overexpressed in breast cancer (Ruan et al., 2012). Overexpression of hPTTG1 in premalignant lesions induces oncogene induced senescence, and in order for hPTTG1 to exert its oncogenic effects this senescence must be overcome. Similar to the secretory phenotype of HER2 induced senescence, hPTTG1 induces a similar secretory senescence that can promote metastasis in neighboring senescence-evading cells, hypothetically through development of a metastasis-promoting microenvironment. hPTTG1 expression induces senescence through P53-independent activation of *CDKN1A*, and early stage senescence inhibits cancer cell growth through a P21-CXCR2 axis. Other studies in MCF7 and MDA.MB.231 cells elucidated a unique set of P53-dependent and independent mechanisms for P21 expression and senescence (Wang et al., 1999). Wang et al. found that UV radiation down-regulates *CDKN1A* in cancer cells regardless of P53 expression. Conversely, x-ray exposure up-regulated P21 through P53. MCF7 is a P53 competent cell line, whereas MDA.MB.231 is a P53 mutant. Variations were seen in both cell lines, *CDKN1A* was down-regulated more gradually in MCF7 cells compared to MDA.MB.231, indicating that P53 potentially still regulated *CDKN1A* gene expression, albeit not in a dose-dependent manner.

This loss of *CDKN1A* with UV radiation also suggests a role for P21 in DNA-damage response aside from cell cycle inhibition. Other studies have shown the opposite, up-regulation of P21 in response to UV radiation, but these studies are either in other cell lines or another type of UV-radiation, indicating a complex response pathway than can adapt to unique insults (Zhan et al., 1996). In vivo studies in P53 null mice have shown the induction of OIS through P16 in ductal hyperplasias with the overexpression of mitotic oncogenes (Zhang et al., 2008). Ras-driven breast cancer also induced OIS, but only with high levels of Ras expression (Sarkisian et al., 2007). In this model, OIS has to be circumvented in order for tumor progression to proceed, and the authors hypothesized this occurred with later inactivation of *P53*. Later work confirmed this hypothesis (Borgdorff et al., 2010; Swarbrick et al., 2008). After Ras expressing mammary epithelial cells (MECs) form premalignant lesions with OIS, few progressed to tumors; however selective inactivation of *P19*, *P53*, or *CDKN1A* (but not p16) significantly increased progression. Human DCIS samples have also been analyzed for a relationship between senescence and progression (Gauthier et al., 2007; Kerlikowske et al., 2010). P16 induction in DCIS samples correlated with low proliferation and lower changes of progression to invasion. DCIS samples with *P16* defects, however, have high rates of tumor progression. Another master regulator, C/EBPβ, has also been implicated in breast cancer and senescence (LaMarca et al., 2010). Loss of C/*EBPβ* in MECs decreased the stem cell population, and caused premature senescence and differentiation. This resulted in decreased growth and transplant success both *in vivo* and *in vitro*. As previously discussed, our lab has shown that C/EBPβ suppresses *SIM2s* expression. It is possible that this upregulation of senescence with loss of C/*EBPβ* is due to subsequent upregulation of SIM2s. The implications of senescence in regards to DNA damage, early malignancy, cytokine attraction, and modes of activation indicate an intricate pathway in breast cancer with multiple factors needing to be addressed for successful tumorigenesis.

P21 in Metabolism

The role of P21 and senescence in the Warburg effect and metabolism has not been well researched. As previously discussed, mitochondria have been established as involved in aging, and aging with the onset of senescence, however to date senescence and metabolism have often been viewed as two separate mechanisms. Metabolomic analysis of oncogene induced senescence showed a unique metabolic phenotype that differs from normal cells and replicative senescing cells (Quijano et al., 2012). OIS cells have increased fatty-acid oxidation (a mitochondrial metabolic activity), that correlated with a high rate of oxygen consumption, indicating enhance mitochondrial function with senescence. It is this fatty acid metabolism that may be related to the unique inflammatory phenotype previously discussed. This study also provided a unique differentiation between types of cellular senescence, which are normally viewed as equivalent pathways. Other studies have analyzed the role of mitochondrial dysfunction in the onset of senescence (Masgras et al., 2012; Moiseeva et al., 2009). Ras-induced senescence was shown to be preceded by an increase in mitochondria, ROS, and a drop in ATP production. This response depended on intact P53 and RB, indicating a causal tie between metabolic deregulation and senescence. This was verified through direct pharmacological inhibition of oxidative phosphorylation, which was sufficient to trigger senescence. Masgras et al. (2012) showed that senescence itself cause an upregulation in mitochondrial ROS, dependent on *CDKN1A* expression. Interestingly, the senescent response could be abrogated by exposure to antioxidants, and P21 expression in cancer cells correlated with increased sensitivity to oxidants and mitochondrial polarizations changes. Early analysis of metabolic differences in human fibroblasts showed that senescent fibroblasts has lower levels of glycolysis compared to non-senescent cells (Zwerschke et al., 2003). The authors indicated that as fibroblasts aged, depletion of glycolytic enzymes resulted in a metabolic imbalance, decreased

ATP, and subsequently increased AMP, which induced further senescence. This was the first study establishing a causal relationship between metabolic balance and senescence. In tumor cells, inhibition of the PPP has been shown to induce senescence through accumulation of growth inhibitory glucose intermediates (Sukhatme and Chan, 2012). This inhibition and senescence is associated with increased oxygen consumption and mitochondrial activity in glutamate metabolism. While this initial metabolic shift in cancer cells is to try to accommodate G6PD loss, the ultimate induction of senescence inhibits proliferation. Other mitochondrial specific enzymes, specifically the TCA cycle associate malic enzymes, have been shown to regulate *P53* expression and senescence (Jiang et al., 2013). Down regulation of malic enzymes activates *P53*, generating a positive-feedback look between the two as P53 expression represses malic enzymes. This upregulation of P53 correlates with a strong induction of senescence, indicating a direct relationship between metabolic homeostasis and senescence. In addition to glucose metabolite accumulation, glycogen accumulation in cancer cells has also been associated with premature senescence (Favaro et al., 2012). Glycogen metabolism has been shown to be important during hypoxia for cell survival and metabolic reprogramming (Pelletier et al., 2012; Pescador et al., 2010). Favaro et al. found that inhibition of glycogen depletion through knockdown of glycogen phosphorylase (PYGL) resulted in an accumulation of glycogen, corresponding with reduced proliferation and senescence. This senescent phenotype could be partly abrogated through co-depletion of *P53* or addition of an antioxidant, indicating this senescence occurs through ROS-mediation of *P53* expression. This depletion of *PYGL* also leads to a decrease in the PPP, which we previously discussed as causal in cellular senescence as well. While all of these studies indicate a unique and important relationship between metabolism and senescence, to date senescence is evaluated as a response to metabolic insult, and not the inducer of a unique metabolic phenotype.

The unique relationship between differentiation, metabolism, aging, and cancer is complex. While work has been done in all of these areas individually, little work has been performed examining the causal relationships between the four and genes/pathways that may be integral to this cascade of events that result in cancer. Understanding the intricacies of these pathways will enhance our abilities to effectively target tumors, as well as prevent early tumor progression. In the work discussed herein, we seek to test the hypothesis that SIM2s' promotion of differentiation and inhibition of tumor progression are through induction of cellular senescence and a metabolic shift from aerobic glycolysis to oxidative phosphorylation.

CHAPTER II

MATERIALS AND METHODS

In Vitro

Cell Culture

MCF10DCIS cells were generously provided by Dr. Dan Medina (Baylor College of Medicine, Houston, TX, USA) and maintained in DMEM-F12 (Invitrogen, Carlsbad, CA, USA) with 10% horse serum (Atlanta Biologicals, Lawrenceville, GA, USA). SUM159 cells were purchased from Asterand (Asterand, Detroit, MI, USA) and grown in Ham's F12 (Invitrogen) with 5% fetal bovine serum (Atlanta Biologicals) with insulin and hydrocortisone (Sigma Aldrich, St. Louis, MO, USA). HEK-293T Ampho-Phoenix packaging cells were obtained with permission from Gary Nolan at Stanford University and DMEM (Invitrogen) with 10% fetal bovine serum (Atlanta Biologicals). MCF7 cells were obtained from ATCC and maintained in DMEM (Invitrogen) with 10% fetal bovine serum (Atlanta Biologicals). All cells were maintained in 5% CO_2 at 37˚C.

Plasmids and Lentiviral Transductions

Lentiviral transduction of MCF10DCIS.com, MCF7, and SUM159 cells was performed as previously described (Kwak et al., 2007). Lentiviral transduction, SIM2s shRNA and SIM2s overexpression plasmids have been previously described (Gustafson et al., 2009b; Kwak et al., 2007; Laffin et al., 2008). Retroviral plasmids were transfected into 293T Viral Packaging Cells, which stably express amphotrophic envelope proteins and are referred to as 293-Ampho. Up to 10 μg of plasmid was transfected using Gene Juice (Novagen, Merck, Darmstadt, Germany), in a 3:1 ratio of Gene Juice (μL) to DNA (μg). Media was changed 24 hours later, and collected for

infection 48 hours and 72 hours later. Viral media was filtered through 0.45 μM syringe filters and polybrene was added to a final concentrations of 4 μg/mL. Viral media was added to target cells, followed by centrifugation at 1200 rpm for 1 hour. Target cells were incubated at 32^0C 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 (.4μM-MCF10DCIS.com, .4μM-MCF7, 1μM-SUM159). Selection was considered complete when all cells in a mock-infected plate were dead.

Transient Transfections Using siRNA

CDKN1A and Negative Control siRNA was purchased from Invitrogen. siRNA transfection was performed using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's protocol, and cells were analyzed 48 hours after siRNA treatment.

Invasion Assays

Invasion was measured using control and Matrigel-coated invasion chambers (Falcon BD, Franklin Lakes, NJ, USA). A total of 12,500 cells were seeded in serum-free Dulbecco's modified Eagle's medium (DMEM-F12, Invitrogen) in the upper chamber, with serum-containing medium in the lower chamber as a chemo attractant. After 18 hours at 37 °C, cells were scraped from the upper chamber with a cotton swab, and the undersides of the membranes were fixed in 3.8% paraformaldehyde (Sigma, St Louis, MO, USA), stained with DAPI (4′,6′-diamidino-2-phenylindole) (Invitrogen) and counted. The percent invasion was calculated according to the manufacturer's instructions. All experiments were done in triplicate.

Proliferation Assays

Proliferation was measured using a Coulter particle counter. A total of 15,000 cells of each transduction were plated in triplicates on six-well plates, and then every 24 hours the cells were trypsinized and counted in triplicate. The procedure continued for 6 days, or until the cells reached 100% confluency. Cells were imaged in 6 well plates on day 4 of proliferation using an Olympus IX71 inverted fluorescent microscope using Olympus CellSens Software (Olympus, Center Valley, PA, USA).

In Vivo

MMTV-Sim2s Mice

The transgenic mice used in this study were described previously (Wellberg et al., 2010). An MMTV-KCR cassette was used with the *Sim2s* coding sequence to overexpress Sim2s in the mammary gland of FVB mice. All procedures were approved and followed the guidelines set forth by the Texas A&M University Animal Use and Care Committee.

Animals

For involution studies pups were removed from both WT and MMTV-*Sim2s* mice at lactation day 10. Tissue was harvested at 24, 48, and 72 hours after weaning. The fourth inguinal mammary glands were used for histological sectioning, RNA isolation, and protein isolation. Litters were normalized to eight pups at parturition. All animals were housed with litter under a standard 12-hour photoperiod. Males were analyzed for genetic orientation prior to mating (Klar, 2003; Rahman et al., 2009). The animals were given access to food and water ad libitum. Three mice per genotype were analyzed for each time point, and transgene expression was confirmed before further experimentation. Procedures were approved by the University Laboratory Animal Care Committee at Texas A&M University.

Xenograft Studies

For xenograft studies, MCF10DCIS.com cells (50,000) were injected subcutaneously into 8- to 14-week-old female nude mice in 50% Matrigel (BD Biosciences, Bedford, MA, USA). Tumors were allowed to grow for 18 days, and were measured using calipers starting on day 13. Xenografts were weighed at harvest and either snap-frozen in liquid nitrogen and stored at −80 °C for DNA/RNA purification or formalin-fixed and paraffin-embedded. Three tumors for each variable were taken for RNA, and three for histochemical analysis. Animal experiments were conducted following protocols approved by the Texas A&M Animal Care and Use Committee.

Metabolic

Mitochondrial Bioenergetics

Seahorse analysis was performed as previously described (Meerbrey et al., 2011). Briefly, cells were seeded at 10,000 cells/well and allowed to adhere overnight. Mitochondrial bioenergetic analysis was performed using a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Massachusetts, USA). Cells were exposed to mitochondrial drugs oligomycin (30 μM), FCCP (10 μM), and rotenone (50 μM) (Sigma Aldrich).

NMR Spectroscopy

Samples for NMR spectroscopy were obtained in triplicate from cell cultures. Cells were seeded at 80% density and allowed to grow for 24 hours, then media was collected and centrifuged at 15000xg for 10 minutes. Before NMR analysis, 10%(v/v) D2O was added to all media samples. NMR spectra were measured on a 400 MHz spectrometer (Bruker, Billerica, MA, USA) equipped with a 5 mm triple resonance (TXI) probe. The spectra were measured with 128 scans, a spectral width of 15 ppm and an acquisition time of 1.36 s, with 13C decoupling

(decoupling bandwidth of 9600 Hz). Measurement temperature was 298 K. NMR data were processed and peaks were integrated using Bruker Topspin 3.0. Chemical shifts were referenced indirectly to 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) signal at 0 ppm. The concentrations of lactate in different samples were determined by comparing the signal integrals from the media samples to those of a standard sample of 20mM lactate. The concentrations of glucose remaining in the media samples were determined by comparing the signal integrals to that of unused control media. For each sample, 1H pulse lengths were measured, and based on these parameters the concentrations obtained were corrected for differences in sensitivity.

DCFH Assay

Cellular ROS was measured as previously described (Eruslanov and Kusmartsev, 2010). Briefly, Cells were seeded at 10,000 cells per well in a 9-well plate 12 hours prior to experimentation. Cells were washed with 1xPBS and incubated with 2.5μM CM-H_2DCFDA (Invitrogen) for 30 minutes. Cells were allowed to recover for 15 minutes and then fluorescence was measure in a fluorimetric plate reader at 495 nm excitation/521 nm emission.

ATP-lite Analysis

Intracellular ATP analysis was performed using the ATP-lite luminescence detection assay according to manufacturer's protocol (Perkin-Elmer, Waltham, Massachusetts, USA). Briefly, cells were seeded at a density of 10,000 cells per well in a 96-well plate. Cells were adhered for twelve hours then exposed to the compounds of interest (See Reagents). ATP was measured 24 hours post treatment on a chemiluminescent plate reader. Protein concentrations of cells were determined for signal normalization.

Lactate Measurements

Extracellular lactate analysis was measured using the Lactate Assay Kit according to manufacturer's protocol (Sigma-Aldrich). Briefly, spent media from cells in 6-well plates was harvested and spun through a molecular weight cut off column at 10kD (EMD Millipore, Billerica, Massachusetts, USA) for 30 minutes to remove lactate dehydrogenase. Flow through was collected. 5µl of media was used per sample. Experimental triplicate samples were analyzed in duplicate. Lactate was measured using a fluorimetric plate reader at 535nm excitation/567nm emission.

Mitochondria Isolation

All chemicals were purchased from Sigma-Aldrich. Mitochondria were isolated using the Abcam Mitochondrial purification protocol (MitoSciences, Eugene, Oregon, USA). Briefly, cells were collected by centrifugation (370xg for 10min) and resuspended in NKM buffer (1mM Tris-HCl pH 7.4, .13M NaCl, 5mM KCl, 7.5mM $MgCl_2$), and washed in NKM buffer 3 times. Cells were then resuspended in 6 cell volumes of homogenization buffer (10mM Tris-HCl, pH 6.7, 10mM KCl, .15mM $MgCl_2$, 1mM PMSF, 1mM DTT) and incubated on ice for 10 minutes. Using a glass homogenizer, cells were homogenized for 30 strokes or until cell breakage was approximately %60. Homogenate was mixed with a 2M sucrose solution and mixed gently. Cellular debris was pelleted through centrifugation (1200xg for 5min) and supernatant was removed, containing mitochondria. Mitochondria was pelleted through centrifugation at 7000xg for 10 minutes, and then resuspended in mitochondrial suspension buffer (10mM Tris-HCl pH 6.7, .15mM $MgCl_2$, .2M sucrose, 1mM PMSF, 1mM DTT). Mitochondria were frozen at -20^0C until use.

Microscopy

Electron Microscopy

Cells were fixed in 2% glutaraldehyde, 2.5% formaldehyde in sodium cacodylate buffer for 24 hours, then washed in 0.1 M sodium cacodylate buffer (Sigma-Aldrich). Briefly, the buffer-washed cells were stained en bloc with 1% osmium tetroxide and 0.5% potassium ferrocyanide then dehydrated in an ascending alcohol series and embedded in epoxy resin. Ultrathin sections were examined with an FEI Morgagni 268 transmission electron microscope operating at an accelerating voltage of 80 kV (FEI, Hilssboro, OR, USA). Digital images of were acquired with a MegaViewIII camera operated with iTEM software (Olympus Soft Imaging Systems). Work was performed in the Texas A&M University College of Veterinary medicine & Biomedical Sciences Image Analysis Laboratory.

Live Cell Imaging

Cells were grown on coverslip chamber slides (Nunc) for 12 hours before experimentation. Cells were incubated with MitoTracker Deep Red (100nM) or LysoTracker (200nM) (Invitrogen) for 30 minutes at 37˚C. Images were acquired with a 63x oil plan-apochromat objective. Lysotracker images were taken at 488nm excitation and 500-550nm emission. Mitotracker deep red images were taken at 633nm excitation and 565-615nm emission. Image area was 142.9µm x 142.9µm. Images were taken on a Zeiss 510 confocal microscope (Carl Zeiss Microscopy, Jena, Germany). At least 10 images per specimen were collected for analysis. Quantification of these images was done using Adobe Photoshop by analyzing the mean histogram of color specific channels.

B-Galactosidase Staining

Senescence was determined by β-Galactosidase Staining Kit (Cell Signaling, Beverly, Massachusetts, USA) according to manufacturer's protocol. Cells were grown and stained on coverslips and then imaged on the Zeiss AxioImager.v1 using a 40x objective lens (25.2x magnification) using Zeiss Axiovision software (Carl Zeiss Microscopy).

Immunohistochemical Analysis

Immunostaining was carried out as previously described (Kwak et al., 2007). Samples were incubated in blocking solution for 1 h, followed by incubation in primary antibody overnight at 4 °C. Antibodies used with dilution and antigen retrieval information are included in Table 2 and Table 3. Tissue preparation and hematoxylin and eosin staining were carried out by the Histology Core Facility at Texas A&M University College of Veterinary Medicine and Biomedical Sciences. Immunostaining for SIM2s was performed on DCIS and IDC tissue sections provided by the University of Kansas Cancer Center Biospecimen Share Resources at the University of Kansas Medical Center. Statistical analysis of positive staining was performed using the ImageJ (National Institutes of Health, Bethesda, MD) cell-counting feature and has been previously described (Koodie et al., 2010; Stockmann et al., 2008). Images were taken on a Zeiss AxioImager .v1 with a 10x (6.3x) and 40x oil objective lens (25.2x magnification) using Zeiss Axiovision software (Carl Zeiss Microscopy).

Table 2. Immunohistochemical Antibodies

Target	Source	Dilution	Ag Retrieval
CDH1	Cell Signaling	1:2000	5min high pressure
Cleaved Caspase 3	Cell Signaling	1:100	5min high pressure
GLUT1	Cell Signaling	1:250	5min high pressure
HK2	Cell Signaling	1:250	5min high pressure
KER14	Covance	1:250	5min high pressure
KER18	Neomarkers	1:500	5min high pressure
KER5	Covance	1:250	5min high pressure
Mucin-1	Neomarkers	1:250	5min high pressure
p21	Cell Signaling	1:100	5min high pressure
p63	Neomarkers	1:250	5min high pressure
PECAM-1	Santa Cruz	1:250	5min high pressure
Perilipin	Cell Signaling	1:250	5min high pressure
Phopho-STAT3	Cell Signaling	1:250	5min high pressure
SIM2	Millipore	1:100	5min high pressure
SIM2s	Santa Cruz	1:250	5min high pressure
SMA	Sigma	1:1200	5min high pressure
STAT3	Cell Signaling	1:200	5min high pressure
Vimentin	Sigma	1:1000	5min high pressure
β-Casein	Santa Cruz	1:100	5min high pressure

Table 3. Immunofluorescent Antibodies

Target	Source	Dilution	Ag Retrieval
SIM2s	Santa Cruz	1:500	5min high pressure
VIM	Covance	1:1000	5min high pressure
KER5	NeoMarkers	1:500	5min high pressure

RNA

RNA Isolation from Cells

Cells were washed with PBS and RNA was isolated using the High Pure RNA Isolation Kit (Roche Applied Science, Indianapolis, Indiana, USA) following the manufacturer's protocol for spin isolation, including DNase digestion of RNA contaminants. RNA was eluted in 30 to 50µL RNAse-free H2O and stored at -80 C.

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 H2O. Rehydrated RNA was subjected to purification according to that described in the previous section for RNA isolation from mammalian cells.

Reverse Transcription

For cDNA synthesis, depending on the RNA concentration, 1-2 µg of RNA was used for reverse transcription reactions. cDNA was formed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) according to the manufacturer's protocols. One µL of each 10 mM dNTPs and Oligo dT were 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 Inhibitor, and 1 µL Superscript Reverse Transcriptase 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. These samples were then used for Q-PCR Analysis.

PCR

For chromatin immunoprecipitation, DNA samples were analyzed via PCR to determine binding at response elements on the proximal promoter of *CDKN1A* (P21) as previously described (Wellberg et al., 2010). Briefly, 2µl of DNA were combined with 2.5 µl 10X Taq Buffer (Invitrogen), 1 µl 50mM $MgCl_2$ (Invitrogen), 1 µL 10mM dNTPs (Invitrogen), 2.5µL Primers (See Table 4) (Sigma-Aldrich), .25 µl Taq polymerase (Invitrogen), and water for a total reaction volume of 25 µl. PCR reactions were run on a thermal cycler for 33 cycles with an annealing temperature of 60^0C. Products were analyzed by electrophoresis on an 8% agarose gel with ethidium bromide (Sigma-Aldrich).

Table 4. ChIP RT-PCR Primers

Gene	Sense	Anti-sense
CDKN1A TATAA Box	TCTAGGTGCCCAGGTGCTT	ACATTCCCCACGAAGTGAG
CDKN1A 3'p53RE	CCAGGTCTTGGATTGAGGAA	TGTTAAGGTGGTGGCATTGA

Quantitative Real-time PCR

One µL of each cDNA sample were mixed with 5.0 µL 2x SyberGreen master mix (Roche Applied Science), 3 µL H2O, and 1 µL of both sense and antisense primers (Tables 5 and 6) and added to a 384 well plate (Applied Biosystems). Reactions were run according to the following cycle conditions: 95^0C for 10 minutes, and 40 cycles of 95^0C for 10 seconds followed by 60^0C for 1 minute. Analysis was performed using the ΔΔCT method as previously described (Hettinger et al., 2001). For mouse mammary tissue samples, expression of Claudin 7 was used

to normalize mRNA levels of assayed genes (Blackman et al. 2005). For cell culture, expression of TBP and/or β-Actin was used to normalize mRNA levels.

Table 5. Human Q-PCR Primers

Gene	Sense	Anti-sense
ACO2	TGTCACGTCCCCAGAGATTG	CGGTCTCTGGGTTGAACTTGA
AKT	GGGCGAGCTGTTTTTCCAT	TGTAGATAGTCCAAGGCAGAGACAA
B2Globulin	GGCTGGCAACTTAGAG	GCCTTACTTTATCAAATGTAT
B-Actin	GCAACGAGCGGTTCCG	CCCAAGAAGGAAGGCTGGA
BNIP3	TTCCCCCCAAGGAGTTCCT	CGCTCGTGTTCCTCATGCT
CDH1	CACAGACGCGGACGATGAT	GATCTTGGCTGAGGATGGTGTAA
CDKN1A	CCTAATCCGCCCACAGGAA	AAGATGTAGAGCGGGCCTTTG
CHOP	AGAACCAGGAAACGGAAACAGA	TTCATGCGCTGCTTTCCA
CSN2	TGTGCTCCAGGCTAAAGTTCACT	GGTTTGAGCCTGAGCATATGG
G6PD	GCCTTCTGCCCGAAAACAC	TGCGGATGTCAGCCACTGT
GATA3	CTG-GCT-CGC-AGA-ATT-GCA	AAC-TGG-GTA-TGG-CAG-AAT-AAA-ACG
GLUT1	CAGCTGACGTGACCCATGAC	CCTTCTTCTCCCGCATCATC
GLUT3	GACTCTTCGTCAACCGCTTTG	TGACAGCCAACAGGTTGACAA
GLUT4	GCTTCGTGGCATTTTTTGAGA	AGCTCGGCCACGATGAAC
HK1	TCTTATTTGAAGGGCGGATCA	TTTCGATGGCTGACACATCAC
HK2	GCATCTTTGAAACCAAGTTCTTGTC	GGTGCTCTCAAGCCCTAAGTG
KER18	GAGGCTGAGATCGCCACCTA	CCAAGGCATCACCAAGATTAAAG
LC3	GTGAACCAGCACAGCATGGT	CGTCTTTCTCCTGCTCGTAGATG
LDHA	AAATTGAAGGGAGAGATGATGGAT	AGTTACATTATAGTCTTTGCCAGAGA
MDH2	GGAGTGGCCGCAGATCTG	TCAGGTCCGAGGTAGCCTTTC
MFN1	TTCTACTCCCACTGCTCCTACCA	TCATGAGTTCTTCCTGTGATGCA
MFN2	ACCATGCAGCAGGACATGATAG	GACTCCGCACAGACACAGGAA
MTOR	AGGCGGCATTGTCTCTATCAA	GCAGTAAATGCAGGTAGTCATCCA
OPA1	CTGTGGATGCTGAACGCAGTA	CTCCTTCCATGAGGGTCCATT
P63	CCT-TCT-GTG-AGC-AG-CTT-ATC-A	CAT-CAG-GAA-TGG-TTG-TAG-GAG-TGA
PARKIN	TCCCAGTGGAGGTCGATTCT	GCTTAGCAACCACCTCCTTGA
PGC1a	TGTCACCACCCAAATCCTTATTT	TGTGTCGAGAAAAGGACCTTGA
PINK1	GGACACGAGACGCTTGCA	CTTACCAATGGACTGCCCTATCA
SCO2	CTTCACTCACTGCCCTGACA	TGAGCAGGTAGATGGCAATG
SIM2s	GCTGAGAACAAACCCTTACC	GAAGCAGAAAGAGGGCAAGTT
SLUG	GGCTGGCCAAACATAAGCA	CTGCAAATACTGCAACAAGGAATAC
SMA	CAA-GTG-ATC-ACC-ATC-GGA-AAT-G	AGC-AA-CTC-CAT-CCC-GAT-GA
SMO	CAC-CCT-GGC-CAC-ATT-CGT	CGC-ATT-GAC-GTA-GAA-GAG-AAT-AAC-A
SOD2	TTGGCCAAGGGAGATGTTACA	TGATATGACCACCACCATTGAAC
TBP	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA
TFAM	GTGCACCGGCTGTGGAA	TGAAAACCACCTCGGTAAATACAC
TIGAR	CTCCAGTGATCTCATGAG	AGACACTGGCTGCTAATC
TSC2	AGCTCTACCATTCCCCCTTCTT	CTGCACCGACCGCTCAA
VIM	TTCTCTGCCTCTTCCAAACTTTTC	GGGTATCAACCAGAGGGAGTGA
XBP1	GTGAAGGAAGAACCTGTAGAAGATGA	GGGCAGTGGCTGGATGAA

Table 6. Mouse Q-PCR Primers

Gene	Sense	Anti-sense
CEBP/D	CGC-CGC-AAC-CAG-AT	GCT-GAT-GCA-GCT-TCT-CGT-TCT
Claudin 7	TCCCTGGTGTTGGGCTTCT	ACAGCGTGTGCACTTCATG
CSN2	TGTGCTCCAGGCTAAAGTTCACT	GGTTTGAGCCTGAGCATATGG
GAPDH	CTA-ACA-CA-AAT-GGG-GTG-AGG	TCA-TAC-TTG-GCA-GGT-TTC-TCC
IGFBP5	GAT-GAG-ACA-GGA-ATC-CGA-ACA-AG	TTG-AAC-TCC-TGG-AGG-GAA-GCT
IKK2	CAG-CGA-GCA-GCC-ATG-ATG	GGA-GGC-CAT-GGC-GTT-CT
KER18	CATCGTCTTGCAGATCGACAA	GACTGGCGCATGGCTAGTTC
NFKB1	GCC-GTG-GAG-TAC-GAC-AAC-ATC	TGT-CCA-CGT-GGG-CAT-CAC
NFKB2	GGG-CAG-ACT-GGT-GTC-ATT-GA	GGT-TGA-TGA-CGC-CGA-GGT-A
RELA	GCC-CAT-GGA-GTT-CCA-GTA-CTT-G	GTC-CTT-TTG-CGC-TTC-TCT-TCA
SIM2s	AACCAGCTCCCGTGTTTGAC	ACTCTGAGGAACGGCGAAAA
STAT3	GGA-GTA-CGT-GCA-GAA-GAC-ACT-GA	TCC-GAT-GCA-GCG-GAT-CT
TBP	TGC ACA GGA GCC AAG AGT GAA	CAC ATC ACA GCT CCC CAC CA

Protein

Protein Isolation from Cells

Cells were washed once with PBS and scraped in PBS containing 25x protease inhibitor cocktail (Roche Applied Science). Cells were pelleted by spinning in a pre-cooled Eppendorf centrifuge at 2000 rpm for 4 minutes. 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 4^0C for 30 minutes. Debris was pelleted by spinning in a cooled Eppendorf centrifuge at 16.1K X g for 10 minutes. Aliquots of 200 µL were stored at -20^0C. If used immediately, they were stored at 4^0C. Protein content was estimated using the RCDC Protein Assay (BioRad, Hercules, CA, USA) according to the manufacturer's protocol.

Western Blot Analysis

Protein samples were diluted in 30 µL of H2O per sample. 6 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) was added and samples were boiled for 5 minutes, followed by 5 minutes 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 hours and transferred to PVDF membranes for 1.5 to 3 hours at 110mA (constant mA). After a 5 minute wash in PBS + 0.05 % Tween 20 (PBST), membranes were blocked for 1 hour or overnight in PBST + 5 % milk (BioRad). See Table 7 for antibody sources and incubation conditions. Proteins were visualized using the Amersham ECL Plus western blotting detection reagent (GE Healthcare) on Amersham Hyperfilm (GE Healthcare, Waukesha, WI, USA). All films were scanned using an HP All-in-one scanner.

Table 7. Western Blot Antibodies

Target	Source	Catalog #	Dilution
Actin	Sigma	A5441	1:5000
p21	Cell Signaling	2947	1:1000
LC3B	Cell Signaling	3868	1:4000
Total OXPHOS Cocktail	MitoSciences	MS601	1:100

Microarray Analysis

Gene Expression Microarray

The Whole Mouse Genome CodeLink Bioarray (Amersham, GE Healthcare, Piscataway, NJ) was used to determine differential gene expression between WT and MMTV-Sim2s transgenic females at 72 hours of involution. Sample preparation and hybridization were performed according to the manufacturer's protocols and as previously described (Davidson et

al., 2004) . RNA samples were confirmed for *Sim2s* overexpression via qPCR before microarray analysis.

The Whole Human Genome CodeLink Bioarray (Applied Microarrays, Tempe, AZ, USA) was used to determine differential gene expression between Scr and *SIM2si* MCF7 breast cancer cells. Sample preparation and hybridization were performed according to the manufacturer's protocols and as previously described (Davidson et al., 2004). RNA samples were confirmed for loss of *SIM2s* expression via qPCR prior to microarray analysis.

Microarray Analysis

Raw microarray data were initially normalized and analyzed using CodeLink Expression Analysis Software version 4.1.0.29054 (GE Healthcare). A median normalization method was used, with a 20% threshold trim percentage. Microarray data were analyzed, and functionally analyses were generated through the use of Ingenuity Pathways Analysis (Ingenuity Systems. Redwood City, CA, USA). A data set containing gene identifiers and corresponding expression values was uploaded into the application. The identifiers were mapped to their corresponding objects in Ingenuity's Knowledge Base. A P value cutoff of 0.05 was set to identify the expression of which pathway was significantly differentially regulated. These molecules were integrated into a molecular network developed from information in Ingenuity's Knowledge Base. The data were then analyzed for the biological functions and/or diseases that were most significant.

Accession Numbers

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE44187(MCF7 array) and GSE27012 (Involution) (Edgar et al., 2002).

Mechanistic Assays

Luciferase Promoter Assay

CDKN1A-Luciferase constructs were obtained from Dr. Zhi-Min Yuan (Harvard School of Public Health). Luciferase assays were performed as previously described (Yuan et al., 1996). Briefly, MCF7 breast cancer cells were transiently transfected with a *CDKN1A*-luciferase construct, then exposed to increasing amounts of SIM2s protein. Luciferase was measured in a luciferase plate reader as an indicator of gene expression.

Chromatin Immunoprecipitation Assay (ChIP)

Chromatin Harvest from Cells

Formaldehyde (Sigma Aldrich) was added to fresh culture media (final concentration 1%) and cells were incubated at room temperature (RT) for 10 minutes with gentle rocking. Glycine (Sigma Aldrich) was added to a final concentration of 125 mM and allowed to quench formaldeyhde for 5 additional minutes at RT. Cells were washed 2 times with ice cold PBS and scraped in cold PBS containing 25x Complete protease inhibitors (Roche Applied Science). 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 minutes. Using a sonicator (Heat Systems Ultrasonics Inc.), DNA was sheared in 20 second pulses, 16 times, allowing the lysate to cool on wet ice for 1 minute after every pulse. Debris was pelleted by spinning at 13,200 rpm (16.1K x g) in an Eppendorf 5415D centrifuge for 10 minutes at 4˚C. Chromatin was stored at -80˚C in 100 µL aliquots.

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. 100µL of normal rabbit serum (5 µL Santa Cruz) conjugated Protein A Dynabeads (Invitrogen) was added for 1 hour and chromatin was precleared at 4^0C with agitation. Beads were pelleted by magnet and lysate was precleared once more with 100 µl magnetic beads. Antibody conjugated beads (4µg SIM2 Rbd antibody, Millipore) were added and chromatin was agitated at 4^0C overnight. Beads were pelleted and washed consecutively 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 4^0C. Immune complexes were eluted from beads in 1 % SDS and 0.1 M NaHCO3, adding 250µL 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 65^0C for 5 hours to reverse formaldehyde crosslinks. Two and one half volumes of 100 % EtOH were added to each sample and they were placed at -20^0C overnight. On the third day, chromatin was pelleted by spinning in an Eppendorf centrifuge at 16.1K x g for 10 minutes. Supernatant was removed and the pellet resuspended in 100 µL H2O, 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 Aldrich). Samples were incubated at 45 C for 2 h, and then purified using a Qiagen PCR purification kit (Qiagen). DNA was eluted in 50 µL elution buffer (supplied with kit). PCR was performed according to conditions listed above.

Miscellaneous

Statistical Analyses

All differences were analyzed with Student's t test unless otherwise specified. Student's T-Tests were performed in Microsoft excel. A p-value less than 0.05 was considered to be statistically significant. For involution samples Fischer's exact test was also used to test for significance. Tissue microarrays and pathology reports were analyzed using categorical analysis accompanied with the Chi^2 Test of homogeneity with Likelihood Ratio and Pearson Tests. Tissue microarray analysis and Fischer's exact tests were performed using JMP statistical software (JMP, SAS Cary, North Carolina, US). Microarray heat maps were generated using R statistical software (R, GNU project).

Reagents

Cells were treated with rotenone (1μM–24hrs), oligomycin (2μM-24hrs) and 2-deoxy glucose (2-DG, 10mM-24hrs) (Sigma-Aldrich) for ATP and ROS assays. Cells were treated with rotenone (50μM), oligomycin (30μM), and carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP, 10μM) (Sigma-Aldrich) for seahorse flux analysis. All compounds were reconstituted in DMSO (Sigma-Aldrich).

CHAPTER III

MANUSCRIPT 1: SINGLEMINDED-2S (SIM2S) PROMOTES DELAYED INVOLUTION OF THE MOUSE MAMMARY GLAND THROUGH SUPPRESSION OF STAT3 AND NFκB[*]

Synopsis

Post-lactational involution of the mammary gland following lactation provides a unique model to study breast cancer susceptibility and metastasis. We have shown that the short isoform of Singleminded-2 (Sim2s), a bHLH/PAS transcription factor, plays a role in promoting lactogenic differentiation, as well as in maintaining mammary epithelial differentiation and malignancy. *Sim2s* is dynamically expressed during mammary gland development, with expression peaking during lactation, and decreasing in early involution. To determine the role of Sim2s in involution, we used transgenic mice expressing Sim2s under the mouse mammary tumor virus (*MMTV-Sim2s*) promoter. Over-expression of *Sim2s* in the mouse mammary gland resulted in delayed involution, indicated by a lower proportion of cleaved caspase-3 positive cells and slower re-establishment of the mammary fat pad. Immunohistochemical and quantitative RNA analysis showed a decrease in apoptotic markers and inflammatory response genes, and an increase in anti-apoptotic genes, which were accompanied by inhibition of signal transducer and activator of transcription 3 (Stat3) activity. Microarray analysis confirmed that genes in the Stat3 signaling pathway were repressed by Sim2s expression, along with NFκB and other key pathways involved in mammary gland development. Multiparous *MMTV-Sim2s*

[*] Reprinted with permission from "Singleminded-2s (Sim2s) promotes delayed involution of the mouse mammary gland through suppression of Stat3 and NFκB" by Scribner, K. C., Wellberg, E. A., Metz, R. P., and Porter, W. W., 2011. *Mol Endocrinol,* Apr; 25(4):3-44, Copyright 2011 by The Endocrine Society.

females displayed a more differentiated phenotype compared to wild-type controls, characterized by enhanced β-casein expression and alveolar structures. Together, these results suggest a role for Sim2s in the normal involuting gland, and identify potential down-stream pathways regulated by Sim2s.

Introduction

Mammary gland involution is the regression of a lactating mammary gland to its quiescent state following weaning and is characterized by a decrease in milk protein, collapse of alveolar structures, apoptosis of epithelial cells, and re-establishment of the fat pad (Baxter et al., 2007; Furth, 1999; Thangaraju et al., 2004; Walker et al., 1989; Wilde et al., 1999). Involution has been shown to proceed in two separate phases; first an acute response phase characterized by a decrease in milk protein synthesis, and epithelial cell apoptosis (Baxter et al., 2007; Bierie et al., 2009; Clarkson and Watson, 2003; Furth, 1999; Henson and Tarone, 1994; Jaggi et al., 1996; Lund et al., 1996; Marti et al., 1999; Quarrie et al., 1996). This initial phase is reversible and occurs 1-3 days post weaning. Acute phase involution is characterized by a drop in Stat5, followed by an increase in Stat3 signaling. The second, irreversible, phase begins at 72 hours after pup removal, and is typified by a collapse in the alveoli, extensive epithelial apoptosis, and breakdown of the basement membrane by matrix metalloproteinases (MMPs) (Stein et al., 2007). Involution is a unique process with a wound healing signature and controlled inflammation, both of which are associated with breast cancer progression, metastasis, and survival. Studies investigating differences in gene expression, extracellular matrix composition, and signaling associated with involution and breast cancers have shown that metastatic breast cancer shares characteristics of the involuting mammary gland (Clarkson and Watson, 2003; Clarkson et al., 2004; Come et al., 2004; Henson and Tarone, 1994; Jager et al., 1997; Lefebvre et al., 1992;

Lyons et al., 2009; McDaniel et al., 2006; O'Brien and Schedin, 2009; Pensa et al., 2009; Radisky and Hartmann, 2009). Therefore, defining the mechanisms regulating involution will identify potential pathways involved in breast cancer progression.

Singleminded-2s (Sim2s) is a member of the bHLH/PAS family of transcription factors that has been implicated in normal mammary gland development and is frequently lost or reduced in primary human breast tumors. We have shown that *Sim2s* is expressed in mouse luminal mammary epithelial cells and is developmentally regulated with highest expression observed in mid-lactation (Kwak et al., 2007; Wellberg et al., 2010). To further define the role of Sim2s in mammary gland development, we generated a transgenic mouse expressing *Sim2s* under control of the Mouse Mammary Tumor Virus promoter (*MMTV-Sim2s*) (Wellberg et al., 2010). Analysis of mammary glands from staged virgin *MMTV-Sim2s* and WT mice, showed that mRNA levels of *Csn2* and *Wap*, were significantly increased, implying that Sim2s expression is associated with enhanced differentiation (Wellberg et al., 2010). While Sim2s is expressed in mammary epithelial cells, it is down regulated in a majority of breast tumors and breast cancer cell lines and forced expression of *Sim2s* in invasive breast cancer cells inhibits growth and motility. In addition, loss of *Sim2s* in the mouse mammary gland and in normal human breast epithelial cells results in an epithelial-mesenchymal transition (EMT), inducing an aggressive basal like phenotype (Gustafson et al., 2009a; Kwak et al., 2007; Laffin et al., 2008). Mammary glands from *Sim2*-null mice show impaired development, as the epithelial ducts do not properly form and differentiate (26).

To further elucidate the function of Sim2s in breast cancer progression and metastasis, as well as the function of Sim2s in normal mammary development, we analyzed the effect of *Sim2s* over-expression on involution following forced weaning (Wellberg et al., 2010). We found that mammary gland involution is disrupted in *MMTV-Sim2s* mice as demonstrated by a delay in the

reoccurrence of the fat pad and decrease in apoptosis. Furthermore, over-expression of *Sim2s* inhibited Stat3-mediated signaling and other pathways involved in the acute phase response. These studies extend the role of Sim2s in mammary gland development and identify Sim2s-regulated gene expression signatures that correlate with involution.

Results

Constitutively Active Sim2s Delays Mouse Mammary Gland Involution

To determine the effect of Sim2s on mammary involution, we analyzed glands from 3 *MMTV-Sim2s* transgenic mice force-weaned at lactation day 10 and harvested at 24, 48, and 72 hours after pup removal. Histological analysis showed clear differences starting at 24 hours post weaning in the number of apoptotic cells shedding into the alveolar lumen (Figure 12 A-D). Transgenic *Sim2s* mammary glands maintained distinct alveolar structures with copious amounts of milk at 72 hours of involution (Figure 12 K & L), while the wild-type glands had larger adipocytes and breakdown of luminal structures (Figure 12 I & J). Consistent with the observation that Sim2s promotes alveolar differentiation (Wellberg et al., 2010), we also observed an increase in β-casein (*Csn2*) and Whey Acidic Protein (*Wap*) gene expression 24 hours after pup removal as compared to controls (Figure 12 M & N). However, we observed no changes in phospho-Stat5a staining (data not shown), suggesting that Stat5a signaling is not involved in mediating this phenotype. Immunohistochemical staining of involuting mammary glands with anti-perilipin revealed distinct differences in adipocyte size between the transgenic and wild type glands 48 and 72 hours after pup removal. Figure 13 shows both fewer and smaller adipocytes in the fat pads of MMTV-Sim2s mice compared to wild type mice, indicating a delay in adipocyte lipid synthesis and repopulation of the mammary fat pad resulting from *Sim2s* over-expression.

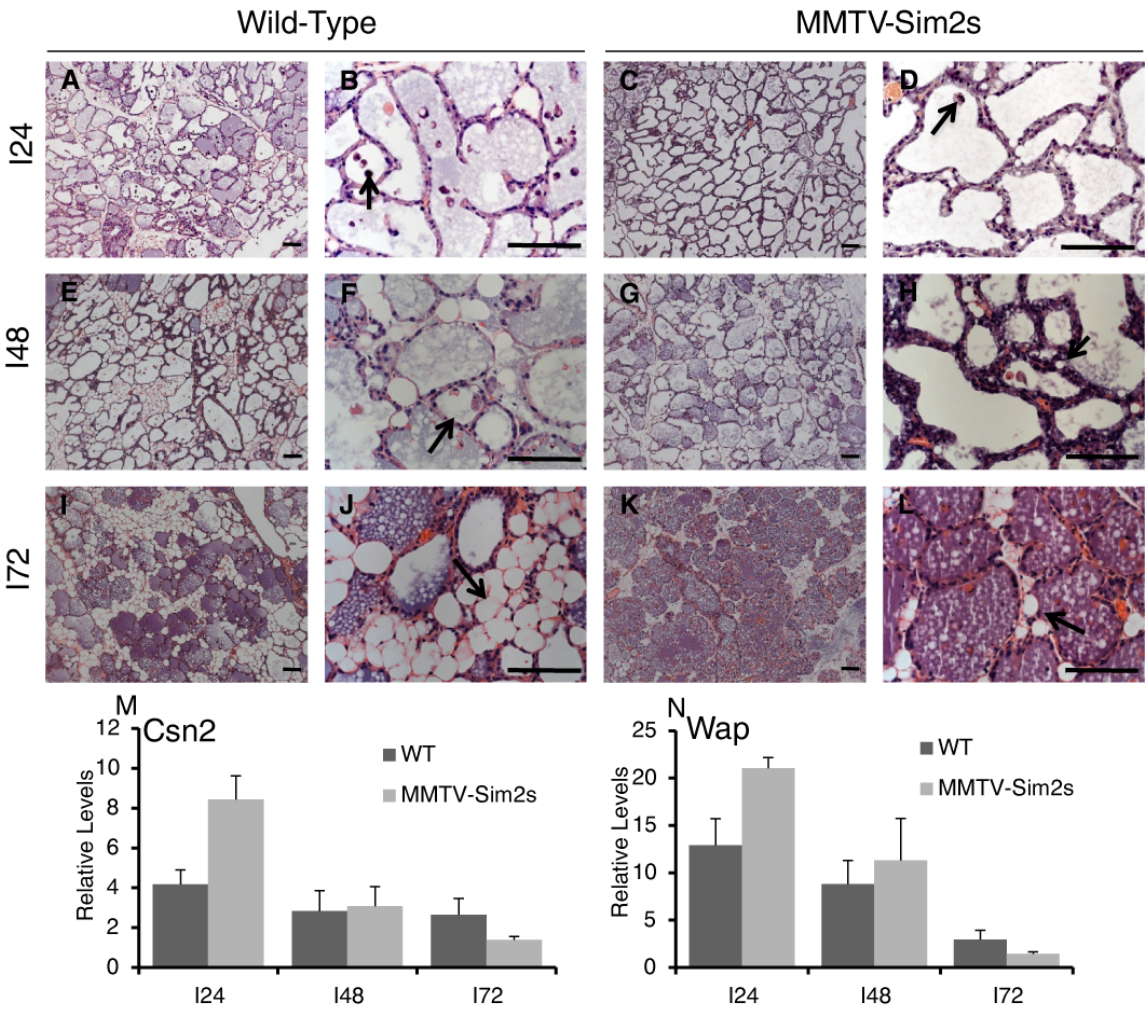

Figure 12. Involution is Delayed and Milk Protein mRNA Levels Are Increased in MMTV-Sim2s Transgenic Mice.Normal (FVB) and MMTV-Sim2s mice were harvested at 24, 48, and 72 hours involution (I24, I48, I72). A,B,E,F,I, and J - Hematoxylin & eosin (H&E) stained mammary glands from wild-type FVB mice. C, D, G, H, K, and L - H&E stained mammary glands from MMTV-Sim2s transgenic mice. Transgenic mammary glands show less apoptotic cell shedding, slow fat pad reoccurrence, and slower alveolar regression (Indicated by arrows). A-D - 24 hours involution. E-H - 48 hours involution. I-L - 72 hours involution. Magnification bar represents 100μm on all images. Images are representative of all samples collected. M – Quantitative PCR analysis of involuting mammary gland β-casein (Csn2). N – Quantitative PCR analysis of involuting mammary gland Whey Acidic protein (Wap). Transgenic mammary glands have an increased trend of milk protein mRNA expression throughout involution when normalized to an epithelial specific control gene (Claudin 7).

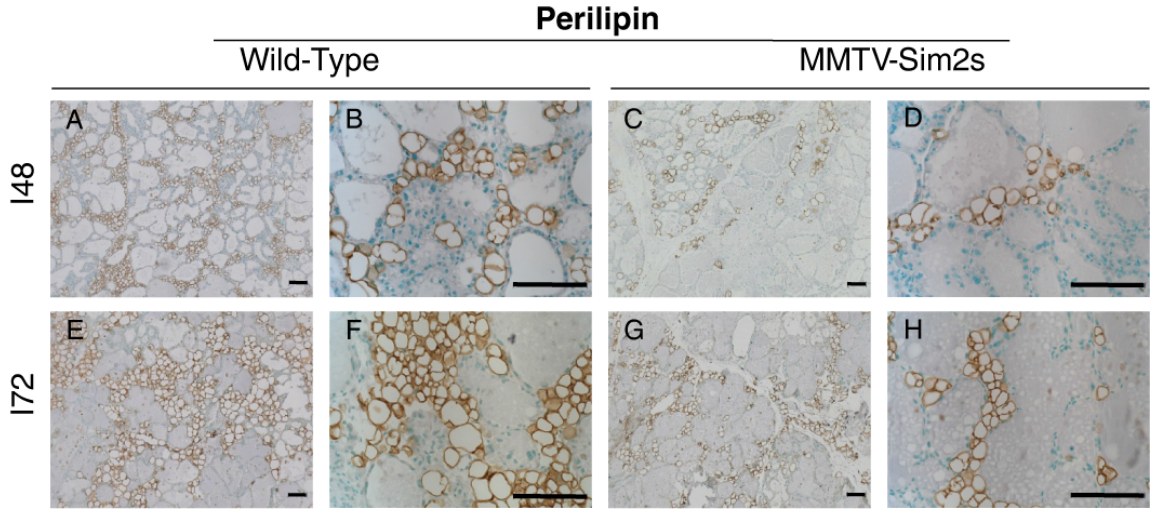

Figure 13. Delayed Fat Pad Regeneration in MMTV-Sim2s Mice During Involution. Perilipin staining of wild-type FVB and transgenic MMTV-Sim2s mice. A-D - mammary gland 48 hours involution. E-H - Mammary glands I72 hours involution. A, B, E, and F - Wild-type FVB mammary glands. C, D, F, and H - MMTV-Sim2s mammary. Transgenic mammary glands show less fat pad regeneration post forced weaning. Magnification bars represent 100 µm. Images are representative of all samples.

The acute phase of involution is largely characterized by shedding of apoptotic cells into the lumen of mammary gland alveoli. To evaluate differences in apoptosis between transgenic and WT involuting mammary glands, we used immunohistochemistry to evaluate cleaved caspase-3, which is normally confined to those cells that have undergone apoptosis and shed into the lumen. The results showed a significant increase in caspase-3 positive cells in involuting glands from *MMTV-Sim2s* mice at 24, 48, and 72 hours compared to controls (Figure 14).

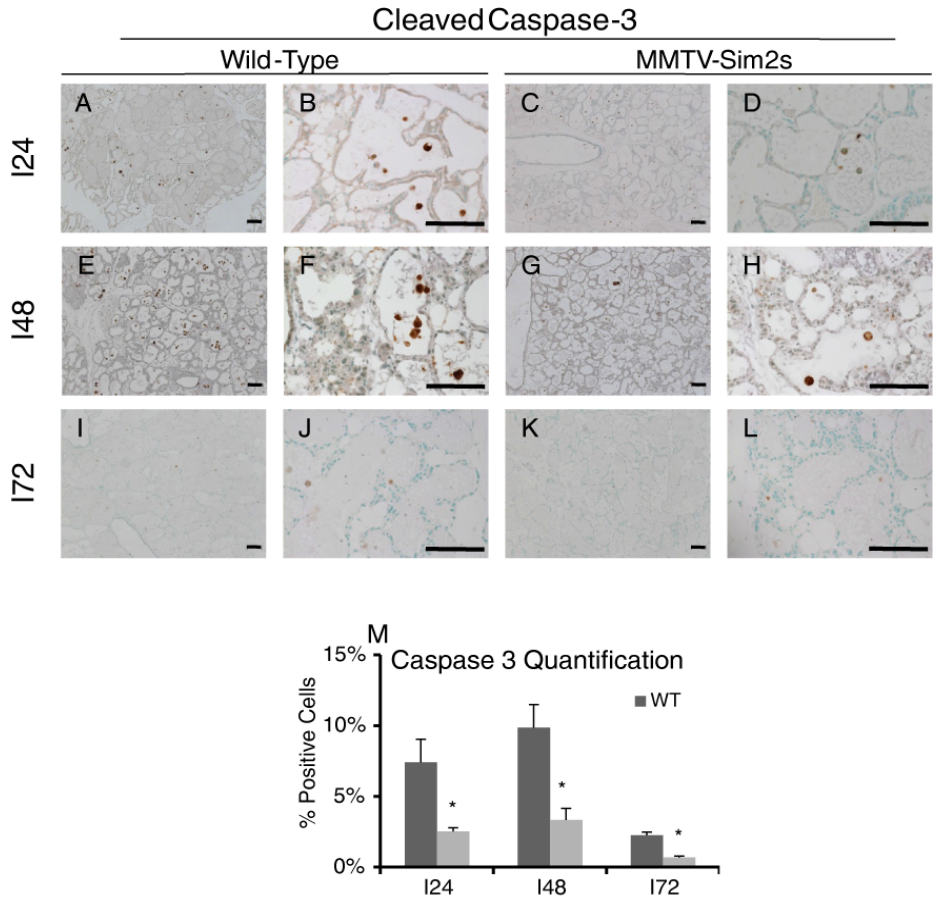

Figure 14. MMTV-Sim2s Transgenic Mice have Significantly Lower Levels of Apoptotic Cells During Involution. A-L - Wild-type and MMTV-Sim2s mammary glands stained for cleaved caspase 3. A-D - 24 hours involution. E-H - 48 hours involution. I-L - 72 hours involution. A, B, E, F, I and J - Wild-type FVB mammary glands. C, D, G, H, K and L - MMTV-Sim2s mammary glands. Magnification bars represent 100 µm in all images. Images are representative of all samples. M - Quantification of cleaved caspase 3 images, %Positive cells taken from five images for each mouse, counted and averaged. MMTV-Sim2s transgenic mammary glands have significantly lower levels of cleaved-caspase 3 positive cells. * = *p-value*<.05

Multiparous MMTV-Sim2s Mammary Glands Exhibit Alveolar Structure and Milk Protein Expression

To determine the effect of Sim2s over-expression on multiple rounds of involution, mammary histology of transgenic and WT mice that had gone through at least 3 pregnancies each was evaluated. Analysis of H&E staining shows a distinct alveolar phenotype in the

MMTV-Sim2s mammary glands with a more differentiated morphology and lipid accumulation (Figure 15 B, C, E, F) as compared to control animals (Figure 15 A and D). Furthermore, immunohistochemical analysis showed that WT glands have little β-casein expression, whereas the *MMTV-Sim2s* gland has distinct staining within the alveolar structures (Figure 15 H-M).

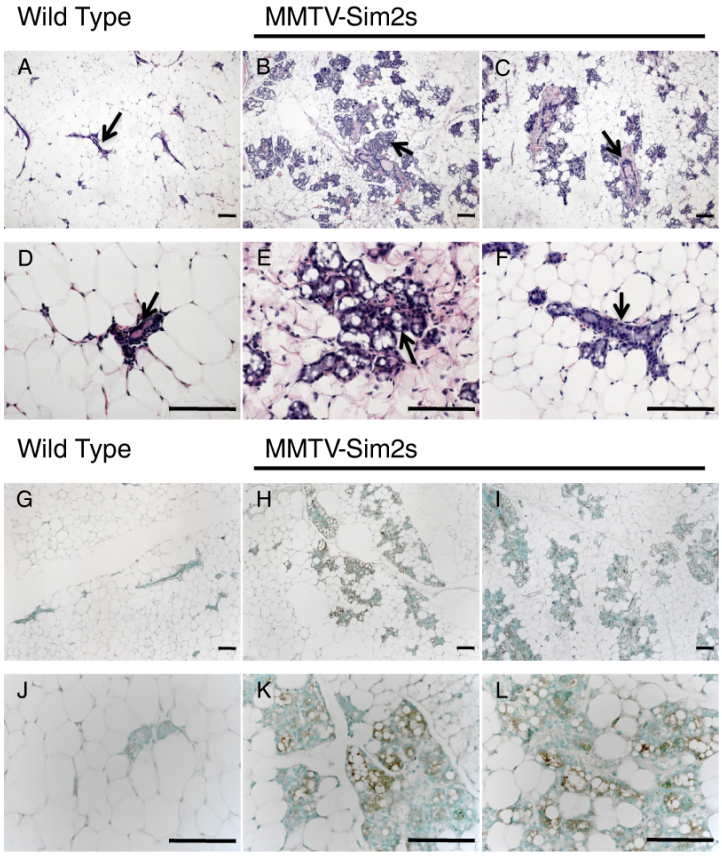

Figure 15. Multi-parous MMTV-Sim2s Females Have More Alveolar Structures in the Non-lactation Gland, and Higher β-casein Expression. A-F - H&E staining for multi-parous (+1 litter) WT and MMTV-Sim2s mice. A & D, WT multiparous, non-lactating mammary glands have small ductal branching similar to the virgin gland. B, C, E, and F – MMTV-Sim2s multi-parous, non-lactating mammary glands have larger alveolar glands indicating an incomplete involution (Indicated with arrows). Magnification bars represent 100μm. H-M - β-Casein Immunohistochemical staining of multi-parous wild-type and MMTV-Sim2s mice. H & K - WT multiparous, non-lactating mammary glands show little to no detectable levels of β-casein. I, J, L, and M - MMTV-Sim2s multi-parous, non-lactating mammary glands show much higher levels of β-casein in the alveolar structures still present in the quiescent gland. Magnification bars represent 100μm. Images are representative of at least 60% of samples.

Sim2s Expression Inhibits Activation of Stat3

Stat3 signaling plays an important part in regulating mammary gland involution by inducing genes involved in inflammation and the acute phase response. Activation of Stat3 through phosphorylation promotes translocation into the nucleus, where phospho-Stat3 (pStat3) functions as a transcriptional regulator. Analysis of pStat3 staining in WT and *MMTV-Sim2s* mice showed chimeric staining patterns throughout the mammary epithelium, but glands from transgenic mice appeared to have far fewer pStat3-positive cells than WT glands (Figure 16 A-L). Quantification of pStat3-positive cells revealed a significant decrease in *Sim2s* glands at 48 and 72 hours of involution (Figure 16 M). Pan-Stat3 immunohistochemistry was also performed to ensure pStat3 changes seen are due to altered activation of Stat3. No changes are seen between wild-type and transgenic mammary glands in levels of Stat3 (Figure 17 A-L). To determine if the reduction of active Stat3 seen in *MMTV-Sim2s* mice correlated with a decrease in down-stream Stat3 target genes, we performed qPCR analysis for *C/EBPδ* and *IGFBP5* and found a distinct downward trend in gene expression in *Sim2s* transgenic mice at 72 hours of involution (Figure 16 O and P). Together, these results suggest that the delayed involution observed in mammary glands from *MMTV-Sim2s* mice is mediated, in part, by Sim2s-dependent suppression of the Stat3 pathway.

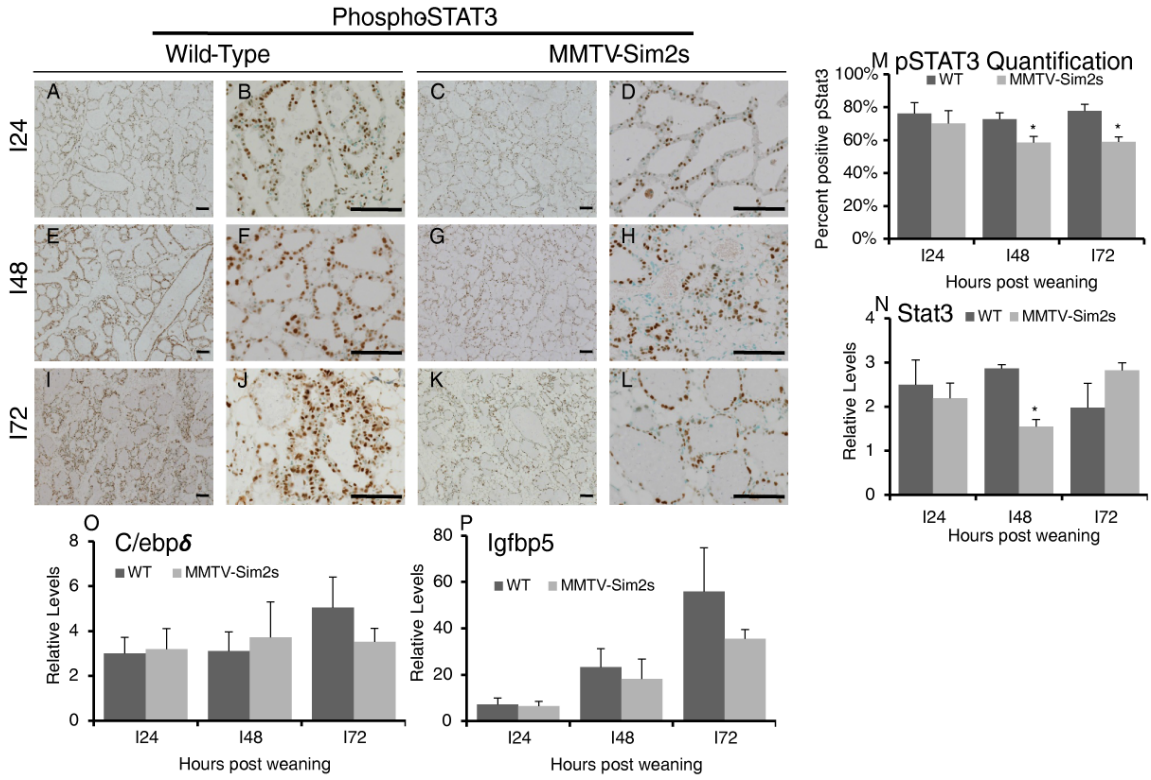

Figure 16. Active Stat3 and Stat3 Target Genes Are Significantly Lower in MMTV-Sim2s Mice During Involution. A-L - Immunohistochemical staining of phosphorylated Stat 3 in wild-type and MMTV-Sim2s mice. A-D - 24 hours involution. E-H - 48 hours involution. I-L - 72 hours involution. A, B, E, F, I, and J - Wild-type mammary glands have very high levels of pStat3, especially in the epithelial secretory cells. C, D, G, H, K, and L - MMTV-Sim2s transgenic mammary glands exhibit significantly lower levels of pStat3 staining. Heterogeneous mixture of p-Stat3 staining is consistent with heterogeneous expression of MMTV-LTR. No difference was seen in pan Stat3 staining (Supp. 1). Magnification bars represent 100µm. Images are representative of at least 60% of samples. M – Quantification of pStat3 staining, four images were taken for each gland harvested and counts were averaged. N – mRNA expression levels of pan Stat3 in involuting mammary glands. O and P – Stat3 target genes C/EBPδ and IGFBP5 have downward trends in mRNA expression at 72 hours in transgenic mice as shown by quantitative PCR. * = *p-value*<.05

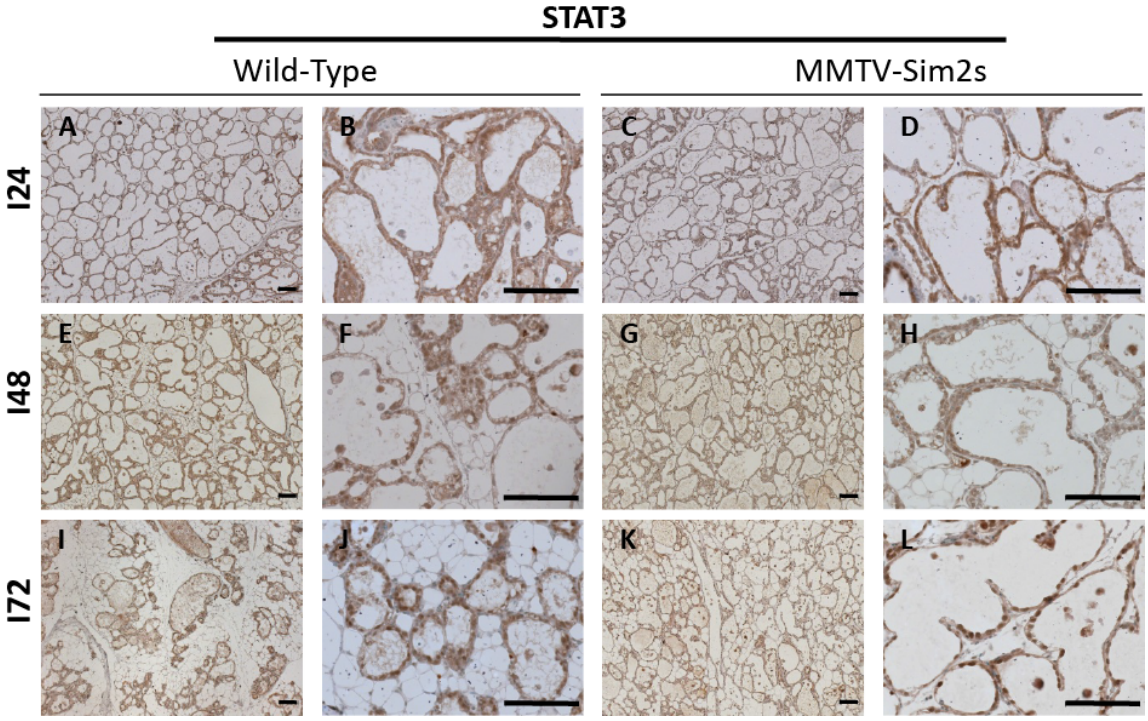

Figure 17. Immunohistochemical Analysis of pan-Stat3 During Involution Reveals No Change in Overall Expression. A-L - Immunohistochemical staining of Stat 3 in wild-type and MMTV-Sim2s mice. A-D - 24 hours involution. E-H - 48 hours involution. I-L - 72 hours involution. A, B, E, F, I, and J - Wild-type mammary glands. C, D, G, H, K, and L - MMTV-Sim2s transgenic mammary glands. Both wild-type and transgenic mammary glands express pan-stat3 at comparable levels. Stat3 is detected both as a cytosolic and nucleic stain, which is expected with non-active Stat3. Magnification bars represent 100µm. Images are representative of at least 60% of samples.

Microarray Analysis Revealed Changes in Pathways Regulating Involution, Specifically Stat3 and NFκB

Microarray analysis was performed using mammary glands from WT and *MMTV-Sim2s* transgenic females harvested 72 hours after force-weaning to identify additional pathways regulated by Sim2s during involution. Data was analyzed using the Ingenuity Pathway Analysis Software. The significance of the association between the data set and the canonical pathway was measured by taking a ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway displayed. Fisher's exact test was used to calculate a p-value to determine the probability that the association between the genes in the data set and the canonical pathway is explained by chance alone. As expected, one of the most significantly affected pathways identified in the involuting *MMTV-Sim2s* mammary gland was the Jak/Stat signaling pathway, which is involved in regulating mammary gland growth, differentiation, migration, and apoptosis. Other pathways identified, including Notch, Wnt/β-catenin, PI3K/Akt, and NFκB, also play roles in involution and breast cancer progression. Changes in multiple genes involved in NFκB signaling, identified in the microarray studies, were verified using qPCR (Figure 18 B-E). In transgenic mammary glands, *Ikk2*, an activator of NFκB, showed reduced expression compared to wild type glands throughout the time series. NFkB2 expression was gradually upregulated in wild type glands, showing maximal expression by 72 hours; however, the levels remained low in glands from transgenic females. These data suggest that Sim2s regulates multiple pathways involved in normal mammary gland function and malignancy.

A. Statistically Significant Pathways Affected at 72 hours involution by constitutively active Sim2s

Ingenuity Canonical Pathways	-log(p-value)
JAK/Stat Signaling	2.11E00
Growth Hormone Signaling	1.71E00
Prolactin Signaling	1.53E00
FGF Signaling	9.98E-01
EGF Signaling	6.68E-01
TGF-β Signaling	8.09E-01
Estrogen-Dependent Breast Cancer Signaling	6.5E-01
Notch Signaling	5.09E-01
AMPK Signaling	7.82E-01
ERK/MAPK Signaling	7.7E-01
Wnt/β-catenin Signaling	3.28E-01
PI3K/AKT Signaling	4.3E-01
NF-κB Signaling	3.07E-01

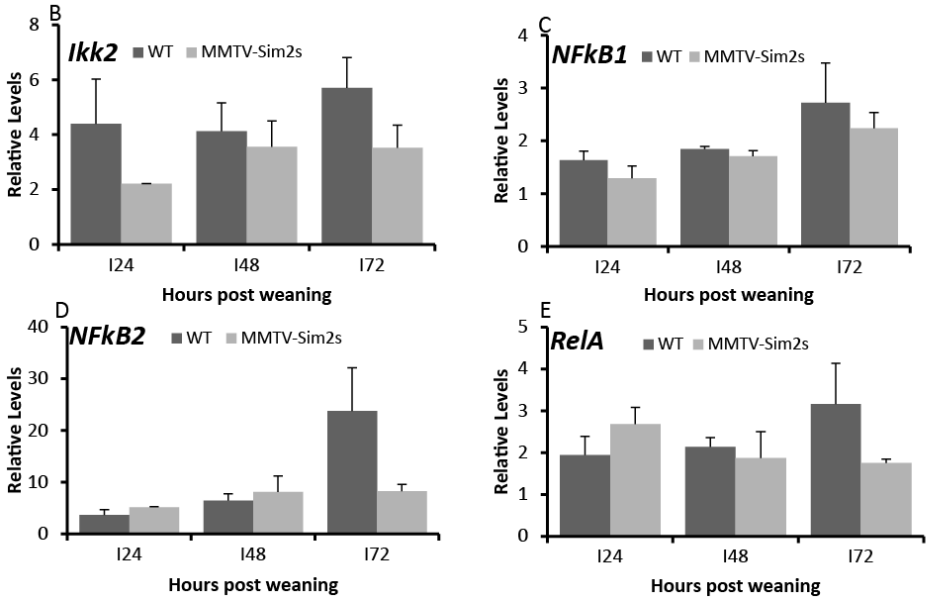

Figure 18. Canonical Pathway Analysis of 72 Hour Involuting Glands Reveals Multiple Pathways that Are Affected by MMTV-Sim2s Over Expression. A- Canonical Pathway Analysis was performed using Ingenuity Pathway Analysis Software. Statistically significant data (p-value <.05) from the Codelink Microarray was analyzed to determine what pathways were significantly altered by Sim2s expression. B-C - Quantitative PCR analysis of Ikk2 and NFκB 2 show downward trends in MMTV-Sim2s mice.

Discussion

We have shown that Sim2s promotes mammary lactogenic differentiation when over expressed in virgin mice (Wellberg et al., 2010). The objective of this study was to evaluate the effect of *Sim2s* over expression on mammary involution following forced weaning. Here, we demonstrate that *MMTV-Sim2s* transgenic mice experience delayed involution, characterized by reduced epithelial cell apoptosis and lower Stat3 activation. Microarray studies performed on tissue from involuting glands revealed that Sim2s inhibited pathways associated with inflammation and apoptosis, both of which are hallmarks of involution and cancer progression. Previously, our lab has shown that Sim2s is required for proper development and differentiation of the mammary ductal tree. Additionally, down-regulation of *Sim2s* in MCF7 breast cancer cells and MCF10A breast epithelial cells results in the loss of epithelial characteristics and the acquisition of a basal phenotype (Gustafson et al., 2009a; Laffin et al., 2008; Rudolph et al., 2009; Wellberg et al., 2010). The results from this study support a role for Sim2s in maintaining epithelial differentiation and suppressing involuting pathways.

Recent studies have utilized the involuting mouse mammary gland as a model to identify pathways involved in breast cancer progression (Ackler et al., 2002; Atabai et al., 2007; Chapman et al., 2000; Clarkson et al., 2000; Clarkson and Watson, 1999; Clarkson and Watson, 2003; Desrivieres et al., 2006; Flanders and Wakefield, 2009; Gordon et al., 2000; Heermeier et al., 1996; Humphreys et al., 2002). Special interest has focused on the regulation of apoptosis during the acute phase of involution, and on controlled inflammation, which are part of the wound-healing signature. Bax, Akt, Il6, Stat3, and other pro apoptotic genes associated with involution have been conditionally deleted in the mammary gland to determine their roles in involution (Ackler et al., 2002; Bierie et al., 2009; Chapman et al., 2000; Clarkson et al., 2000; Flanders and Wakefield, 2009; Heermeier et al., 1996; Humphreys et al., 2002; Jager et al.,

1997; Jerry et al., 2002; Jerry et al., 1998; Kritikou et al., 2003; Schorr et al., 1999; Stein et al., 2004; Thangaraju et al., 2005; Tonner et al., 2000; Zhao et al., 2002a). Gene expression analysis of the involution signature shows that Stat3 and NFκB pathways are induced during involution, while the Stat5 pathway is inhibited. From these results, we know that a substantial number of factors up-regulated during involution play various roles in inflammation and the acute phase of involution. These pathways have also been analyzed in conjunction with various breast cancer subtypes, and correlate with an increase in metastasis and poor prognoses. In addition, the microenvironment of the involuting mammary gland has been shown to promote metastasis due to active basement membrane degradation, controlled inflammatory signaling, activation of fibroblasts, and other signals also involved with a wound healing signature (Baxter et al., 2007; Clarkson and Watson, 2003; Come et al., 2004; Henson and Tarone, 1994; Lyons et al., 2009; McDaniel et al., 2006; Pensa et al., 2009; Schedin et al., 2007).

The acute phase of involution, which occurs during the first 72 hours post weaning, is characterized by an increase in apoptosis, an enhanced immune response, and decreased milk protein synthesis (Abell et al., 2005; Chapman et al., 2000; Clarkson and Watson, 2003; Hennighausen et al., 1997; Kritikou et al., 2003; Stein et al., 2004; Walker et al., 2009; Watson, 2001). Many of these processes are dependent on Stat3, which mediates apoptosis of secretory epithelial cells, as well as their removal from the gland during involution. Stat3 is also the key regulator of the controlled inflammatory response observed in the involuting mammary gland and is constitutively active in many breast cancer cell lines and primary tumors (Pensa et al., 2009; Walker et al., 2009; Watson, 2001; Watson and Neoh, 2008; Yu et al., 2009; Zhou et al., 2007). Stat3 regulates both pro and anti-inflammatory responses, based on LIF and OSM signaling, and is often activated by tumor cytokines, oncogenes, and growth factors. In Stat3 null mice, involution is delayed and the secretory alveolar epithelial cells maintain functional

integrity up to 6 days post weaning in the absence of lactogenic stimuli (Chapman et al., 2000; Chapman et al., 1999; Humphreys et al., 2002; Pensa et al., 2009; Thangaraju et al., 2005; Zhao et al., 2002a). We observed a similar phenotype in our *MMTV-Sim2s* mice with a significant inhibition of pStat3 activity and reduced expression down-stream targets genes IGFBP5 and C/EBPδ (Thangaraju et al., 2005).

Stat3 and NFκB are known to interact at multiple levels, and similar to Stat3, NFκB is constitutively active in cancers (Yu et al., 2009). *NFκB* expression is high during the acute phase of mammary gland involution, and has been shown to play a role in milk clearance (Connelly et al., 2010). Conditional deletion of *IKK2*, a NFκB activator, resulted in delayed involution and a decrease in cleaved-caspase 3 positive apoptotic cells (Baxter et al., 2006). These studies indicate that NFκB plays a role in pro-apoptotic signaling, in addition to the commonly described anti-apoptotic regulation of Bcl2 and $BclX_L$. Transgenic mice with a doxycycline inducible *NFκB* construct undergo rapid loss of milk and alveolar collapse shortly following pup weaning, in addition to an increase in cleaved-caspase 3 positive cells compared to wild-type counterparts (Connelly et al., 2010). Similarly, induction of *NFκB* expression during lactation resulted in decreased milk protein levels and alveolar collapse. NFκB has been implicated in tumorigenesis, as constitutive activation results in increased proliferation, inhibition of apoptosis, increased metastasis and angiogenesis (Cao and Karin, 2003; Clarkson and Watson, 1999; Haffner et al., 2006; Kozlow and Guise, 2005; Pensa et al., 2009; Pratt et al., 2009; Yu et al., 2009; Zhou et al., 2008; Zhou et al., 2005b). A pro-inflammatory target of NFκB, interleukin-6 (IL-6), is a known activator of Stat3, and forms a positive feedback loop that is a major regulator of inflammation (Yu et al., 2009). *IL-6* null mice also have delayed involution and decreased epithelial cell death; however, Stat3 remains activated in these mice, indicating that Stat3 is activated through multiple cytokines (Zhao et al., 2002a). Stat3 also

inhibits anti-tumorigenic pathways that are typically activated by NFκB Rel, allowing only the expression of pro-oncogenic Rel A (Yu et al., 2009). In tumors, this results in a reciprocal relationship between Stat3 and NFκB because many cytokines and growth factors encoded by RelA activate Stat3. Similar to Stat3, active NFκB signaling has also been indicated in maintenance of mammary stem cells (Pratt et al., 2009; Zhou et al., 2008).

We have shown that mammary gland involution is delayed in *MMTV-Sim2s* mice, and that Sim2s inhibits gene signatures associated with involution including Stat3 and NFκB signaling pathways. This suggests that Sim2s expression in breast cancer cell lines might suppress the pro-oncogenic activities of these transcription factors. Further studies into the inhibition of these pathways by Sim2s may significantly contribute to the understanding and therapeutic treatment of metastatic breast cancer. Increasing evidence indicates that the differentiation status of a tumor correlates with its aggressiveness, thus showing the importance of finding factors that induce terminal differentiation not only in the mammary gland, but also in primary tumors. Based on the studies shown here, and our previous work showing that Sim2s promotes mammary alveolar differentiation, we hypothesize that over-expression of Sim2s will inhibit tumor progression, metastasis, and render breast tumors more susceptible to therapeutics, leading to a better prognosis.

CHAPTER IV

MANUSCRIPT 2: REGULATION OF DCIS TO INVASIVE BREAST CANCER PROGRESSION BY SINGLEMINDED-2S (SIM2S)*

Synopsis

Singleminded-2s (SIM2s) is a member of the bHLH/PAS family of transcription factors and a key regulator of mammary epithelial cell differentiation. SIM2s is highly expressed in mammary epithelial cells and down regulated in human breast cancer. Loss of *Sim2s* causes aberrant mouse mammary ductal development with features suggestive of malignant transformation, whereas over-expression of *SIM2s* promotes precocious alveolar differentiation in nulliparous mouse mammary glands, suggesting that SIM2s is required for establishing and enhancing mammary gland differentiation. To test the hypothesis that SIM2s regulates tumor cell differentiation, we analyzed SIM2s expression in human primary breast ductal carcinoma in situ (DCIS) samples and found that SIM2s is lost with progression from DCIS to invasive ductal cancer (IDC). Utilizing a MCF10DCIS.COM progression model, we have shown that *SIM2s* expression is decreased in MCF10DCIS.COM cells compared to MCF10A cells and re-establishment of *SIM2s* in MCF10DCIS.COM cells significantly inhibits growth and invasion *in vitro* and *in vivo*. Analysis of *SIM2s*-MCF10DCIS.com tumors showed that SIM2s promoted a more differentiated tumor phenotype including the expression of a broad range of luminal markers (CSN2 (β-casein), CDH1 (E-cadherin), and KER18 (keratin-18)) and suppressed genes associated with stem cell maintenance and a basal phenotype (SMO (smoothened), p63, SLUG (snail-2), KER14 (keratin-14) and VIM (vimentin)). Furthermore, loss of *SIM2s* expression in

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MCF10DCIS.COM xenografts resulted in a more invasive phenotype and increased lung metastasis likely due to an increase in hedgehog signaling and matrix metalloproteinase expression. Together, these exciting new data support a role for SIM2s in promoting human breast tumor differentiation and maintaining epithelial integrity.

Introduction

Ductal Carcinoma In Situ (DCIS) has been shown to be a precursor to invasive ductal cancer (IDC) (Burstein et al., 2004) with 20-30% of DCIS showing evidence of invasion upon diagnosis (Cody, 2007; Maffuz et al., 2006). Though the progression of DCIS to IDC is believed to be an important aspect of tumor aggressiveness, prognosis and molecular markers that can predict progression are poorly understood. Analysis of biomarkers and molecular profiles of IDC and DCIS have failed to identify progression-specific pathways (Chin et al., 2004; Cocker et al., 2007; Ma et al., 2003; Porter et al., 2003; Yao et al., 2006). Therefore, determining the mechanisms by which some DCIS progress is critical for future breast cancer diagnostics and treatment.

There is increasing evidence that DCIS are heterogeneous tumors, which enhances complexity when attempting to define the mechanisms that promote progression to IDC in *in vivo* models. Recent studies utilizing the MCF10DCIS.COM cell line, which was derived from the non-cancerous MCF10A cell line, have shown that these cells contain a unique bipotent progenitor ability that forms a myoepithelial cell layer in addition to luminal-type cells *in vivo,* which results in basal-like DCIS with high similarities to human DCIS samples (Behbod et al., 2009; Hu et al., 2008; Miller et al., 2000; Shekhar et al., 2008; Tait et al., 2007). Intraductal and flank injections have shown that MCF10DCIS.COM cells not only form DCIS like structures, but also spontaneously progress to invasive breast cancer (Behbod et al., 2009; Hu et al., 2008).

These observations suggest that MCF10DCIS.COM cells are a unique model to study DCIS, as well as the role of different factors in regulating the progression to IDC.

We have previously shown that the basic helix-loop-helix/PER-ARNT-SIM (bHLH/PAS) transcription factor Singleminded-2s (SIM2s) plays a role in normal mammary gland development as well as in promoting tumor cell differentiation (Gustafson et al., 2009b; Kwak et al., 2007; Laffin et al., 2008; Scribner et al., 2011; Wellberg et al., 2010). Loss of *Sim2s* expression in the mouse mammary gland and in normal breast and breast cancer cell lines is associated with an epithelial mesenchymal transition(EMT), whereas over-expression of *Sim2s* under the Mouse Mammary Tumor Virus (MMTV) promoter induces precocious alveolar differentiation in nulliparous mice and delayed forced involution (Gustafson et al., 2009b; Kwak et al., 2007; Laffin et al., 2008; Scribner et al., 2011; Wellberg et al., 2010). *SIM2s* is down-regulated in primary human breast cancer samples, and re-establishment of *SIM2s* in human breast cancer cell lines inhibits cell proliferation and invasion (Gustafson et al., 2009b; Kwak et al., 2007). Moreover, we have found that *SIM2s* mRNA gene expression is inhibited by activation of C/EBPβ and NOTCH signaling, two known EMT promoters, in RAS-transformed MCF10A cells (Gustafson et al., 2009b). Both C/EBPβ and NOTCH expression have been shown to have to play a role in breast cancer progression through mediation of breast cancer stem cells, cellular proliferation, and oncogenesis (Grimm and Rosen, 2003; Kiaris et al., 2004; LaMarca et al., 2010; Politi et al., 2004; Stylianou et al., 2006).Together, these observations suggest that SIM2s is a tumor suppressor gene that is required to maintain epithelial integrity by inhibiting EMT-like pathways and promoting differentiation.

In the studies here, we examined the role of SIM2s in regulating the progression of DCIS to IDC and metastasis, while promoting the less aggressive, luminal-like breast cancer subtype. Based on our studies of SIM2s as a breast cancer tumor suppressor, we hypothesize

that *SIM2s* expression will decrease spontaneous metastasis seen in the MCF10DCIS.COM model, and play a role in the inhibition of DCIS progression.

Results

SIM2s is Lost During DCIS Progression

To determine the role of SIM2s in progression from DCIS to IDC, fourteen human primary DCIS and IDC samples were analyzed for SIM2s expression by immunohistochemistry. We have previously shown that SIM2s is localized in the nuclei of human breast and mouse mammary ductal epithelial cells (Kwak et al., 2007; Laffin et al., 2008), and similar punctate staining was observed in normal ductal structures surrounding the tumors analyzed (Figure 19 A). In these studies, we found that SIM2s expression is prominent in both the nucleus and cytoplasm in over 75% of DCIS samples (Figure 19 B), suggesting a loss in localization at the onset of progression. In contrast, as DCIS progresses to IDC, SIM2s staining is dramatically down-regulated with no evidence of nuclear expression in over 80% of IDC samples (Figure 19 C), supporting a role for loss of SIM2s in breast cancer progression.

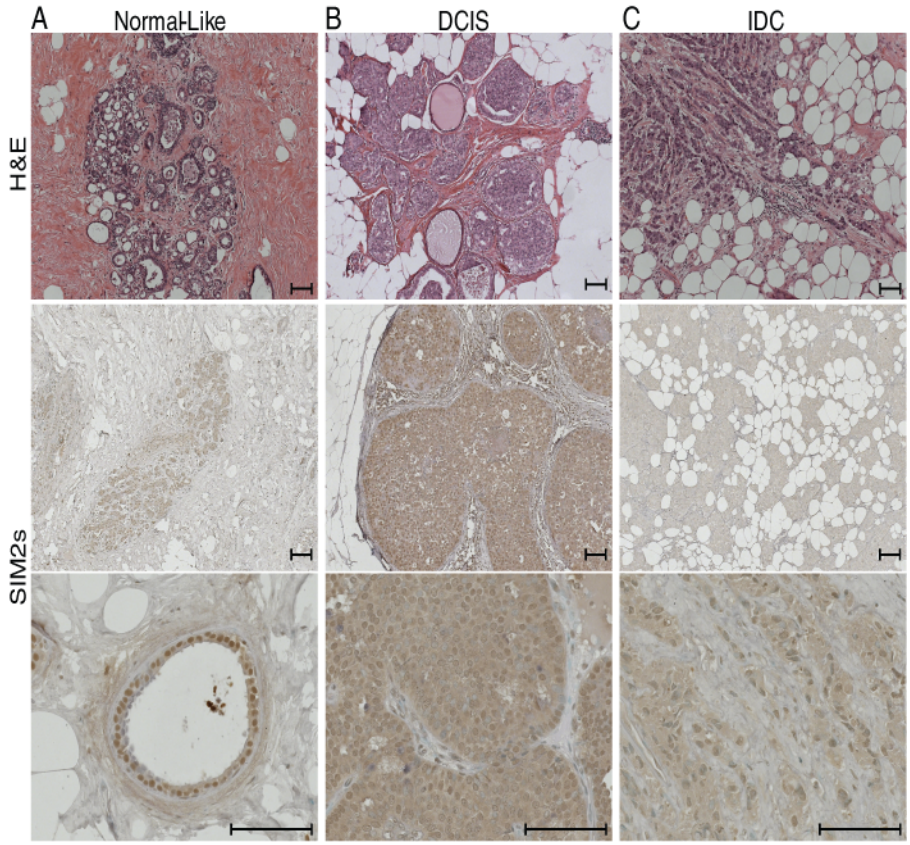


Figure 19. SIM2s Expression is Progressively Lost in Human Ductal Carcinoma In Situ (DCIS) Transition to Invasive Ductal Cancer (IDC). A – Human Normal-like tissue, B – Human DCIS, C – Human IDC. Top Row – H&E staining of Normal, DCIS, and IDC samples. Bottom Rows - SIM2s immunohistochemistry of Normal, DCIS and IDC samples. Normal-like structures show clean, punctate nuclear staining. DCIS samples show nuclear and cytoplasmic SIM2s staining in over 75% of samples, and this expression is lost in over 80% of IDC samples. Images were taken at 10x and 40x objective (6.3x and 25.2x), scale bars represent 100µm. Images are representative of all samples. An n=14 was used for each tumor classification (DCIS and IDC).

Re-establishment of SIM2s in the MCF10.DCIS.com Cell Line Induces Genetic and Morphologic Changes In Vitro

MCF10DCIS.COM cells are a unique human breast cancer cell line which form DCIS-like lesions in *in vivo* mouse models, similar to primary human DCIS lesions, and spontaneously progress to invasive cancer *(Tait et al., 2007)*. In addition, MCF10DCIS.COM cells are thought to contain a bipotent progenitor population that generate both myoepithelial and luminal

cells, mimicking the heterogeneity observed in human DCIS tumors (Behbod et al., 2009; Hu et al., 2008). Quantitative real-time PCR (Q-PCR) analysis of *SIM2s* levels showed that *SIM2s* is significantly down-regulated in MCF10DCIS.COM cells compared to parent MCF10A cells, however, *SIM2s* levels are still higher in MCF10DCIS.COM cells than levels found in luminal MCF7 and basal MDA.MB.231 cell lines, suggesting that SIM2s expression is lost with progression (Figure 20 A). To determine the effect of *SIM2s* loss and gain of function, we stably transduced MCF10DCIS.COM cells with *SIM2s* and previously validated *SIM2s*-shRNA (*SIM2s*i) lentiviruses (Gustafson et al., 2009b; Laffin et al., 2008). *SIM2s* levels and localization were confirmed using Q-PCR and immunofluorescence (Figure 20 B, C, & D) (Figure 21). Q-PCR analysis of *SIM2s* mRNA levels show an approximate 80% loss of expression in *SIM2si* cells compared to scrambled controls (Figure 20 D). In growth assays, SIM2s inhibited cell proliferation, whereas loss of *SIM2s* led to a significant increase in proliferation as compared to scrambled controls (Figure 20 E). We observed no change in invasive potential with *SIM2s* over-expression in Boyden chamber assays; however, there was a significant increase in invasion in the *SIM2si* cells (Figure 20 F). Q-PCR analysis also showed a significant increase in E-Cadherin (*CDH1*) expression with *SIM2s* expression, as well as a decrease with *SIM2si* (Figure 20 G). Similarly, *p21*, an important senescence and cell cycle regulator, was also significantly altered in response to SIM2s (Figure 20 H).

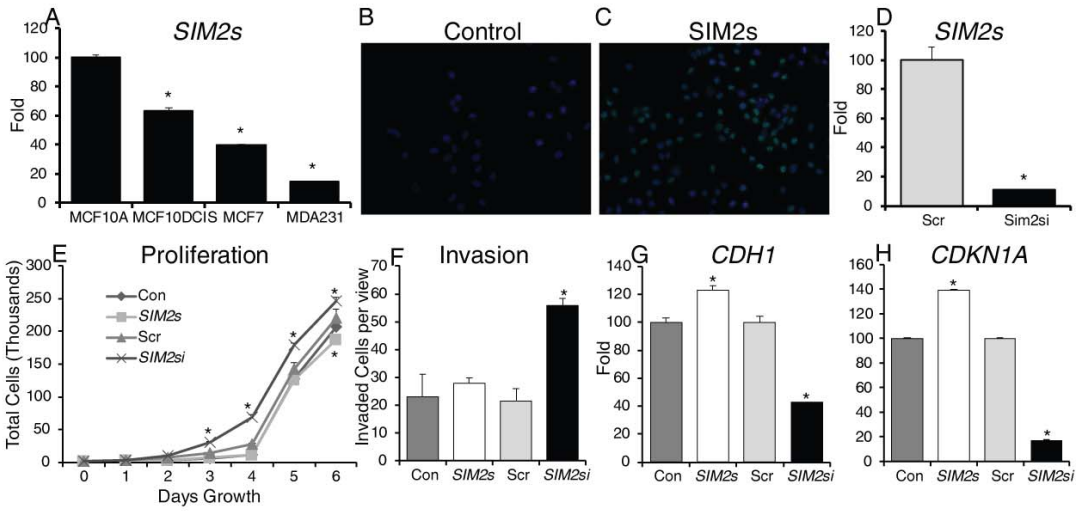


Figure 20. Analysis of MCF10DCIS Cell Transductions In Vitro Show Changes in Proliferation, Invasion, and Differentiation Markers.A – Q-PCR analysis of *SIM2s* mRNA levels in MCF10DCIS.COM and parent MCF10A cells, as well as commonly used breast cancer cell lines MCF7 and MDA.MB.231. B, C - Immunofluorescent staining of SIM2s to confirm nuclear SIM2s overexpression. D – Q-PCR analysis of *SIM2s* in the MCF10DCIS.com cell line confirming an approximate 80% loss of expression in adhered *SIM2si* cells. E – Proliferation assays confirm that *SIM2s* expression inhibits breast cancer cell proliferation, while loss of *SIM2s* increases growth. The values shown are the mean ± SE of triplicate samples. F - Boyden chamber invasion assay shows significantly more *SIM2si* cells were able to invade and migrate compared to controls. Values are the average number of cells per five fields per membrane of three separate plates. G & H - Q-PCR analysis of differentiation markers CDH1 and p21. * = p-value < .05.

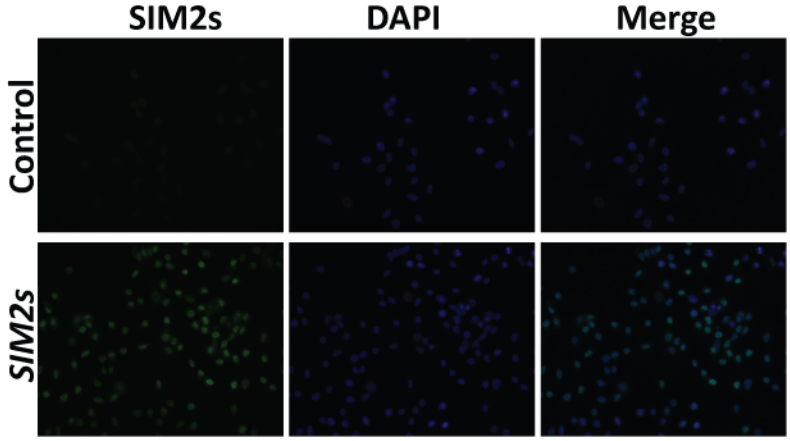


Figure 21. Channel Images of the SIM2s Fluorescence Previously Shown (Figure 20). Adhered cells were stained with SIM2s (green) and DAPI (blue). Images were taken using a 40x objective (25.2x).

In Vivo Analysis of Transduced MCF10DCIS.com Cell Xenografts Show Changes in Growth and Morphology

Similar to the *in vitro* results, we found that *SIM2s* xenograft tumors grew significantly slower than controls (Figure 22 A & B). In contrast to our *in vitro* observations, we were surprised to find that down-regulation of *SIM2s* led to a decreased trend in tumor size and weight as compared to scrambled controls; however statistical significance was not obtained (Figure 22 A & B). To determine if *SIM2s* expression affected changes in tumor morphology, we analyzed H&E sections from *SIM2s* and *SIM2si* tumors. Histological analysis confirmed a distinct phenotypic differences with *SIM2s* expression (Figure 22 D): *SIM2s* tumors had a more differentiated phenotype including lobular-like structures with intact myoepithelial layers whereas *SIM2si* tumors, despite growing at a slower rate, were more invasive and had large necrotic areas as compared to scrambled controls (Figure 22 D, invasion shown by arrows). These observations are similar to previous studies that found no correlation between MCF10DCIS.COM growth *in vivo* and invasive potential and support the hypothesis that SIM2s inhibits DCIS progression by promoting and maintaining a luminal phenotype (Behbod et al., 2009; Hu et al., 2008; Shekhar et al., 2008). Immunohistological analysis of tumors confirmed that *SIM2s* and *SIM2si* tumors continued to overexpress or knock down SIM2s protein levels *in vivo* (Figure 22 E). Q-PCR analysis of *SIM2s* levels confirmed an approximate 50% knockdown of *SIM2s* expression in *SIM2si* xenografts (Figure 22 C).

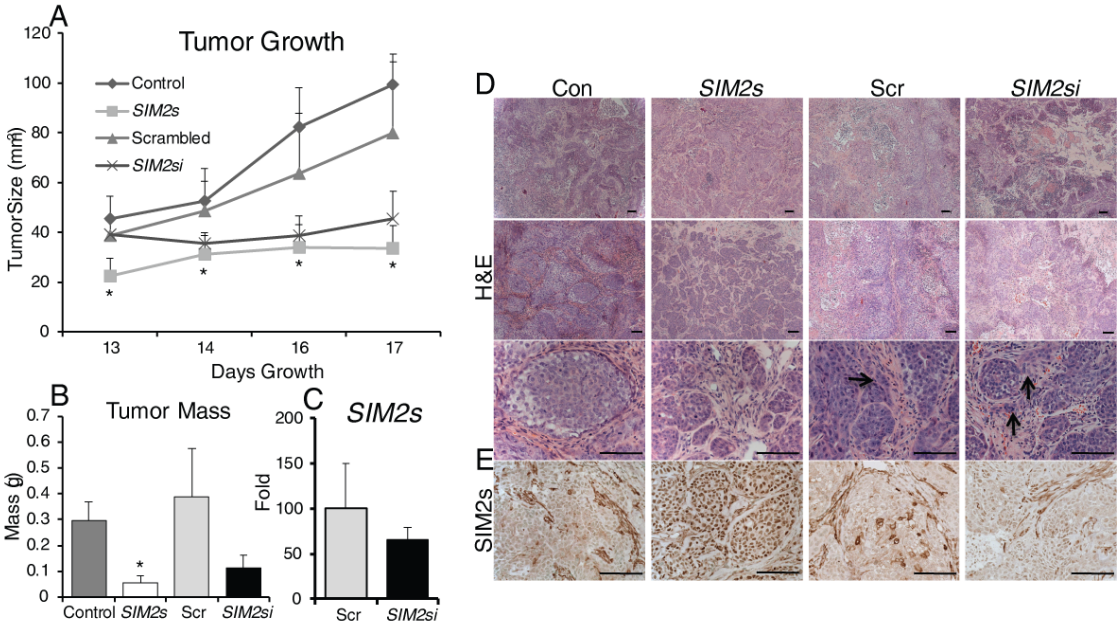


Figure 22. Differential SIM2s Expression Regulates Growth In Vivo. A & B – Analysis of xenograft tumor growth and mass shows that over-expression and loss of SIM2s expression inhibits xenograft growth. C – Q-PCR analysis of SIM2s mRNA expression in SIM2si tumors shows an approximate 50% loss of expression in vivo. D – H&E histological analysis shows that SIM2s expressing tumors exhibit smaller, more lobulo-like structures throughout the tumor, with less necrosis and inflammation. Images were taken using a 5x objective (5x) 10x objective (6.3x) and a 40x objective (25.2x). Scale bars represent 100µm. Arrows indicate areas of invasion. D – Histological analysis of SIM2s expression in xenografts confirms SIM2s overexpression and loss of expression. Images were taken using a 40x objective (25.2x). Scale bars represent 100µm. * = p-value < .05.

SIM2s Inhibits Expression of Basal Breast Cancer Markers

We have previously established that SIM2s is a negative regulator of EMT and promotes mammary gland differentiation *in vitro* and *in vivo* (Kwak et al., 2007; Scribner et al., 2011; Wellberg et al., 2010). To determine if the morphological changes associated with *SIM2s* expression in MCF10DCIS.COM xenografts are similar to our previous gain and loss of function studies in the mouse mammary gland, we examined basal markers involved in breast cancer progression and EMT via Q-PCR and immunohistochemical analysis. Immunostaining for basal markers including keratin 14 (KER14), alpha-smooth muscle actin (□SMA), vimentin (VIM) and p63 show a decrease in staining in *SIM2s* over-expressing MCF10DCIS.COM tumors, and

up-regulation with loss of *SIM2s* (Figure 23 A, B, C, & D). Moreover, indicative of enhanced tumor aggressiveness and progression, we observed an increase in co-localization of KER5 and VIM in *SIM2si* tumors (Figure 23 E, Figure 24). Further analysis of EMT and basal markers, including the EMT transcription factor, *SLUG,* which we have previously shown is directly regulated and suppressed by SIM2s, was significantly decreased in *SIM2s* tumors along with *SMA* and *p63* (Figure 23 F, G, & I). These results are consistent with SIM2s's role in mammary gland differentiation as p63, SMO and SLUG regulate cell differentiation and stem cell maintenance, which suggests that re-establishment of *SIM2s* is sufficient to promote a decrease in basal breast cancer markers (de Biase et al., 2010; Du et al., 2010; Dubois-Marshall et al., 2011; Kallergi et al., 2011; Moraes et al., 2007; Visbal et al., 2011; Yalcin-Ozuysal et al., 2010). Analysis of *p21* mRNA levels also showed a decrease in *SIM2si* xenografts, similar to what was seen *in vitro* (Figure 23 H). p21 is an important cell cycle regulator and is involved in the p53 stress response pathway as well as senescence (Bond et al., 1996; Malkin et al., 1990; Zuo et al., 2012).

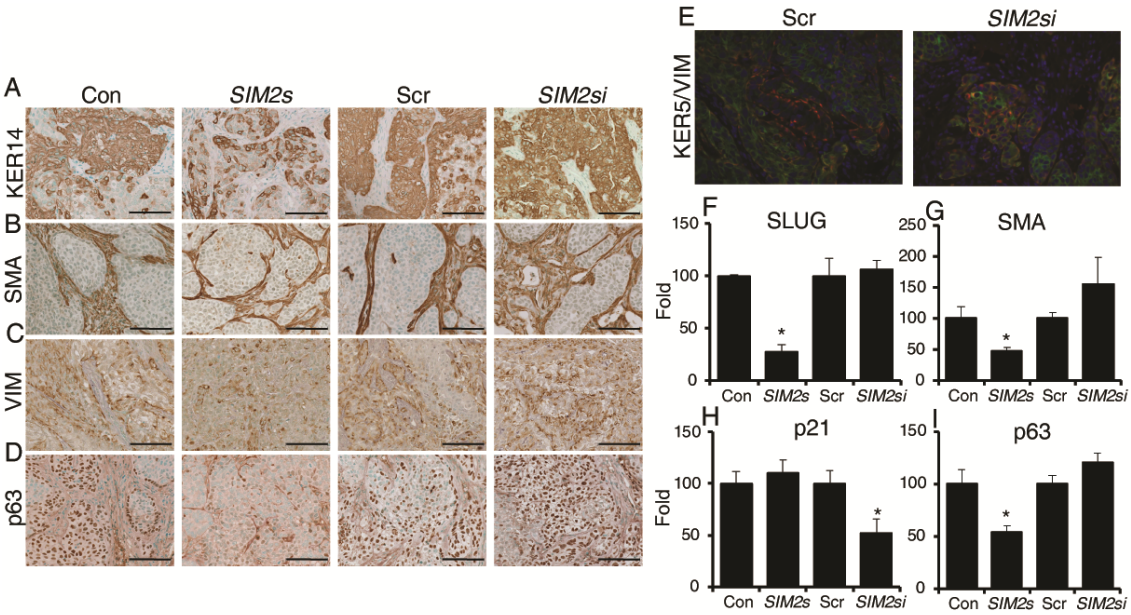


Figure 23. SIM2s Decreases Markers Associated with Basal Breast Cancer in MCF10DCIS.COM Xenografts.A, B, C, & D – Immunohistochemical staining for basal markers including KER14, SMA, VIM, and p63. Images were taken using a 40x objective (25.2x). Scale bars represent 100µm. E – KER5/VIM immunofluorescence shows increased overlap of KER5 and VIM in SIM2si xenografts, which is associated with enhanced invasive potential and aggressiveness. F, G, H, & I – Q-PCR analysis of basal markers SLUG, SMA, and p63, as well as cell cycle regulator p21. * = p-value < .05.

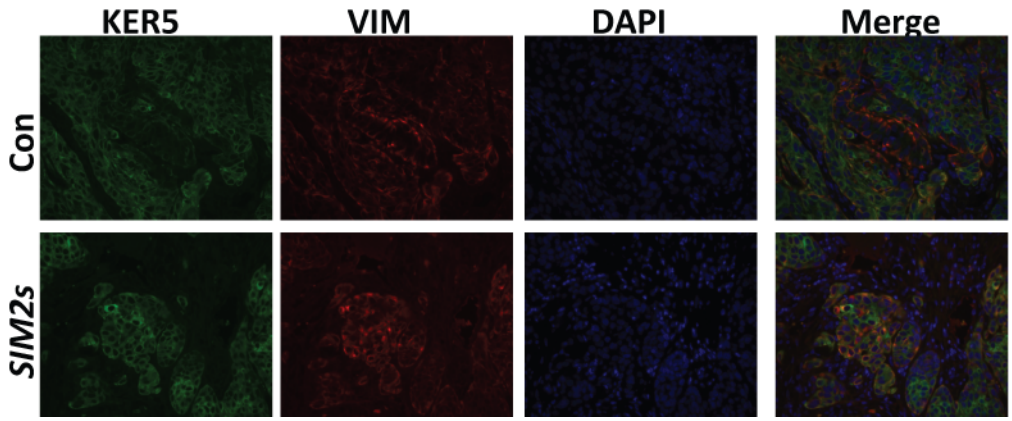


Figure 24. Channel Images of the KER5/VIM Fluorescence Previously Shown (Figure 23). Tumors were stained with KER5 (green), VIM (red), and DAPI (blue). Images were taken using a 40x objective (25.2x).

SIM2s Promotes Expression of Luminal Markers

To determine whether differential expression of *SIM2s* regulates prominent luminal markers in DCIS xenografts, we evaluated the expression of E-cadherin (CDH1), keratin-18 (KER18), and Mucin-1 (MUC1). Immunostaining results for CDH1 showed increased trends and localization to the cellular membrane in the *SIM2s* over-expressing tumors, and a decrease in localized staining with *SIM2si tumors* (Figure 25 A). Interestingly, Q-PCR analysis of *CDH1* showed no significant changes with *SIM2s* overexpression; however *SIM2si* tumors had significantly lower levels of *CDH1* mRNA compared to controls (Figure 25 D). Analysis of keratin 18 (KER18) showed a significant positive relationship with *SIM2s* expression; KER18 protein and mRNA levels were elevated in *SIM2s* tumors and protein appeared decreased with loss of *SIM2s* (Figure 25 B & E). MUC1, an apical luminal marker that is often mis-localized in cancer, showed an increase in apical staining in *SIM2s* xenografts, while a loss of localization is seen in *SIM2si* tumors (Figure 25 C). In addition, we also examined changes in the transcription factor GATA3, a prognostic factor associated with positive breast cancer outcome and regulator of breast tumor cell differentiation (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006). Analysis of *GATA3* mRNA expression showed no differences in with *SIM2s* expression, indicating that the differentiation phenotype associated with *SIM2s* expression appears to be *GATA3* independent in this model (Figure 25 G). To determine whether *SIM2s* tumors undergo partial lactogenic differentiation, we also examined the expression of the milk protein β-casein (CSN2). Immunostaining for β-casein in *SIM2s* tumors not only showed increased protein expression over controls, but also secretory globule formation indicative of milk protein expression as seen in the lactating mammary gland (Figure 25 H, arrows) and a distinct trend in *CSN2* mRNA gene expression in *SIM2s* tumors (Figure 25 F & H). Overall these data support

the hypothesis that expression of *SIM2s* induces a luminal phenotype, including expression of milk proteins, while loss of *SIM2s* significantly decreases the expression of luminal markers.

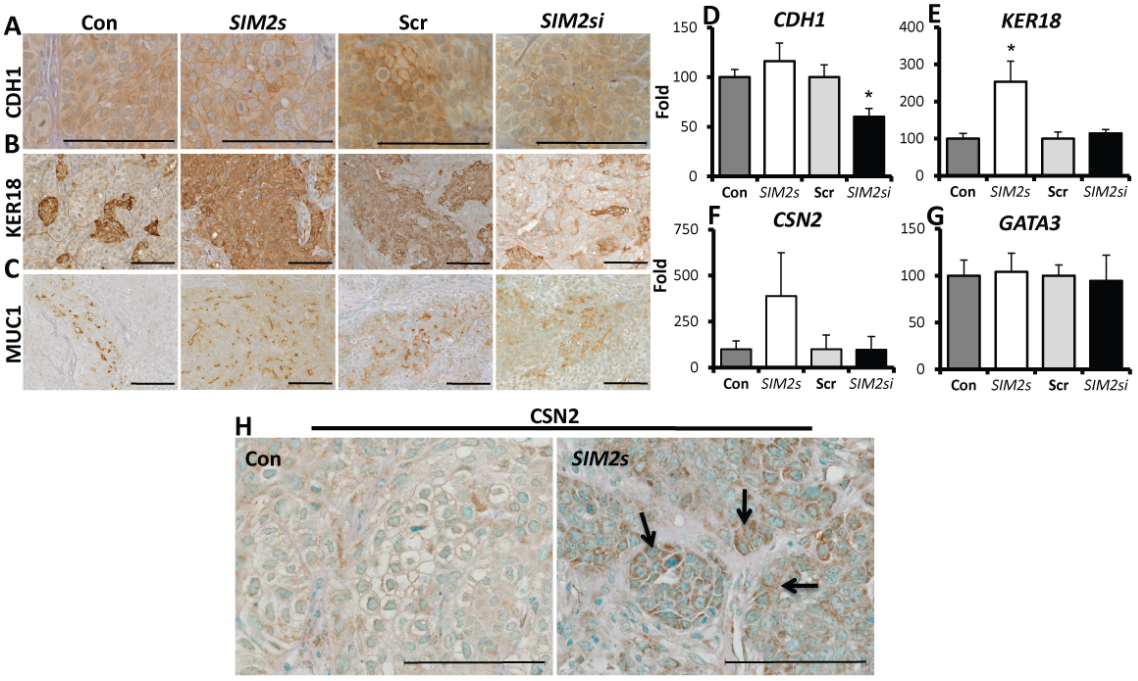


Figure 25. SIM2s Xenografts Have Increased Levels of Luminal Markers and Express β-Casein. A, B, & C –Re-establishment of SIM2s promotes apical localization of CDH1 and increased KER18 expression, while SIM2s loss results in a decrease in expression. SIM2s expression also promotes apical expression of luminal marker MUC-1, whereas loss of SIM2s causes a loss of localization. D, E, F, & G – Q-PCR analysis of luminal markers CDH1, KER18, CSN2, and GATA3. H – Immunohistochemical analysis for CSN2 shows elevated levels in SIM2s tumors (see arrows). Images were taken using a 40x objective (25.2x) and 63x objective (63x). Scale bars of images represent 100µm. * = p-value < .05.

Angiogenesis and Metastasis Are Inhibited by SIM2s

We have shown that gain and loss of *SIM2s* expression in MCF10DCIS.COM correlates with phenotypic changes in invasive behavior and expression of luminal and basal differentiation markers. To investigate if these phenotypic differences affect cancer progression and metastasis, we analyzed lungs from tumor bearing mice for vimentin (VIM), which is expressed in MCF10DCIS.COM cells, but not in normal lung tissue (McDaniel et al., 2006). The results showed positive VIM staining in lungs from control and scrambled tumors; however, we did not detect VIM expression in lungs from mice with *SIM2s*-expressing tumors (Figure 26 A & C). To confirm *SIM2s*-dependent changes in progression, we performed Q-PCR analysis for human specific β-2-globulin (*β2M*) gene expression in mouse lungs, which has been previously shown as an indicator of human cells in mouse lung tissues either due to metastasis or circulating tumor cells (McDaniel et al., 2006). While moderate levels of *β2M* expression were observed in control tissues, we did not detect *β2M* expression in lung tissue from mice with *SIM2s* over-expressing tumors (Figure 26 B). In contrast, the majority of the lungs from mice with *SIM2si* tumors had high levels of *β2M* expression (Figure 26 B). Consistent with differences in metastatic potential and *SIM2s* expression, we observed a decrease in angiogenesis in *SIM2s* tumors compared to controls (Figure 26 D and E) Interestingly, this difference is seen without taking into account the drastic change in tumor size (Figure 26 E). In contrast, no significant change in angiogenesis was seen with loss of *SIM2s*, indicating that the increase in metastasis is due to other metastatic processes.

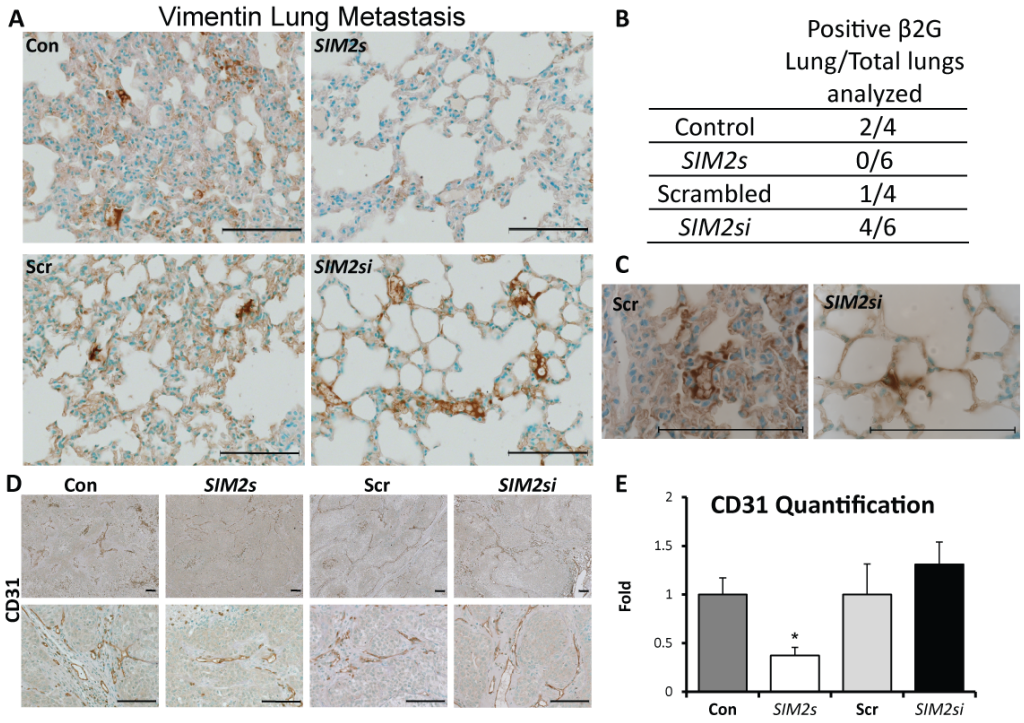


Figure 26. SIM2s Inhibits Metastasis and Alters Angiogenesis. A – Immunohistochemical analysis of lung tissue for vimentin (VIM) positive micrometastases showed decreased staining in SIM2s tumors, whereas loss of SIM2s enhanced lung metastasis. Images were taken with a 40x objective (25.2x). B – Q-PCR analysis for human β-2-Globulin as an indicator of lung metastasis confirmed the effect of SIM2s on metastasis with a decreased in β-2-Globulin expression in SIM2s tumors and increased expression with loss of SIM2s. Data is shown as the number of β-2G positive samples out of the total number of samples analyzed. C – Increased magnification (63x) of VIM staining to indicate the presence of vimentin positive cells in Scrambled controls and SIM2si tumors. D – Immunostaining for CD31 expression showed that SIM2s tumors have smaller blood vessels that remained on the outer perimeter of the tumors, whereas SIM2si tumors had an increased trend in angiogenesis. E – Quantification of CD31 staining by measuring blood vessel length confirms trends seen with CD31 immunohistochemistry. Images were taken using a 10x objective (6.3x) and a 40x objective (25.2x). Scale bars in images represent 100μm. *= p-value < .05

SIM2s-dependent Regulation of Matrix Metalloproteinase and Hedgehog Signaling

We have previously shown cell type specific SIM2s-dependent regulation of matrix metalloproteinase (MMP). These studies found SIM2s binds to the *MMP3* promoter and inhibits *MMP3* expression in MDA.MB.435 cells (Kwak et al., 2007), while *MMP2* expression is increased in MCF7-*SIM2si* cells and mouse *Sim2s* knockout mammary glands (Laffin et al., 2008). Using Q-PCR, we examined *MMP* gene expression in *SIM2s* over and under-expressing tumors (Figure 27). The results show that *SIM2s* xenografts have decreased *MMP* expression, whereas *SIM2si* tumors have increased *MMP* levels (Figure 27 A, B, C, & D). In addition, the hedgehog signaling pathway has been shown to be overexpressed in breast cancer and play a role in proliferation and differentiation (Moraes et al., 2007; Visbal et al., 2011). Analysis of Indian Hedgehog (*iHH*) and *SMO* by Q-PCR showed a drastic increase in *iHH* and *SMO* mRNA levels with loss of *SIM2s* (Figure 27 E & F).

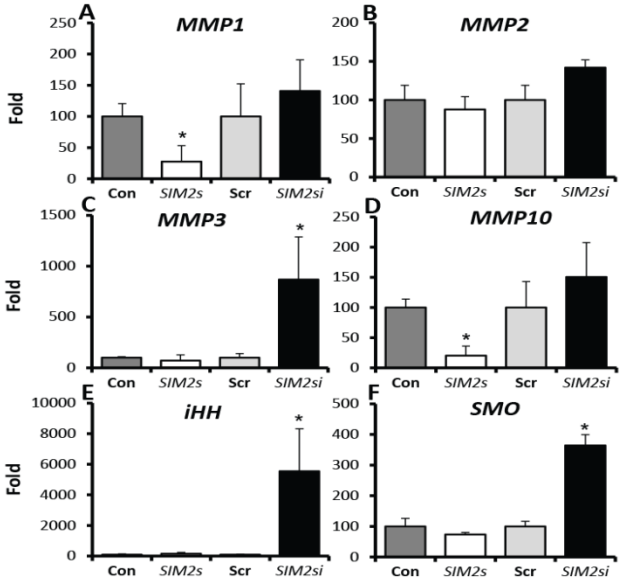


Figure 27. Loss of SIM2s Increases Tumor Invasiveness through MMP Expression and Hedgehog Signaling. A, B, C & D – Q-PCR analysis of various MMPs showing a decrease in expression with SIM2s tumors and an increase in SIM2si tumors, a likely mechanism for increased tumor invasiveness and metastasis. E & F – Q-PCR analysis of Indian Hedgehog (IHH) and Smoothened (SMO) show elevated levels of expression in SIM2si tumors. * - p-value < .05.

Discussion

There is significant evidence that relates the differentiation status of a tumor with its metastatic potential (Bloom and Richardson, 1957; Liu et al., 2007; van 't Veer et al., 2002). Analysis of luminal markers by microarray analysis and immunohistochemistry have confirmed that loss of epithelial characteristics correlates with an increase in cancer progression (Perou et al., 2000; Sorlie et al., 2001; West et al., 2001; Zajchowski et al., 2001). Though pathways have been identified that promote differentiation, few molecules have been identified that maintain and enhance differentiation potential. We have previously shown that SIM2s is a negative regulator of EMT in normal breast, breast cancer cell lines and the mouse mammary gland by suppressing *SLUG* and *MMP2* gene transcription (Kwak et al., 2007; Laffin et al., 2008). In contrast, overexpression of *Sim2s* in the mouse mammary gland under the *MMTV* promoter induces precocious lactogenic differentiation in virgin mice and delayed involution following forced weaning (Scribner et al., 2011; Wellberg et al., 2010). Together, these observations led us to hypothesize that expression of *SIM2s* in breast cancer would inhibit tumor growth by regulating differentiation potential. We report here that *SIM2s* expression is lost in human DCIS progression to invasive breast cancer and, utilizing the MCF10DCIS.com progression model, we demonstrate that re-establishment of *SIM2s* promotes a more luminal-like phenotype, whereas down-regulation of *SIM2s* leads to an increase in invasive potential. These new data support a role for SIM2s in regulating epithelial identity and a potential novel molecular target for differentiation therapy.

At the onset of xenograft studies, as expected we observed that tumors over-expressing *SIM2s* grew at a slower rate, as compared to controls, with lower amounts of necrosis. Surprisingly, we found that *SIM2si* tumors exhibited a decreased trend in growth compared to scrambled controls. However, upon histological analysis, we observed a more invasive

phenotype and large necrotic areas in the *SIM2si* tumors. In comedo DCIS, necrotic centers have been implicated as a more rapidly progressing DCIS with a worse prognosis compared to DCIS lacking necrosis (Shekhar et al., 2008). Further analysis of luminal and basal-like breast cancer markers showed distinct trends regarding *SIM2s* gain and loss of function. We observed that basal markers were inhibited in *SIM2s* tumors, with upward trends occurring in *SIM2si* tumors. Conversely, when examining luminal markers, we found an increase either in the appropriate localization (CDH1 and MUC1) or increased expression with *SIM2s* tumors and a loss of localization and expression with *SIM2si.* Another unique expression pattern was the presence of αSMA in the SIM2s over-expressing tumors. We anticipated that re-establishment of *SIM2s* in MCF10DCIS.COM cells would inhibit the bipotent progenitor capabilities of the cell line, and thus prevent the development of a myoepithelial layer *in vivo*. Surprisingly, however, SIM2s did not affect the bipotent progenitor ability. The significant decrease in *SMA* mRNA expression seen in *SIM2s* tumors could possibly be due to the smaller size of the tumors and lobular units rather than a biologically significant change in *SMA* expression. Finally, the expression of CSN2 in *SIM2s* tumors further indicates that not only does re-establishment of *SIM2s* maintain epithelial integrity, but also promotes functional differentiation.

Recent studies have identified a number of transcription factor cascades that control key events in regulating mammary epithelial differentiation including GATA3, ELF5, NOTCH and C/EBPβ (Asselin-Labat et al., 2007; Grimm and Rosen, 2003; Politi et al., 2004; Zhou et al., 2005a). For instance, analysis of *Gata3* conditional knockout mammary glands found an increase in luminal progenitor cells during alveolar differentiation and a defect in virgin ductal morphogenesis as a result of compromised estrogen responsiveness (Kouros-Mehr et al., 2006) , whereas loss of *Elf5* has no effect on virgin development and exclusively regulates alveolar cell fate (Oakes et al., 2006). Although these factors play key roles in promoting differentiation

along the luminal lineage, it begs the question: what maintains mammary ductal epithelial cells in a differentiated state and keeps them from de-differentiating and acquiring stem cell characteristics? It can be hypothesized that this/these factor(s) may target pathways regulating tumor initiating cell self-renewal by blocking induction of EMT and maintaining epithelial integrity. Interestingly, studies with GATA3 have shown that while overexpression is sufficient to promote epithelial differentiation, forced loss of *GATA3* is not tolerated (Asselin-Labat et al., 2007). With *SIM2s* we observed that while overexpression is sufficient to induce differentiation, loss of expression promotes malignant transformation. Moreover, the lack of change in *GATA3* in the *SIM2s* tumors, which has been shown to promote differentiation and inhibit breast cancer growth and metastasis, indicates that SIM2s is operating independently of GATA3. This has significant implications for our understanding of normal mammary development and breast cancer progression by introducing a novel pathway that may play a role in maintaining tumor initiating cells or in promoting functional differentiation.

One of the most interesting phenotypes of this study was the dramatic increase in lung metastasis with loss of *SIM2s*. MMPs have long been attributed as playing a key role in breast cancer invasion and (Ioachim et al., 1998; Liu et al., 2012; Mendes et al., 2005; Pellikainen et al., 2004; Walsh et al., 2012) are integral to the degradation of the basement membrane during normal biology functions including mammary gland involution and lobular development (Ioachim et al., 1998; Kessenbrock et al., 2010; Schedin et al., 2004; Scribner et al., 2011; Sims et al., 2011; Sternlicht et al., 1999; Wang et al., 2011; Witty et al., 1995). Previous work in our lab has shown that SIM2s differentially regulates *MMP2* and *MMP3* gene expression (Kwak et al., 2007; Laffin et al., 2008). In the study here, we observed a *SIM2s*-dependent regulation of *MMP* gene expression in *SIM2s* over and under-expressing DCIS xenografts. We showed that *MMP1* and *MMP10* were significantly decreased by *SIM2s* expression, whereas *MMP3* was

significantly increased by loss of *SIM2s*, accompanied by increased trends in other MMPs analyzed. This data indicates that a likely mechanism for SIM2s' effect on metastasis may be through a global regulation of MMPs in a complex and varied manner. Hedgehog signaling has also been implicated in breast cancer progression and invasion as well as the maintenance of cancer stem cells (Kasper et al., 2009; Kasperczyk et al., 2009; Moraes et al., 2007; Visbal et al., 2011). In our study the decrease in *SMO* expression in *SIM2s* tumors is unique since *SMO* up-regulation in tumors causes a phenotype similar to that seen in *SIM2si* tumors and *Sim2s* knockout mammary glands, including increased proliferation and altered differentiation, possibly indicating interaction between these genes (Moraes et al., 2007). Studies in gastric and ovarian cancer have also connected hedgehog signaling with invasion and *MMP* expression (Liao et al., 2009; Yoo et al., 2011), suggesting a potential mechanism of action by which SIM2s inhibits invasion and metastasis *in vivo*.

These observations provide a possible mechanism for the promotion of tumor differentiation through re-establishment of *SIM2s*, as well as possible roles for SIM2s in breast cancer progression. Determining what point of the metastatic cascade that SIM2s functions will be key in understanding SIM2s' role in breast cancer progression. Moreover, the use of established metastatic models and the impact of SIM2s on breast cancer subtypes will help elucidate the mechanism by which SIM2s affects tumor progression as well as its possible effect on tumor initiating cells. Together, these results suggest that SIM2s has the potential to be a novel target for differentiation therapy by inhibiting or reversing breast cancer progression.

CHAPTER V

MANUSCRIPT 3: SIM2S INHIBITS DCIS BREAST CANCER PROGRESSION BY REGULATING SENESCENCE-DEPENDENT METABOLIC EQUILIBRIUM

Synopsis

Dysregulation of cellular metabolism is a defining hallmark of breast cancer progression and is associated with metastasis and therapeutic resistance. However, there is a gap in our understanding of the mechanisms by which cells undergo the switch from respiration to glycolysis and its impact on tumorigenesis. Here, we show that the bHLH/PAS transcription factor Singleminded-2s (SIM2s) inhibits ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC) progression by regulating metabolic homeostasis and cell senescence. We found that SIM2s promotes oxidative phosphorylation and inhibits aerobic glycolysis through direct up-regulation of CDKN1A gene expression. These findings suggest that SIM2s is a tumor suppressor that blocks DCIS progression by regulating the equilibrium between glycolysis and senescence.

Introduction

Ductal Carcinoma in situ (DCIS) are a heterogeneous group of diseases that are characterized by a neoplastic mammary lesion that is confined to the ductal-lobular system of the breast (Cocker et al., 2007; Polyak, 2010; Porter et al., 2003; Tait et al., 2007). Due to improved detection methods, the diagnosis of DCIS has risen from less than 1% of diagnosed breast cancers to 15-25% (Jones, 2006; Norton et al., 2010). Studies have yet to find reliable

biomarkers that dictate the progression of DCIS to invasive ductal carcinoma (IDC). Pathological markers such as nuclear grade, comedo necrosis, and size have been associated with increased risk of DCIS progression; however these features are confounded when addressing treatment and follow-up (Fujii et al., 1996; Gur, 2010; Liao et al., 2011; Nofech-Mozes et al., 2008; Shekhar et al., 2008). Many confirmed oncogenes and tumor suppressors such as PTEN, ERBB2, and MYC have been analyzed in DCIS and IDC samples; all showing no clear change with progression (Behling et al., 2011; de Biase et al., 2010; Lu et al., 2009; Miron et al., 2010; Rajan et al., 1997; Schmidt et al., 2010; Yao et al., 2006). Emerging evidence indicates that response to metabolic stress and hypoxia promotes the progression of DCIS to IDC (Hu et al., 2008; Lee et al., 2012; Schmidt et al., 2010). First described by Otto Warburg, tumor cells preferentially utilize glycolytic pathways for energy generation while down-regulating oxidative phosphorylation, even in the presence of abundant oxygen (Cairns et al., 2011b; Warburg, 1956a). This metabolic shift is driven by the tumor cells' need to meet increased metabolic demands for proliferation and survive insults to mitochondrial respiration (Bensinger and Christofk, 2012; Dang, 2012; Warburg, 1956a; Yeung et al., 2008). Increased glycolysis provides tumors with a selective growth advantage by supplying ATP and the precursors required for nucleotide, amino acid and lipid biosynthesis. Previous work has shown that altered metabolism in breast cancer is associated with increased tumor progression and therapeutic resistance, supporting a role for alterations in metabolism in malignant transformation (Gambhir, 2002; Gatenby and Gillies, 2004; Hama and Nakagawa, 2010; Jones and Thompson, 2009; Kroemer and Pouyssegur, 2008; Tennant et al., 2010).

Tumor cell metabolism is dependent upon the convergence of oncogenic and tumor suppressor pathways to support growth and survival (Levine and Puzio-Kuter, 2010; Yeung et al., 2008). The p53 and hypoxia inducible factor-1 alpha (HIF1α) pathways play a major role in

metabolic adaptation since selective loss of *p53* and activation of HIF1α promote a metabolic switch. p53 has been implicated in metabolic control by influencing the balance between glycolysis and oxidative phosphorylation through inhibition of glucose transporters and promoting mitochondrial respiration (Madan et al., 2011; Yeung et al., 2008; Zawacka-Pankau et al., 2011). A major role of p53 is the promotion of cellular senescence, which is a major barrier to breast cancer progression (Kuilman et al., 2010; Peeper, 2010; Smit and Peeper, 2010). Senescent cells remain metabolically active yet fail to proliferate, and induction of senescence through chemotherapy or early oncogenic induction is considered inhibitory to tumor progression (Schmitt et al., 2002; te Poele et al., 2002). While past work has indicated an intricate relationship between AMP, glycogen consumption, and senescence; little research has been done to define the potential role of senescence in tumor progression through mediation of metabolism (Favaro et al., 2012; Kim et al., 2010; Zwerschke et al., 2003).

We have previously shown that the bHLH/PAS transcription factor Singleminded-2s (SIM2s) plays an important role in normal mammary gland development and tumor differentiation (Gustafson et al., 2009b; Kwak et al., 2007; Laffin et al., 2008; Metz et al., 2006; Scribner et al., 2012; Scribner et al., 2011; Wellberg et al., 2010). SIM2s expression is lost in primary breast tumors compared to normal mammary glands (Kwak et al., 2007; Scribner et al., 2012) and loss of *SIM2s* expression is associated with an epithelial mesenchymal transition (EMT), both in normal mammary glands and breast cancer cell lines, using multiple shRNA constructs. Re-establishment of *SIM2s* in breast cancer cell lines and xenografts inhibits growth and metastasis, while promoting luminal gene and milk protein expression (Kwak et al., 2007; Scribner et al., 2012). Enhanced *Sim2s* expression in the mouse mammary promotes lactogenic differentiation and delays involution (Scribner et al., 2011; Wellberg et al., 2010). The expression of beta casein milk protein expression in *SIM2s* expressing tumors and the normal

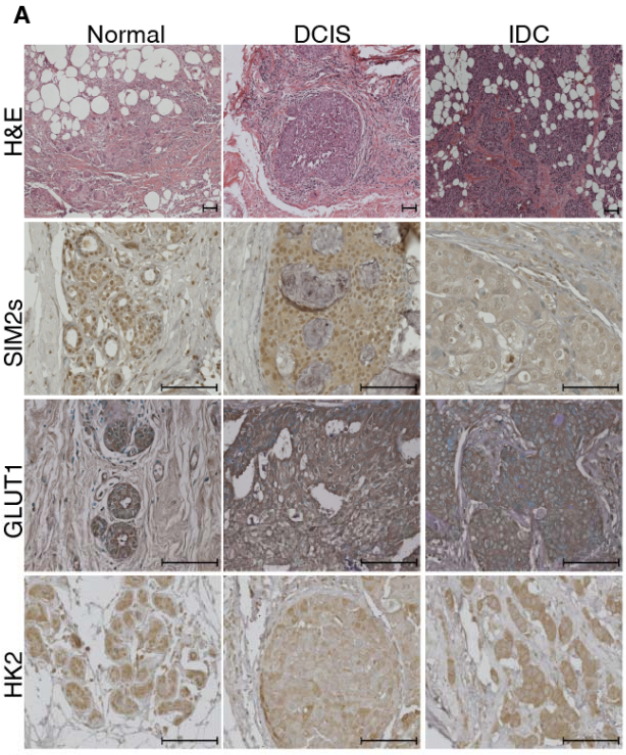
expression of *SIM2s* during lactation indicates a role for *SIM2s* in mediating the metabolic demands of the mammary gland during lactation. The loss of *SIM2s* expression with breast tumor progression and EMT prompted us to examine the potential role of *SIM2s* in early DCIS progression in regards to cellular metabolism and the Warburg effect.

Results

SIM2s Expression Correlates with Early Stage DCIS and Luminal Breast Cancer

To confirm SIM2s expression with breast cancer progression, we analyzed normal breast, DCIS and IDC for differences in SIM2s by IHC. Tumor microarrays were stained for H&E, SIM2s, GLUT1, and HK2 (Figure 28 A). The results show that SIM2s expression is high and nucleic in normal tissue, but decreases and begins to translocate to the cytoplasm in DCIS, and is lost in IDC, verifying previous work (Scribner et al., 2012). Statistical analysis of SIM2s staining shows a significant correlation between SIM2s expression and the state of breast disease (Normal, DCIS, or IDC) (Figure 28 B). GLUT1 and HK2 expression increased with tumor progression, indicative of the increased aerobic glycolysis, confirming previous work showing a glycolytic shift with tumor progression (Schmidt et al., 2010; Vander Heiden et al., 2010; Young et al., 2011) (Figure 28 A).

We performed further analysis for correlations within DCIS and SIM2s. SIM2s expression significantly correlated with estrogen and progesterone receptor expression in DCIS samples, but not HER2, indicating a significant relationship between SIM2s and luminal stage breast cancer (Figure 28 C). SIM2s expression was also significantly aligned with p53 expression and inversely related to Ki67 expression in DCIS samples (Figure 28C). Importantly, loss of SIM2s in DCIS correlated with increased microinvasion and metastasis, supporting a role for SIM2s in breast cancer progression (Figure 28 D).



B

Breast Cancer Type	SIM2s expression Nuclear	Both	Cyto	None	Test of Homogeneity	
Normal	17	3	0	5		*p-val*
DCIS	1	6	7	5	*Likelihood Ratio*	6.17E-07 *
IDC	0	1	5	4	*Pearson*	2.02E-05 *

C

Biomarkers

Variable	Sim2s Positive	Negative	Test Homogeneity	
ER				*p-val*
Positive	5	6	*Likelihood Ratio*	0.0197 *
Negative	0	6	*Pearson*	0.0493 *
PR				*p-val*
Positive	5	4	*Likelihood Ratio*	0.0041 *
Negative	0	8	*Pearson*	0.0121 *
HER2				p-*val*
High	1	7	*Likelihood Ratio*	0.1377
Normal	4	5	*Pearson*	0.1491
p53				*p-val*
Positive	0	7	*Likelihood Ratio*	0.0095 *
Negative	5	5	*Pearson*	0.026 *
Ki67				*p-val*
Positive	1	10	*Likelihood Ratio*	0.0124 *
Negative	4	2	*Pearson*	0.0128 *

D

Metastasis

Variable	Sim2s Positive	Negative	Test Homogeneity	
Invasion				*p-val*
Positive	1	9	*Likelihood Ratio*	0.0079 *
Negative	6	3	*Pearson*	0.0106 *
Metastasis				*p-val*
Positive	0	4	*Likelihood Ratio*	0.0386 *
Negative	7	8	*Pearson*	0.0856

Figure 28. SIM2s Expression Correlates with DCIS and Luminal Breast Cancer. (A) Immunostaining of human normal, DCIS, and IDC samples for H&E, SIM2s, GLUT1, and HK2. Samples were imaged at 25.2x. (B) Statistical analysis of breast type with SIM2s expression. Tissue microarrays were analyzed using categorical analysis comparing SIM2s staining versus the type of tissue (C) Statistical Analysis of DCIS specific pathology reports (n=17). Common biomarkers ER, PR, HER2, p53, and Ki67 were categorically analyzed compared to binomial SIM2s staining (nucleic/both = positive, cytoplasmic/none = negative). (D) Prognosis of micro invasion and/or metastasis was compared with binomial SIM2s staining. Likelihood Ratio and Pearson Chi Squared tests were performed to test correlations. *=p-value <.05. Scale bars = 100μm.

Loss of and Re-establishment of SIM2s Modulates Metabolic Shifts in Breast Cancer Cells

We have shown that loss of *SIM2s* in MCF7 and MCF10A cells and the mouse mammary gland induces an EMT (Laffin et al., 2008). These experiments analyzed multiple *SIM2s* targeting shRNA constructs to verify the effect of *SIM2s* knockdown in MCF7 and MCF10A cells. Potential downstream targets were identified by microarray analysis of MCF7 *SIM2si* cells using Ingenuity Pathway Analysis. Pathways critical to tumor metabolism and cellular function, as well as canonical pathways including p53 signaling, mitochondrial dysfunction, and oxidative stress were altered with loss of *SIM2s* (Figure 29 A). These results correlated with our observation that MCF7 *SIM2si* cells rapidly acidify their media compared to controls, indicative of enhanced glycolytic activity and lactate production (Figure 29 B). To determine if the MCF7-*SIM2si* cells had undergone a metabolic shift, we analyzed media from control and MCF7-*SIM2si* cells by ^{1}H nuclear magnetic resonance (NMR). MCF7 *SIM2si* cells significantly increased glucose consumption and lactate production under normal culture conditions (Figure 29 B). Metabolic flux was determined by measuring changes in oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) using a Seahorse XF-24 extracellular flux analyzer. These results confirmed that the basal respiration rate in *SIM2si* cells was significantly lower than controls, whereas there was an increased trend in ECAR (Figure 29 B). Surprisingly, MCF7 *SIM2si* cells did not respond to mitochondrial drugs oligomycin (ATP

Synthase inhibitor), rotenone (Complex I inhibitor), and FCCP (electron transport uncoupler), indicating that the mitochondria are almost completely inert (Figure 29 B). Together, these data support a role for SIM2s in energy homeostasis.

To further determine the effect of SIM2s on tumor metabolism, we analyzed *SIM2s* gain and loss of expression using basal p53 mutant SUM159 cells and MCF10DCIS.com cells (referred to hereafter as DCIS), which are an early stage cell line derived from MCF10A cells that mimics the natural progression of DCIS to IDC *in vivo* (Miller et al., 2000). Over-expression of *SIM2s* in SUM159 cells caused a significant loss in lactate production along with a decreased trend in glucose consumption. However, we found that DCIS *SIM2s* cells showed significantly higher glucose consumption, with no change in lactate – indicating that glucose consumption was being shunted to other pathways than lactate production (Figure 29 C).

For these initial studies, cells were grown in excess glucose under normal conditions (10mM-25mM), which according to the Crabtree effect, increases glycolysis while inhibiting reliance on oxidative phosphorylation (Crabtree, 1929). To determine if this glycolytic switch represented a homeostatic response to proliferation, we examined the ability of *SIM2s* over and under-expressing cells to turn to oxidative phosphorylation for energy provision upon nutrient starvation. In normal glucose containing media, we have shown that *SIM2si* cells proliferate more rapidly as compared to controls, whereas SIM2s inhibits proliferation (Gustafson et al., 2009b; Kwak et al., 2007; Laffin et al., 2008; Scribner et al., 2012). When we limited glycolysis and forced oxidative phosphorylation by either substituting galactose as an energy source or

under glucose starvation, we found that loss of *SIM2s* impaired adaptation to nutrient stress, whereas re-establishment of *SIM2s* promoted resistance to low glucose as compared to controls (Figure 29 D-F and Figure 30).This response is likely due to the *SIM2s* cells' ability to utilize their mitochondria more efficiently than control and *SIM2si* cells.

Further analysis of SUM159 *SIM2s* cells showed significantly higher levels of oxidative phosphorylation in both glucose concentrations, along with decreased ECAR rates (Figure 29 G). DCIS *SIM2s* cells also showed higher OCR rates in glucose rich media, but interestingly this significance was lost in low glucose. This is likely due to the control DCIS cells being able to mount a mitochondrial response to the stressed environment. DCIS *SIM2s* cells' ECAR rates were insignificant in glucose rich media, but were significantly decreased in low glucose. DCIS *SIM2si* cells showed similar trends to the MCF7 *SIM2si* cells, with decreased OCR and increased ECAR, varying with glucose levels (Figure 29 G). *SIM2s* expression thus has a significant effect on the metabolic adaptation of breast cancer cells, and this effect varies with breast cancer aggressiveness and progression.

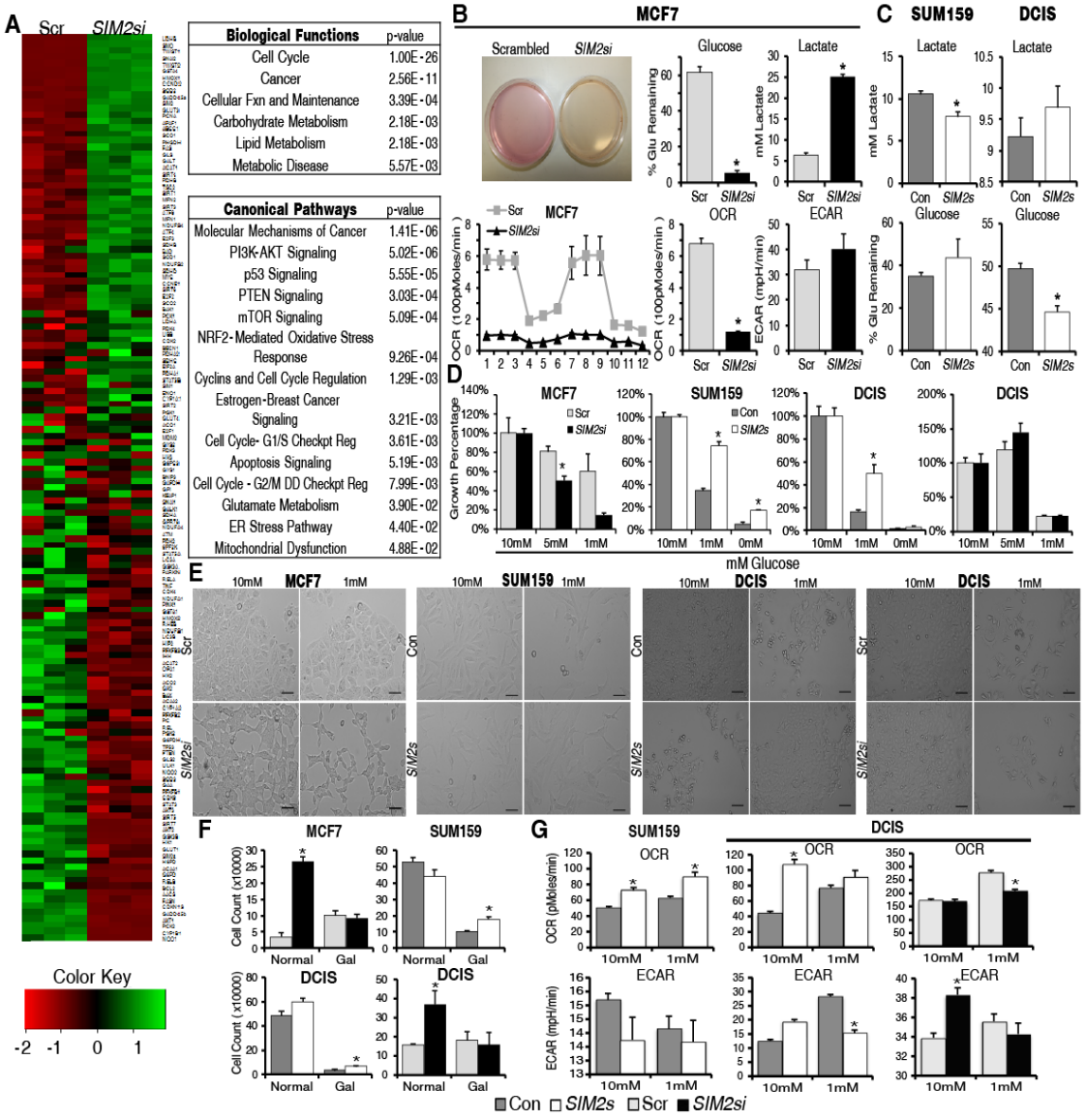


Figure 29. Loss of and Re-establishment of SIM2s Modulates Metabolism in Breast Cancer Cells. (A) Microarray Analysis of MCF7 SIM2si cells shows that pathways common to cell cycle, metabolism, and oxidative phosphorylation are significantly affected by loss of SIM2s. (B) Enhanced media acidification caused by MCF7 SIM2si cells. NMR analysis of % glucose remaining and mM lactate produced. Seahorse flux analysis of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of MCF7 SIM2si cells, along with response graph showing OCR cellular response to oligomycin (points 4-6), FCCP (points 7-9), and rotenone (points 10-12). (C) NMR analysis of SUM159 and DCIS SIM2s cells for mM lactate produced and % glucose remaining. (D) Proliferation assays of MCF7, SUM159, and DCIS cells in decreasing concentrations of glucose. 10mM cell counts were set to 100%, and decreasing glucose cell counts are normalized to their respective 10mM control. (E) Images of MCF7, SUM159, and DCIS cells in high and low glucose. Images were taken using a 20x objective lens. (F) Proliferation assays of MCF7, SUM159, and DCIS cells grown in normal media (10-25mM glucose) and galactose (10mM) containing media. (G) Seahorse flux analysis of SUM159 and DCIS cells in high and low glucose media. *=p-value<.05. Data are shown as mean + SEM. Scale bars = 100µm.

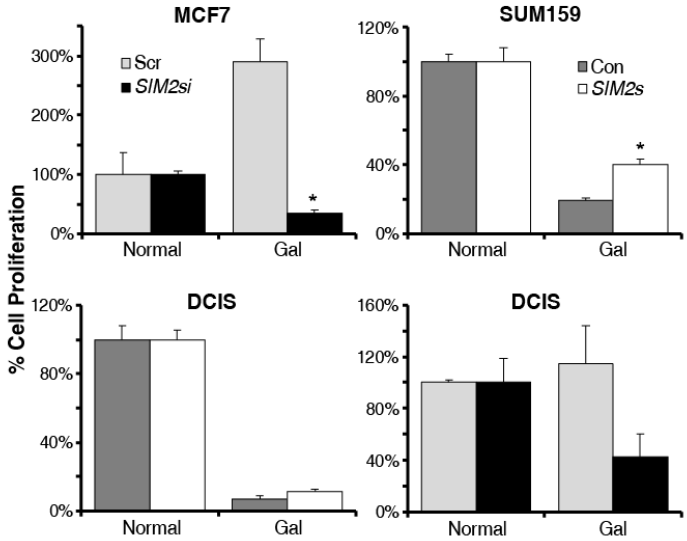


Figure 30. Proliferation Assays of MCF7, SUM159, and DCIS Cells in Galactose Containing Media. 10mM cell counts were set to 100%, and galactose cell counts are normalized as a percentage to their respective 10mM control. Student's T-test was performed to test correlations. *=p-value <.05. Data are shown as mean + SEM.

SIM2s Induces Intracellular Changes in Metabolic Constituents

To further address SIM2s dependent regulation of oxidative phosphorylation, we assessed various parameters of mitochondrial content in each cell type. Despite the significant differences in glycolysis, we found no change in mitochondrial membrane potential in *SIM2s* over and under-expressing cells (data not shown). When we measured mitochondrial and lysosome content using live cell imaging of cells labeled with MitoTracker and LysoTracker, we saw a significant increase in lysosomes with *SIM2s* over-expression in DCIS and SUM159 cells, and a decrease in MCF7-*SIM2si* cells. Similarly, we found an inverse relationship between *SIM2s* expression and mitochondrial content (Figure 31 A and 32 A-B). The observed increase in lysosomal activity accompanied with a decreased trend in mitochondria is likely due to enhanced mitophagy. Increased LC3B protein was seen with *SIM2s* expression in both SUM159 and DCIS cells and decreased in MCF7 *SIM2si* cells (Figure 32 B). To further

characterize the effect of SIM2s on mitochondria function, mitochondria morphology was examined by transmission electron microscopy. *SIM2s* expression correlated with shortened mitochondria, well-defined cristae and an electron-dense cytoplasm in DCIS cells, whereas loss of *SIM2s* resulted in elongated mitochondria and electron-lucent cytoplasm as compared to controls (Figure 31 B and Figure 32). In SUM159 cells, *SIM2s* expression also caused shortened mitochondria with well-defined cristae, however the cytoplasm was more electron-lucent than controls. Loss of *SIM2s* in MCF7 cells reduced mitochondria definition and cristae organization (Figure 31 B and Figure 32).

The endpoints of mitochondrial oxidative phosphorylation are ATP and reactive oxygen species (ROS). Therefore using ATP-lite luminescence and DCFH fluorescent assays we analyzed cellular capacity for energy production with *SIM2s* expression. In DCIS cells, *SIM2s* expression was associated with significantly higher ATP and ROS levels, regardless of glucose conditions. In contrast, down-regulation of *SIM2s* in DCIS cells significantly decreased ROS levels under glucose rich conditions, and decreased ATP production in low glucose (Figure 31 C). MCF7 *SIM2si* cells showed significantly lower levels of ATP regardless of glucose levels, as well as decreased trends in ROS that reached statistical significance in low glucose media. SUM159 *SIM2s* cells also showed significantly decreased ATP levels, with no significant change in ROS (Figure 31 C). Cells were treated with 2-deoxy-glucose (2-DG), which blocks glycolysis, and the ATP synthase inhibitor, Oligomycin (Oligo). Inhibition of glycolysis by 2-DG had little effect on *SIM2s* related differences; however the decreased ATP seen in *SIM2si* DCIS cells was lost. Trends remained similar with ROS as well, although blocking glycolysis in MCF7 *SIM2si* cells significantly reversed their ROS expression in glucose rich media. Inhibition of ATPase also saw little effect on ATP trends, except for during stressed, low glucose conditions – then ATP levels were reversed – indicating that during low glucose conditions a

majority of ATP is from oxidative phosphorylation, supporting the Crabtree effect (Figure 31 C). Since all trends were not reversed by 2-DG or oligomycin, it appears there is a complex balance between oxidative phosphorylation and glycolysis that is constantly adapting to the environment. These results also indicate that *SIM2s* plays an important role in the cells' ability to adapt to nutrient changes in the environment.

Key Glycolytic Enzymes Are Inhibited by SIM2s Expression

Tumor metabolism is dependent on the convergence of numerous signaling cascades and transcription factors. Minor changes in key pathways can have a significant impact on metabolic homeostasis (Figure 33 A). Analysis of key metabolic genes by qPCR in DCIS and SUM159 cells found that *SIM2s* expression correlated with significant decreases in glycolytic genes including *HK1* and *HK2*, as well as glucose transporters *SLC2A1*, *SLC2A3*, and *SLC2A4* (Figure 33 B). DCIS *SIM2si* cells showed a significant increase in *SLC2A3* and *HK1*, and increased trends in *SLC2A* and *HK2* gene expression. Similar trends were also observed in MCF7 *SIM2si* cells (Figure 34). Additionally, we saw no distinct trends with pentose phosphate pathway Glucose-6-phosphate dehydrogenase (*G6PD*), but did see significant decreases in lactate dehydrogenase (*LDHA*) in DCIS *SIM2s* cells (Figure 34).

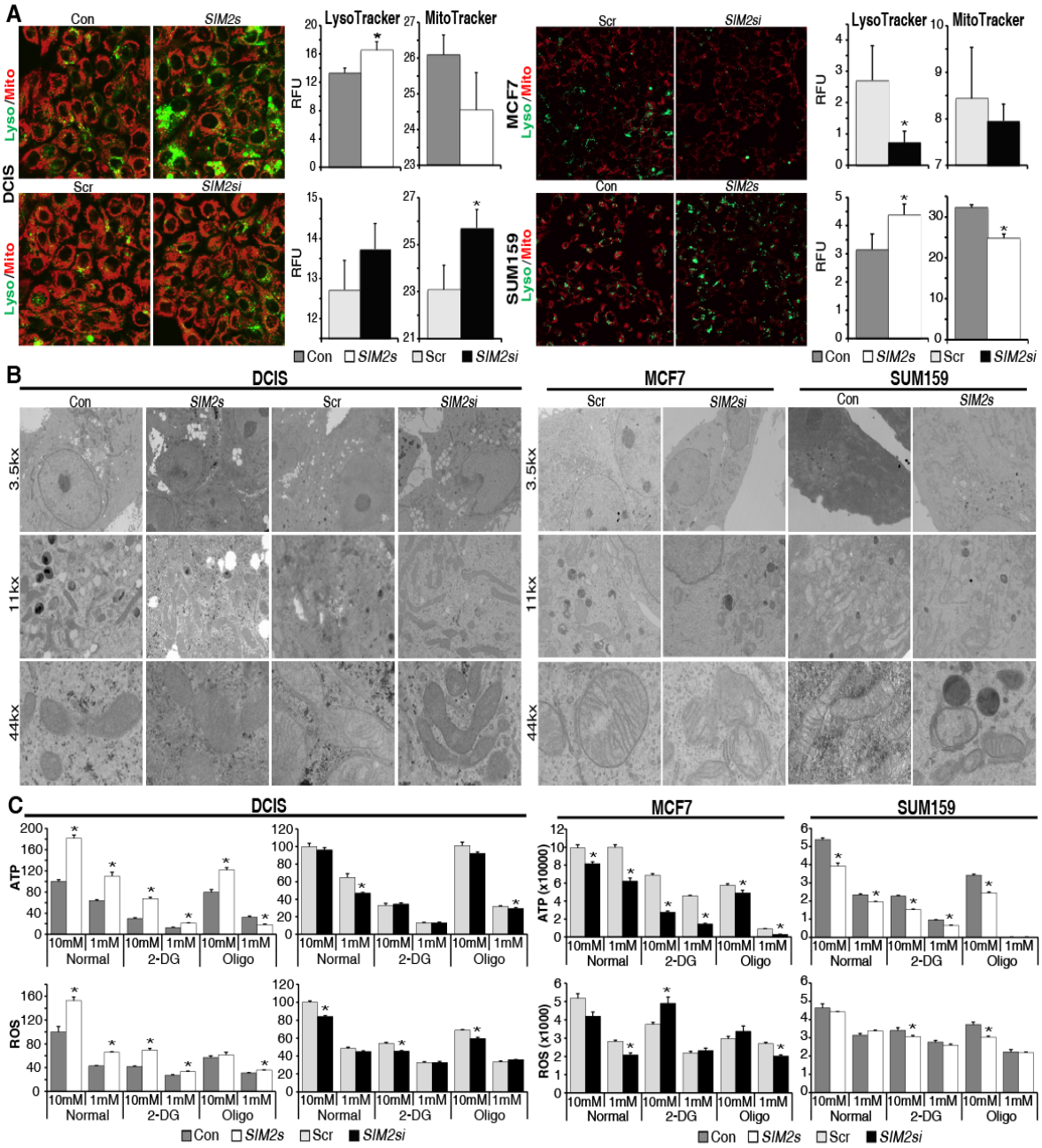


Figure 31. SIM2s Induces Intracellular Changes in Metabolic Constituents. . (A) Imaging of DCIS, MCF7, and SUM159 cells using LysoTracker and MitoTracker fluorescent probes. Images were taken using 63x objective lens. (B) Electron microscopy images of DCIS, MCF7, and SUM159 cells under glucose rich conditions. Images taken at 3.5kx, 11kx, and 44kx. (C) ATP and ROS quantification for DCIS, MCF7, and SUM159 cells. Cells were plated, adhered for 12 hours, and then were dosed for 24 hours prior to experiments. *=p-value<.05. Data are shown as mean + SEM.

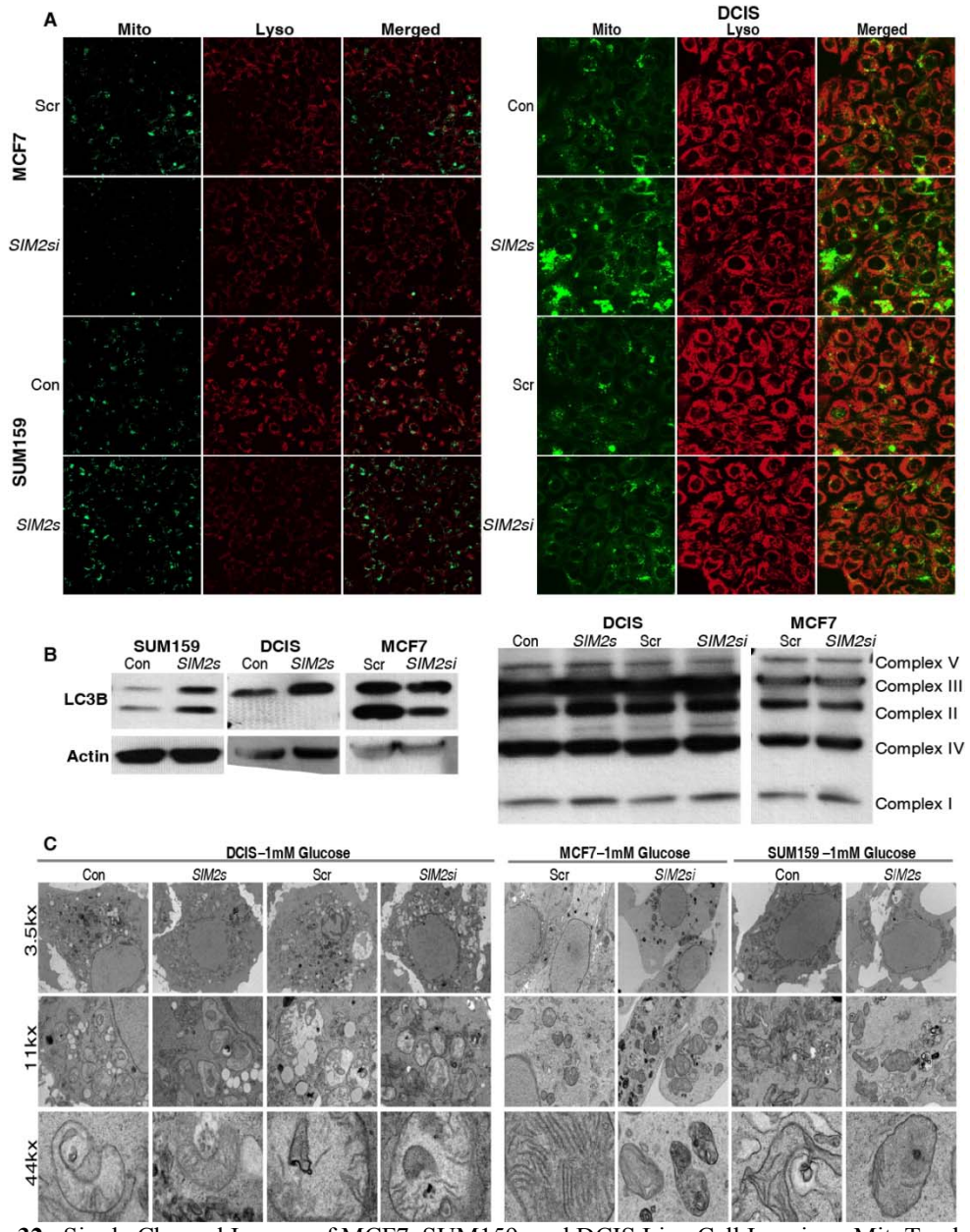


Figure 32. Single Channel Images of MCF7, SUM159, and DCIS Live Cell Imaging. MitoTracker is in red, LysoTrackers is in green. Images were taken using 63x objective lens. (B) Western blot analysis of LC3B and oxidative phosphorylation complexes using isolated mitochondria. Actin was used as loading control. . (C) Electron microscopy images of MCF7, SUM159, and DCIS cells grown in 1mM glucose media. Cells were grown in 1mM glucose for 24 hours prior to fixation and preparation. Images were taken at 3.5kx, 11kx, and 44kx.

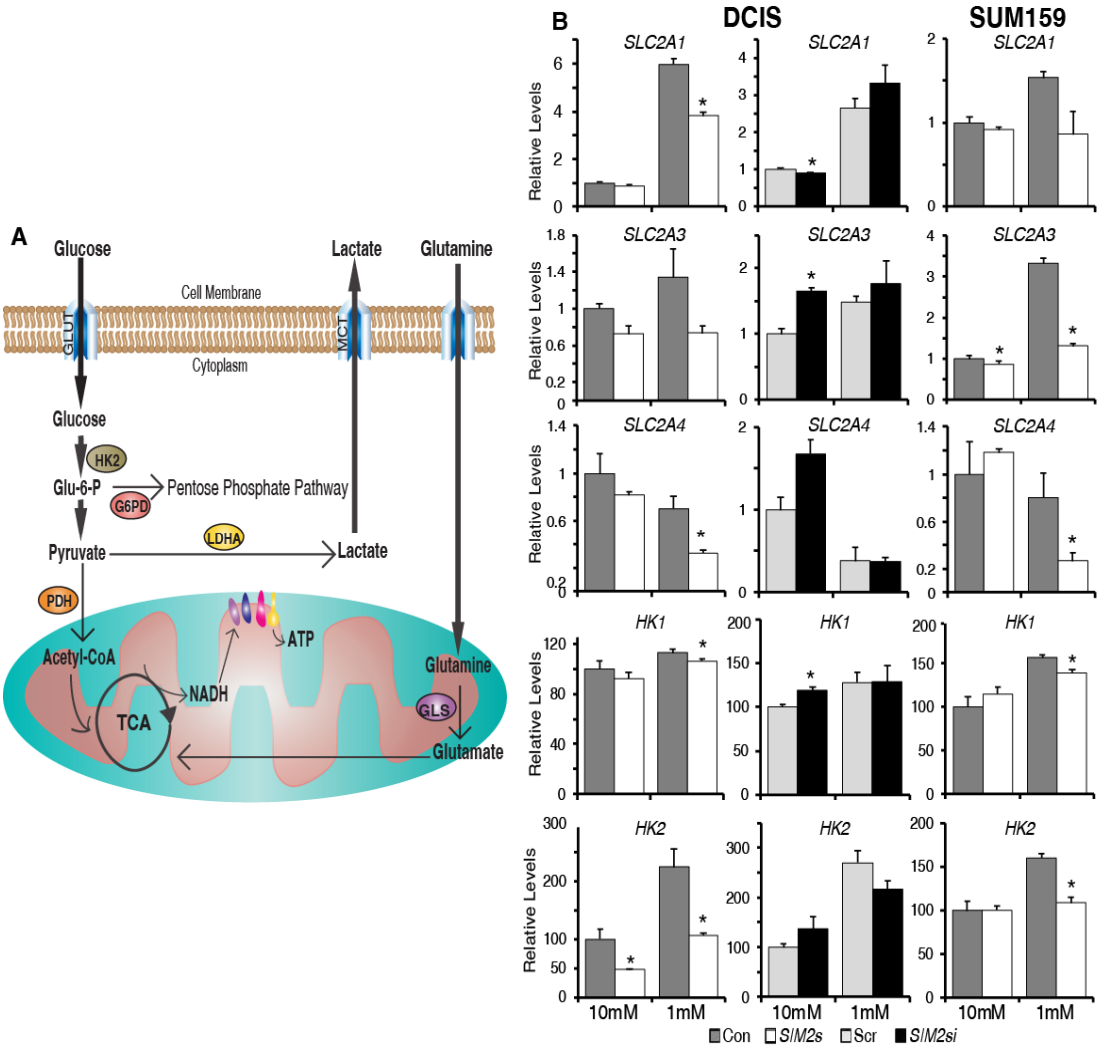


Figure 33. Key Glycolytic Enzymes Are Inhibited by SIM2s Expression. (A) Simplified model of key pathways involved in cellular metabolism, along with key, rate-limiting enzymes involved in each pathway. (B) Q-PCR analysis of gene expression of key glycolytic enzymes in MCF7 and SUM159 cells. Samples are normalized to the Control, 10mM samples. *=p-value<.05. Data are shown as mean + SEM.

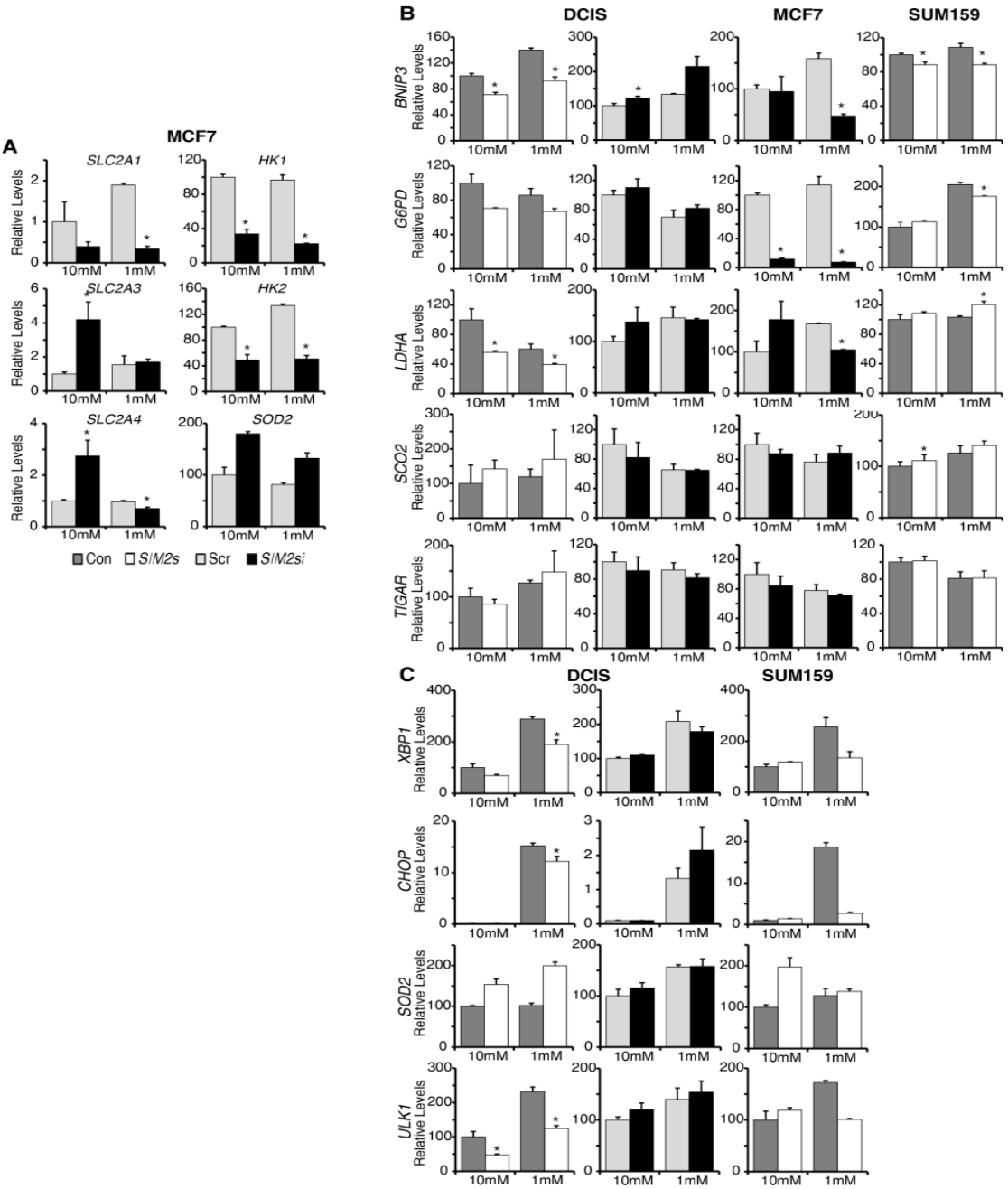


Figure 34. Q-PCR Analysis of Breast Cancer Cells for Metabolic and Stress Response Genes. (A) Q-PCR analysis of MCF7 cells for key glycolytic genes. (B) Q-PCR Analysis of MCF7, SUM159, and DCIS cells for alternative metabolic pathways and stress response genes. *=p-value<.05. Data are shown as mean + SEM.

DCIS Xenograft Analysis Confirms In Vitro Data and Induces P53 Stress Response

We have shown that SIM2s regulates MCF10DCIS.com progression and invasion in *in vivo* xenograft assays by promoting luminal gene expression and inhibiting matrix metalloproteinases (MMPs) (Scribner et al., 2012). The glycolytic trends observed *in vitro* were analyzed *in vivo,* DCIS control*, SIM2s,* and *SIM2si* tumors were stained for SIM2s, GLUT1, and HK2 (Figure 35 A). Consistent with the *in vitro* results, we found that GLUT1 and HK2 staining increased with loss of SIM2s, along with shifts in localization to the cellular membrane (Figure 35 A). qPCR analysis showed decreased *HK1* and *HK2* with *SIM2s* expression, as well as *SLC2A1* (Figure 35 B). We saw a significant increase in the insulin-regulated *SLC2A4* with *SIM2s*, and no change in the NFκB regulated *SLC2A3* (Figure 35 B). Also, DCIS xenografts showed a significant increase in p53-regulated oxidative phosphorylation genes *SCO2* and *TIGAR* with *SIM2s* expression (Figure 35 B) (Bensaad et al., 2006; Won et al., 2012). This change, not seen *in vitro*, is likely due to the unique stressed and hypoxic environment that occurs in the tumor. We found that *SIM2s* expression significantly increased nucleic p21 expression in DCIS xenografts, and that p21 expression decreased under normal breast cancer progression from DCIS to IDC (Figure 35 C). Additionally, we saw a significant decrease in key metabolic signalers *AKT* and *MTOR*, as well as *TSC2,* which is often involved in autophagy (Figure 35 B) (Alexander et al., 2010; Zhu et al., 2012). However, we still see the increased trend in *LC3* gene expression in *SIM2s* tumors. We did not see changes in genes involved in mitochondrial fusion, fission, and function, as well as key enzymes in the TCA cycle and pentose phosphate pathway (Figure 36).

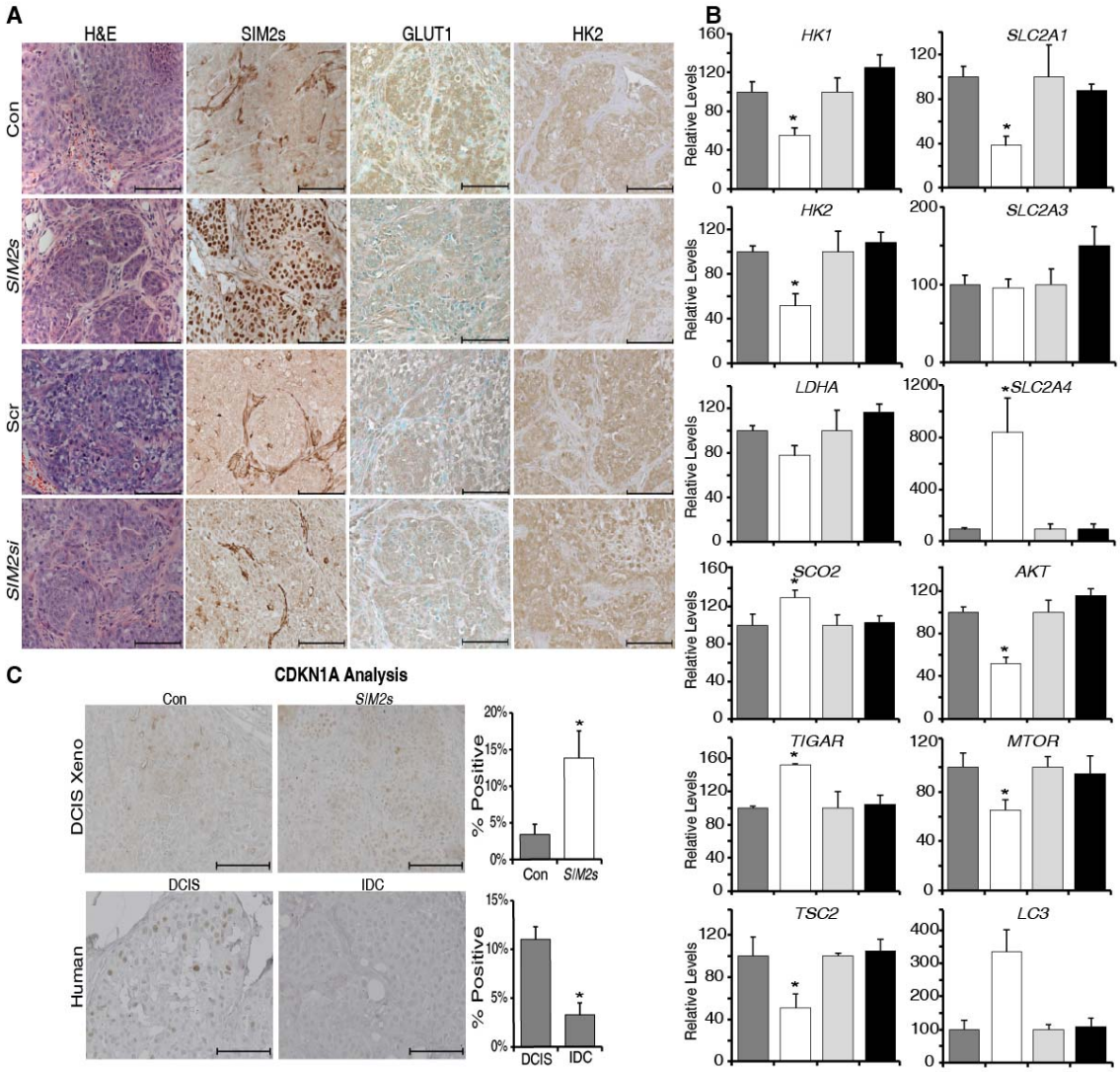


Figure 35. DCIS Xenograft Analysis Confirms In Vitro Data and Induces P53 Stress Response. (A) Immunohistochemical analysis of DCIS xenografts. Immunostaining for H&E, SIM2s, GLUT1, and HK2. Images were taken at 25.2x magnification. (B) Q-PCR analysis of key metabolic and stress response genes in DCIS xenografts (C) p21 immunostaining and quantification. Quantification was done by counting positive and negative nuclei and calculating percentage. *=p-value<.05. Scale bars = 100μm. Data are shown as mean + SEM.

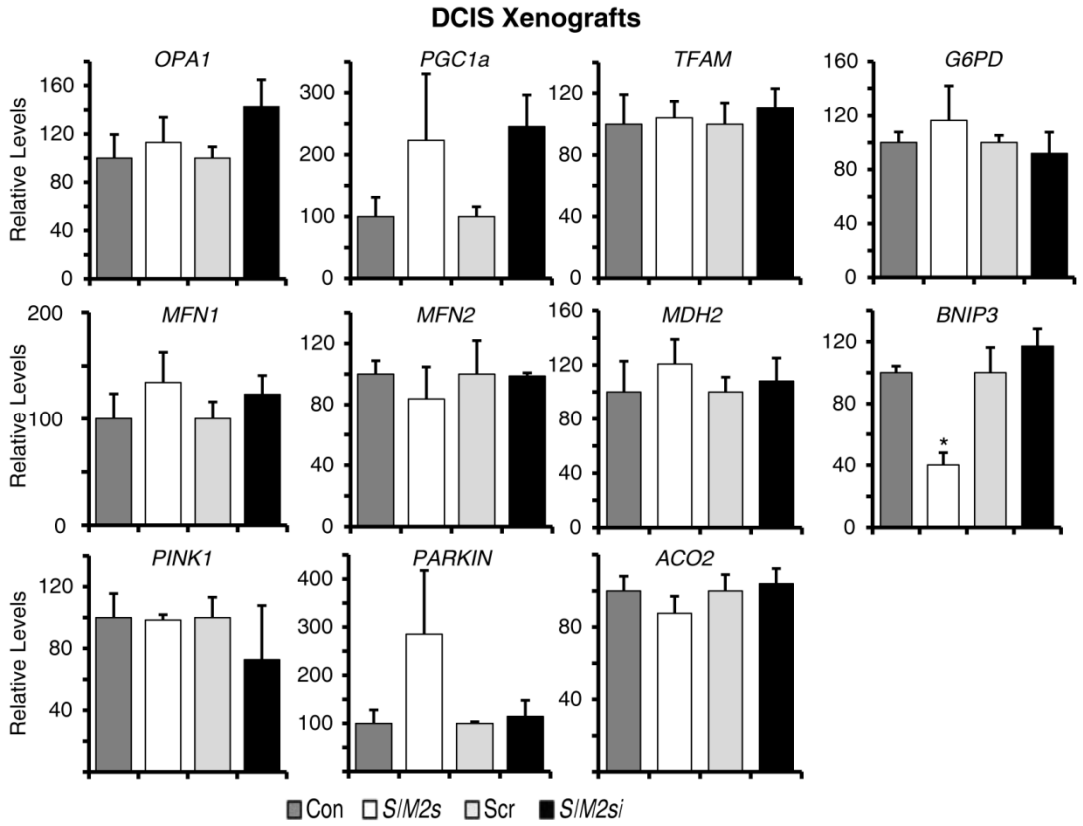


Figure 36. Q-PCR Analysis of DCIS Xenografts of Key Mitochondrial Genes and Alternative Metabolic Pathways. *=p-value<.05. Data are shown as mean + SEM. Control and SIM2s represent PLPCX lentiviral transductions (Empty plasmid and SIM2s overexpression). Scrambled and SIM2si represent shRNA transductions (Scrambled Control and SIM2s targeting).

SIM2s Regulates P21 Expression and Induces Cellular Senescence and Inhibits Glycolysis

The significant increase in p21 expression seen in DCIS *SIM2s* xenografts indicated a potential mechanism by which *SIM2s* may inhibit proliferation and tumor metabolism. Analysis of basal *CDKN1A* gene expression by qPCR showed that *CDKN1A* is highly expressed in DCIS cells as compared to MCF7 and SUM159 cells (Figure 37 A). Over-expression of *SIM2s* enhanced basal *CDKN1A* expression in DCIS, MCF7, and SUM159 cells, whereas *CDKN1A* levels decreased in *SIM2si* cells (Figure 37 A). p21 expression is associated with cellular senescence, and cell cycle regulation was significantly effected in our microarray analysis.

Analysis of senescence-associated β-Galactosidase (SaβGal) activity showed a increase in SaβGal staining in *SIM2s* cells (Figure 37 B). The observation that *SIM2s* induces senescence in MCF7 and SUM159 cells is significant, since these cells have lost several nodal tumor suppressive pathways. MCF7 cells lack the *INK4A*/*ARF* locus that encodes *p16* and *p14*, whereas SUM159 cells have a defective p53, suggesting that *SIM2s*-induced senescence is independent of these pathways (Deng et al., 2002; Wasielewski et al., 2006).

To determine the molecular mechanism of *SIM2s* induction of *CDKN1A* gene expression, we co-transfected a 2.4kb *CDKN1A* promoter luciferase into MCF7 cells with increasing amounts of *SIM2s*, showing that *SIM2s* induced *CDKN1A* promoter activity in a dose-dependent manner (Figure 37 C). Analysis of the *CDKN1A* promoter identified two potential *SIM2s* central midline binding elements (CME) near the 3'-p53 response site and TATA box (Figure 37 C). Chromatin immunoprecipitation (ChIP) analysis showed that SIM2s differentially bound the 3'-CME in MCF7 cells under basal conditions (Figure 37 C). Since the p53 response element is often a stress response element, the MCF7 cells were irradiated and ChIP analysis was performed, showing increased SIM2s binding to the *CDKN1A* promoter in response to cellular stress (Figure 37 C). These results confirm that SIM2s directly binds and regulates basal and IR-induced *CDKN1A* promoter activity.

To determine if silencing *CDKN1A* was sufficient to reverse the metabolic phenotype seen in cancer cells with *SIM2s* expression, we knocked down *CDKN1A* using a confirmed siRNA and analyzed changes in senescent and metabolic behavior. Down-regulation *CDKN1A* gene expression was verified by qPCR (Figure 37 D). Loss of *CDKN1A* reversed the increased SaβGal staining seen in *SIM2s* cells (Figure 37 D). We also found that *SIM2s*-induced ATP production was significantly affected by inhibition of *CDKN1A*, whereas control cells had no response to loss of *CDKN1A* (Figure 37 E). Additionally, we measured lactate levels in spent media of cells after *CDKN1A* knockdown. DCIS control and *SIM2s* cells showed significant increases in lactate production with loss of *CDKN1A*, likely due to the high levels of *CDKN1A* already present in the parent cell line (Figure 37 E). SUM159 *SIM2s* cells also showed a significant increase in lactate production with *CDKN1A* knockdown, while control cells showed no response to loss of *CDKN1A* (Figure 37 E). Together, these data show that SIM2s mediates the glycolytic shift of breast cancer cells through direct up-regulation of p21-induced senescence.

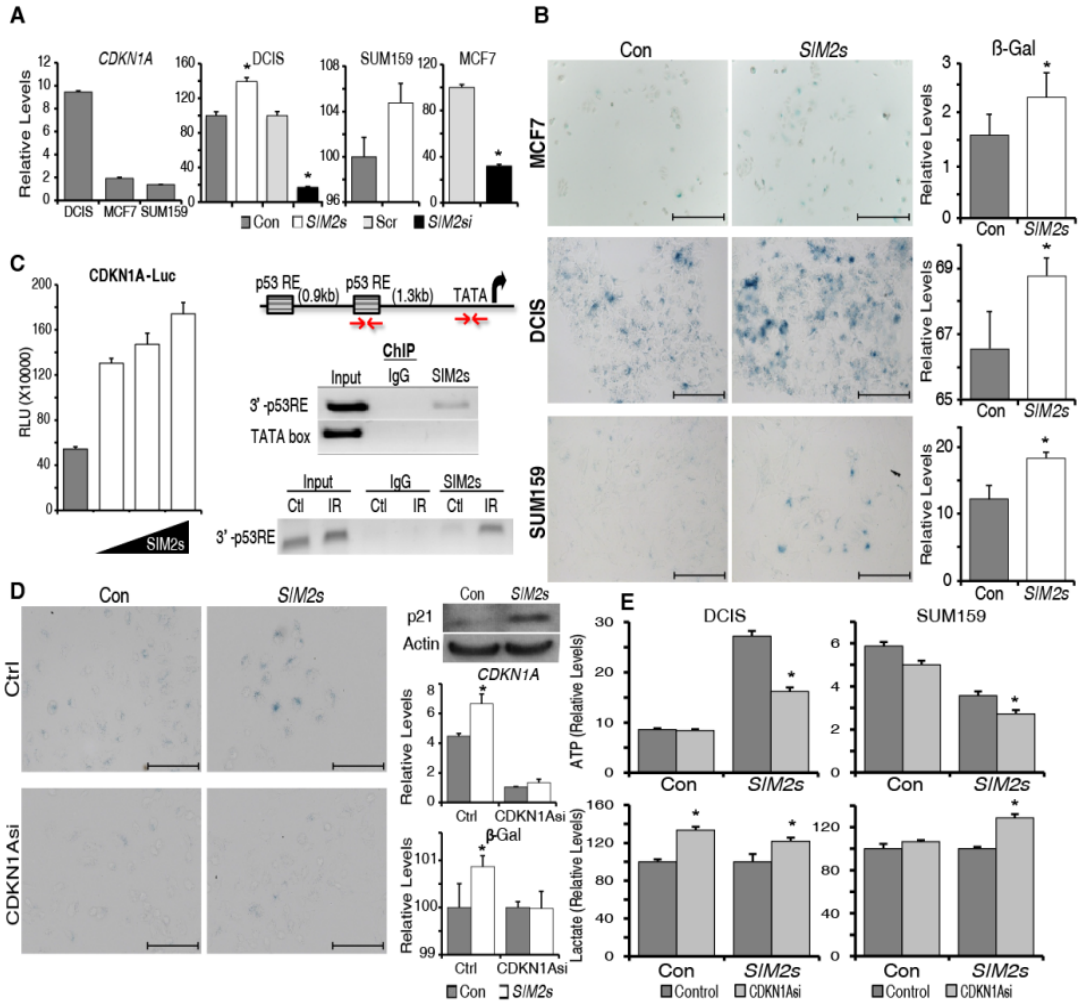


Figure 37. SIM2s Regulates P21 Expression, Induces Cellular Senescence, and Inhibits Glycolysis. (A) Q-PCR analysis of CDKN1A in DCIS, SUM159, and MCF7 cells. (B) β-Gal staining for cellular senescence in MCF7, DCIS, and SUM159 SIM2s cells. Images were taken at 25.2x magnification. Quantification was taken as average of mean blue staining on cells. (C) Luciferase assay to determine SIM2s regulation of CDKN1A activity. Gene diagram showing RT-PCR primer designs. ChIP analysis of MCF7 cells confirms that SIM2s binds the p53RE in the promoter of CDKN1A, and is increased with stress induction through irradiation. (D) β-Gal staining of Control and CDKN1A siRNA transfected cells. Western blot showed increased p21 expression with SIM2s expression. Q-PCR analysis confirmed loss of CDKN1A in transfected cells. Quantification of β-Gal staining showing loss of increased cellular senescence with loss of CDKN1A. (E) ATP and lactate analysis of DCIS and SUM159 SIM2s cells transfected with control and CDKN1A siRNA, resulting in significantly decreased ATP levels in SIM2s cells, as well as increased lactate production. *=p-value<.05. Scale bars = 100μm. Data are shown as mean + SEM

Discussion

In this study we have demonstrated that SIM2s plays a key role in DCIS progression by regulating the switch between senescence and glycolysis. Previously, we have shown that SIM2s expression inhibits tumor growth and metastasis *in vivo*, and loss of *SIM2s* induces an EMT through SLUG (Gustafson et al., 2009b; Kwak et al., 2007; Laffin et al., 2008; Scribner et al., 2012). Our current study shows that SIM2s plays a role in maintaining cellular metabolic homeostasis under normal circumstances, and this system is hijacked during cancer progression. SIM2s promotes normal mitochondrial function and oxygen consumption while decreasing extracellular acidification and glycolytic enzyme activity, whereas loss of *SIM2s* induces mitochondrial dysfunction and enhances glycolysis. This correlates with our observation in human breast tumors: *SIM2s* expression is lost as glycolytic activity is gained. Taken together, these data provide a mechanism by which SIM2s actively inhibits tumor progression in early stage DCIS.

The functional interface between senescence and tumor metabolism is not well understood. Previous work has alluded to this relationship, as p53 has been well established as a regulator of both cellular metabolism and senescence, as well as the unique roles of reactive oxygen species (ROS) and low glucose in their ability to induce cellular senescence (Bensaad et al., 2006; Feng and Levine, 2010; Ferbeyre et al., 2002; Kawauchi et al., 2008; Ma et al., 2007; Malkin et al., 1990; Matoba et al., 2006; Yeung et al., 2008; Zhou et al., 2003). Previous work in fibroblasts has also established that senescence cells have lower levels of glycolysis than their proliferating counterparts (Zwerschke et al., 2003). Glycogen metabolism has also shown to be up-regulated in tumors and inhibition of glycogen consumption induces senescence and impairs tumorigenesis (Favaro et al., 2012). Our study demonstrates that SIM2s induces p21 expression independently of p53, and subsequently promotes senescence and loss of glycolytic ability.

Down-regulation of p21 in *SIM2s* expressing cells is sufficient to inhibit oxidative phosphorylation and promote lactate production, thereby blocking the SIM2s related effect, indicating that promotion of senescence is the primary mechanism through which SIM2s regulates metabolism. The up-regulation of p21 and subsequent inhibition of glycolysis raises the question whether senescence is involved in differentiation. Comparison of DCIS and SUM159 cell lines show complex responses to *SIM2s* expression depending on cell type. We have previously shown that re-establishment of SIM2s promotes terminal differentiation and milk protein expression in DCIS xenografts (Scribner et al., 2012). This supports our previous work showing that increased *Sim2s* expression in the mouse mammary gland induces precocious lactogenic differentiation and delayed involution (Scribner et al., 2011; Wellberg et al., 2010). This suggests a role for senescence in the normal metabolic function of the mammary gland. Based on this we hypothesize in early stage DCIS, overexpression of *SIM2s* induces a terminal differentiation, lactational phenotype. Recent research has made similar connections with oxidative phosphorylation, ROS, and senescence in the phenotype of Alzheimer's and Downs syndrome (Chatterjee et al., 2013; Helguera et al., 2013; Piccoli et al., 2013). SIM2s has been shown to be elevated in the brains of Downs syndrome populations, indicating a potential relationship between SIM2s and brain metabolic activity (Chatterjee et al., 2013; Chrast et al., 2000; Ema et al., 1999; Spellman et al., 2013).

The demand for understanding metabolic homeostasis in tumor cells and how to maintain normal metabolic activity is significant (Ferreira et al., 2012; Levine and Puzio-Kuter, 2010; Lunt and Vander Heiden, 2011). Many effects have been seen and studied in both normal and cancerous environments (DeBerardinis et al., 2007; Marroquin et al., 2007; Pavlides et al., 2009; Swerdlow et al., 2013; Warburg, 1956a). Cells can alter their metabolism in response to available nutrients, however with tumorigenesis, this pathway is derailed and the cells become

metabolic powerhouses to maintain proliferation and avoid apoptosis. Our study shows that *SIM2s* expression is necessary for cells to competently operate their metabolic machinery in response to changing environments.

CHAPTER VI

CONCLUSIONS

In conclusion, we have developed a new and novel mechanism of action through which SIM2s inhibits early breast cancer progression through upregulation of p21-induced senescence, and subsequently a reverse Warburg effect. The relationship between senescence and metabolism has been alluded to earlier, however no studies have been done to determine the possibility of an upstream role in senescence in metabolic regulation. Our studies point towards this as the potential mechanism for SIM2s-regulated cellular differentiation. The relationship we have shown between differentiation, metabolism, aging, and disease has far reaching implications in breast cancer, as well as other diseases.

SIM2s in Mammary Gland Lactation and Metabolism

Previous work in our lab has elucidated the importance of Sim2s in mammary gland differentiation and lactation (Wellberg et al., 2010). Lactation is the most metabolically active state of mammary gland development, and is characterized by metabolically active, non-proliferating epithelial cells. This is interesting due to the fact senescent cells are also defined as metabolically active and non-proliferating (Rodier and Campisi, 2011). The role of senescence and p21 in induction and maintenance of lactation has not been studied, however prolonged lactation has been associated with increased ROS, oxidative damage, and mitochondrial dysfunction (Hadsell et al., 2011; Hadsell et al., 2006; Hadsell et al., 2005). We hypothesize that the natural induction of Sim2s during lactation actually promotes this lactational phenotype through senescence, and that overexpression of Sim2s could potentially prolong lactation be helping maintain mitochondrial integrity during enhanced metabolic activity. A study done in

2004 examined the expression of p19ARF (another senescence associated protein) during normal mammary gland development (Yi et al., 2004). Yi et al. found that p19ARF increased significantly during pregnancy and plateaued during lactation, eventually decreasing during the second phase of involution. Loss of p19ARF in the mouse mammary gland delayed the first phase of involution, similar to the overexpression of Sim2s, and decreased p21 expression and apoptosis. This aligns well with our hypothesis for the potential role of senescence in lactational metabolism. Thus Sim2s is responsible for metabolic homeostasis not in normally mammary gland function, and this regulation must be lost for tumor progression.

SIM2s and Full Body Metabolism

We have shown that SIM2s can regulate metabolic homeostasis in breast cancer. We hypothesize that likewise, SIM2s plays a role in normal metabolism throughout the body. This is especially important with current health concerns like diet-induced obesity and insulin resistant diabetes. Additionally, this has important implications in muscle development and training. Obesity is defined as a metabolic imbalance: energy input is greater than energy output, likely through enhanced energy storage, with a chronic inflammatory phenotype (Johnson et al., 2012). Many studies have evaluated different risk factors associated with obesity: sleep, genetic predisposition, maternal diabetes, diet, exercise, etc (Bell et al., 1995; Goularte et al., 2012; Johnson et al., 2012; Klingenberg et al., 2012). In general, calorie restriction and exercise are the biggest inhibitors of obesity, and obesity associated diseases. Similarly, caloric restriction has also been shown to lower breast cancer risks through increased insulin sensitivity and decreased proliferation (Frankenberry et al., 2006; Harvie and Howell, 2006; Harvie and Howell, 2012; Howell et al., 2009).

Interestingly, people diagnosed with Down syndrome have a high occurrence of obesity and type 2 diabetes (Cento et al., 1999; Cusi, 2010; Fonseca et al., 2005). Down syndrome individuals are prone to obesity at an earlier age, have a lower resting metabolic rate, and low dietary intake. Hypothyroidism and increased leptin levels in people with DS have also been implicated in contributed to the higher rates of obesity. Studies have indicated this predisposition toward metabolic deregulation is through increase adipocyte inflammatory signaling, as well as deregulated endocrine homeostasis, which also has systemic effects in the brain (Misiak et al., 2012). We have previously discussed that upregulation of SIM2 in DS, and its contribution to the etiology of DS. Based on our current studies linking SIM2s to metabolism, we hypothesize that SIM2s also plays an important role in diet induced obesity and diabetes. Initial studies using a systemic, heterozygous knockdown of Sim2s, have shown that loss of Sim2s significantly inhibits weight gain on a high fat diet (Figure 38).

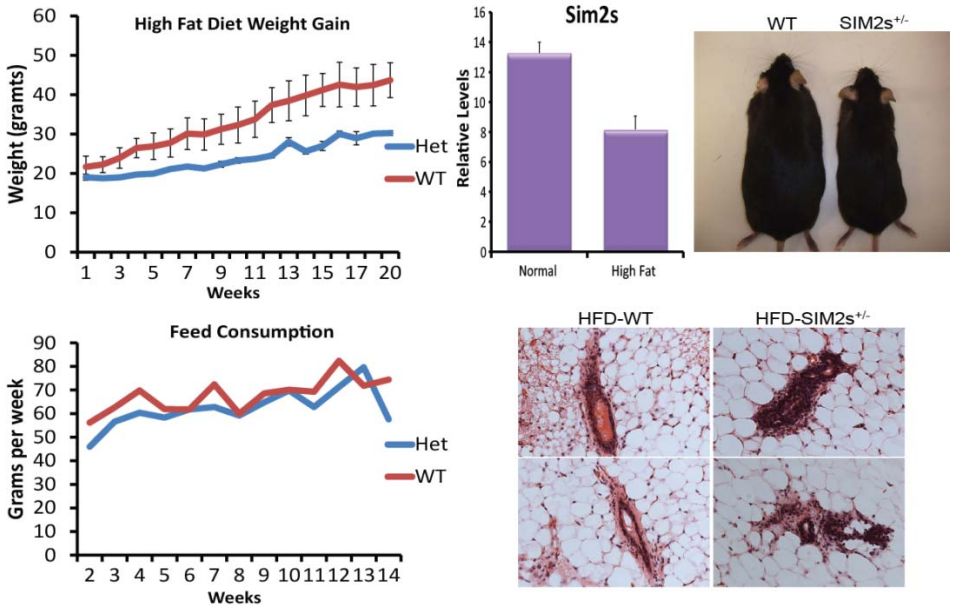


Figure 38. High Fat Diet Reduces SIM2 Expression, and Loss of SIM2s Affects Weight Gain and Mammary Gland Development. WT and *Sim2s*$^{+/-}$ mice were fed a 40% high fat diet for 14+ weeks, mice were weighed weekly and food consumption was measured weekly. High fat diet significantly reduced *Sim2s* expression. *Sim2s*$^{+/-}$ mice gained significantly less weight, despite equal energy uptake, and developed pre-malignant mammary lesions.

Although energy intake between wild-type mice and Sim2s$^{+/-}$ is equivalent, the Sim2s$^{+/-}$ mice do not gain weight compared to wild-type, and high fat diet also down regulates *Sim2s* expression in the mammary gland. We hypothesize that the Sim2s heterozygous mice lose their limited expression of Sim2s with high-fat diet, inhibiting weight gain, and increasing breast cancer risk. Initial studies have shown increased hyperplasias in the mammary gland of Sim2s+/- mice fed a high-fat diet compared to wild-type (Figure 38). This translates the effect SIM2s has on the mammary gland to a systemic regulation of metabolism.

In addition to adipocytes and obesity, another major organ involved in metabolic homeostasis and weight is skeletal muscle. SIM2 has already been identified to be integral to myogenic differentiation (Havis et al., 2012; Zhao et al., 2002b). *SIM2* expression in myogenic progenitor cells prevents premature entry into the myogenic differentiation program, and helps to maintain muscle integrity. Using the same Sim2s$^{+/-}$ mice we just discussed, we allowed these mice to run at will and measure distance run and time run. Sim2s $^{+/-}$ mice ran significantly less than their WT counterparts – both in distance and time (Figure 39). This also associated with an increase in glycolytic enzyme RNA levels in Sim2s$^{+/-}$ muscle tissues. In skeletal muscle, muscle fibers are divided into two groups based on their metabolic activity (Figure 39). Type I muscles are slow twitch, used for endurance such as marathon running, and are highly oxidative – primarily using mitochondria and oxidative phosphorylation of energy. Type II fibers are fast twitch, used for strength and sprinting, and are highly glycolytic – with fewer mitochondria than Type I fibers and increased glucose uptake. Based on this we hypothesize that SIM2s expression in muscle fibers is important to the muscle fiber phenotype, with higher expression seen in Type I fibers, and lower expression in Type II fibers. We also hypothesize that under conditions of endurance training, athletes' muscle-specific levels of SIM2s increase, due to the increased demand for slow twitch muscle fibers.

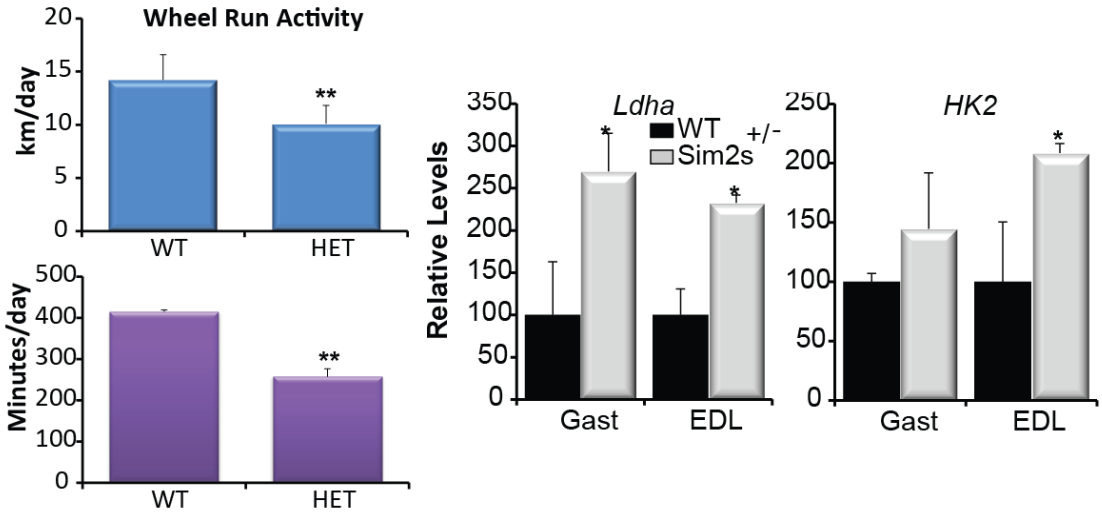


Figure 39. Sim2s Expression Affects Muscle Development and Aerobic Activity. WT and Sim2s$^{+/-}$ were given access to an activity wheel to run on *ad libitum*. Sim2s$^{+/-}$ mice ran significantly less, both in distance and time. Sim2s$^{+/-}$ mice also had significantly higher levels of glycolytic genes *Ldha* and *HK2* in both the gastrocnemius and EDL muscles.

SIM2s in Brain Development and Neurodegenerative Disease

As previously discussed, SIM2s expression is upregulated in the brain of people with Down Syndrome, and contributes to the etiology of DS (Chrast et al., 2000; Spellman et al., 2013). Altered metabolic activity and oxidative stress have long been associated with DS (Arbuzova et al., 2002; Brooksbank and Balazs, 1984; Busciglio et al., 2002). DS neurons generate high levels of ROS which can compromise neuronal survival, and altered mitochondrial activity has been measured in DS fibroblasts (Busciglio et al., 2002; Coskun and Busciglio, 2012). Mitochondria are more fragmented in the DS brain, similar to the shortened mitochondria we found with overexpression of SIM2s, several of the pathways we discussed using microarray analysis are also altered in DS brains compared to normal brains :oxidative stress and mitochondrial dysfunction (Helguera et al., 2013). These symptoms point to prolonged mitochondrial stimulation, ultimately resulting in mitochondrial dysfunction and cell death. It is

this metabolic shift that promotes an increased susceptibility to neurodegenerative diseases in DS populations (Coskun and Busciglio, 2012; Coyle et al., 1988; Scholl et al., 1982).

Mitochondrial dysfunction is the key causative effect in the brain for Alzheimer's, Parkinson's, and other neurodegenerative diseases (Ramalingam and Kim, 2012). The accumulation of oxidative stress and disrupted mitochondrial respiration initiate apoptotic cascades in neurons. Cell death in neurons is the hallmark of these diseases, as healthy neurons are not able to proliferate to accommodate neuron loss. Depending on the region of the brain affected, this apoptosis results in the symptoms of Parkinson's, such as loss of muscle control, or Alzheimer's, such as loss of memory, or other diseases such as stroke and Huntington's (Ramalingam and Kim, 2012). Under normal circumstances, the brain is a rapid glucose consuming organ that is easily identified using 18-fluorodeoxyglucose signaling. The onsets of these diseases are often diagnosed by decreased glucose consumption in specific regions of the brain. We have shown that the upregulation of SIM2s decreases glucose uptake and promotes mitochondrial function and ROS, and since it has been shown to be upregulated in DS, we hypothesize that the upregulation of SIM2s in neuronal and astrocyte cells is part of the initial event that induces neurodegenerative disease. Also, since neuronal cells are unable to proliferate, yet remain highly metabolically active, we again hypothesize that SIM2s is necessary for the induction of a senescence phenotype that promotes normal neuronal function. A recent study has already shown that Sim2 expression in neurons is necessary for hyperglycemia-induced injury – and down regulation of Sim2 inhibited subsequent neuronal damage (Wang et al., 2013). Patients suffering from diabetes mellitus (a metabolic disorder) are at high risk for hyperglycemia-induced brain injury, which can result in stroke or intracerebral hemorrhage (ICH). We hypothesize that an upregulation in SIM2s would promote obesity and diabetes,

which would also indicate a higher susceptibility to neuronal injury. This is just another indicator of the important role Sim2s potentially plays in full body and brain metabolism.

Other proteins have had similar implications in the combination of aging, metabolism, and disease (Bonuccelli et al., 2010). Caveolin-1 has been shown to induce oxidative stress in tumor associated stroma, through which it promotes metastasis. Analysis of the transcription profile of this stroma showed a close association with the genetic signature of Alzheimer's disease. Termed the "Reverse Warburg Effect," the authors indicate that oxidative stress, hypoxia, and mitochondrial dysfunction not only contribute to breast cancer metastasis, but also to neurodegeneration. While this conflicts with our studies of these effects being tumor suppressive in the breast, it is important to note that SIM2s is expressed in the luminal epithelial cells, not the stroma and myoepithelial cells – indicating a unique cell compartment effect for mitochondrial function and oxidative stress.

Conclusion

In conclusion, these new studies have elucidated a new and complex role for SIM2s in the global regulation of differentiation, metabolism, and senescence. In breast cancer, SIM2s must be lost for cells to induce metabolic adaptations for survival and progression, and re-establishment of SIM2s promotes cellular senescence and differentiation, which reverses the Warburg effect (Figure 40). These effects have exciting implications in normal mammary gland lactation, diet-induced obesity, muscle development, and neurodegenerative diseases. These are all examples of metabolic homeostasis, and how the loss of metabolic regulation can induce disease. Our hypothesis is that SIM2s will be a causal regulator of metabolic homeostasis in multiple environments in the body, and thus be implicated in further metabolic disorders.

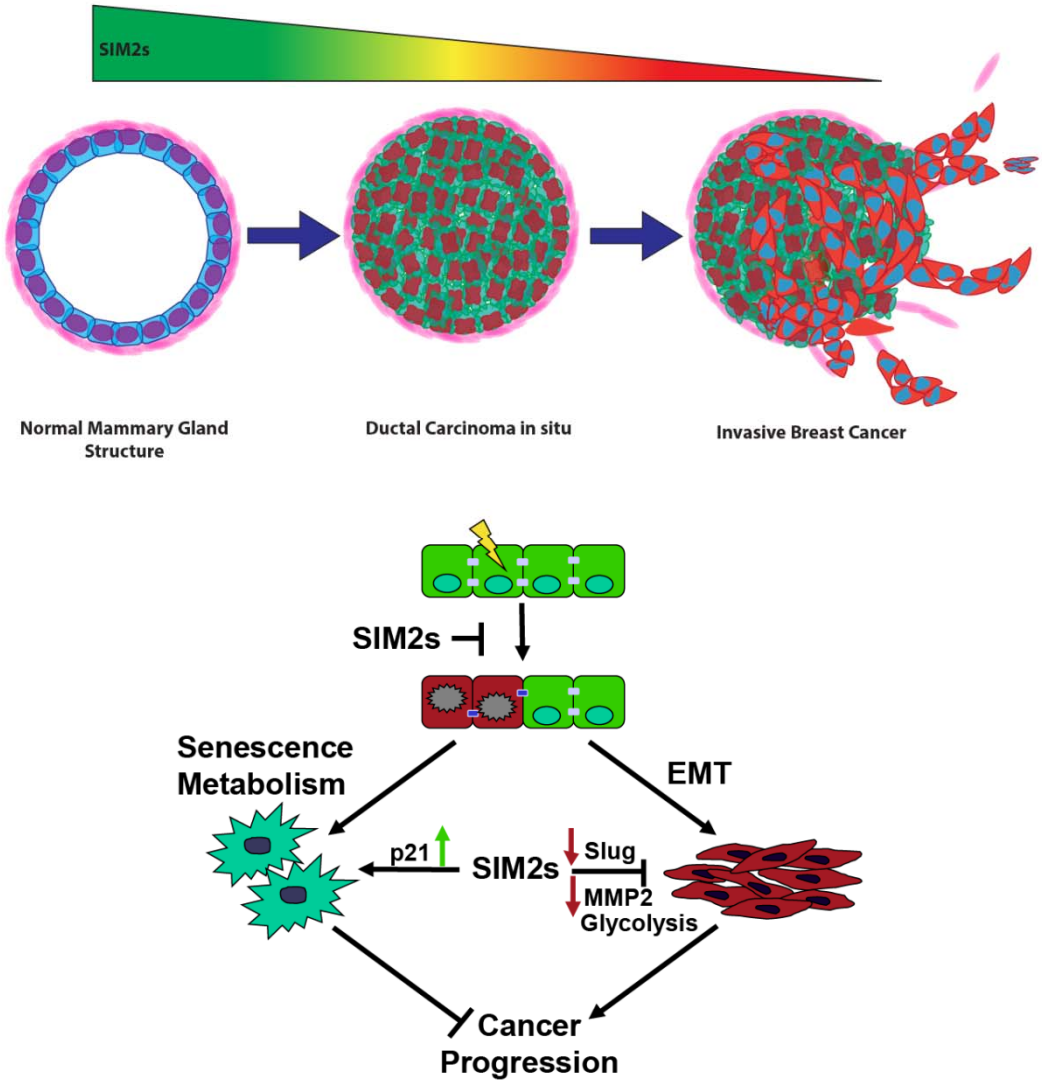


Figure 40. Model of SIM2s Regulation of DCIS Progression through P21 and Metabolic Homeostasis.

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APPENDIX I

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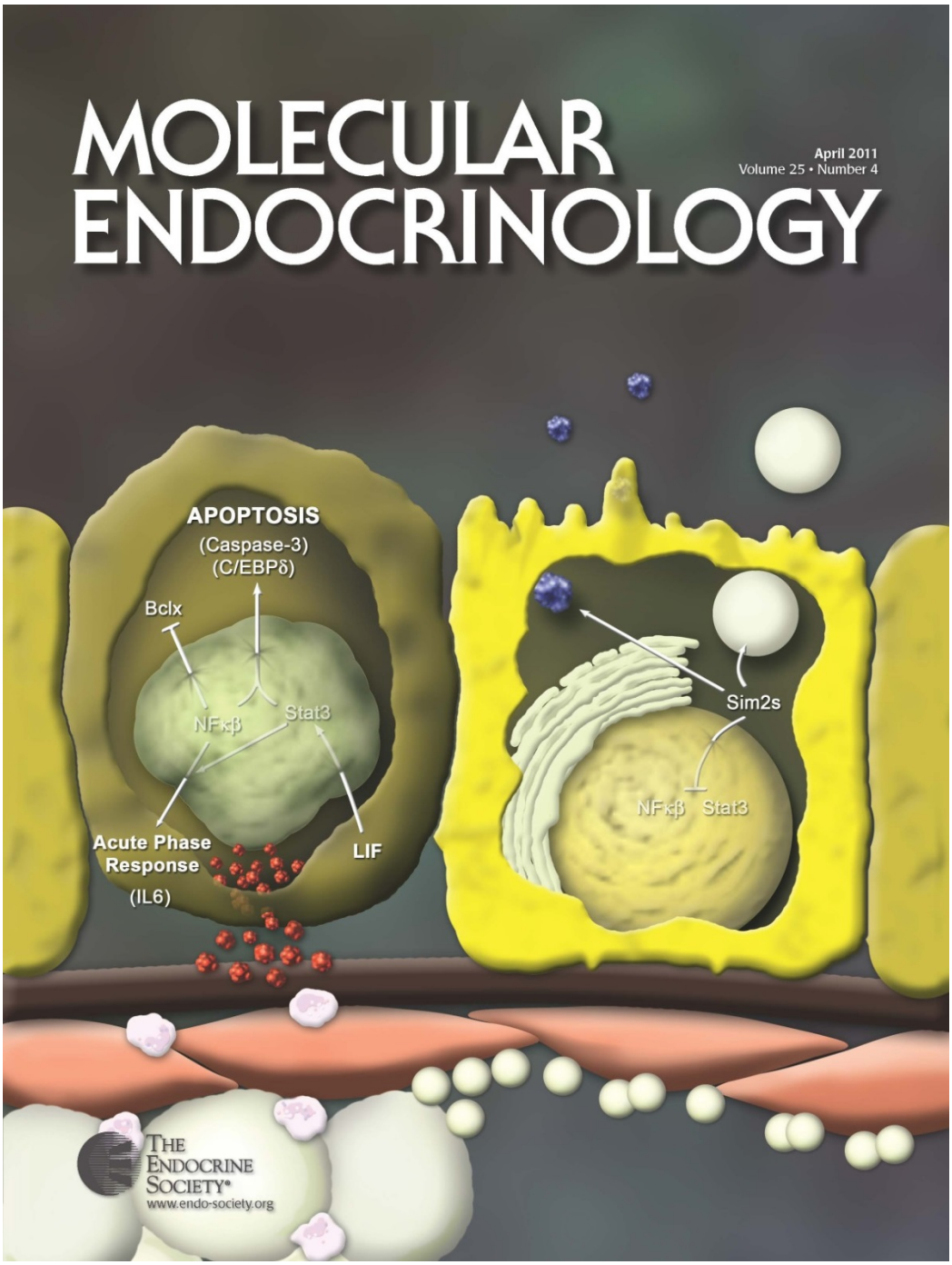
APPENDIX II

MOLECULAR ENDOCRINOLOGY COVER



APPENDIX III

NOMENCLATURE

2-DG	2-Deoxy Glucose
AKT	Protein Kinase B
ALDH	Aldefluor Dehydrogenase
APP	Amyloid Precursor Protein
ARNT	Aryl hydrocarbon Receptor nuclear translocator
ATP	Adenosine Triphosphate
BCL2	B-cell lymphoma 2
BECN1	Beclin-1
bHLH	basic helix-loop-helix
BNIP3	Bcl2/adenovirus E1B 19kDa protein-interacting protein 3
BRCA1/2	Breast cancer 1/2
C/EBP	CAAT/Enhancer Binding Protein
CBF1	C-Repeat Binding factor 1
CC3	Cleaved-Caspase 3
CCDN1	Cyclin-D1
CDH1	E Cadherin
ChIP	Chromatin Immunoprecipitation
CK5/6	Cytokeratin 5/6
CK8/18	Cytokeratin 8/18

CME	Central Midline Element
CNS	Central Nervous System
CoIP	Coimmunoprecipitation
COX	Cytochrome c complex
Csn2	β-Casein
CTNNB1	β-Catenin
DCIS	Ductal Carcinoma in situ
DS	Down Syndrome
DSCR	Down Syndrome Critical Region
dSim	drosophila, Single-minded
dTgo	drosophila, TANGO
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial Mesenchymal Transition
ER	Estrogen Receptor
ETC	Electron Transport Chain
FDG	FluorDeoxyGlucose
FiSH	Flurescent in situ hybridization
G6PD	Glucose-6-phosphate dehydrogenase
GADD	Growth arrest and DNA damage
GLUT	Glucose Transporter
GR	Glucocorticoid Receptor
H&E	Hematoxylin and Eosin
HER2	Human epidermal growth factor receptor 2

HIF1α	Hypoxia-inducible factor 1-alpha
HK	Hexokinase
hPTTG1	Human pituitary tumor-tranforming gene 1
HRE	Hypoxic Response Element
IDC	Invasive ductal cancer
IGFBP5	Insulin-like Growth Factor Bdinging Protein
JAK2	Januse Kinase 2
LCIS	Lobular Carcionoma in situ
LDHA	Lactate Dehydrogenase
MAPK	Mitogen-activated protein kinase
MIND	mouse intraductal injection
MMP	Matrix Metalloproteinase
MMP	Matrix Metalloproteinase
MMTV	Mouse Mammary Tumor Virus
mtDNA	Mitochondrial DNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NCAD	N-Cadherin
NFκB	Nuclear Factor -kappa B
NICD	NOTCH intracellular domain
OIS	Oncogene Induced Senescence
OXPHOS	Oxidative Phosphorylation
PAS	Per-Arnt-Sim
PAS	Per-Arnt-Sim
PET	Positron Emission Tomography

PPP	Pentose Phosphate Pathway
PR	Progesterone Receptor
Prl	Prolactin
PTEN	Phosphatase and tensin homolog
PYGL	glycogen phosphorylase
PyMT	Polyoma Middle-T
Q-PCR	Quantitative PCR
RB	Retinoblastoma
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcriptase PCR
S2RE	SIM2 Response Element
SA-βGal	Senescence Associated β-Galactosidase
SCO2	Synthesis of cytochrome c oxidase
SIM2s	Singleminded-2 short isoform
SNP	single nucleotide polymorphisms
STAT	Signal transducer and activator
STAT	Signal transducer and activator of transcription
TBP	TATA Binding Protein
TCA	Tricarboxylic Cycle
TEB	Terminal End Bud
TIC	Tumor Initiating Cell
TIGAR	TP53-induced glycolysis and apoptosis regulator
TIMP	Tissue Inhibitor of metallo proteinase
UPR	Unfolded Protein Response

VEGF	Vascular endothelial growth factor
VIM	Vimentin
WAP	Whey acidic protein
WT	Wild Type
XRE	Xenobiotic response element

APPENDIX IV

VITA

Education

Ph.D. – Toxicology (Defense: March 25, 2013)

Texas A&M University – College Station, TX

Committee: Weston Porter, Ph.D. (Chair), Stephen Safe, Ph.D., Les Dees, Ph.D., Tim Phillips, Ph.D., and Scott Dindot, Ph.D.

Dissertation: "Metabolic Demands in Mammary Gland Development and Breast Cancer"

B.S. - Veterinary Science, Biomedical Option (Minor: Biochemistry)

University of Nebraska – Lincoln, NE

Research Experience

Aug 2008 – Current **Graduate Research Assistant**

Texas A&M University College of Veterinary Medicine (CVM)

Veterinary Integrative Biosciences/Interdisciplinary Faculty of Toxicology

Principle Investigator: Weston W. Porter, Ph.D.

Jan 2008 – May 2008 **Undergraduate Research Assistant (Independent Study)**

University of Nebraska – Lincoln

Veterinary Diagnostic Lab – Toxicology

Principal Investigator: Michael Carlson, Ph.D.

Apr 2004 – Mar 2005 **Student Assistant**

University of Nebraska – Lincoln

Animal Research Facility

Jan 2003 – May 2003 **Student Assistant**

University of Nebraska – Lincoln

Ruminant Nutrition Lab

Awards & Honors

2013 TAMU Auxiliary Graduate Student Award

2013 High Impact Research Achievement Award – First Author Publication, Texas A&M

2012 Ethel Ashworth-Tsutsui Memorial Award for Research

2012 3rd Place, Platform Presentation, Breast Cancer Research and Education Program, Baylor College of Medicine

2012 High Impact Research Achievement Award, Texas A&M University

2012 1st Place, Platform Presentation, CVM - Graduate Student Association Symposium

2012 TAMU Academic Excellence Award

2012 George T. Edd's Outstanding Toxicology Student Award, TAMU Interdisciplinary Faculty of Toxicology

2011 CVM Graduate Student Research Trainee Grant

2011 CVM-GSA Travel Award

2011 CVM Award for Exemplary Work during Graduate Career

2011	5th Place, Poster Presentation, CVM-GSA Spring Symposium
2011	Pinnacle Honor Society, Induction
2010	Who's Who among Students in American Colleges & Universities, Induction
2010	San Antonio Breast Cancer Symposium Basic Science Scholars-In-Training Scholarship, American Association for Cancer Research
2010	2nd Place, Platform Presentation, Society of Toxicology, Gulf Coast Chapter Annual Meeting
2010	Gulf Coast Society of Toxicology Annual Meeting Travel Award
2010	1st Place, Platform Presentation, Toxicology Research Forum
2010	3rd Place, Poster Presentation, Texas Forum on Reproductive Science
2010	CVM-GSA Travel Award
2009	Honorable Mention, Poster Presentation, Toxicology Research Forum
2009	CVM-GSA Travel Award
2008-2009	Toxicology Regents Fellow (full support)

Research Support

BC100880 (Scribner, KC) 1/15/2011 – 1/31/2014

DOD BCRP Pre-Doctoral Traineeship Award

"Regulation of Mammary Gland and Breast Tumor Differentiation by Singleminded-2s"

Role: **Principle Investigator**

CVM Graduate Student Research Trainee Grant 1/01/2012 – 12/01/2012

"The Role of Singleminded-2s (Sim2s) in breast cancer cell metabolism and energy homeostasis"

Role: **Principle Investigator**

Publications

1. Scribner K.C., Metz R.P., Schilling L., Ragavan M., Fan Y.Y., Hilty C., Chapkin R., Payne H.R., Mouneimne R., Behbod F., Medina D., Porter W.W. "SIM2s inhibits DCIS progression by regulating senescent-dependent metabolic equilibrium." *Cancer Research*. (Submitted)
2. Dyer L.M., Hu J., Reinhard M.K., Izumchenko E., Scribner K.C., Leeuwenburgh C., Porter W.W., McKinnon P.J., Brown K.D. "Atm is required for homeostasis within the lactating mammary gland." *Breast Cancer Research*. (Submitted).
3. Scribner K.C., Behbod F., and Porter W.W. "Regulation of DCIS to invasive breast cancer progression by Singleminded-2s (SIM2s)." *Oncogene.* 2012 Jul 9. [Epub ahead of print].
4. Romoser A.A., Figueroa D.E., Sooresh A, Scribner K.C., Chen P.L., Porter W.W, Criscitiello M.F., and Sayes C.M.(2012) "Differential NF-κB competency mediates

nanoparticle toxicity in normal human dermal cells." *Toxicology Letters,* May 5;210(3):293-301.

5. Scribner K.C., Wellberg E.A., Metz R.P., Porter W.W. (2011) "Singleminded-2s (Sim2s) promotes delayed involution of the mouse mammary gland through inhibition of Stat3 and NFκB." *Molecular Endocrinology,* Apr;25(4):635-44.(**Cover**)

National Presentations

1. *Mediation of a Metabolic "Switch" from DCIS to IBC*

   Platform, 8th Annual Breast Cancer Research and Education Program, September 2012, Montgomery, TX (3rd Place)

2. *Singleminded-2s (Sim2s) plays a unique role in mammary gland and breast cancer autophagy and energy homeostasis*

   Poster, American Association for Cancer Research Special Conference on Metabolism in Cancer, October 2011, Baltimore, MD

3. *Singleminded-2s (Sim2s) plays a unique role in mammary gland and breast cancer autophagy and metabolism homeostasis*

   Poster, 7th Annual Breast Cancer Research and Education Program, September 2011, Montgomery, TX

4. *Singleminded-2s (Sim2s) induces metabolic and autophagic changes in the functioning mammary gland and breast cancer*

   Poster, Mammary Gland Biology Gordon Research Conference, Salve Regina University, June 2011, Newport, RI

5. *Singleminded-2s (Sim2s) in Breast Cancer*

Seminar, Interdisciplinary Faculty of Toxicology, Texas A&M University, February 2011, College Station, TX

6. *Inhibition of DCIS progression through promotion of differentiation*

   Poster, CTRC-AACR Breast Cancer Research Symposium, December 2010, San Antonio, TX

7. *Singleminded-2s (Sim2s) inhibits MCF10DCIS.COM progression in vivo by promoting differentiation*

   Platform, Gulf Coast Society of Toxicology Annual Meeting, October 2010, Houston, TX (2nd Place)

8. *Singleminded-2s (Sim2s) inhibits MCF10DCIS.COM progression and metastasis in vivo by promoting differentiation*

   Platform, 6th Annual Breast Cancer Research and Education Program, September 2010, Montgomery, TX

9. *Singleminded-2s (Sim2s) inhibits MCF10DCIS.COM progression and metastasis in vivo by promoting differentiation*

   Poster, MRS-AACR Metastasis and The Tumor Microenvironment Conference, September 2010, Philadelphia, PA

10. *Singleminded-2s (Sim2s) delays apoptosis and involution of the mammary gland through inhibition of phospho-Stat3*

    Poster, Texas Forum on Reproductive Sciences, April 2010, Houston, TX (3rd Place)

11. *MDF10DCIS.COM Breast Cancer Cell Lines are Attenuated by Expression of Singleminded- 2s(Sim2s)*

Platform, Gulf Coast Society of Toxicology Annual Meeting, October 2009, Austin, TX

12. *Aberrant Involuting Pathways in MMTV-Singleminded-2s (Sim2s) Mice*

    Poster, 5th Annual Breast Cancer Research and Education Program, September 2009, Houston, TX

13. *Differential Induction of Involution in MMTV-Singleminded-2s (Sim2s) Mice*

    Poster, Mammary Gland Biology Gordon Research Conference, June 2009, Newport, RI

Local Presentations

1. *SIM2s inhibits DCIS progression by regulating senescent-dependent metabolic equilibrium*

   Seminar, Baylor College of Medicine, February 2013, Houston, TX.

2. *Singleminded-2S (SIM2S) mediates breast cancer cell metabolism and mitochondrial bioenergetics*

   Platform, CVM-GSA Spring Research Symposium, April 2012, College Station, TX. (1st Place)

3. *Singleminded-2s (Sim2s) plays a unique role in breast cancer metabolism and mitochondrial bioenergetics*

   Platform, Student Research Week, March 2012, College Station, TX.

4. *Singleminded-2s (Sim2s) induces metabolic and autophagic changes in the functioning mammary gland and breast cancer*

   Poster, Toxicology Research Symposium, August 2011, College Station, TX.

5. *Inhibition of DCIS Progression through the Re-establishment of Sim2s Expression*

Poster, CVM-GSA Spring Research Symposium, April 2011, College Station, TX (5th Place)

6. *Singleminded-2s (Sim2s) inhibits MCF10DCIS.COM progression and metastasis in vivo by promoting differentiation*

   Platform, Toxicology Research Symposium, August 2010, College Station, TX. (1st Place)

7. *Differential Induction of Involution in MMTV-Singleminded-2s (Sim2s) Mice*

   Poster, Toxicology Research Symposium, August 2009, College Station, TX. (Honorable Mention)

Professional Affiliations

2011-Current	Society of Toxicology
2011-Current	Metastasis Research Society – Member
2010-Current	American Association of Cancer Researchers – Associate Member
2009-Current	Lone Star Society of Toxicology – Student Member

Volunteering & Service

2011-2012	TAMU Veritas Forum Planning Committee – Member
2009-Current	Texas A&M CVM Graduate Student Association Spring Symposium
2009-Current	Texas A&M CVM Open House Volunteer
2008-Current	TAMU CVM Graduate Student Association – Member
2011-2012	TAMU Veritas Forum Planning Committee – Member
2010-2012	American Cancer Society (College Station, TX) – Volunteer

2010-2012	American Cancer Society Reach to Recovery Program East Texas – Coordinator
2010-2012	Executive Toxicology Committee - Student Representative
2010-2012	TAMU Student Service Fee Advisory Board – Board Member
2009-2012	TAMU Medical Sciences Library Student Advisory Board - Board Member
2012	TAMU Student Research Week – Poster Judge
2011	TAMU Women's Leadership Conference
2011	Expanding Your Horizons Program (Bryan, TX) – Workshop Leader
2010-2011	Cattle Barron's Ball Planning Committee – Food and Beverage Chair
2010-2011	TAMU GSC Legislative Relations Committee –Committee Member
2010-2011	TAMU Rec Sports Advisory Committee– Student Representative
2010-2011	Graduate Student Council – Toxicology Representative
2010	Harmony Science Academy (Bryan, TX) – Science Fair Judge
2009-2010	TAMU CVM Graduate Student Association - President
2009-2010	TAMU CVM Graduate Instruction Council - Graduate Student Representative
2008	Texas A&M/BYD Kennels Equine Theft Prevention Clinic

Mentorship/Teaching

- Graduate Panel, VIBS 650: Education in a Veterinary Medical and Biomedical Environment
    - (Faculty Coordinator: Dr. Jane Welsh) Fall Semester 2012.
- Sydney Duckworth, Undergraduate, 485 Directed Studies January-May 2012

- Substitute Lecturer, Tumor Cell Biology (Professor: Weston Porter) Spring Semester 2012.
- Expanding Your Horizons: Motivating young women in math and science, 2011
  Fall workshop for 6th grade girls interested in math and science.
- Graduate Panel, VIBS 650: Education in a Veterinary Medical and Biomedical Environment
  - (Faculty Coordinator: Dr. Jane Welsh) Fall Semester 2010.
- Graduate Panel, VIBS 650: Education in a Veterinary Medical and Biomedical Environment
  - (Faculty coordinator: Dr. Jane Welsh) Fall Semester 2009.
- Mansi Gaitonde, Undergraduate researcher, (Directed Studies) Aug-Dec 2009.
  Currently a medical student at UT Southwestern in Dallas, TX.
- Sara Filliben, Veterinary student, Summer Research Program 2009
  Poster Presentation: "Role of Singleminded-2s in tumorigenicity of MCF10DCIS.COM Cancer Cell Line." Sarah Filliben, Kelly Scribner, and Weston Porter at the Merck-Merial NIH Veterinary Scholars Symposium in North Carolina. August, 2009.
- Nebraska Women in Science Conference, 2008.
  Conference for junior high and high school girls interested in careers in science.