DEVELOPMENT AND CHARACTERIZATION OF MOUSE MODELS OF HUMAN BREAST CANCER

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“Ah! How harshly the youth of the student passes,

While all around her, with passions ever fresh,

Other youths search eagerly for easy pleasures!

And yet in solitude

She lives, obscure and blessed,

For in her cell she finds the ardor

That makes her heart immense.”

“But the blessed time is effaced.

She must leave the land of science

To go out and struggle for her bread

On the grey roads of life.

Often and often then, her weary spirit

Returns beneath the roofs

To the corner ever dear to her heart

Where silent labor dwelled

And where a world of memory has rested.”

- Marie Curie

ABSTRACT

MALINI MUKHERJEE: Development and Characterization of Mouse Models of Human Breast Cancer
(Under the direction of Terry A. Van Dyke)

While previous studies using genetically engineered mice (GEM) have indicated potential effects of several aberrations observed in human breast cancer, the combined role of loss of *RB*$_f$, *P53* and *BRCA1* has not been assessed before. As these events frequently occur together in human breast cancer, we use GEMs to show that these pathways have a synergistic tumor suppressor role in the mammary gland. We show that loss of *Rb*$_f$ alone is not enough to promote mammary tumors, but combined loss of *Rb*$_f$ and *p53* can lead to mammary adenocarcinoma with a reduction in apoptosis and low latency. In addition to *p53* we studied the role of *Brca1* loss either in conjunction with loss of *Rb*$_f$ or with both *Rb*$_f$ and *p53*. We found that these three important tumor suppressors have a synergistic effect in mammary tumor progression and combined loss of all three further reduces mammary tumor latency and leads to progression to distant metastasis. Genomic analysis suggests that the combined loss of *Rb*$_f$, *p53* and *Brca1* results in increased genetic instability, overexpression of metastasis promoting genes and an altered micro RNA profile that may also contribute to increased malignancy.

So far analysis of genetic lesions in human cancers has focused on the accumulation of multiple events within the epithelial cell. However it is still unresolved as to why human *BRCA1* mutated cancers have their characteristic “basal” like features. We show here for the

first time, that the target cell of origin of the *Brca1* mutation determines the nature of the tumor that evolves and hence the right combination of genetic mutations and cell of origin is important in modeling human cancers. All of our mouse models generated in this study can be used as preclinical animal models, which both genetically and biologically model the initiation and progression of human breast cancer.

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LIST OF ABBREVIATIONS

AAV Cre	Adeno Associated Viral Vector containing Cre
BMP	Bone morphogenetic protein
BRCA1	Breast Cancer Associated Gene 1
BRCT	Breast Cancer Associated Gene 1 C Terminal
CCND1	Cyclin D1
CDK2	Cyclin Dependent Kinase 2
CDK4	Cyclin dependent kinase 4
CK5	Cytokeratin 5
CK8	Cytokeratin 8
CK14	Cytokeratin 14
CML	Chronic Myelogenous Leukemia
COP-1	Constitutively Photomorphogenic 1
CRYAB	Crystallin Alpha B
CXCR4	C-X-C Chemokine Receptor 4
DCIS	Ductal Carcinoma In Situ
EMT	Epithelial Mesenchymal Transition
ER	Estrogen Receptor
eGFP	Enhanced Green Fluorescent Protein
EGFR	Epidermal Growth Factor Receptor
ESA	Epithelial Specific Antigen
GADD45	Growth Arrest and DNA Damage Inducible Gene 45
GEMM	Genetically engineered mouse model

GIST	Gastrointestinal Tumor
H&E	Hematoxylin and eosin
HDAC	Histone deacetylase
HDR	Homology Directed Repair
HER2	Human Epidermal Growth Factor Receptor 2
HR	Homologous Recombination
IBC	Inflammatory Breast Cancer
IDC	Invasive Ductal Carcinoma
IHC	Immunohistochemistry
ILC	Invasive Lobular Carcinoma
LCIS	Lobular Carcinoma In Situ
LOH	Loss of heterozygosity
MEC	Mammary Epithelial Cell
miRNA	Micro RNA
MMHCC	Mouse Models of Human Cancers Consortium
MMP	Matrix Metalloproteinase
MMTV	Mouse Mammary Tumor Virus
NHEJ	Non Homologous End Joining
PCR	Polymerase Chain Reaction
PI-MEC	Pregnancy Induced Mammary Epithelial Cells
PR	Progesterone Receptor
PTEN	Phosphatase and Tensin Homology on Chromosome Ten
Rb	Retinoblastoma

Rbl-1	Retinoblastoma Like 1
Rbl-2	Retinoblastoma Like 2
Rb_f	Retinoblastoma family members
RT-PCR	Reverse transcription- polymerase chain reaction
SDF1	Stromal derived factor 1
SMA	Smooth Muscle Actin
SV40	Simian virus 40
T_{121}	First 121 amino acids of SV40 large T antigen
TEB	Terminal End Bud
TGF	Transforming growth factor
TNF	Tumor Necrosis Factor
TUNEL	Terminal deoxynucleotidyltransferase mediated dUTP-end labeling
VEGF	Vascular endothelial growth factor
WAP	Whey Acidic Protein
Wt	Wild type

CHAPTER ONE

INTRODUCTION

1.1 What is Cancer?

In a simplistic way cancer can be defined as a disease where cells undergo uncontrolled proliferation. This leads to a breakdown of the normal differentiation pattern of the cells and the burden of tumor cells affects all organs within the body. This results in organ failure finally leading to death. Almost any cell type within the body can be affected by cancer and as such more than 100 different types of human cancer have been identified. Some of the common "hallmarks" of cancer cells that have been identified through prolonged research on this disease are: (i) increased proliferation through positive growth signaling pathways (ii) decreased sensitivity to growth-inhibitory signaling pathways (iii) decreased sensitivity to cell death (apoptotic) pathways (iv) infinite ability of the cells to self-replicate (v) increased supply of blood vessels (angiogenesis) (vi) ability to travel to distant sites through blood and lymph nodes and form colonies (metastasis) (Hanahan, Weinberg 2000).

Even after several centuries of sustained research in every aspect of cancer development and progression, the complexity of this disease remains mind-boggling. The complicated molecular circuitry of cancer signaling pathways has had many new additions in the last century. A current idea of some critical pathways that are involved frequently in human cancer is shown in **Figure 1.1**.

Second only to lung cancer, breast cancer is the biggest killer in the modern world (American Cancer Society, Breast cancer facts and figures, 2007). The goal of this work is to understand some molecular and genetic aspects of human breast cancer that are currently not well understood with the hope that this work will count towards improved cancer treatment of patients in the real world.

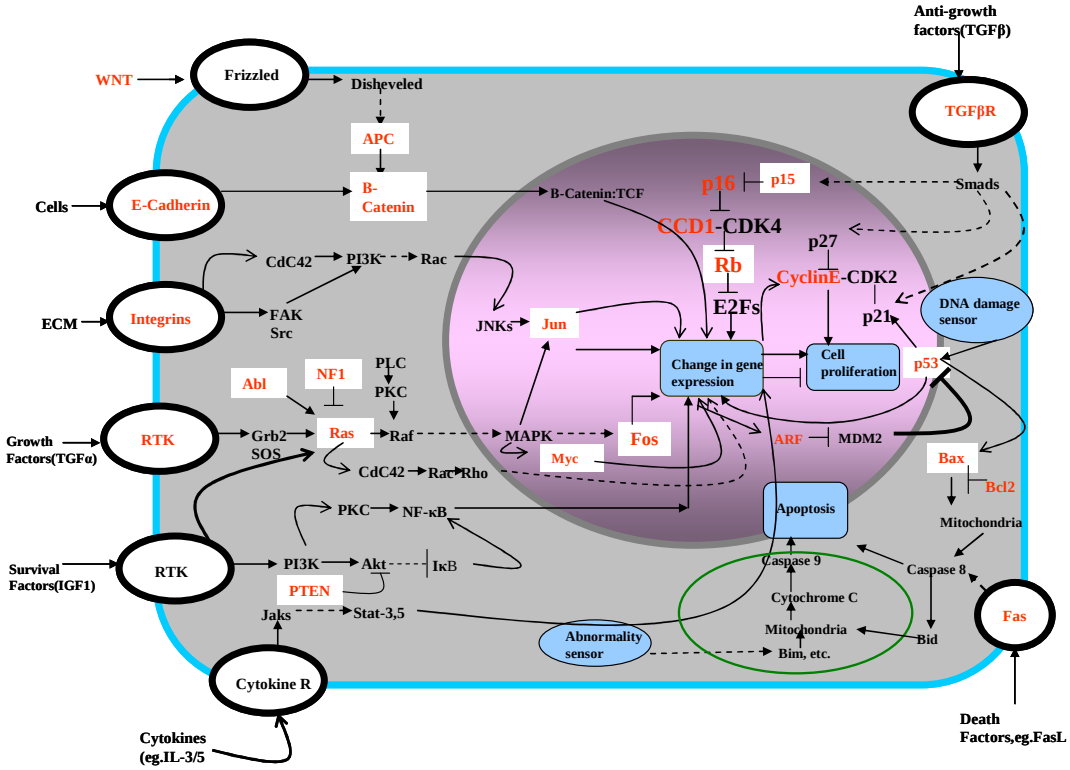

Figure 1.1. Multiple pathways of tumor suppressors and oncogenes are involved in the initiation and progression of cancer. The genes and pathways that are commonly involved in cellular signaling and are often involved in multiple cancer types are shown here. The commonly altered tumor suppressor genes and oncogenes are highlighted in red. (Figure adapted from Hanahan and Weinberg, 2000). This figure is incomplete in portraying the intricacy of cancer pathway circuitry. However it gives an idea of how complex this network is.

1.2 Cancer Cause and Statistics.

Cancer is a disease of abnormal proliferation within any cell type of the body. The list of cancers that affect human beings exceed a hundred different types of cancer, classified

based on the cell type affected (American Cancer Society, http://www.cancer.org/docroot/home/index.asp?promo=gaw, Weinberg, 2007). The causes of cancer are manifold and include life style factors like smoking or intake of tobacco products and alcohol (Ames, Gamble 1983, Hamajima et al. 2002), personal factors like obesity and diet (Ames 1983, Gridley et al. 1990), environmental factors (Augenlicht et al. 2002) and genetic factors like familial retinoblastoma (Leiderman, Kiss & Mukai 2007, MacPherson, Dyer 2007), etc. While the former factors can be somewhat controlled by altering one's life style, it is impossible to control genetic factors that pre-dispose to cancer (Augenlicht et al. 2002, Bickers, Lowy 1989, Greenlee et al. 2000, Parkin, Pisani & Ferlay 1999, Peto 2001, Pisani et al. 1999, Preston-Martin et al. 1990). The focus of this work is to understand the molecular etiology of a sub type of familial breast cancer pre-disposed by inherited genetic mutations.

Cancer is staged clinically to gauge the extent of the disease at the time of diagnosis and to predict outcome (www.cancer.gov, (Greene, Sobin 2008). Various staging nomenclatures exist (like the TNM classification) but largely staging is usually done based on the following three criteria, (i) size of the primary tumor (T) at the time of diagnosis, (ii) amount of local advancement of the disease to surrounding tissues and lymph nodes (N) (iii) extent of distant metastasis (M), that is spreading of the cancer from primary site to distant secondary sites. Based on these criteria the cancer could be stage I (early and more curable) or stages III or IV that indicate metastatic cancer with a poor prognosis.

Even after a decade of research spent in understanding cancer etiology and developing new cancer drugs, the figures for predicted cancer cases in the near future present a bleak picture. The American Cancer Society predicts 71,030 new cases of lung cancer only

in women and 182,460 new cases of breast cancer in 2008 (www.cancer.org). In 1970, in the United States, only 7% percent of patients that had lung cancer were alive after 5 years. This survival percentage increased to 14% three decades later (Weinberg, 2007, Dubey, Powell 2007). This indicates that significant progress has been made in the early diagnosis of cancer, which improves survival. However, the treatment of cancer is still sadly behind. The translation from basic science to clinical manifestation of new drugs has been painfully slow. It is clear that the complexity of cancer requires treatment that is tailored to the individual patient needs and not just to the disease (Linn, Jonkers 2007). A large amount of patient –to-patient variation exists in cancer, based on the race as well as genetic makeup of the individual patient (Daley et al. 2008). Specific genetic modifiers make some races very susceptible to some types of cancers (Lenoir, Narod & Ponder 1990, Ponder 1990, Ponder 1990). The common method of cancer treatment is still chemotherapy using drugs that were discovered several years ago when the understanding of the molecular patterns of cancer was quite poor. However, scientific research has identified some very good cancer targets some of which have resulted in the discovery of novel targeted drug molecules. Some of the examples of significant developments using drugs that target specific molecular pathways that are deregulated in cancers are Trastuzumab in HER2 positive breast cancers (Doyle, Miller 2008, Paik, Kim & Wolmark 2008)and Gleevec (chemical name Imatinib) in treating chronic myelogenous leukemia (CML) and gastrointestinal tumors (GIST) (Deininger et al. 2003, Deininger, Druker 2003, Druker, Lydon 2000). Both these drugs have had tremendous success in treating specific types of cancer by targeting a very specific receptor (HER2 receptor by Trastuzumab) or enzyme (bcr-abl tyrosine kinase by Gleevec) that is overexpressed in these cancers. This comes from an understanding of the molecular pathway

that is upregulated in these cancers. The focus of this research is to identify specific molecular mechanisms that are altered in a sub type of familial breast cancer with *BRCA1* mutations that are currently not well understood.

1.3 Breast Cancer Types and Statistics

With more than 178,480 predicted new cases of invasive breast cancer in women in the United States estimated by the American Cancer Society in the year 2007 (accounting for about 7% of all cancer deaths), breast cancer is the second greatest threat for women in the Western world.

[http://www.cancer.org/docroot/STT/content/STT_1x_Cancer_Facts__Figures_2007.asp, (Desantis et al. 2008)]. Specific races around the world have showed greater incidence rates of breast cancer, for example the Ashkenazi Jewish population have a great susceptibility to familial breast cancers (Pereira et al. 2007, Spannuth, Thaker & Sood 2007), indicating the role of genetic modifiers in the etiology of this disease.

The most common type of breast cancer is ductal carcinoma in situ (DCIS) that is thought to originate in the mammary luminal epithelial cells and gradually spread to the local mammary lymph nodes (Fisher et al. 2007, O'Sullivan, Morrow 2007, Patani, Cutuli & Mokbel 2007). If it goes undetected this can become invasive ductal carcinoma (IDC) (Goldstein et al. 2007, Nielsen et al. 2004, Wiechmann, Kuerer 2008) or invasive lobular carcinoma (ILC) (Arpino et al. 2004, Vo et al. 2006). Early detection and treatment are the keys to breast cancer cure right now. Advances in early diagnostic techniques, such as routine self-examination and mammography (Cruz et al. 2008, Kohrt et al. 2008), new therapeutic interventions (like using Trastuzumab to treat *HER2* positive cancers)(Hicks,

Kulkarni 2008, Paik, Kim & Wolmark 2008), and improved therapy regimens may have contributed to declines in breast cancer mortality in recent years, but breast cancer still remains the second greatest life threat for women following lung cancer (ACS). Currently, numerous treatments are available to patients suffering from breast cancer depending on the grade, histopathology and hormone receptor status of their tumor. Advances in treating estrogen and/or progesterone receptor positive (ER^+, PR^+) breast cancers have greatly increased the life expectancy of patients (Cuzick 2008a, Cuzick 2008b). In contrast, a relatively rare form of breast cancer often referred to as "triple negative" because of the absence of three growth factor receptors, ER^-, PR^-, and $HER2^-$, are particularly hard to treat, and often require an aggressive chemotherapeutic regimen to elicit regression (Livasy et al. 2006, Rakha et al. 2008). Long-term treatments with drugs like Tamoxifen or Trastuzumab fail for this category of cancers since they lack the respective drug targets, ER or HER2. Triple negative cancers tend to metastasize quickly to distant organs like the brain, liver, bone and lungs, resulting in especially poor patient prognosis. It has been identified now that familial *BRCA1* mutations in women make them susceptible to the triple negative breast cancers. Recent studies with molecular classification of breast cancers using microarrays have shown that the triple negative sub-type of breast cancer forms a distinct class of its own and includes both sporadic and *BRCA1* mutated cancers (Perou et al. 2000, Sorlie et al. 2001, Sorlie et al. 2003). Genetic testing of families with more than one incidence of breast or ovarian cancer for *BRCA1* mutation followed by counseling on possible prophylactic options have increased awareness about this more malignant variety of breast cancer(Metcalfe et al. 2008a, Metcalfe et al. 2008b, Metcalfe et al. 2008c). However very little is known about the molecular etiology and pathways involved in the initiation and progression of this cancer sub

type and there is a great need for animal models that can accurately model distinct features of this disease. The current work focuses on developing and characterizing multiple mouse models to help understand some of the critical pathways that are deregulated in this type of breast cancer, which include the *P53*, *BRCA1* and the *RB* pathways. In depth molecular characterization of this malignant cancer can potentially identify diagnostic markers that can be used to identify and treat them early on.

It is worth mentioning that mouse models provide an avenue to investigate the molecular mechanisms underlying the pathways involved in tumor evolution. But due to the heterogeneity of breast cancers, no single mouse model is able to depict all aspects of human cancer. Rather, mouse models seem to recapitulate certain key features of the human disease histopathologically and also in terms of the tumor "behavior" e.g. metastatic or non-metastatic. Engineered mouse models which incorporate genetic changes observed among human breast cancers more closely mimic their human counterparts with regard to histology and tumor progression than mouse tumors generated by older methods such as random viral insertion or carcinogen induction (Cardiff, Kenney 2007). Transcriptional profiling of mouse tumors shows that some mouse models more closely resemble ER^+/PR^+ adenocarcinomas while others share features of triple negative tumors, and confirm that none completely mimics the human pathology (Herschkowitz et al. 2007).

1.4 Landmarks of Cancer Research

Theodor Boveri, a German biologist who lived in the nineteenth and early twentieth century first proposed a mechanism by which cancer arises. During his work with sea urchins he noticed that abnormal chromosomal distribution resulting from the fertilization of a single

egg by more than one sperm gave rise to cell death or the creation of an abnormal cell with aberrant chromosomal numbers. This led him to surmise that the individual chromosomes retain their "individuality" even after cell division occurs, in the daughter cells. So he proposed that aberrant chromosomal distribution might be the cause for abnormal cell behavior that ultimately results in cancer. His ideas gave rise to the earliest concept of "genetic instability" in cancer. In his book "The Origin of Malignant Tumors" published in 1914 he proposed many ideas regarding cancer, tumor progression and cell cycle that have since then been confirmed and accepted (discussed in http://en.wikipedia.org/wiki/Theodor_Boveri).

In 1971 Knudson proposed his "two hit hypothesis" from his study of the familial childhood cancer retinoblastoma (Knudson 1971). He observed that children born to parents with retinoblastoma do not always develop the disease themselves but may later have their children who develop retinoblastoma. From this observation he suggested that loss of single allele of a gene may pre-dispose to a specific cancer type but the cancer only occurs upon loss of the second wild-type allele (thus "second hit"). This hypothesis was also later confirmed to be true.

The discovery of the retinoblastoma gene *RB1* in 1986 was the beginning of a long era of great progress made in cancer research. Two important tumor suppressors that play a big role in cancer suppression, *TP53* (Lane, Crawford 1979, Linzer, Levine 1979, Linzer, Maltzman & Levine 1979) and *BRCA1* (Miki et al. 1994) were also discovered around this time. A timeline of the great discoveries that marked the early path of cancer research are shown in **Figure 1.2**.

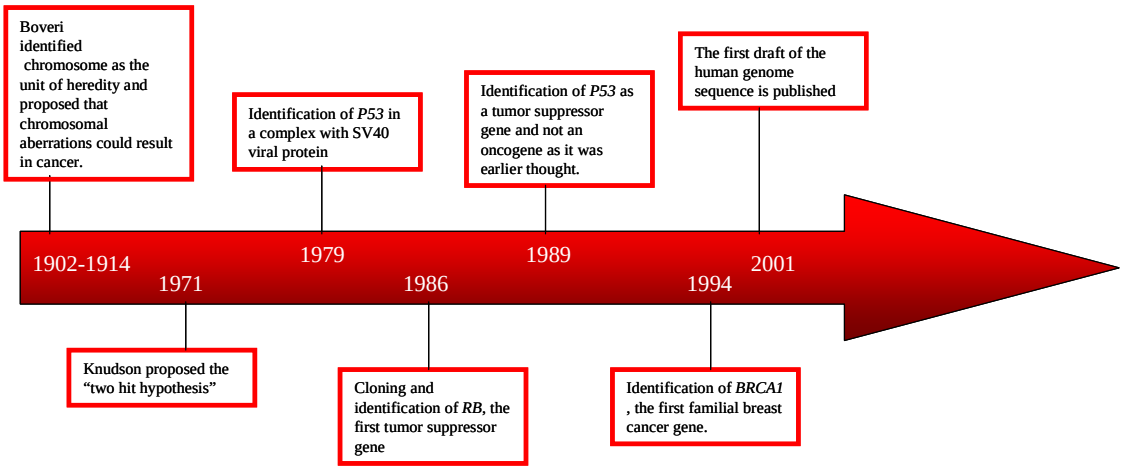

Figure 1.2. A timeline for cancer research is shown here. A timeline for some of the landmark historical discoveries that fuelled cancer research are shown here. Adapted from Balmain, 2001

It was my goal in this project to study the role of three major tumor suppressor genes, *RB*, *TP53* and *BRCA1* in the molecular etiology of breast cancer. I used GEMs to study the role of loss of each of these genes either alone or in combination, in the initiation and progression of breast cancer. This work has resulted in the generation of several mouse models of clinical importance that can be used in the future for drug targeting studies in breast cancer.

1.5 Biology of the Mammary Gland

The mammary gland is a branched secretory epithelium with a bilayered structure (**Figure 1.3**). The inner layer comprises the luminal mammary epithelium cells that are responsible for milk secretion. The outer layer is composed of the myoepithelial cells that provide scaffolding to the luminal epithelial cells as well as contract to help in the ejection of milk by the luminal epithelial cells. The myoepithelial cells have dual properties of muscle

cells (and express smooth muscle actin) as well as epithelial cells (and express epithelial cytokeratins 5 and 14). Myoepithelial cells are thought to regulate cross talk between the luminal epithelial cells and the surrounding stroma by paracrine signaling pathways. Surrounding the mammary gland are the mammary stroma and fat pad.

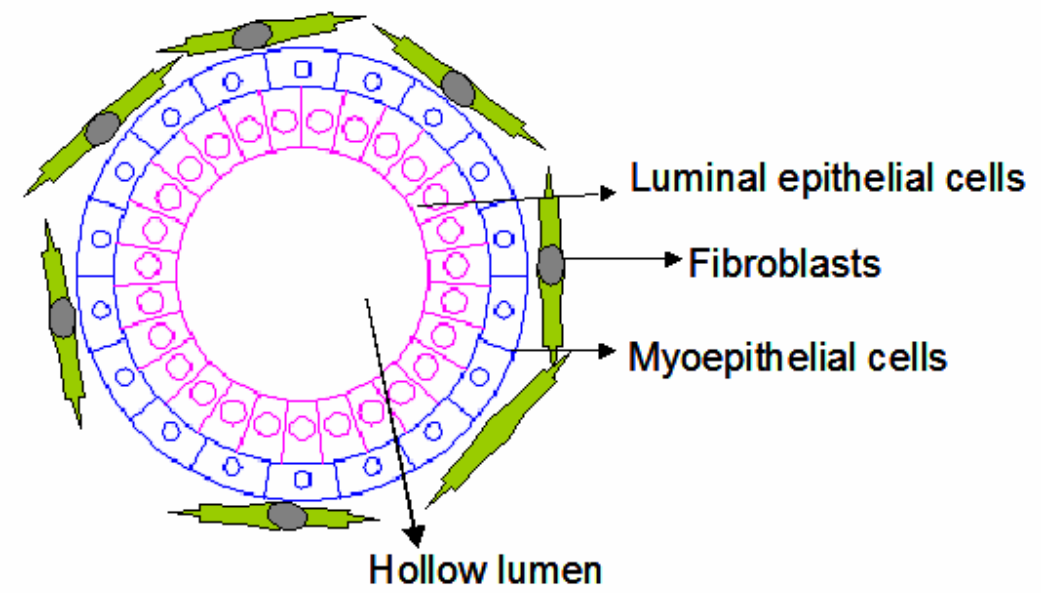

Figure.1.3. A schematic representation of the components of the mammary gland is shown here. The mammary gland is composed of two cell types. The luminal epithelial cells are responsible for milk secretion. The outer myoepithelial cells are contractile in nature and help in milk secretion. The ductal structure is embedded in fibroblasts and mammary fat pad.

The mammary gland is unique among other organs in the mammalian system in that it undergoes most of its development in the adult animal rather than in the embryo. In most mammals the earliest development of the mammary gland is marked by the existence of the "milk line" which is a thickening of the ectoderm layer on external body wall (Lanigan et al. 2007, Robinson 2007). A variety of signaling cues then causes the milk line to disperse into the future locations of the mammary gland to form lens shaped structures called "placodes". These placodes then proliferate and form small buds of mammary epithelial cells in the

mammary fat pad. In the mouse the rudimentary mammary gland exists and can be detected in 10-11 day old embryos as an epithelial bud, but the major development of the mammary gland occurs in adulthood. Interestingly early mammary gland development is strikingly similar to the development pattern of other secretory organs like hair follicles and salivary gland (Mikkola, Millar 2006). The early developmental signaling cues for the morphogenesis of these glands are very similar, though the subsequent events leading to the formation of the adult glands are unique and different. Postnatally the mammary gland forms ductal structures and lobules but this growth is very slow. By 4-7 weeks post natal, the mammary gland terminal end buds (TEBs) (**Figure 1.4**) have formed a ductal pattern within the mammary gland fat pad. At this stage apoptosis is an important regulator of ductal morphogenesis and helps maintain the TEB structure with a hollow lumen (as shown by arrow in **Figure 1.4**). The TEB is made up of cap cells and body cells. The cap cells are highly proliferative and generally differentiate to form myoepithelial cells. The cap cells have also been implicated to have progenitor (stem) cell properties. The body cells form the inner layer of the TEB and differentiate to form luminal epithelial cells. It is only at puberty that the mammary glands undergo a big growth spurt and estrogen secretion along with high mitotic activity causes the mammary ductal structure to completely fill up the mammary fat pad (Wiseman, Werb 2002). At pregnancy there occurs a second spurt of very rapid proliferation and lateral branching morphogenesis of the mammary gland. At this time the combined role of estrogen, progesterone and other pregnancy related hormones have an important role in the gland development. During lactation milk secretion occurs into the hollow mammary lumen and is controlled by prolactin, insulin and glucocorticoids. Lactation is followed by involution of the mammary gland. This phase has two parts. In the first part

p53 mediated apoptosis clears the milk secreting luminal epithelial cells (Strange et al. 1992). In the second phase there occurs a collapse of the extensive ductal structures formed during late stage pregnancy and lactation, including break down of the mammary extra cellular matrix and basement membranes. The second phase is p53 independent (Medina 2005).

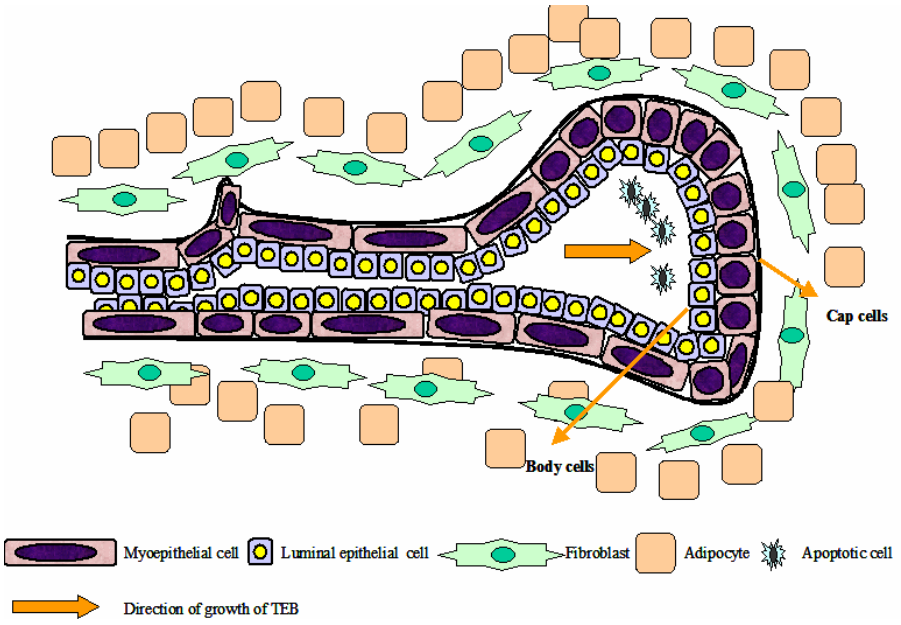

Figure 1.4. Structure of the mammary gland terminal end bud is shown here. The TEB of the mammary gland is composed of the leading edge of rapidly proliferating cap cells and the inner mass of more differentiated body cells. (Figure adapted from Lanigan et al. 2007).

A lot of comparisons can be drawn between the growth spurt in the mammary gland at pregnancy and the development of a tumor. At pregnancy the mammary gland undergoes rapid proliferation and invades into the surrounding fat pad and stroma. The gland also generates anti-apoptotic signals during this time to prevent pre-mature involution. There is also significant amount of angiogenesis to support the rapidly proliferating mammary epithelial cells. During breast cancer development, many similar features are seen, like a

spurt of proliferation, anti-apoptotic signals, stromal invasion and angiogenesis. So it is not surprising that many of the signaling pathways involved in normal mammary gland development are hijacked by tumor cells to help in mammary tumor progression (Lanigan et al. 2007). Some of the pathways that are involved in mammary gland development and are also frequently altered in breast cancer are indicated in **Figure 1.5.**

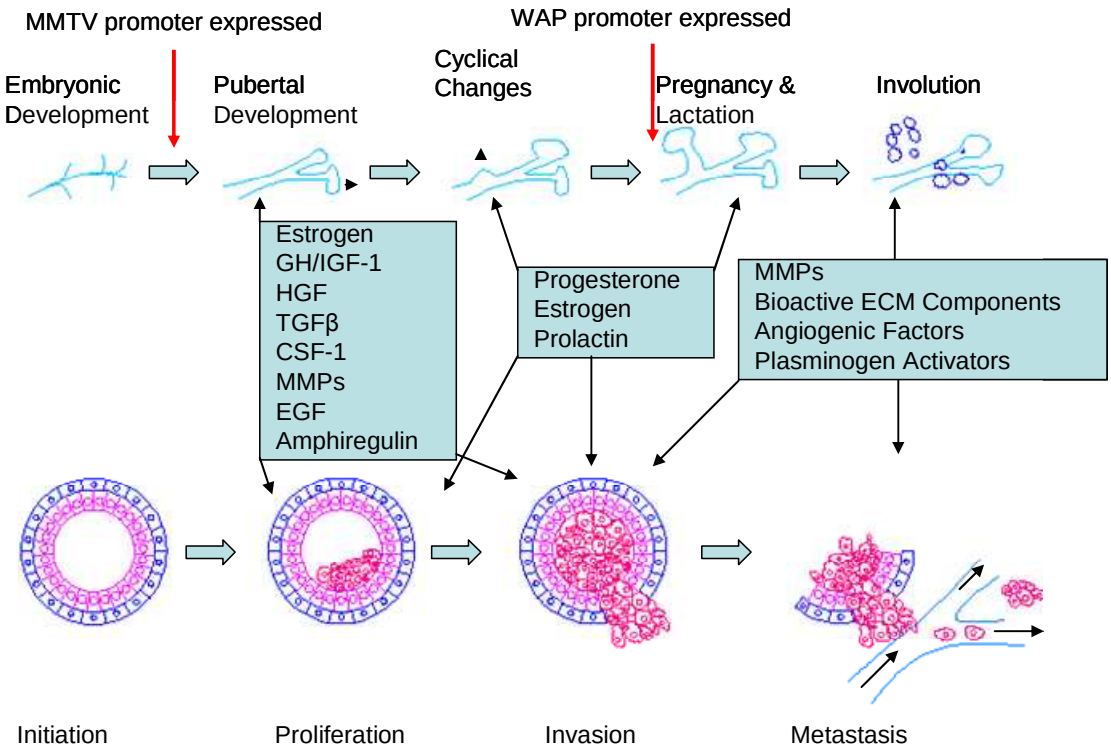

Figure 1.5. **Signaling pathways involved in the development of mammary gland at several stages is frequently altered in breast cancer.** Hormones like Estrogen, Progesterone and Growth hormones play an important role in mammary gland proliferation and ductal morphogenesis during puberty. However overexpression of these hormones can lead to breast cancer and ER and PR are frequently overexpressed in breast cancers. Similarly, while matrix metalloproteases (MMPs) play a critical role in mammary gland remodeling during involution, overexpression of MMPs is frequently found in metastatic breast cancer. MMPs act like molecular scissors and help mammary epithelial cells invade through the stroma to distant sites. The various promoters used frequently in targeting transgenes and knockouts to the mammary gland are turned on at different stages of mammary gland development. The two most common promoters used are shown in this figure. Figure adapted from (Lanigan et al. 2007)

The tight regulation of mammary gland ductal morphogenesis by multiple hormones is disrupted in cancer resulting in abnormal and uncontrolled cell proliferation. The effects of pregnancy hormones like estrogen and progesterone on the mammary gland (both long term and short term) have been the theme of breast cancer research for a long time now. It has been observed statistically, that women who are multiparous and have full term pregnancies with extended breast feeding periods earlier in life have a reduced risk of having breast cancer (Albrektsen et al. 2005, Althuis et al. 2004, Harris 1992, McPherson, Steel & Dixon 2000). Also women who have a late onset of menarche tend to reduce breast cancer risk significantly (Bernstein et al. 1991, Harris 1992). This suggests that exposure of the mammary gland to hormone stimulation induced by pregnancy has a long term or permanent protective effect on the cells that prevent them from becoming undifferentiated and proliferative tumor cells. There are various theories about the nature of this protective effect. One hypothesis is that pregnancy and lactation promote terminal differentiation of cells in the leading edge of the TEB of the mammary gland (Russo, Russo 1987, Russo, Russo 1997). As the terminal edge of the TEB has highly mitotic cells undergoing rapid cell division, these are also most susceptible to carcinogenesis. So pregnancy results in the replacement of the highly susceptible cell population by a differentiated and less susceptible population. This hypothesis does not entirely explain the phenomenon. Another hypothesis is the mammary gland hormonal environment is permanently altered by pregnancy and lactation. Supporting this hypothesis are studies that have reported lower levels of growth hormone and also reduced levels of the estrogen and epidermal growth factor receptors in the parous mammary gland (Thordarson et al. 1995) compared to the age matched virgin mammary glands. As both ER and EGFR overexpression have been correlated to increase breast cancers this is a

likely hypothesis. Finally the "cell fate hypothesis" suggests that exposure to pregnancy hormones induce a permanent change in the mammary gland. This prevents proliferation of mammary epithelial cells upon exposure to carcinogens (Medina 2005, Sivaraman et al. 1998) prevents proliferation specifically in the ER positive cells and it results in the emergence of a new cell lineage referred to as the "parity-induced mammary epithelial cells" (PI-MECs, (Wagner et al. 2002). Overall it is now clear from gene expression profiling studies that pregnancy and lactation results in an altered gene expression profile in the mammary gland compared to the virgin mammary gland. This altered state of the gland includes many different regulatory pathways that are different from the virgin mammary gland and that make the gland less susceptible to cancer. While drug induced rodent carcinoma models (both mouse and rat) have been used to study the above hypotheses, transgenic mouse models have been limited by the use of mammary gland specific promoters like Whey Acidic Protein (WAP) that require multiple cycles of pregnancy and lactation to turn on gene expression. This is a paradox between mouse models and humans, where pregnancy is required in the mouse model to promote cancer. This has precluded a thorough study of the protective effects of pregnancy and lactation on the mouse mammary gland and also the effects of cancer causing genes in the virgin mammary gland. Also, it is unclear from current research if pregnancy confers the same protective effect on all types of breast cancer. There is conflicting evidence that women with *BRCA1* germline mutations may get significant protective effects from prophylactic mammoplasty and hysterectomy (Rodriquez, Domchek 2007). Mouse models that allow testing the effects of these genetic mutations in virgin as well as multi-parous mammary gland will help find answers to these questions. In our report we have attempted to get around this problem by using the Mouse Mammary

Tumor Virus (MMTV) promoter that is expressed earlier in development and does not require multiple cycles of pregnancy and lactation to be turned on (Wagner et al. 2001).

1.6 Breast Cancer Research – A History

Two common classifications of breast cancer are ductal and lobular carcinomas, which are based on morphological features shared by these tumor cells and their presumed cells of origin, either the milk ducts of the mammary gland or the mammary gland lobules, respectively. Although most human breast cancers are thought to be comprised of luminal epithelial cells and predicted to arise in the ductal compartments of the mammary gland, the cell of origin is impossible to determine at the final stage of the disease due to the great heterogeneity of the tumor. Depending on whether they are local or have spread to surrounding lymph nodes or other organs breast cancer is classified as "*in situ*" (local) or invasive. Progress in understanding this disease has been done by carrying out primarily "reverse engineering", that is by trying to recreate those changes seen in patients presenting clinical forms of breast cancer, in either cell culture systems or animal models.

1.6.1 Cell culture studies of breast cancer

Cell lines used for study of changes involved in the progression of breast cancer, like proliferation, apoptosis and invasion and also signaling pathways involved have provided much useful information. Both human and mouse cell lines have been used though human cells are thought to represent the human disease better. Cell lines are easy to grow and maintain and produce highly reproducible results that make them a very powerful tool. Human cell lines can also replicate the presence or absence of estrogen receptor (ER), a key

receptor involved in human breast cancer treatment and can thus be used for studying ER positive breast cancers. Also, cell lines have been used to look for the potential breast cancer "stem cell niche" and researchers have identified cells with self renewal properties by using cell sorting methods and cell surface markers like CD44 and ESA (epithelial surface antigen) (Dontu et al. 2003). Some cell populations sorted in this manner have shown self-renewing properties and have formed well-differentiated tumors when transplanted in nude mice. Research in this area has been seminal in understanding the potential stem cell properties of some mammary epithelial cells and targeting those cells therapeutically may be the next step in breast cancer drug development. However, most cell lines undergo random mutations when maintained over many years and these mutations can lead to potentially confounding results. This is a caveat for any work using cell lines that researchers have not been able to overcome. The more recent advent of **three-dimensional cell culture** has brought many improvements to traditional cell culture systems. Three dimensional cell culture systems allow cells to grow in a more "in-vivo" like environment by embedding the cells in matrigel and hence prevent the accumulation of random changes necessary for the cell's survival on a flat surface, like a traditional cell culture plate. Breast cancer research has been further advanced by work done by Bissell and Brugge and colleagues using three-dimensional culture of mammary cell lines (Debnath, Brugge 2005, Lee et al. 2007, Paszek, Weaver 2004, Shaw, Wrobel & Brugge 2004). Several key signaling pathways have been identified by this method that have an effect in breast cancer progression and poor prognosis, for example identification of the "autocrine loop" signaling pathway involving EGFR (epidermal growth factor receptor), TGF-alpha (transforming growth factor alpha), TNF-alpha (tumor necrosis factor alpha) and amphiregulin (Vargo-Gogola, Rosen 2007). It was shown for the first time

in a three dimensional mammary epithelial culture system that this signaling pathway acts to increase cellular proliferation and can promote cancer progression (Dillon et al. 2007, Dillon, White & Muller 2007). However, the caveat still is, that the three dimensional cultures use mammary cell lines like MCF-10A that have been grown and maintained for several years and have already accumulated unknown genetic changes. It has been a constant effort in the field to make the cell culture systems as "life like" as possible and current efforts are underway to use primary mammary epithelial cells for short-term three-dimensional cultures that would preserve the "in vivo" characteristics of the cells as much as possible. We have made significant progress in trying to establish primary mammary epithelial cells in three-dimensional culture. In this work we have shown that short term cultures with cells derived directly from the mouse mammary gland can indeed be grown in 3-D and these cells also express specific transgenes in a hormone dependant manner in culture. Such a system can be used as a high throughput assay for studying cooperating lesions without setting up multiple mouse crosses. This work will be reported in a later chapter in this thesis and the significant conclusions from it will be identified.

1.6.2 Mouse models of breast cancer

Mouse researchers believe that genetically engineered mouse models (GEMMs) are the closest we have come to modeling human breast cancer. The original GEMMs for studying breast cancer were relatively simple and used the MMTV or WAP promoters to target the overexpression of relevant oncogenes in mammary epithelial cells, like Myc (Schoenenberger et al. 1988), Her2 (Guy et al. 1992), Wnt (Kwan et al. 1992, Tsukamoto et al. 1988) and H-Ras (Nielsen et al. 1991). These mouse models have resulted in the

overexpression of a single oncogene in the entire mammary gland. This is unlike the scenario in human breast cancers where initial changes most likely affect a single cell in the mammary gland and the gradual accumulation of mutations lead to a stochastic progression of tumorigenesis. The timing as well as sequence of genetic changes after the initiation event decide the long term pattern of cancer progression, for example, metastatic or not, as well as the clinical outcome. For studying the effects of loss of tumor suppressors in mammary tumorigenesis, classical mouse models have looked at the germline loss of *p53* and *Brca1*. A big problem with these models was that they often had phenotypes that precluded mammary tumor analyses at later times, like embryonic lethality in *Brca1* knock out mice(Gowen et al. 1996) or a predisposition to an unrelated tumor type (lymphomas, in particular for *p53* null mice, (Donehower et al. 1992). Significant advances in mouse modeling have helped overcome many of the difficulties mentioned above. It is now possible to target precise cell types within the mammary gland (e.g. luminal or myoepithelial, using specific Keratin promoters (K8/18 or K5/14 respectively). It is also possible to turn “on” gene expression at a precise time in development and then to turn it off (using multiple available mechanisms like the Cre-Lox-P system, the Cre-ER system, the Tet inducible system and by viral introduction of transgenes). This “on-off” mechanism has made it possible to look at roles of specific genes not only in tumor initiation but also tumor maintenance and progression. Specifically the Cre-Lox-P system has been widely used to turn on transgene expression in a tissue specific manner as well as knockout genes in specific tissues. The Cre enzyme is a 38kDA bacterial protein isolated from phage P1. Cre mediates intra and intermolecular site-specific recombination between two loxP (locus of X-ing over) sites. A single loxP site has two 13 bp inverted repeat sequences with an 8bp spacer in between them. Recombination by Cre takes

place in this spacer region leading to the precise removal of DNA in between the sites leaving a single loxP site behind (Weinberg, 2007, Van Dyke, Jacks 2002). By using this technology only those cells within the mammary gland that have undergone the recombination event will express the transgene of interest. It is possible by using the Cre-Lox-P technology to study many complex and compound genetic interactions that take place in a stochastic fashion over time and better reflect the human breast cancer scenario. We used this system to our advantage by generating the *TgMMTV-Floxed-T_{121}* (*$TgMFT_{121}$*) (**Figure 1.6**) mice that expressed eGFP specifically in the mammary glands. Upon mating with mammary specific Cre strains (WAP or MMTV), excision of the eGFP cassette resulted in placing T_{121} expression directly under the control of the MMTV promoter. So while all cells within the mammary gland that expressed the MMTV promoter showed eGFP expression prior to crossing with Cre strains. Upon Cre introduction, only a subset of cells within the mammary gland that expressed the WAP promoter also expressed T_{121}, depending upon the efficiency of Cre mediated recombination. This served two purposes. First, it allowed healthy and wild type mouse lines to be maintained in the colony that did not express T_{121} and could be visually screened for strong transgene expression by the robustness of their eGFP expression. Second, it allowed somatic deletion of eGFP and expression of T_{121} in a specific sub set of cells within the mammary gland that underwent the recombination event, that had not been possible in a similar model using the direct WAP-T_{121} transgene (Simin et al. 2004) and allowed us to study the role of loss of pRb family in conjunction with other co-operating genetic lesions in the developed mammary gland.

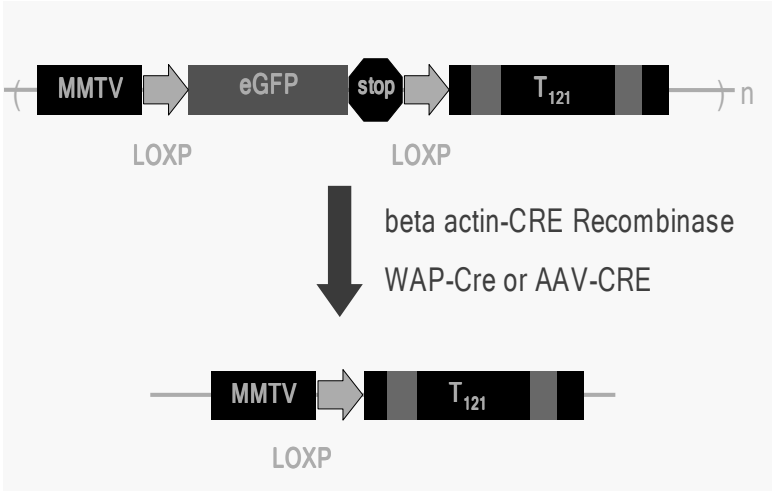

Figure1.6. MFT_{121} transgene design. The MMTV promoter drives expression of a floxed eGFP cassette prior to crosses with mammary specific Cre strains. Upon introduction of a Cre strain (mammary or non mammary specific as indicated by WAP, beta Actin or AAV-Cre), eGFP is excised leading to the expression of T_{121} under the control of the MMTV promoter in all cells that underwent recombination. The target cells that express T_{121}would depend on the expression of Cre. (Hua Wu in the Van Dyke Lab made the transgene).

Finally, GEMMs still have problems that need to be overcome to make them the most "ideal" system for cancer study. Some of these are the differences between mouse and human biology that make most mouse mammary tumors hormone independent while human tumors are often not. In addition, interspecies differences have made mouse mammary tumor metastasis patterns very different from human mammary tumors. While most human mammary tumors metastasize to the bones, brain and liver, most mouse tumors rarely metastasize and if they do, it is usually to the lungs. The reason for this is not clear and could be a fundamental difference in mouse and human mammary gland. Or it could be that a specific combination as well as sequence of events is necessary in a specific cell type within the mammary gland to see progression to metastasis. Modifier gene effects in the specific genetic background of mice used for these studies may also play a role (Balmain, Nagase

1998). However it is clear that developing mouse models for breast cancer metastasis are the next barrier to be overcome to allow for good pre-clinical testing of anti-metastatic drugs. In this thesis we have described the establishment of a metastatic mouse model for breast cancer by layering on mutations within the mouse mammary gland, that occur with great frequency in human breast cancer.

1.6.3 Mammary specific promoters play an important role in mouse models of breast cancer

To target specific genetic events to a specific cell type in the mouse tissue specific promoters are required to drive expression of the gene of interest. Researchers have used several promoters to target gene expression specifically to the mouse mammary gland. The use of the Cre-Lox –P technology allows the narrowing down of gene expression or loss to a specific cell type by using a cell specific Cre recombinase. Each of these promoters has some advantages to their use, based on the scientific questions being asked. Some of the commonly used mouse mammary gland promoters and their specific advantages and disadvantages are listed below.

Whey Acidic Protein (WAP) promoter

The WAP promoter uses the promoter elements of the WAP gene that is expressed intrinsically in the mouse mammary gland. Lactogenic hormones like insulin, hydrocortisone and prolactin activate the WAP gene expression to almost a 1000 fold at mid-pregnancy (between day 15 and day 17) of the animal (Burdon et al. 1991) and therefore the WAP promoter expression in the mouse is not detected prior to at least a single cycle of pregnancy and lactation in the mouse (Pittius et al. 1988, Triplett et al. 2005). Many mouse models of

cancer have been developed using the WAP promoter. The biggest advantage of this promoter is its expression is tightly regulated by mammary hormones and found to be present mainly in the mammary luminal epithelial cells. This prevents leaky expression of transgenes that may often pre-dispose to non-mammary phenotypes. WAP expression in the mammary gland is absent in virgin animals and peaks at around day one of lactation. Expression persists through day 10-post lactation and even after the mammary gland has undergone involution. WAP expression has also been reported in the brain but at negligible levels (Wagner et al. 1997).

The disadvantage of using the WAP promoter is the mice must undergo at least one (preferably multiple) cycles of pregnancy and lactation to turn on the transgene. So WAP promoters cannot be used to target virgin mammary epithelial cells. Also, hormonal regulation of this promoter can turn on other developmental cues that may play a role in the phenotype observed (Jonkers, Derksen 2007). This can be a confounding effect. Nevertheless, WAP has remained the promoter of choice for many mouse mammary gland researchers (Andres et al. 1987, Gallahan et al. 1996, Nielsen et al. 1991, Schoenenberger et al. 1988, Schulze-Garg et al. 2000, Simin et al. 2004) and is used in this study for restricting Cre expression to the mammary luminal epithelial cells.

Mouse Mammary Tumor Virus (MMTV) promoter

The MMTV promoter uses promoter elements from the mouse mammary tumor retro virus that infects mammary glands specifically and causes a wide spectrum of tumors in mice, including mammary tumors. So this promoter uses the viral promoter and enhancer sequences to drive the expression of specific transgenes or knockout genes in the mouse mammary gland (Stewart, Pattengale & Leder 1984). Some of the distinct advantages of the

MMTV promoter are it shows strong expression in both the virgin and lactating mammary gland. Studies have detected expression of the MMTV promoter as early as 6 days post partum and strong expression in the entire mammary ductal tree is detected by 5 weeks in some mice (Wagner et al. 2001). The level of expression of MMTV peaks during pregnancy and lactation.

The disadvantage of the MMTV promoter is that it shows frequent leaky expression in other secretory tissue types, especially in the salivary glands, skin, hair follicles and seminal vesicles. The temporal and spatial expression pattern of this promoter seems to follow early developmental cues that are similar for several secretory organs like the mammary gland, hair follicles and salivary glands (Mikkola, Millar 2006, Wagner et al. 1997). This predisposes the mice to non-mammary gland tumors like salivary gland tumors. However, the MMTV promoter has been frequently used in mouse models of breast cancer due to its pre-dominant expression in both the virgin and lactating mammary glands (Guy et al. 1992, Tsukamoto et al. 1988).

In the current work, it was our goal to target T_{121} expression both in the virgin and lactating mammary glands. For this we used the MMTV promoter to drive the floxed transgene expression. However, leaky expression of MMTV-Cre resulted in early lethality of the *Rb*$_{f}$*/p53* inactivated mice. This ruled out the use of the MMTV-Cre for follow up studies and only WAP-Cre was used that expresses only in the lactating mammary epithelial cells and thus restricts T_{121} expression to these cells as well.

Keratin 14 Promoter

While the use of mammary specific promoters listed above has been successful in targeting the mammary luminal epithelial cells, very few promoters have targeted the

mammary myoepithelial cells. The myoepithelial (basal) like profile of human *BRCA1* mutated familial breast cancers made it necessary to target this cell type in the mouse, in an effort to model this cancer sub type. To do this the Cytokeratin 14 promoter that targets the basal cells of many organs, including mammary gland, skin, lung epithelium, etc. was used. This promoter was successfully used to target the mammary myoepithelial cells and was been able to recapitulate some features of the human basal like breast cancer upon loss of *BRCA1* (Liu et al. 2007). The advantage of this promoter is it does not require pregnancy of the mice to turn on expression of the gene of interest. The disadvantage is, this promoter is not mammary gland specific and the mice get a high percentage of non-mammary (particularly skin) tumors. Other keratin promoters that target specific cell types within the mammary gland like the Keratin 8/18 promoter to target the luminal epithelial cells and Keratin 5 promoter to target the mammary myoepithelial cells are currently being developed by many labs and may be used in the near future.

Some of the other promoters used to target gene expression to the mammary gland are the Bovine beta-lactoglobulin, BLG, (Whitelaw et al. 1992), Rat prostate-steroid binding protein C(3)1 (Allison, Zhang & Parker 1989), Metallothoinin, MT, (Palmiter et al. 1993) and H19 (Turksen et al. 1992) promoters. All of these promoters have been used with less frequency due to their significant non-mammary expression. It is clear that the need of the day is to find a mammary specific promoter that is hormone independent which would allow the study of critical genetic interactions in all stages of mammary gland development, without the confounding effects of hormonal stimulation.

1.7 Microarray Classification of Breast Cancer

The advances in DNA microarray technology in breast cancer stratification have made it possible to predict the outcomes of the different classes of breast cancer. Instead of the older method of histological stratification of breast cancers that was often deceptive and did not reflect the true nature of the cancer (Bertucci et al. 2008), this method uses the molecular signature of each cancer subtype to predict its outcome (Bertucci et al. 2008). Using this technology Perou et al identified four broad categories of human breast cancer (Perou et al. 2000). They were either ER positive or ER negative. Among the ER positive tumors were the Luminal A type that resembled the normal mammary gland closely and had a good prognosis. Also in the ER positive tumors were the Luminal B tumors that had higher proliferation levels and were more aggressive. However both these sub types expressed the cell lineage markers keratin 8 and 18 that are normally expressed in the mammary luminal epithelial cells. These tumors also respond well to hormonal treatment by Tamoxifen. In the ER negative tumors, there are the Her2 (erb-B-2) positive tumors that respond well to Trastuzumab. The basal tumors are the second group within the ER negative tumors that do not express ER, PR or Her 2 and have very poor prognosis. These basal tumors were also found to express the mammary myoepithelial cell markers, keratins 5 and 14. The basal type tumors also show frequent mutations in *BRCA1* and will be the focus of much of our studies in this thesis.

Microarray technology has also made it possible to compare and contrast human and mouse tumors (Herschkowitz et al. 2007). These studies have shown that some mouse tumors model certain aspects of human mammary tumors. For example the highly proliferative human basal tumors co-clustered with the mouse tumors that had inactivated *Rb* and *p53*

pathways. As *Rb* pathway inactivation leads to higher expression of E2F target proliferation genes this is a highly relevant result of mouse models reflecting human cancer biology. It also indicates that loss of *RB* pathway, *P53* and *BRCA1* may have a potential synergistic role in the promotion of familial human cancers. Also significant is similar profiles of cell lineage markers in mouse and human tumors when both sets of tumors are compared by array analysis. Both mouse and human basal-like tumors showed high expression of keratins 5, 14, 15, c-KIT and CRYAB. Some of these results were confirmed by immunohistochemistry, for example keratin 5 staining identified keratin 5 as marker for both human and mouse basal-like tumors. A subset of GEMMs display heterogeneous phenotypes and do not represent any single subtype of human tumor. However these mouse models may be useful for appreciating the scope of possible outcomes, and helpful for identifying new pathways involved in the biology of mouse mammary progression that may also play a key role in human mammary tumorigenesis. Some types of human breast cancer still remain very difficult to model in mice due to inherent mouse-human differences, like the ER^+ human cancers. The sporadic "basal-like" and *BRCA1* mutated familial breast cancers have also been difficult to replicate in mice due to their unknown cell of origin and the possible complex genetic events necessary to generate these tumors. Several groups have tried to model this class of breast cancer, and some have been able to replicate certain key features (Liu et al. 2007).

One of the goals of this project is to use microarray technology to (i) classify the new mouse models generated in this study into a sub class of human cancer it best represents using molecular marker characterization (ii) identify molecular markers associated with a

specific combination of genetic lesions in the mouse models generated to further understand the etiology for breast cancer progression.

1.8 Genetic Instability and Cancer

Genetic instability in the germline of living organisms has been a common phenomenon since the earliest evolution of eukaryotic cells. According to Darwinian laws of evolution, cells that accrued mutations that conferred them better survival potential were carried on in the germline to create new organisms. However a great increase in genetic instability could lead to loss of viability of cells and therefore the organisms. Especially instability in somatic cells (cells that form specific tissues with the organism) results in the accumulation of mutations that can ultimately fuel cancer (discussed by Weinberg, 2007).

One of the important ways in which normal somatic cells are prevented from undergoing uncontrolled proliferation is by the process of replicative senescence. Chromosomes are capped by specific DNA sequences called “telomeres” that prevent chromosomes from fusing with each other. The telomeres undergo gradual shortening during the lifetime of a cell and the ultimate loss of this protective chromosomal cap causes the cell to undergo crisis resulting in cell death or apoptosis (Stindl 2008). However mutations in cells resulting in loss of the critical apoptotic machinery can cause cells to have random chromosomal fusions after their telomeres are eroded. These cells then accumulate large amounts of genetic instability and can ultimately result in cancer (Cheung, Deng 2008). Telomeric crisis resulting in genetic instability has been stated to be a frequent occurrence in human breast cancer (DePinho, Polyak 2004).

Mouse cells have much longer telomeres than human cells. Because of this mouse cells rarely undergo the “crisis” caused by complete telomere erosion in human cells. Also the much shorter lifespan of mice results in their rarely undergoing complete telomere erosion in a lifetime. So it has been suggested that the mouse genome is more stable than the human genome and does not easily undergo genetic instability (Artandi et al. 2000, Artandi et al. 2002).

Genetic instability is a common feature in human solid tumors (Pihan, Doxsey 2003). The cause for genetic instability is primarily damage of the fundamental genetic material within cells, DNA. DNA damage in cells can occur through multiple mechanisms. Chemically reactive molecules within the cells like reactive oxygen species can affect DNA bases. External agents like chemicals in consumed food products and environmental pollutants can cause slow but significant DNA damage. Radiation in any form can cause major DNA damage if given in large doses (Ayouaz et al. 2008). Besides damage to DNA structure chromosomal instability (CIN) within cells can also be created by alterations (increase or decrease) in their chromosome number, leading to aneuploidy (which means a deviation from the normal or euploid karyotype of a cell). This is caused by the missegregation of chromosomes due to an ineffective mitotic checkpoint control in the M phase of cell cycle. A large contribution to genomic instability and aneuploidy in human cancers is made by double strand break repair defects where damaged DNA is replicated without being repaired.

Several “tumor suppressor” and “caretaker” genes in the eukaryotic genome act as watchdogs and prevent the frequent accumulation of genetic instability. Some of the noteworthy players in this caretaking process are *P53* (often referred to as “guardian of the

genome") and *BRCA1* that is an important DNA damage repair protein. *P53* acts as a cell cycle checkpoint control protein at both the G1/S and G2/M phases. Cells that lack *P53* display an elevated level of genetic instability (Chin et al. 1999, Sharpless et al. 2002). Similarly, *BRCA1* functions by forcing cells that have undergone DNA double strand breaks to be repaired by the error-free Homologous Recombination Process (HR). In the absence of *BRCA1* cells are repaired by the error-prone Non Homologous End Joining Process (NHEJ) that leads to the accumulation of mutations leading to widespread genetic instability and ultimately cancer (Deng 2006). An ongoing debate in the cancer community is whether genetic instability is the cause or the consequence of cancer. It is possible that random mutations resulting in the accumulation of genetic instability sets the stage for cancer progression. Support for this comes from studying early polyps in colorectal cancers that have abundant genetic instability both as aneuploidy as well as microsatellite instability (Bardi et al. 1997, Lengauer, Kinzler & Vogelstein 1997). This suggests that genetic instability may be a process that cancer cells that have undergone mutations acquire to get a growth advantage. However future work is required to find out if this is the case for all cancer types.

Mouse mammary tumor models with loss of *p53* alone or combined loss of *Rb1* pathway and *p53* have been unable to recapitulate the genetic instability seen in human cancers (Liu et al. 2007, Simin et al. 2004). The reason for this could be the more stable genome of the mouse caused by their longer telomeres. To mimic genetic instability seen in human cancers the mouse may require an additional loss of *Brca1* resulting in accumulation of unrepaired damaged DNA (Deng 2006). Liu et al., 2007, showed that mouse mammary tumors with loss of both *p53* and *Brca1* exhibited widespread genomic instability compared

to mammary tumors generated by *p53* loss alone. This gave us reason to believe that loss of *Brca1* in addition to the *Rb1* pathway inactivation and loss of *p53* in mice would lead to genetic instability and result in mammary gland tumors that better emulate human tumors in numerous aspects. We show here that the loss of *Rb1-family, p53* and *Brca1* in the mammary gland results in widespread genetic instability along with deletions on chromosome 4 and 10. Significant instability is not observed in mice with loss of *Rb1* and *p53* only. This finding further suggests that a weakened genome is more susceptible to the development of aggressive cancers and genetic instability could be the cause for tumorigenesis.

1.9 Making a Good Pre-clinical Mouse Model

An ideal preclinical mouse model would incorporate several important characteristics (Sharpless, Depinho 2006). First, it would reflect human genetic events as closely as possible. Second, the mouse model should have a high penetrance and a reasonable tumor latency (at most a few months). Very long tumor latencies and low penetrance would rule out using the model for drug testing studies. Third, the model would have an "easy readout" (like shrinking of tumor that can be easily measured) for testing drug efficacy. Finally, and perhaps most importantly, a model should reflect the true complexity of human cancer and be modified by factors like immune response, tumor-stromal interaction, genetic background of the mouse and other testable factors. We have generated a GEMM for human breast cancer that meets most of these criteria. Our model, as elucidated in later chapters, also achieves several of the important criteria for the "ideal" GEMM for drug development studies,

including complete penetrance, short latency, easy readout and mutations highly relevant to human cancer.

I will summarize next the roles played by the *RB1* pathway, *P53* and *BRCA1* in human breast cancer and their potential synergistic effect in promoting breast cancer that we aim to model.

1.10 Common Genetic Alterations in Human Breast Cancer

1.10.1 The *RB1* pathway is frequently mutated in human breast cancer.

The retinoblastoma tumor suppressor (*RB1*) gene is an important regulator of the G1-S phase of cell cycle control and is frequently mutated in many different types of cancer. *RB1* also regulates the cell's response to diverse external signals, mitogenic factors and therapeutic agents. For example, DNA damage induced G1 and S phase cell cycle arrest are mediated by *RB1* following exposure of the cells to DNA damaging agents. In quiescent cells, *RB1* is hypophosphorylated and assembles transcriptional repressor complexes on the promoters of *E2F*-regulated genes to block progression through the cell cycle. When *RB1* is hyperphosphorylated by Cyclin D1-CDK4 and Cyclin E-CDK2 it is rendered inactive and this inactivation releases the E2f proteins from their pRb bound state and thus releases the brake on transcription, allowing cell cycle progression (**Figure 1.7**). In response to mitogenic factors like estrogen in breast cancer cells, pRb is inactivated through hyperphosphorylation catalyzed by the Cyclin D1–cyclin-dependent kinase 4 (CCND1–CDK4) and Cyclin E–CDK2 complexes (Broceno, Wilkie & Mittnacht 2002, Sutherland, Musgrove 2004, Yu, Foster & Dean 2001). Loss of pRb mediated G1-S arrest results in abnormal proliferation that can lead to cancer. Loss of *RB1* often leads to activation of *P53*, a major tumor

suppressor protein that then induces cell cycle arrest and cell death. So *RB1* mutations in many human cancers are also coupled with mutations/inactivation of *P53* (Sage 2007, Simin et al. 2005).

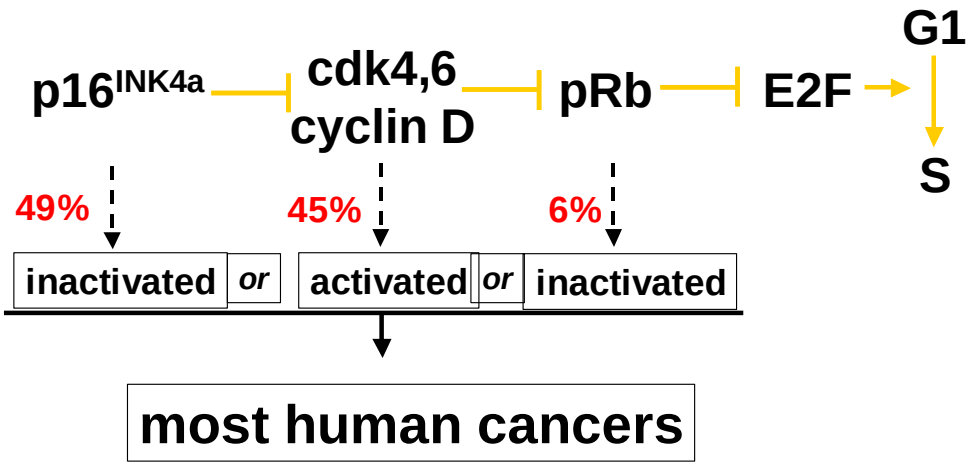

Figure 1.7. Common cancer causing mutations in the pRb pathway are shown here. Mutations in the whole pRb pathway including mutations to inactivate p16, activate cdk4.6 and inactivate pRb are very common in all human cancers. The frequency of these mutations in breast cancer is shown in red (Buckley et al. 1993, Geradts, Wilson 1996). These mutations result in abnormal cell proliferation and are very often coupled with mutations in p53, resulting in loss of cell cycle checkpoint control and decreased apoptosis.

1.10.2 Rb family proteins show functional overlap in multiple cell types.

Two proteins that are similar to pRb in structure and function, retinoblastoma-like 1 (Rbl-1, previously known as p107) and retinoblastoma-like 2 (Rbl-2, previously known as p130), have very similar binding properties to pRb and also bind E2F transcription factors (Mulligan, Jacks 1998, Mulligan, Wong & Jacks 1998). There is significant functional overlap between these three proteins, hence loss of all three of these genes may be required in cell types where all three are expressed, to elicit aberrant cell cycle progression and proliferation. Most human cancers including retinoblastoma do not exhibit mutations or loss in p107 and p130, though mutations in pRb are very common (MacPherson, Dyer 2007,

Scambia, Lovergine & Masciullo 2006). This suggests that the Rb family proteins may have distinct functions in different species. But it has been shown in mouse models that all three of the *Rb* family genes are expressed in the mammary gland and *Rb1* alone is dispensable in normal mammary gland development (Maandag et al. 1994). So loss of pRB alone in the mouse does not result in mammary gland tumors, probably due to functional compensations (Robinson, Wagner & Hennighausen 2001).

1.10.3 pRb mutations in breast cancer

RB1 gene mutations have been reported in about 20-35 % breast cancers (Fung, T'Ang 1992, Oesterreich, Fuqua 1999, Pietilainen et al. 1995). *RB1* mutations in breast cancers have been consistently correlated with poor patient outcome. Also, loss of heterozygosity of *RB1* has been commonly observed in primary breast cancer specimen and seems to be the rate-limiting step for cancer initiation (Borg et al. 1992, Chano et al. 2002). Finally, over expression of Cyclin D1, Cyclin E (which are negative regulators of *RB1*) (Malumbres, Ortega & Barbacid 2000) and up regulation of E2F target genes have been correlated with poor prognosis in some breast cancer patients (Van't Veer, Weigelt 2003). In spite of a significant amount of clinical evidence for correlations between the pRb pathway inactivation and poor prognosis in breast cancer, there has been no real mechanistic study on how the disrupted pathway leads to breast cancer initiation and progression and what therapeutic strategies could be targeted to the pathway. Mice overexpressing Cyclin D1 in the mammary gland and thus inactivating pRb by hyperphosphorylation, developed mammary tumors with a long latency of over one year (Wang et al. 1994) suggesting the need for co-operating lesions besides pRb pathway inactivation, in the development of full blown cancer.

Simin et al showed for the first time that Rb_f mutation in the mammary gland could induce an apoptotic response from P53 on day one lactation. Subsequent tumor progression occurs through selective pressure for loss of *p53* (Simin et al. 2004). However this model targeted the loss of Rb_f in the entire mammary gland and not in a single cell or a subset of cells within the normal mammary gland. This is not similar to how human breast cancer begins in a single cell and progresses stochastically with the accumulation of further co-operating lesions. The possible co-operation between Rb_f inactivation and *p53* loss could not be studied in this model as most of the *p53* germline inactivated mice (*p53* knockout mice) developed non-mammary tumors (lymphomas and sarcomas). The tumor latency in this model was extremely long (median of ten months). To overcome these drawbacks we sought to induce somatic and conditional inactivation of both *Rb* pathway and *p53* in the mammary gland.

1.10.4 T_{121} specifically binds to and inactivates pRb_f

Simian Virus 40 (SV40) is a DNA polyoma virus that can cause various forms of cancer in mice, humans and monkeys (discussed by Weinberg, 2007). In 1988 research revealed that one of the important ways oncogenic DNA viruses can cause cancer is by binding to and inactivating the pRb protein. The SV40 virus oncogene - T antigen was found to behave in the same way. Once inside the cells, the T antigen specifically sequestered and inactivated all three hypophosphorylated pRb family proteins (pRb, p107 and p130), thereby removing the growth-inhibitory effects of pRb in G1 phase. The T antigen did not bind to already inactive hyperphosphorylated pRb in late G1 and S phases. Crystallography studies showed that the specific binding of the viral oncoprotein SV40 T antigen to the B groove of pRb (Lee, Russo & Pavletich 1998) occurred through a conserved LXCXE motif that is

shared by SV40 T antigen and human papilloma virus E7 (that causes human cervical cancer). This binding results in a conformational change in the pRb protein and prevents its binding to the E2F transcription factors.

Interestingly, SV40 T antigen also has a p53-binding domain and can specifically bind to and inactivate p53. The discovery of the mechanism by which SV40 T antigen functioned had a profound impact in understanding the cancers caused by these viruses. It also provided researchers with a powerful tool that they could now use to their advantage to specifically inactivate RB and P53 in multiple cells in the mouse and ask a variety of questions about their roles in tumorigenesis. We used this tool to our advantage by using a truncated version of the SV40 T antigen that only inactivates the pRB_f but not P53. The truncated T antigen referred to as T_{121} in the rest of this work, contains the first 121 amino acid sequence of SV40 T antigen. By leaving an active P53 we were able to specifically study the role of pRB_f inactivation in mammary tumor initiation. To specifically target the mammary gland we used the MMTV promoter that expressed early in development in the mammary luminal epithelial cells (Wagner et al. 2001). To generate an inducible expression of T_{121} we inserted an eGFP cassette within floxed stop sequences (**Figure 1.8**). Upon crossing to mammary specific Cre strains the bi transgenic mice would express T_{121} and hence have functional pRB_f inactivation in the mammary glands only. We also hoped by using the MMTV promoter to uncouple T_{121} expression from regulation by pregnancy hormones and thus exclude the necessity of multiple cycles of pregnancy and lactation for transgene expression. A second goal in using this model was to grow the primary mouse mammary epithelial cells in a three-dimensional cell culture system and use the expression of eGFP as an initial readout for the efficacy of this system.

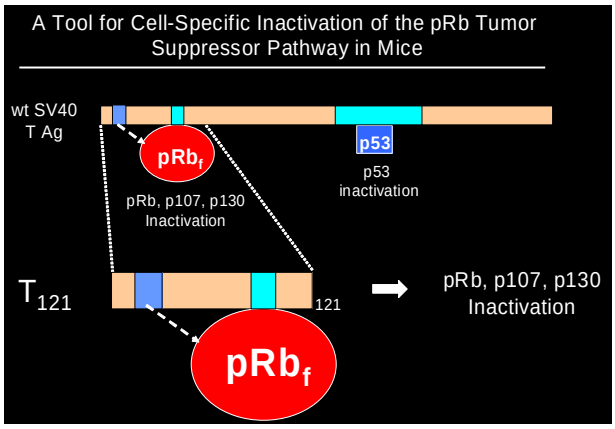

Figure 1.8. The truncated SV40 T antigen binds to pRB_f and inactivates it functionally. The pRB_f can be specifically inactivated by T_{121} that is the truncated (first 121 amino acids) of the SV40 T Antigen. This molecule was originally reported by (Saenz Robles et al. 1994) and has since then been used to target many cell types in the mouse to study the loss of pRB_f inactivation in a cell specific manner (Hill et al. 2005a, Hill et al. 2005b, Lu et al. 2001, McLear et al. 2006, Simin et al. 2004, Simin et al. 2005, Xiao et al. 2002, Xiao et al. 2005).

1.11 The *P53* Tumor Suppressor Gene is Frequently Mutated Concomitant to *RB1* Pathway Mutations in Human Breast Cancer

P53 has been in the focus of cancer research ever since its discovery in 1979 (DeLeo et al. 1979, Kress et al. 1979, Lane, Crawford 1979, Linzer, Levine 1979, Linzer, Maltzman & Levine 1979) as the transcription factor binding to SV40 large T-antigen (DeLeo et al., 1979, Lane and Crawford, 1979) and acting as a cell cycle checkpoint control gene. Almost 50% human cancers show *P53* mutations alone or in combination with other gene mutations like *RB1* (Soussi 2005). The p53 activating pathways (**Figure 1.9**) in a cell can be broadly classified under the following categories:

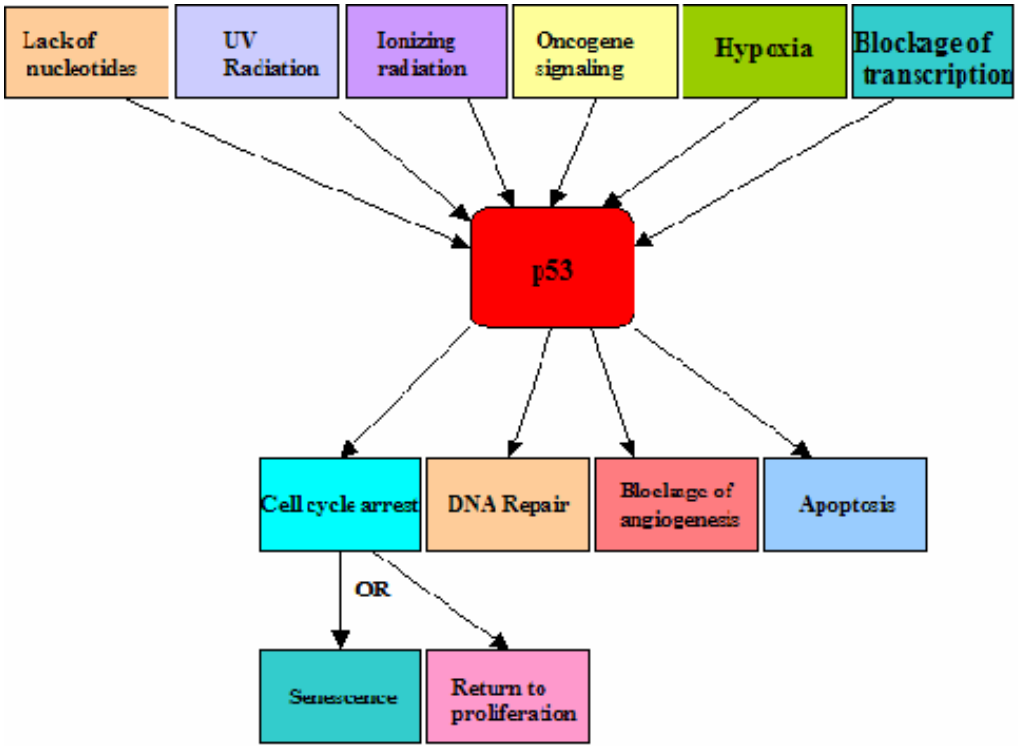

Figure 1.9. The signals activating p53 and their downstream effects are shown here. A variety of physiologic stress in the cell can induce p53 activation. The resulting p53 undergoes a variety of posttranslational modifications and induces many cellular responses. The response to cell cycle arrest can either be irreversible, called “senescence” or reversible and the cells can start proliferating again. In certain circumstances p53 can trigger apoptosis. (Adapted from Weinberg, 2007)

(i) p53 pathway activating signals, for example, DNA damaging agents like UV radiation, cellular hypoxia, cellular glucose starvation (Feng et al. 2005, Jones et al. 2005)

(ii) mediator signals upstream to p53 that sense the activation signals and as a result can increase or decrease the levels and functional state of the p53 protein in the cell (MDM2, COP-1, and PIRH-2)

(iii) p53 and its binding partner proteins that regulate its activity (p53-Mdm2 binding that regulates p53 levels and activity in the cell)

(iv) downstream target genes of p53 activation like genes resulting in cell cycle (G1-S and G2-M) arrest (p21, GADD45), genes regulating apoptosis (Fas, Caspase 8,9.3, Bid, Bax, Noxa, Puma, Bcl-2, Bcl-XL and genes regulating cellular senescence

(v) effects of the downstream gene activation resulting in responses like cell cycle arrest, apoptosis and / or senescence (Levine, Hu & Feng 2006).

P53 mutations in cancer are often missense mutations (Jerry et al. 1993, Ozbun et al. 1993, Ozbun, Butel 1995) or loss of heterozygosity in the later more advanced stages of cancer progression. Frameshift mutations resulting in premature stop codons also occur, and more infrequently there occur a separate missense mutation on each allele. Human beings with the Li-Fraumeni syndrome have one copy of mutant *P53* and another wild type copy of the gene. These people are predisposed to multiple cancers during their lifetime, including breast and ovarian cancer (Greenblatt et al. 1994, Hollstein et al. 1991, Hollstein et al. 1996, Malkin 1994a, Malkin 1994b, Malkin 1994c, Petitjean et al. 2007).

That *P53* gene is commonly mutated in human breast cancer(Borresen-Dale 2003) and has long been observed but the stage of disease progression when this gene is lost and the mechanistic effects of loss of *P53* in the mammary gland have not been clearly elucidated. The fact that *P53* mutations are frequently presented in the advanced breast disease and in recurrent tumors more often than in the initial primary tumor seems to suggest a role of *P53* mutation in tumor progression (Lozano, Liu 1998, Norberg et al. 2001). *P53* also seems to play a role in the initiation of breast cancer as patients with Li Fraumeni syndrome often have breast cancer early in their lives (Varley 2003).

Several studies using both cell culture and mouse models have been able to recapitulate one or more features of specific types of human breast cancer that show *P53* loss or mutation (Attardi, Jacks 1999, Blackburn et al. 2004, Blackburn, Jerry 2002, Derksen et al. 2006, Dittmer et al. 1993, Jerry et al. 1998, Jerry et al. 1999, Jerry et al. 2000, Jonkers et al. 2001, Koch et al. 2007, Kuperwasser et al. 2000, Lin et al. 2004a, Olive et al. 2004, Seluanov et al. 2001, Sigal, Rotter 2000, Zambetti et al. 1991). Studies in knockout mice have shown that mice with one mutant *p53* allele have an increased risk of developing spontaneous tumors (Donehower et al. 1992) similar to the Li-Fraumeni patients. But these mice developed breast cancers at a very low frequency. Conditional mouse models were developed to target loss of *p53* in the mammary gland alone. While loss of *p53* alone pre-disposed to mammary adenocarcinomas, the very long latencies in these models suggested accumulation of additional lesions. But *p53* loss in mouse mammary tumor models initiated by the loss or expression of other genes resulted in a drastic reduction in tumor latency. For example, it was shown that loss of *p53* in conjunction with loss of *E-Cadherin* can recapitulate several features of human lobular breast cancer and the combined loss of *p53* and *Brca1* can lead to human "basal like" cancers often associated with familial loss of *BRCA1* (Liu et al. 2007). This suggested that loss of *p53* had a role in the progression rather than initiation of breast cancer.

We had shown before that Rb_f inactivation leads to a cellular response in the form of increased apoptosis. However the pathway for this response depends on the cellular context. While in the choroid plexus (brain epithelium) the apoptotic response is through *p53*, in the brain astrocytes and the prostate epithelium this response is through *Pten*. We also showed that loss of Rb_f in the mammary epithelium resulted in a *p53* mediated apoptotic response

(Simin et al. 2004). The germline loss of *p53* made these mice very prone to lymphomas and sarcomas and hence it was impossible to use this model to study the complete loss of *p53* in mammary tumor progression. In this project it was our goal to study the effect of complete loss of *p53* in a Rb_f inactivated mammary tumor model and also study the co-operating role of Rb_f, *p53* and *Brca1* in mammary tumorigenesis.

1.12 Loss of *BRCA1* Combined with Loss of *P53* and RB_f Pre-disposes to Highly Malignant Breast Cancers

Brca1 (**Figure 1.10**) is a large protein composed of 22 coding exons over 100 kb genomic DNA. It encodes 1863 amino acids. Since its discovery in 1994 on chromosome 17q21 (Futreal et al. 1994, Miki et al. 1994), *BRCA1* has been the focus of intense research, especially to elucidate its role in breast and ovarian cancer. Brca1 is a multifunctional protein and all of its functions have not yet been defined. It plays a role in DNA double strand break repair, homologous recombination, cell cycle checkpoint control (Deng 2006) and transcription (Lane 2004). Women with mutations in the *BRCA1* gene allele have a 80% lifetime risk of developing breast cancer, a 40% risk of developing ovarian cancer (Narod, Foulkes 2004). *BRCA1* mutations have also been associated with an increased lifetime risk of having colon and prostate cancer. LOH of the wild type allele of *BRCA1* is a very common event in breast cancers arising in patients with a familial *BRCA1* mutation. Breast cancers in familial *BRCA1* mutated patients are typically high grade invasive ductal carcinomas and are clinically identified by their "triple negative" (ER, PR and HER2) status (Chappuis, Nethercot & Foulkes 2000, Livasy et al. 2006, Phillips 1999). These cancers have a histological similarity to sporadic human basal like breast cancer (Sorlie et al. 2003). In

general patients with this class of breast cancer have a poor diagnosis and rate of survival is low . Long term treatments with tamoxifen fail in these cancers as they are ER negative. Also, breast cancers arising from *BRCA1* mutations tend to occur in younger women and early pregnancy is not a good predictor of good prognosis for this type of breast cancers as it is for some other types.

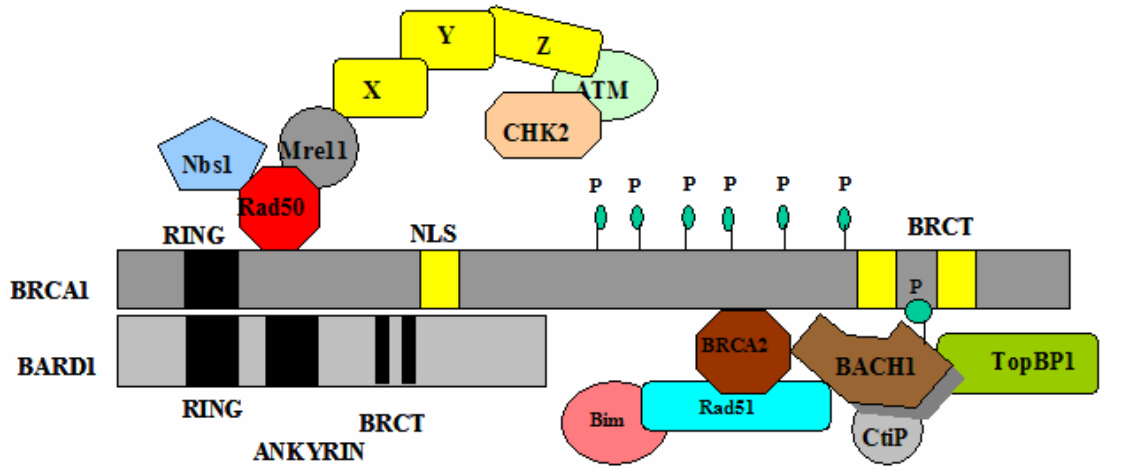

Figure 1.10. BRCA1 protein and its binding partners are shown here. The BRCA1 protein acts as a scaffolding molecule. It binds to and assembles other DNA repair proteins to form large protein complexes. These protein complexes can then help the repair of dsDNA breaks by homology directed repair (HDR). The loss of the binding partners of BRCA1 affects specific cell cycle checkpoint controls and also compromise homologous recombination and HDR. (Adapted from Weinberg, 2007)

Human breast cancers arising from *BRCA1* mutations often have a higher frequency of *P53* mutations (Crook et al. 1997). Earlier work with GEMMs of *Brca1* failed due to embryonic lethality of the *Brca1* null homozygous mice (Gowen et al. 1996). So it became necessary to target *Brca1* mutations only to the mammary gland, to study its role in breast cancer. GEMMs were developed that targeted loss of *Brca1* to the mammary gland using the WAP or MMTV promoters. The observation was that loss of *Brca1* alone did not promote mammary tumorigenesis in the mice (Hakem et al. 1996, Liu et al. 1996, Ludwig et al. 1997,

Shen et al. 1998). However, the combined loss of *Brca1* and *p53* resulted in a wide spectrum of mammary tumors with varying latencies (Brodie et al. 2001, Brodie, Deng 2001). It was recently shown by Liu et al., 2007 that tumors with concomitant loss of *Brca1* and *p53* have much higher levels of genetic instability compared to those with loss of *Brca1* or *p53* alone. However, studies have shown that embryonic lethality induced by loss of *Brca1* in mice is incompletely rescued by the loss of *p53* (Xu et al. 2001). This suggests that loss of *p53* alone is not sufficient for the survival of *Brca1* mutated cells and other genetic alterations are most likely necessary for promoting tumor progression. Additional apoptotic pathway, possibly through the Fas ligand could be playing a role in the early lethality of these mice. We hypothesize that loss of the Rb_f pathway that promotes increased proliferation by activation of E2F target genes, along with loss of *p53* may be the two other genetic lesions necessary for a synergistic effect on the loss of *Brca1* in promoting mammary tumorigenesis.

Some mouse models for breast cancer have shown that *Brca1* and *Trp53* loss act synergistically in promoting certain basal like characteristics like the expression of Keratin 5 in breast cancer (Liu et al. 2007). These basal tumor characteristics are similar to the keratin expression profiles seen in human familial *BRCA1* mutated cancers. The reason for the basal nature of these cancers has been a confounding question as the cell of origin for these tumors is not known. Very recent studies using human cells and SCID mice (Severely Compromised Immune Deficient) have indicated that the regulation of mammary stem cell differentiation into the luminal lineage is controlled by *BRCA1* and mediated through an Estrogen Receptor pathway (Liu et al. 2008). So loss or mutation of *BRCA1* forces the progenitor cells to differentiate only into the myoepithelial lineage and not the luminal lineage. These results suggest that the initial mutation event takes place in the mammary progenitor cells.

A direct interaction between *RB1* and *BRCA1* has been implicated by some studies. The BRCT domain in the C terminal of Brca1 is thought to bind to pRb and cooperate with pRb in the suppression of E2F transcription factors (Yarden, Brody 1999). Brca1 also has the conserved LXCXE motif required for pRb pocket protein binding that further suggests a direct interaction between them (Aprelikova et al. 1999). However their potential co-operation in breast cancer has not been studied before.

As human cancer rarely occurs from a single mutation event and *BRCA1* mutated familial breast cancers often harbor concomitant mutations in *P53* as well as have a highly proliferative E2F positive signature, we hypothesized that the synergistic effect of loss of Rb_f, *p53* and *Brca1* could promote familial like breast cancer in mice. We were also interested in understanding the cause for the basal characteristics of these cancers. We hypothesized that increasing genetic instability in the mammary gland by the combined loss of Rb_f, *p53* and *Brca1* could turn on non cell autonomous pathways that possibly play a role in *Brca1* mutated familial breast cancers. In this report we attempt to address these complex questions and to model the synergistic effect of Rb_f, *p53* and *Brca1* loss in the initiation and progression of breast cancer in the luminal mammary epithelial cells by performing a mechanistic study using GEMM.

1.13 Transgenic Mice Strains Used in Current Project

The $TgMFT_{121}$ mice were generated and maintained on a BDF1 genetic background in the Van Dyke lab.

The WAP-Cre mice were obtained from MMHCC (strain name is B6.Cg-Tg (WAP-Cre) 11738 Mam) and were on a C57BL6 background. The donating investigator of this

strain is Dr. Lothar Hennighausen (Wagner et al. 1997). Strain has been backcrossed to B6.Cg at least 7 times and was maintained on a B6 background in the lab.

The *p53* conditional mice (strain name FVB; 129-Trp53<tm2Brn>) were obtained from MMHCC, strain (# 01XC2). Donating investigator for this strain is Dr. Anton Berns (Jonkers et al. 2001, Marino et al. 2000) This strain carries a conditional mutation in the endogenous *p53* locus. LoxP sites were inserted into intron 1 and intron 10 of the *p53* locus. These mice were maintained on a FVB background in the lab. These mice will be referred to as $p53^{cf/f}$ mice with the specific Cre strain mentioned where they have been crossed to Cre (like *WAP-Cre;* $p53^{cf/f}$).

The *Brca1* conditional mice (strain name FVB; 129-Brca1<tm2bRN>) were received from MMHCC (#01Xb8) and the donating investigator is Dr. Anton Berns (Liu et al. 2007). This strain carries a conditional mutation in the endogenous *Brca1* locus with LoxP sites inserted into intron 3 and intron 13 of the *Brca1* locus. These mice were maintained on a FVB strain in the lab. These mice will be referred to as $Brca1^{cf/f}$ mice with the specific Cre strain mentioned where they have been crossed to Cre (like *WAP-Cre;* $Brca1^{cf/f}$).

The resulting experimental mice were on a mixed genetic background. An example of a typical background of mouse used is 20% B6, 4.7% BDF1 and 75% FVB for a representative *WAP-Cre;* $TgMFT_{121+/-}/p53^{cf/f}/Brca1^{cf/f}$ mouse. The predicted percentage contribution of each genetic strain is generated using a calculator developed in the Van Dyke lab. As all experimental mice and controls used in this study were siblings or cousins the diversity in genetic modifier effect if any, was consistent across the experimental animals. But the effect of segregation of specific modifiers that may have affected tumor appearance

and latency cannot be ruled out. The highly susceptible BALB/c strains of mice (Koch et al. 2007) that frequently get spontaneous mammary tumors were not used in this study.

1.14 Dissertation Chapter Sequence

Chapter two of this dissertation describes the establishment and characterization of a conditional mouse model for *Rb_f* inactivation in the mouse mammary gland using the MMTV promoter.

Chapter three describes the establishment and characterization of a conditional mouse model to study the effect of the combined loss of *Rb_f* and *p53* in the mouse mammary gland.

Chapter four of this dissertation describes the establishment and characterization of conditional mouse models to study the effect of combined losses of *Rb_f* and *Brca1* and also of *Rb_f*, *Brca1* and *p53* in the mouse mammary gland.

Chapter five describes the genomic analysis performed to characterize the molecular signature of the mouse mammary tumors established in this work. Three kinds of genomic analysis are described: mRNA array analysis, CGH analysis and micro RNA array analysis.

Chapter six describes the establishment and characterization of a mouse primary mammary epithelial three-dimensional culture system in matrigel.

Chapter seven of this dissertation discusses the major conclusions made from this work and the future directions this work can take.

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CHAPTER TWO

Rb FAMILY INACTIVATION IN MAMMARY TUMORIGENESIS

2.1 Abstract

The Retinoblastoma 1 (*RB1*) gene was identified as the gene responsible for familial retinoblastoma. Since that time however, mutations in this gene have also been identified not only in non-familial cases of retinoblastoma, but also in many other tumors. Extensive *in vitro* study of this gene has shown that loss of *RB1* results in an increase in cell proliferation secondary to increased expression of the E2F target genes. The *RB1* gene is aberrant in about one third human sporadic breast cancers. Importantly, recent genomic studies have revealed loss of *RB1* expression in breast cancer is associated with poor response to long-term tamoxifen therapy and overall poor prognosis. Information from animal models regarding the mechanism by which *RB1* contributes to tumorigenesis of mammary epithelium has been limited for a number of reasons. First, mice lacking *RB1* die during embryogenesis. Second, at least two *RB* family members, *RBL1* (p107) and *RBL2* (p130), which have overlapping functions with *RB1*, have been identified in the mouse. An alternative strategy was developed that takes advantage of the SV40 T antigen protein. SV40 is an oncogenic polyomavirus that encodes a protein (T antigen) that binds to and inactivates all three Rb family members as well as p53. In this study we use a truncated version of the SV40 Large T-antigen domain that binds to and inactivates only the three Rb family

members but not p53. To limit the loss of RB_f to the mammary epithelial tissue, expression of the T antigen is driven by the MMTV promoter. We found that loss of function of the Rb family of proteins did result in increase in proliferation of mammary epithelial cells. In addition, changes in the involution of the gland after cessation of lactation were also observed. However, surprisingly, these alterations in regulation of epithelial cell growth did not result in an increase in tumor incidence. This suggests that while Rb does play an important role in mammary epithelial cell growth, loss of function of this gene family alone is not an initiating event in the formation of mammary tumors, at least in this model system.

2.2 Introduction

Hypophosphorylated pRb binds to E2F transcription factors in the cell nucleus and prevents cell cycle progression from early to late G1 phase (Grana, Garriga & Mayol 1998, Weinberg 1995). After mid G1 phase pRb is hyperphosphorylated by Cyclin D1- CDK4/6 kinase and this results in its disassociation from the E2F transcription factors resulting in cellular proliferation. Under the influence of growth inhibitory signals to the cell, the cyclin-CDK inhibitors (CDKIs) like $p16^{INK4a}$ bind to CyclinD1-CDK4/6 and as a result inhibit pRb hyper-phosphorylation.

Understandably, tumor cells hijack the critical checkpoint control exerted by pRb frequently, by multiple ways including the mutation, loss or promoter methylation of the *RB1* gene as well as RB pathway mutations. This results in loss of the G1 restriction point and contributes to abnormal and uncontrolled cell proliferation.

The RB pathway has been identified as the one of the most frequently mutated pathway in human cancers with at least one critical member of this pathway (Cyclin D1,

CDK4, INK4a, pRb) being deregulated in most cancers (Marshall 1991, Ortega, Malumbres & Barbacid 2002, Sherr 1996). Individuals born with a familial mutation in a single *RB1* gene allele are pre-disposed to the rare childhood cancer retinoblastoma and to other cancer types like osteosarcoma and small cell lung carcinoma as adults (Classon, Harlow 2002). *RB1* is mutated in about 20-30% sporadic breast cancers (Knudson 1993). Loss of heterozygosity and/or other mutations leading to *RB1* functional loss is a common occurrence in breast cancers (Chano et al. 2002). Also loss of function of other proteins in the Rb pathway that interact with Rb in the regulation of cell cycle is a common event in human breast cancers. Examples are the inactivation of $p16^{INK4A}$ (Geradts, Wilson 1996) that prevents Cyclin D1 inactivation under the influence of growth-inhibitory signals and activation of Cyclin D1 (Buckley et al. 1993) that results in pre-mature Rb inactivation and release of the E2F transcription factors. Finally recent genomics studies looking at chromosomal aberrations in human breast cancer have found that several genes that undergo frequent copy number aberrations are targets of E2F thus supporting the hypothesis that the Rb pathway deregulation plays an important role in human breast cancer (Fridlyand et al. 2006).

Several early attempts were made to make mouse models to study the impact of *Rb* inactivation in multiple cells. $Rb^{-/-}$ mice undergo embryonic lethality between day 13-15 of gestation (Clarke et al. 1992, Jacks et al. 1992, Lee et al. 1992) with defects in neuroprogenitor cell development, hematopoesis and skeletal muscle development. These defects were also accompanied by severe apoptosis and differentiation defects. $Rb^{+/-}$ mice developed pituitary tumors (Hu et al. 1994, Jacks et al. 1992, Maandag et al. 1994) and not retinoblastoma like their human counterparts. The early embryonic lethality of the $Rb^{-/-}$ mice

precluded the extensive use of these animals to study the role of this protein in tumorigenesis. A number of strategies have been used to circumvent this problem. First investigators generated ES cell homozygous for the mutant allele. Using these cells they generated chimeric *Rb* mice. These chimeras would be composed of ES cell derived cells that were null for *Rb* in a background of *Rb* wild type cells. However again, these mice survived and also did not develop retinoblastoma (Maandag et al. 1994, Williams et al. 1994). The primary tumor type observed in the chimeras was again pituitary gland tumors. The difference in mouse and human mammary tumor spectrum upon loss of *Rb* was partly explained with the finding of two *Rb* family related members, *Rbl1* and *Rbl2* that showed a great deal of functional overlap (Lee et al. 1996, Mulligan, Jacks 1998, Sage et al. 2000). Later it was shown that the combined loss of *Rb* and *Rbl1* leads to retinoblastoma in mice (Robanus-Maandag et al. 1998). Although orthologs of these genes are found in humans, the role of *RBL1* and *RBL2* in human breast cancers, if any, is still under investigation (Scambia, Lovergine & Masciullo 2006). It can be postulated that the relative ability of these family members to compensate for loss of *RB1* may differ between species. Also other human and mouse differences may play a role in the difference in tumor spectrum, for instance the effect of genetic modifiers can play a dramatic role in tumor pre-disposition in mice (Balmain, Nagase 1998). Also it is possible that co-operating mutations along with loss of *Rb1* are necessary for tumor pre-disposition in mice.

To address the possibility that similar redundancy in gene function between members of the *RB* family will be present in other tissues, specifically in the mammary epithelium we have utilized an alternative strategy for the study of the role of *RB* in this tissue. To functionally inactivate all three members of the pRB family we use a truncated version of the

SV40 Large T antigen that has been shown to bind to all three RB family members using a conserved LXCXE domain (Lee, Russo & Pavletich 1998). As the entire SV40 T antigen also binds and inactivates P53, we use the only the first 121 (referred to as T_{121} in the rest of this thesis) amino acids of this molecule that excludes its P53 binding domain. This allows us to study specifically the effect of pRB_f inactivation in the mammary epithelium without the confounding effects of P53 loss as well. In order to use this mouse in the future to examine the cumulative effect of loss of RB protein function along with mutations in other gene we have designed this transgene in a manner that allows the attenuation of RB_f function to be regulated by expression of a cre transgene in a temporal specific manner.

2.3 Results

2.3.1 Generation of mice with mammary epithelium specific inactivation of pRB_f function

An SV40 T antigen containing two mutations that prevent binding of P53 were used for these studies. The 196 bp amino terminal deletion prevents production of small t antigen and the 1137 deletion results in truncation of the large T antigen protein. The expected T_{121} transcript is shown below with the black boxes indicating coding regions and empty boxes as introns. The J and LXCXE domains that are necessary for pRB_f binding remain intact in the truncated molecule **(Figure 2.1)**. Expression of the gene was placed under the regulation of the mouse mammary tumor virus promoter MMTV. However, in order to allow temporal and tissue specific expression of the T antigen we place a eGFP gene flanked by double flox sites 3' to the promoter thus preventing T_{121} expression in the absence of Cre recombinase expression. In the absence of Cre the MMTV promoter is positioned to drive expression of

eGFP. Cre expression will result in excision of the eGFP cassette resulting in juxtaposition of the promoter and the T antigen coding sequences.

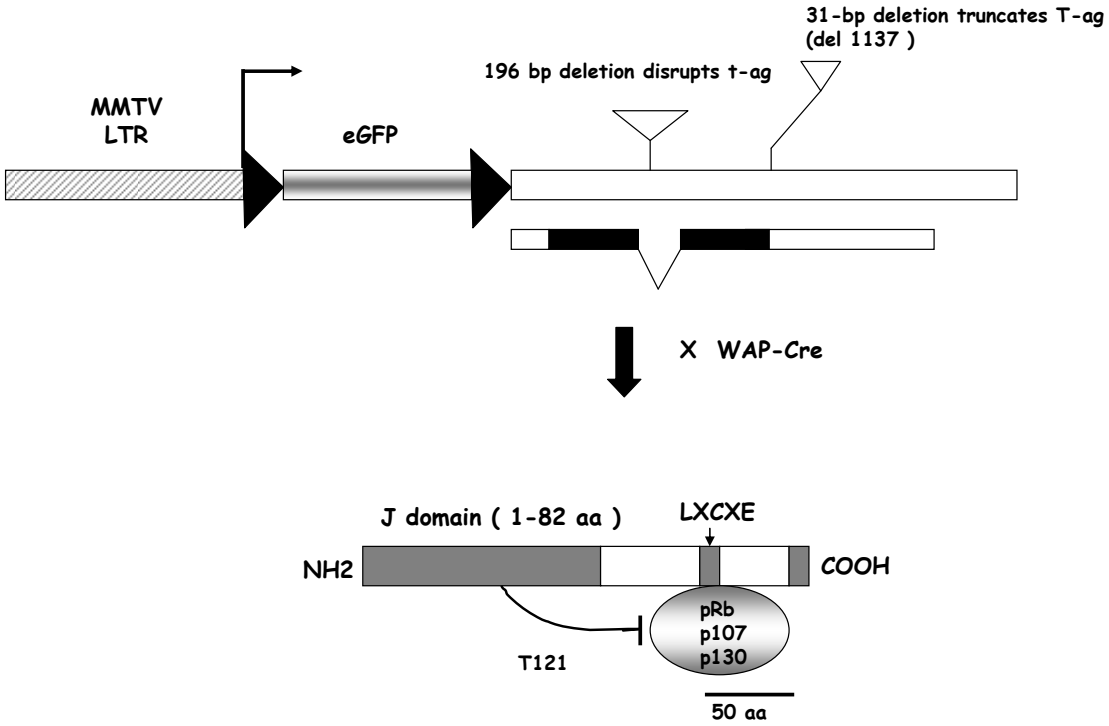

Figure 2.1 Schematic Diagram of the *MFT*$_{121}$ Transgene and Predicted Protein. The transgene consists of the 2.4 kb MMTV promoter (hatched) and the mutant SV40 T-antigen coding region (white box). Two deletions have been introduced into the transgene, the 196-bp amino-terminal deletion, which abolishes small t antigen production, and the dl1137 deletion, which truncates T antigen and prevents its binding P53. A floxed eGFP cassette is placed immediately following the promoter sequences. This prevents T_{121} expression in the mammary glands before crossing to a Cre strain. Upon crossing the transgenic mice to a Cre strain (in this case a mammary gland specific WAP-Cre) the floxed eGFP is spliced out. The expression of T_{121} is then observed only in cells in which both the WAP and MMTV promoters are active. Past studies indicate that this will include primarily the mammary luminal epithelial cells. Also, as the MMTV promoter is active in both virgin and lactating mammary glands, but the WAP promoter is turned on by lactation, eGFP expression in these mice will be observed in virgin mammary glands but T_{121} expression will only be observed post day one lactation. Both the J domain and the LXCXE domain are required for pRb family inactivation.

The transgene was introduced by pronuclear injections into the fertilized BDF1 oocytes. This yielded five mice in which analysis of DNA revealed integration of the transgene into the mouse genome. These mice were then evaluated for expression of the transgene by examination of the mammary epithelium and determining the level of eGFP expression. Out of five mouse lines screened for high eGFP expression, only one was selected for follow up studies based on highest expression of eGFP in the virgin mammary glands of this strain (**Figure 2.2**). Examination of other tissues showed eGFP expression in the salivary glands of these mice indicating a pattern of expression typical for the MMTV promoter. We refer to this mouse line as MFT_{121}.

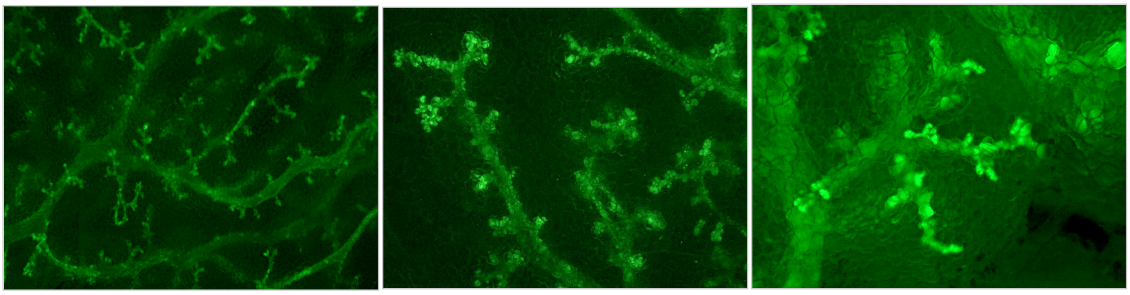

Figure 2.2 eGFP Expression in *$TgMFT_{121}$* (no Cre) virgin mammary glands is shown here. Virgin mammary glands of MFT_{121} mice express eGFP. Total magnification (objective x eyepiece) 2.5x, 5x and 10x are shown here. Consistent with the published characteristics of this promoter expression was broadly observed in both the epithelial cells lining the ducts and in the terminal end buds. Expression was not detected in mammary myoepithelial cells but was restricted only to the luminal epithelial cells.

We next determined, whether, as predicted, expression of Cre would result in excision of the GFP gene, loss of eGFP expression and induction of expression of the mutant SV40 transgene. To do this we intercross the BDF1-MFT_{121} mouse with a mouse line carrying a Cre transgene (in the C57BL/6 genetic background) under the control of the WAP promoter (Wagner et al. 1997). As expected DNA analysis showed that 25% of the offspring

carried both transgenes and examination of the virgin mammary glands showed loss of eGFP expression. To verify that the Cre mediated excision place the T antigen gene under the control of the MMTV promoter sections of mammary gland were prepared and examined by immunofluorescence (IF) in these offspring. For these studies glands prepared from mice 24 hours after initiation of lactation were used when the MMTV promoter expression peaks {{493 Wagner,K.U. 2001}}. As can be seen in **Figure 2.3**, high levels of T_{121} can be detected in the epithelial cells of these glands. In contrast, no expression is observed in littermates carrying the MFT_{121} transgene alone (no Cre expression), in wild type littermates or in the mice expressing only the Cre transgene.

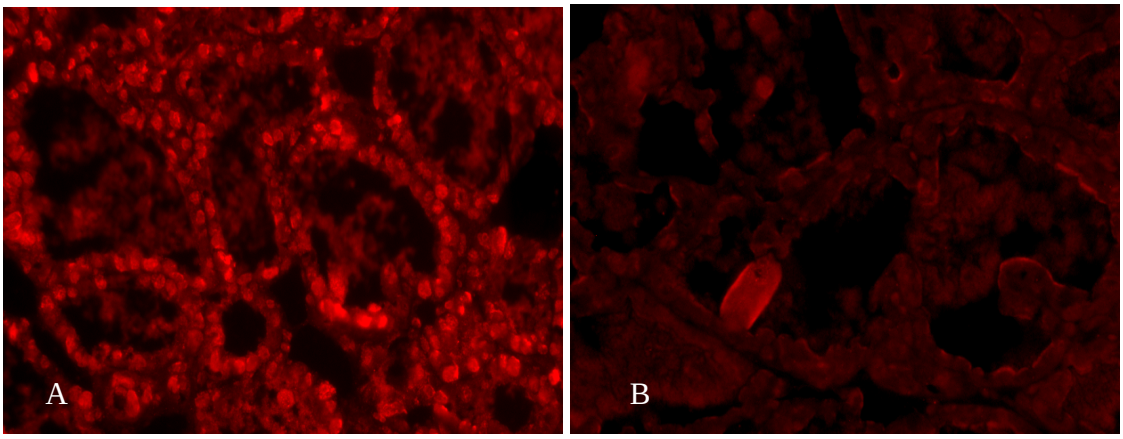

Figure 2.3 T_{121} Expression is detected in Day 1 Lactation Mammary Glands of MFT_{121}/WAP-Cre mice. Unlike the MMTV promoter, activation of the WAP promoter required at least one cycle of lactation and pregnancy. T_{121} expression is detected by using an antibody that targets the N terminal of large T antigen. Here nuclear staining of T_{121} **(A, red)** detection by immunofluorescence is shown in the luminal epithelial cells of day 1 lactation mammary glands but not in non transgenic litter mate mammary glands **(B)**. Magnification is 20X.

To determine the functional effects of pRB_f in the mammary glands we assessed day 1 lactation mammary glands of these mice for proliferation and apoptosis. Previously it was shown using the Wap-T_{121} model that loss of pRB_f leads to increased proliferation and

apoptosis in the mammary glands at day 1 lactation (Simin et al., 2004). In this model however, we see an increase in proliferation but no change in apoptosis compared to controls. Proliferation was determined by performing immunofluorescence (IF) with Ki67 antibody (**Figure 2.4**) that is a specific marker of the S phase of the cell cycle. Apoptosis was determined by performing TUNEL assay. A 10 fold increase in proliferation (**Figure 2.5, a**) was observed but unlike the WAP-T_{121} model, no substantial increase in apoptosis was detected (**Figure 2.5, b**). MFT_{121}/Wap-Cre female mice developed mammary tumors after a very long latency and with a less than 5% penetrance. All animals underwent at least one cycle of pregnancy and lactation as has been shown previously to activate the WAP and MMTV transgenes(Wagner et al., 1997).

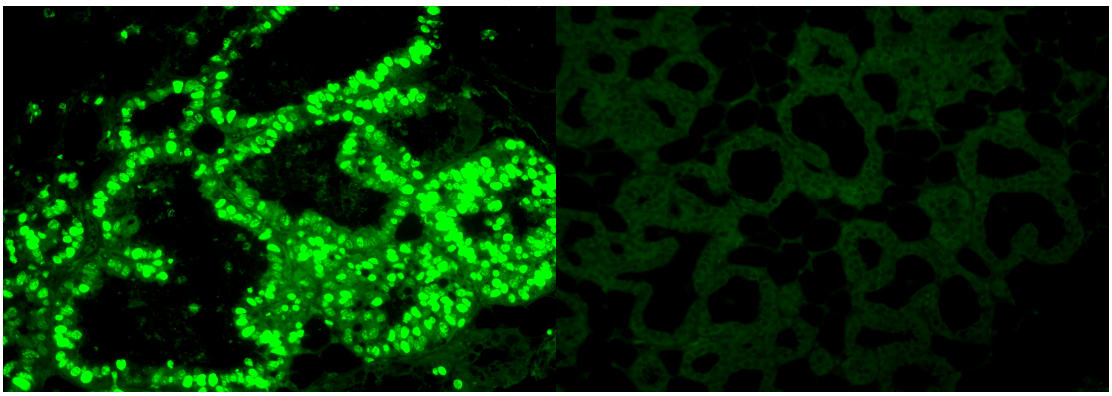

Figure 2.4 T_{121} expression causes increased proliferation on day 1 lactation (a) Representative Ki67 staining on day 1 lactation mammary glands of *$TgMF_{T121+/-}$ / WAP-Cre* show rapidly proliferating cells. **(b)** Representative non-transgenic mammary glands show very low levels (undetectable Ki67) levels of proliferation, which is normal for day 1 lactation. Magnification is 20X. n=5.

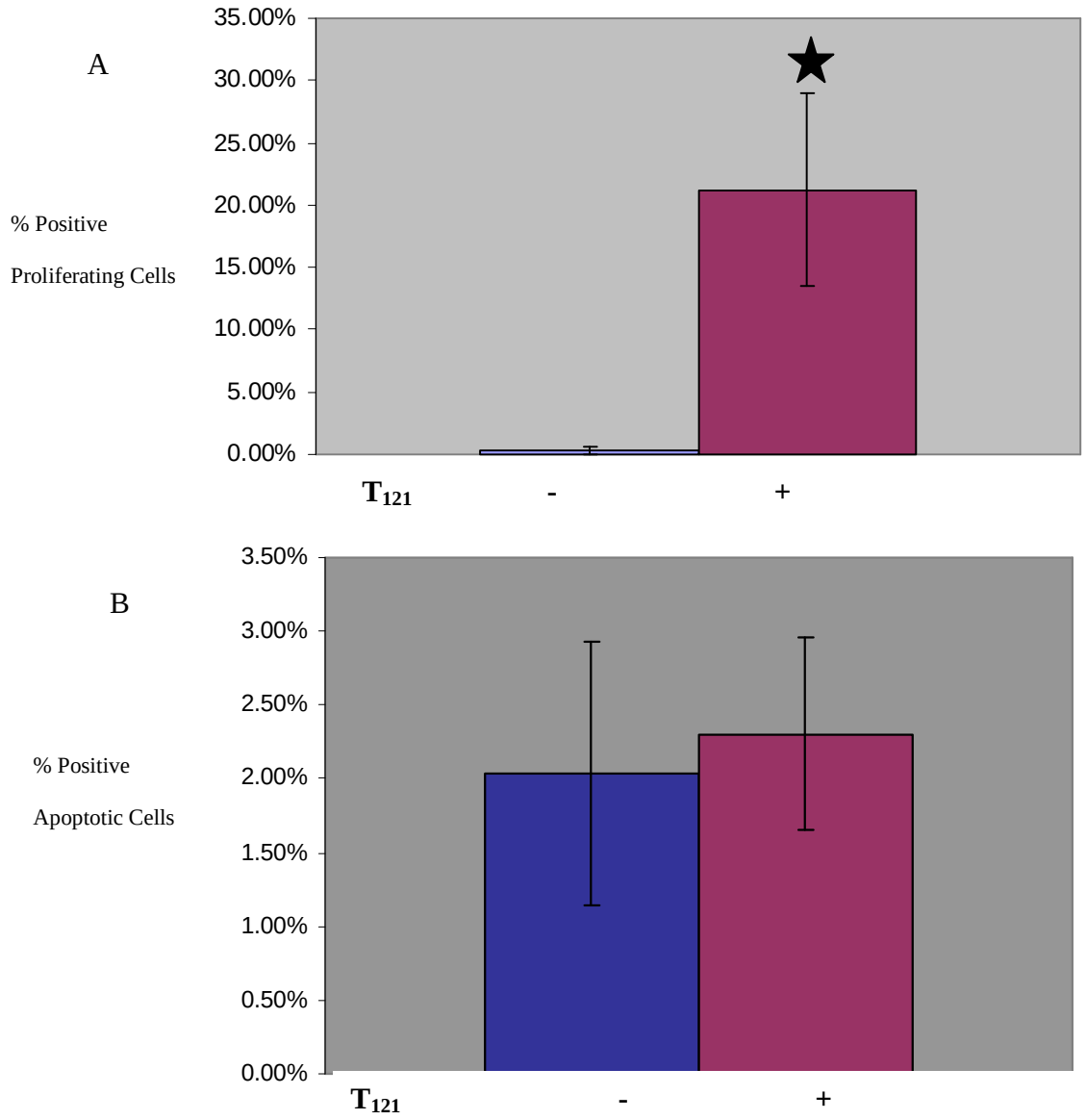

Figure 2.5. Proliferation but not Apoptosis increases in $MFT_{121+/-}$ /WAP-Cre mammary glands on Day 1 lactation. T_{121} expression shows a significant increase in proliferation **(P = 0.00057, as shown by star, a)** but no change in apoptosis **(P= 0.28677, b)**. Two mice for the control ($MFT_{121\ +/-}$) set and three mice for the experimental set ($MFT_{121+/-}$ /WAP-Cre) were used. For each mouse at least three randomly selected sections were counted from at least 2 slides. An average of 200 cells was counted in each section. The nonparametric Wilcoxon rank-sum test (using Van der Waerden normal scores) was used for all pair-wise or two-group comparisons. Nominal or unadjusted p-values reported. All statistical analyses were performed with SAS statistical software, Versions 9.1, SAS Institute Inc., Cary, NC.

2.3.2 Disruption of pRB_f in mammary epithelium induced increased proliferation and delayed mammary gland involution post day 1 lactation

Post weaning the mouse mammary glands, similar to human mammary glands undergoes rapid involution. The process of mammary gland involution is complex and involves multiple steps of a combination of apoptosis, phagocytosis and inflammatory pathways (Stein, Salomonis & Gusterson 2007). Involution results in bringing the mammary glands back to their pre-pregnancy stage. Compared to littermate controls (**Figure 2.6,c**) the mammary glands of the T_{121} expressing mice showed slightly delayed involution with scattered mammary glands with abnormal gland architecture (large glands, expanded lumen) in the mammary fat pad (**Figure 2.6, d**). To monitor the time course for these events mammary gland biopsies were performed on these mice at day 1 lactation, day 14-post day 1 lactation (after weaning pups at day 1) and day 42 post day 1 lactation and compared to non transgenic littermates (MFT_{121} mice not crossed to Cre). The bitransgenic (WAP-Cre/ MFT_{121}) mice showed remnant large gland structures that had not involuted both on day 14 and day 42 post day one lactation.

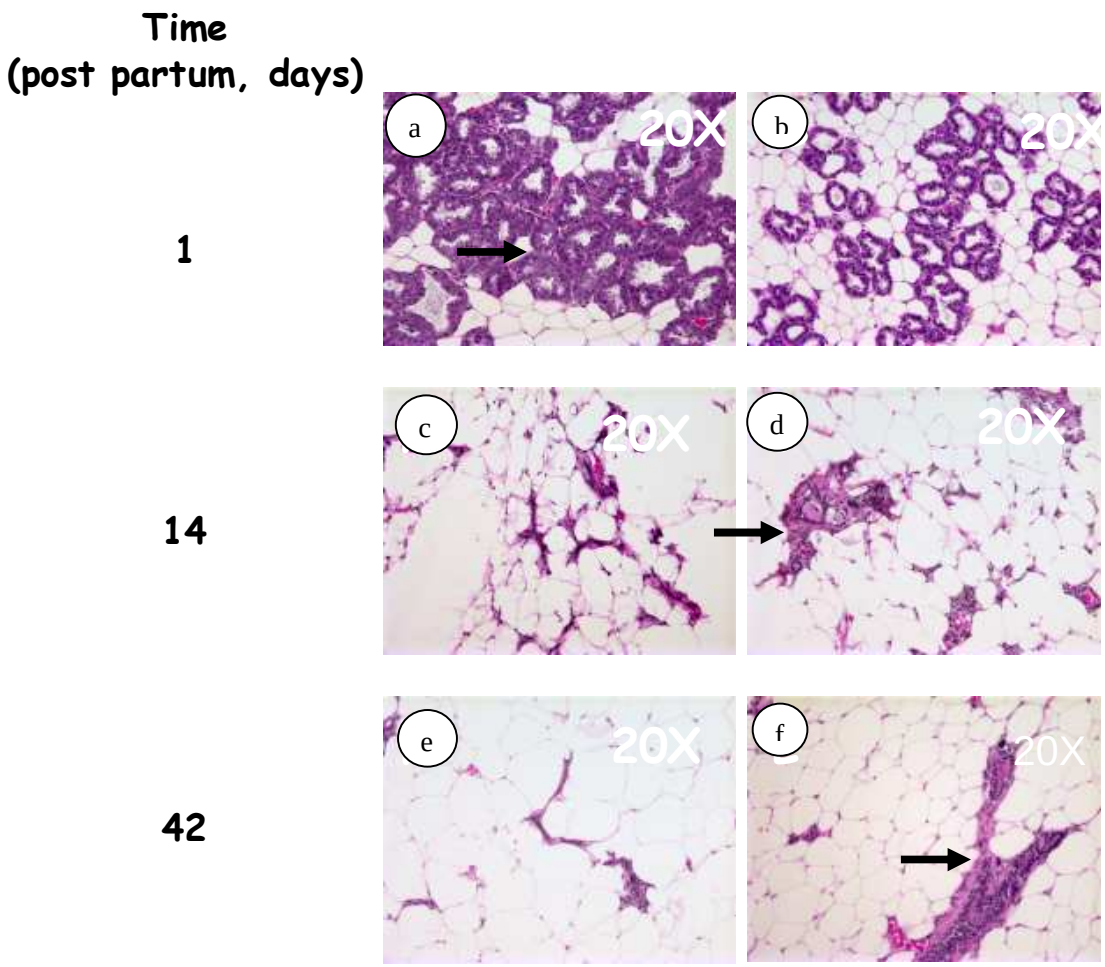

Figure 2.6 Delayed involution is observed in T_{121} expressing mammary glands. Representative mammary morphologies in H&E-stained sections of Day1 Lactation mice are shown. **(a)** *$TgMFT_{121}$* (*WAP-Cre* negative) mice with a normally thick layer of luminal (milk secreting) epithelial (black arrow) on day 1 lactation is shown. **(c)** Mammary glands of *$TgMFT_{121}$* mice show normal regression following day 1 lactation on day 14 (post lactation, pups weaned on day1). Mammary glands have undergone massive apoptosis to reduce mammary glands almost to the level of a virgin mammary gland. **(e)** Mammary glands of *$TgMFT_{121}$* mice show complete regression on day 42-post day- 1 lactation. **(b)** Mammary glands of the transgenic *WAP-Cre; $TgMFT_{121+/-}$* mice show normal ductal pattern on day 1-lactation with no detectable hyperplasias or lesions. **(d)** On day 14 post day 1 lactation the mammary glands of *WAP-Cre; $TgMFT_{121+/-}$* show fairly normal regression pattern (compared to non transgenic controls in **(c)** but there appear remnant large mammary structures **(black arrow)** remaining in the fat pad. These are not seen in the non-transgenic controls. **(c)** On day 42 post day 1 lactation the mammary glands of *WAP-Cre; $TgMFT_{121+/-}$* look normal except for remaining atypical ductal structures **(black arrow, f)** scattered in the mammary fat pad. (n=3 mice for each genotype)

2.4 Discussion

LOH at the 13q14 locus harboring the *RB1* gene has been frequently observed in human breast cancers (Eiriksdottir et al. 1998) and correlates with poor prognosis. However more frequently than not, *RB1* mutations in breast cancers occur together with mutations in other genes, particularly, with *P53* mutations. Given that breast cancer is not generally observed in patients hemizygous for *RB1* mutations, it is more likely that mutations in *RB1* or *RB* family members are not primary events in mammary tumorigenesis. Rather, it is likely that mutations in this gene collaborate with mutations in other tumor suppressors and/or oncogenes as the cells accumulate the full spectrum of genetic changes seen in the malignant disease. Both humans and mice tumor formation requires the accumulation of many mutations. Therefore, in order to study the function of *RB1* loss in tumorigenesis in a model system it is essential to develop a system that allows the examination of lose of *RB1* function in the context of loss or gain of function of other genes implicated in the pathogenesis of breast cancer. In addition, given the overlapping function of many of the *RB* family members it was desirable to use a system in which all *RB* family members could be inactivated with a single genetic manipulation and one in which the loss of Rb could in the future be limited temporally, allowing studies examining its role early and late in tumor formation. The data presented here indicates that we have generated a system ideal for these types of studies.

We show that full *Rb* family inactivation is sufficient to induce abnormal proliferation and delayed gland involution post day 1 lactation but not sufficient to promote mammary tumorigenesis. Our results differ from those reported recently by (Simin et al. 2004). In this model the mutant SV40T antigen (T_{121}) was driven by the WAP promoter. In this study

tumors were observed, albeit with a latency of about 16 months. In theory, the expression pattern of the transgene should be identical to that of the mouse reported here, given that in our model the expression of the T_{121} is limited to WAP expressing epithelial cells. There are a number of possible explanations for these findings. First, and most likely is related to the manner in which transgenes insert into the genome. Many and up to hundreds of copies of a transgene can often be found inserted into the genome and usually these are tandem copies arranged in various tail to tail or head to tail arrangement. With our model we expect that because of the presence of the loxP site this copy number will be reduced as recombination between the loxP sites takes place yielding in theory a single copy of the T_{121} gene. Thus the identification of tumors in the studies by (Simin et al. 2004) might reflect high copy number of T_{121} and therefore complete sequestering of the Rb family of proteins. This possibility could be addressed in the future by examination of the levels of phosphorylated Rb and Rb family members in the two mouse lines.

Alternatively it is possible that difference in the genetic background of the mice used in the two studies results in subtle differences in tumor formation. For example, it has been well documented that different strains of laboratory mice can show dramatic differences in the susceptibility to different tumors (Blackburn et al. 2004, Koch et al. 2007).

We show that full Rb_{f} inactivation is sufficient to induce abnormal proliferation and delayed gland involution post day 1 lactation but not sufficient to promote mammary tumorigenesis. Rb_f inactivation in this model may result in the priming of the cells for further second hit mutations that would lead to breast cancer, like the loss or mutation of *p53* and/ or *Brca1*. This is also consistent with tumor spectrums observed upon loss of Rb_f in other cell types like choroid plexus epithelium. There too, loss of Rb_f resulted in abnormal formation

but loss of *p53* was required for tumors to form (Lu et al. 2001). In the prostate epithelium however, Rb_f loss resulted in increased proliferation similar to the mammary gland but loss of *p53* did not co-operate in tumorigenesis. Rather loss of *Pten* was required for prostate adenocarcinomas to form (Hill et al. 2005a). This indicates that the co-operating lesions required along with Rb_f loss, leading to tumor formation are cell type specific. These models are very powerful in providing insight into the mechanisms of human epithelial cancer progression.

Our results indicate that loss of Rb_f in the mammary glands results in a burst of increased proliferation on day one lactation. Clearly however, this proliferative event is not long lasting, as it does not result in complete loss of mammary gland involution or lead to tumor formation. It is very likely that the peak of proliferation in this model corresponds to the peak of T_{121} expression, which again corresponds with the maximum expression of the MMTV promoter. It has been shown before that the MMTV promoter, though expressed at low levels in both virgin and non-lactating mammary glands, has its peak of expression on day one lactation (Hennighausen et al. 1994, Sinn et al. 1987, Wagner et al. 1997). It is conceivable therefore that sustained and long-term levels of T_{121} expression could result in mammary tumorigenesis in this model.

Also important is the observation that there is no significant P53 mediated apoptosis in the mammary epithelium upon Rb_f inactivation in this model. This could be due to multiple reasons. First, a significant cellular apoptotic response requires pro-apoptotic signals to outweigh the anti-apoptotic signals within a cell. Usually one of the common triggers for increased pro-apoptotic signal within a cell is increased proliferation. We show that Rb_f inactivation in this model results in a short burst of proliferation that cannot induce

tumorigenesis. We hypothesize that the level of proliferation induced here is insufficient to provoke a significant *p53* mediated apoptotic response on day one lactation. Second, increasing evidence suggests that *p53* family proteins, *p63* and *p73* play an important role in apoptosis in certain organs, including the mammary glands (Flores et al. 2002, Flores et al. 2005). Finally *p53* family independent pathways may also play a significant apoptotic role in this model resulting in balancing the increased proliferation and that would result in checking tumor progression pathways. Future experiments designed to test the role of alternate apoptotic pathways can answer these questions.

In summary, we have established two important points. First, Rb_f inactivation models accurately in the mice the initiation of tumorigenic events that are not sufficient in themselves to lead to mammary tumors, which is similar to human cancers where other genetic aberrations occur along with *RB1* mutations in promoting breast cancer. Second, aberrant proliferation in the Rb_f deficient mammary glands most likely predisposes to mammary tumor development.

2.5 Materials and Methods

2.5.1 Derivation of MFT_{121} transgenic mice

Resulting and subsequent generation $TgMFT_{121}$ transgenic mice were identified by PCR amplification of a 215-bp fragment using primers 5'-GCATCCAGAAGCCTCCAAAG -3' and 5'-GAATCTTTGCAGCTAATGGACC-3' complementary to the T_{121} sequence. In the MFT_{121}/*Wap-Cre* mice 5'-TGATGAGGTTCGCAAGAACC-3' and 5'-CCATGAGTGAACGAACCTGG-3' primers were used for the Cre sequence.

2.5.2 Mammary gland whole mount analysis for eGFP expression

Inguinal mammary glands (#4) were isolated from *TgMFT*$_{121}$ female mice and washed with PBS. After brief air-drying the glands were scraped with razor blade to provide a thin section for observation under light microscope. The thin section of the mammary gland was then placed on a glass microscope slide and observed under UV light microscope for eGFP expression. Digital images were captured at 2.5, 5 and 10X magnifications.

2.5.3 Histopathology and apoptosis assays

Mammary gland tissue and tumor samples were dissected from *TgMFT*$_{121}$ transgenic or age matched littermate control animals. Part of each sample was snap frozen for RNA and DNA analysis and a portion was fixed overnight in 10% phosphate buffered formalin, transferred to 70% ethanol, and then embedded in paraffin. To analyze tumor histopathology mammary samples were sectioned for 10 successive layers. Sections at 4-um intervals were taken and stained with hematoxylin and eosin. Histopathological examination of the slides under light microscope was done as previously described. For detection of apoptosis levels in the samples staining of above sections using the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) kit as previously described (Symonds et al. 1994a, Symonds et al. 1994b) was performed. The statistically significant differences in apoptosis levels between mice with varying genotypes was evaluated as described below ($p<.0.05$ considered of statistical significance).

2.5.4 Immunohistochemistry and immunofluorescence

Immunohistochemical analysis for detection of specific markers in the mammary gland samples was performed on the formalin-fixed paraffin sections. For antigen retrieval the slides were boiled in citrate buffer (pH 6.0, Zymed, South San Francisco, CA) for 15 min. Endogenous peroxidase activity was quenched by incubating the slides for 10-min in 3% H2O2 in methanol. For IHC detection was done using the appropriate secondary antibody. The antibodies used here are anti-SV40 (monoclonal Ab2, 1:100, Oncogene, Cambridge, MA) and anti-Ki67 (1:100, Pharmingen). Fluorescence detection for all antibodies was performed using Alexa Flour secondary antibodies.

2.5.5 Statistical Analysis

The nonparametric Wilcoxon rank-sum test (using Van der Waerden normal scores) was used for all pair-wise or two-group comparisons. This method tests for a differences, or shifts, in location between the two groups of interest, with a minimum of assumptions. This is in contrast to the parametric two-group t-test, which compares the means of the two groups, using assumptions that may not hold (i.e. normally distributed, with equal variances). All p-values reported are the nominal or unadjusted p-values. ('Unadjusted' meaning that they have not been adjusted to account for multiple comparisons.) Adjustment was deemed not relevant due to the exploratory nature of this research project. All statistical analyses were performed with SAS statistical software, Versions 9.1, SAS Institute Inc., Cary, NC.

Histological classification of MFT_{121} mammary glands

H&E stained sections were reviewed by M.M., K.S. and TVD. K.S. has extensive experience in murine mammary pathology.

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CHAPTER THREE

p53 HAPLOINSUFFICIENCY CAUSES MAMMARY TUMOR ACCELERATION

3.1 Abstract

While the loss of *RB1* function is one of the most common early events in many solid tumor developments, altered *P53* function is seen as one of the common events associated with late stage progression. People born with the inherited mutation of one copy of *P53* develop Li Fraumeni syndrome and are susceptible to a wide variety of cancers during their lifetime, including breast cancer. *P53* is also mutated in 80% sporadic cancers and 50% sporadic breast cancers. *P53* mutated sporadic breast cancers are often high grade, hormone negative and correlate with poor survival. In vitro and in vivo studies have shown that inactivation of *P53* often results in loss of apoptosis resulting in aberrant proliferation of cells leading to cancer. *RB* pathway mutations and *P53* mutations occur frequently together in human breast cancer. Some mouse models have attempted to study co-operation of these two events in the mammary gland with limited success. First, germline loss of *p53* pre-disposes to non-mammary tumors like lymphomas and sarcomas and precludes study of a mammary phenotype. Second, mouse models using the SV40 Large T antigen to study the combined loss of Rb_f and *p53* in the mouse mammary gland have been unable to identify the individual effects of each of these proteins in the etiology of breast cancer. We used an alternative approach to overcome these problems. In this study we developed a conditional mammary

tumor model using a truncated version of SV40 T antigen that binds to the pRB_f alone and not P53. Crossing to a conditional *p53* mouse and using a mammary specific CRE we are able to study the effect of complete loss of *p53* in the mammary gland both alone and in combination with loss of Rb_f. Using this model system we find that while loss of Rb_f alone pre-disposes to abnormal proliferation in the mammary gland, the combined loss of Rb_f and *p53* play a synergistic role in mammary tumor development. The Rb_f inactivated and *p53* haploinsufficient mice develop mammary tumors with a median latency of 275 days and the complete loss of *p53* reduces the latency to 150 days. All mice develop mammary tumors with a 100% penetrance and the tumors are highly proliferative and luminal epithelial in nature. Several histological features of these tumors resemble human luminal type breast cancers. This suggests that while loss of Rb_f pre-disposes to tumors by increase in proliferation, loss of *p53* is a critical event for mammary tumor progression.

3.2 Introduction

P53 is perhaps the most studied tumor suppressor and is rightly referred to as the "guardian of the genome" (Levine 1997). In normal cells P53 resides in very low concentrations and has a very short half-life of about 20 minutes only. In response to a variety of cellular stress signals P53 undergoes post-translational stabilization and is modified in many different ways (for example phosphorylation, acetylation, methylation, sumoylation, etc) (Giaccia, Kastan 1998). These modifications convert P53 to an active transcription factor that can turn on a variety of downstream effects, all of which are geared to protect the cells from damage. One of the main functions of P53 is sending damaged cells

down the cell death or apoptotic pathway (Green, Evan 2002). For example, cells that have lost a functional pRB pathway and are undergoing abnormal proliferation frequently undergo P53-mediated apoptosis. P53 is controlled at many levels by other proteins. The mouse MDM2 protein (HDM2 in humans) maintains the normal cellular levels of P53 by targeting P53 for proteosomal degradation (Michael, Oren 2003). The ARF protein which is a product of the alternate reading frame of $P16^{INK4a}$ stabilizes P53 levels by binding to and inactivating MDM2, thus preventing P53 degradation (Sherr, Weber 2000, Weber et al. 2000). A feedback loop exists between the E2F transcription factors and ARF, where increased expression of E2F target genes results in up regulation of ARF. This in turn binds to and inactivates MDM2 leading to the stabilization of P53 (Ginsberg 2002).

Given the critical function of P53 in preventing damaged cells from continuing to proliferate, it is not surprising that *P53* is mutated or lost in 80% sporadic human cancers and in about half of sporadic breast cancers (Greenblatt et al. 1994, Lehman et al. 1994). The most common *P53* mutations are point mutations that result in a dysfunctional protein (Harris 1996). Also common are mutations in *ARF* that prevent P53 stabilization (Sharpless et al. 2002, Sharpless, DePinho 2002). A small fraction of cancers also display mutations in *HDM2* leading to overexpression of the protein (HDM2) and thus resulting in the increased degradation of P53. Familial mutations in *P53* cause the Li Fraumeni syndrome, where multiple members of the family inherit one mutant copy of the *P53* gene and are highly susceptible to many different kinds of cancers during their lifetime including breast cancers (Kleihues et al. 1997, Varley 2003).

P53 inactivation resulting in loss of cell cycle checkpoint control and reduced apoptosis a frequent event in mammary cancer progression (Nigro et al., 1989, Greenblatt et

al., 1994, Lozano et al., 1998). In clinical breast cancers, *P53* mutations correlate more frequently with estrogen receptor negative (Borresen-Dale 2003) HER-2 positive breast cancers that also show significant levels of aneuploidy (Tsutsui et al. 2002). Mammary steroid hormones and the estrogen receptor status of the subset of mammary epithelial cells undergoing *P53* mutation could play a role in mammary tumorigenesis. Also, *BRCA1* mutated familial breast cancers show very frequent mutations of *P53* suggesting synergy in the tumor suppression activities of these two proteins (Crook et al. 1997).

Early mouse models devised to model the Li Fraumeni syndrome by inducing a germline mutation in *p53* resulted in mice that were born healthy but were pre-disposed to tumors like lymphomas and sarcomas and died in about five months (Donehower et al. 1992). This indicated that *p53* did not behave like most tumor suppressors, in that complete loss of *p53* did not lead to embryonic lethality in mice. Rather these mice seemed to be very susceptible to cancers arising from other accumulating mutations and unable to protect themselves from abnormal cells. However, mammary tumors were either absent or present in very low frequency in these mouse models (Jacks et al. 1994).

Several mouse models were generated to study the loss of *p53* function in the mammary gland. It was noticed that genetic modifiers in specific mouse strains played a dramatic role in *p53* loss induced mammary tumorigenesis. So while the C57BL/6 strain of mice developed mammary tumors at a very low frequency upon loss of *p53* (Ullrich et al. 1996) the BALB/c strains of mice were highly susceptible to spontaneous and *p53* loss induced mammary tumors (Jerry et al. 1998, Kuperwasser et al. 2000). Since germline loss of *p53* pre-disposed to the early appearance of lymphomas and sarcomas, the early mouse models to study *p53* mediated mammary tumorigenesis transplanted $p53^{-/-}$ cells into the wild

type mammary gland of BALB/c mice (Jerry et al. 2000). These mice developed mammary tumors with a high frequency and carcinogen (DMBA) induced tumor latency in these mice was also greatly reduced. This indicated that loss of *p53* greatly increased the susceptibility of the mammary epithelium to tumorigenesis perhaps by increase in genetic instability. To specifically target *p53* mutation in the mammary luminal epithelial cells, Lin et al., 2004a developed a conditional *p53* model targeting *p53* loss in the mammary glands using both the WAP-Cre and MMTV-Cre recombinase. These mice developed a broad range of mammary tumors albeit with long latencies ranging from 10.5-24 months, depending on the parity status of the mice as well as their genetic backgrounds (Lin et al. 2004a). Jonkers et al., 2001 reported that the conditional loss of *p53* in the mammary gland using a Keratin 14 Cre (that targets the mammary myoepithelial cells) did not result in mammary tumorigenesis. This implied that loss of *p53* alone might not be enough to promote mammary tumorigenesis but other stochastic events were necessary either before or after *p53* loss to co-operate with tumorigenesis. Indeed in human breast cancers *P53* loss is often combined with loss of other tumor suppressors like *RB1, BRCA1* and/or *BRCA2*. Mouse models that combined other relevant genetic lesions in the mammary gland along with loss of *p53* showed a reduced tumor latency, for example, mammary tumors developed much faster in the MMTV-Wnt-1 mammary tumor model upon the loss of *p53* (Donehower et al. 1995, Donehower et al. 1996, Jones et al. 1997). The combined loss of *p53* and *Brca1* or *p53* and *Brca2* in the mammary epithelium resulted in a broad spectrum of mammary tumors (Jonkers et al. 2001, Liu et al. 2007) while the loss of either of these genes alone did not develop mammary tumors. As many human tumors showed the combined loss of *Rb1* (or the *Rb* pathway components) and *p53*, mouse models were derived to study the co-operating activity of these two lesions

(Harvey et al. 1995). As *Rb*$^{-/-}$ mice are embryonic lethal only *Rb* heterozygous mice along with *p53* heterozygous or null mutations could be studied. These mice developed endocrine tumors in the pituitary, thyroid and pancreatic glands, but did not develop mammary tumors. This presented a quandary. Even though human breast cancers frequently presented combined mutations of the *Rb1* pathway and *p53* suggesting potential co-operation between these two lesions, mouse models did not reflect this aspect. It seemed to appear from these studies that the potential co-operation of *Rb1* and *p53* in mammary tumorigenesis required mammary cell specific inactivation of both genes. This was necessary for several possible reasons. First, their co-operation could be context dependent and resulting from complex microenvironmental and hormonal cues within the mammary gland that could not be studied by their germline mutations. Second, mammary tumorigenesis may require other co-operative lesions besides loss of *Rb1* and *p53* and the accumulation of these lesions in a stochastic fashion over time may lead to tumorigenesis in the mammary gland. This could be a slow process and the development of the faster growing lymphomas and sarcomas (arising frequently upon *p53* mutations) and other non-mammary tumors in the germline *Rb1* and/or *p53* mutated mice would preclude study of the mammary phenotype. Mammary specific inactivation of *Rb*$_f$ and *p53* resulted in mammary adenocarcinomas with a latency of about 6 months (Green et al. 2000) in a FVB background and using the C3(1) portion of the rat steroid binding promoter to target the mammary glands. In these mice it was not possible to study the individual contributions of the pRB$_f$ and P53 in both mammary tumor initiation and progression. Also further inhibition of other key tumor suppressor proteins by the large T antigen in this model that may co-operate in mammary tumor formation cannot be ruled out.

Another intriguing aspect of *p53* research has been its cell type specific effect in conducting apoptosis in response to the same oncogenic insult. It was previously demonstrated that in a brain epithelial tumor model, in the absence of Rb_f function, inactivation of *p53* significantly decreases apoptosis and accelerates tumor growth in vivo (Lu et al. 2001, Symonds et al. 1994a, Symonds et al. 1994b). But tumor progression was not accelerated by reduced *p53* activity in astrocytic and prostate tumors, rather the phosphatase and tensin homolog (*pten*) gene regulates the apoptosis, and reduction in its function accelerates tumor growth (Hill et al. 2005a, Xiao et al. 2002). In the mouse mammary gland using the *TgWAP-*T_{121} model (Simin et al. 2004) it was observed that Rb_f loss induced proliferation that was subjected to *p53* dependent apoptosis on day one lactation. However analysis of cooperation of Rb_f and *p53* loss in mammary tumorigenesis was not possible in this model due to pre-disposition of non-mammary tumors in these mice (mainly thymic lymphomas and sarcomas).

We have shown before that the conditional inactivation of Rb_f in the mammary gland does not lead to mammary tumorigenesis. To study the potential co-operation of Rb_f and *p53* in mammary tumorigenesis and circumvent the problem of developing *p53* loss mediated non-mammary tumors here we use a *p53* floxed allele that can be crossed to mammary specific Cre (like WAP or MMTV Cre). Layering on of genetic mutations in this model allows us to study not only the co-operation of genetic events necessary for promoting breast cancer (like *RB1* and *P53*) but also lets us study the precise manner in which each of these lesions contribute to tumor progression. Finally this model provides a powerful tool to study further genetic lesions like loss of *Brca1* and *Brca2*, either alone or in combination with *Rb1* and *p53*. This is the first time that these important and relevant genetic lesions have been

looked at together in a mouse mammary tumor model. We hope that this model will provide future avenues for breast cancer drug testing.

In summary in the current report we discuss the use of GEMs to elucidate the roles of mammary epithelium specific Rb_f loss and the concomitant loss of *p53* using a conditional mouse model to study somatic deletion of Rb_f and *p53*.

3.3 Results

3.3.1 Mammary specific inactivation of Rb_f and *p53* induces mammary tumors.

To study the effect of haploinsufficiency of *p53* in a Rb_f inactivated mammary gland, *p53* heterozygous mice (*WAP-Cre*; $TgMFT_{121+/-}/p53^{\Delta 2\text{-}10/+}$) were generated by mating $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice (FVB, Jonkers et al., 2001) with *WAP-Cre*; $TgMFT_{121+/-}$ mice (C57BL6; B6DF1). These mice were observed over time for mammary tumor appearance. We observed a dramatic reduction in mammary tumor latency in the *WAP-Cre;* $TgMFT_{121+/-}$*/ p53* $^{\Delta 2\text{-}10/+}$ (*p53* haploinsufficient) mice. Analysis of 9 mice of this genotype showed a median mammary tumor latency of 275 days (about 9 months) compared to almost no mammary tumors observed in mice with Rb_f inactivation alone. Due to mammary specific inactivation of *p53* none of these mice developed non-mammary tumors and efficiently circumvented this problem.

An even further reduction in mammary tumor latency was observed with the complete loss of *p53* (obtained by mating $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice to *WAP-Cre;* $TgMFT_{121+/-}$*/ p53* $^{\Delta 2\text{-}10/+}$ mice) (See **Fig 3.1**, Survival Curve). The fourteen *WAP-Cre;* $TgMFT_{121+/-}$*/ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice observed for mammary tumorigenesis developed tumors with a median latency of

150 days. Using the Log Rank Test for significance the median latencies of the *p53* haploinsufficient and *p53* null mice were found to be significantly different (P<0.0001).

Tumor development was hundred percent penetrant in the above genetic combinations (*p53* heterozygous or null) and occurred within a consistent time frame. This indicated that in the mammary gland, loss of Rb_f in physiological levels (lower levels than that caused by multiple copies of T_{121}) is not sufficient to promote mammary tumorigenesis but concomitant loss of *p53* is sufficient to promote advanced mammary adenocarcinoma development and progression. Also loss of *p53* is haploinsufficient for mammary tumorigenesis. The tumors show a very high mitotic index and significant amount of pleomorphic nuclei (**Fig3.2, a, arrow**). All mammary tumors stained positive for T_{121} expression (**Figure 3.2, b**) and showed *p53* deletion upon Cre mediated excision by PCR targeting the single Lox P site left after Cre deletion (**Figure3.3**). *p53* null alone mice (*WAP-Cre*; $Tgp53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$) did not develop mammary tumors in this model. This could be due to the modifier effect of the specific mouse genetic background used for this study. But this is also similar to previous reports where loss of *p53* alone is not sufficient to promote mammary tumors and requires other co-operating lesions.

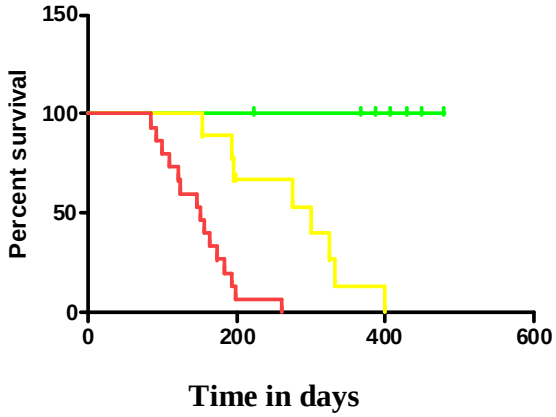


Figure 3.1 Kaplan-Meier Survival curves of cohorts of *WAP-Cre; TgMFT*$_{121+/-}$, *WAP-Cre ; TgMFT*$_{121+/-}$ */p53* $^{\Delta 2\text{-}10/+}$ *and WAP-Cre ; TgMFT*$_{121+/-}$*/ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ **mice. *TgMFT*$_{121+/-}$ mice (top graph) were observed in the mouse colony up to 500 days without significant mammary tumor development. Haploinsufficiency of *p53* in combination with T_{121} reduced tumor latency to a mean of 275 days (middle graph) and one hundred percent penetrance. Complete loss of *p53* (bottom graph) resulted in further loss of latency to a median latency of 150 days and one hundred percent penetrance. $P < 0.0001$ between the survival curves using the log rank test for significance, indicating the survival curves are significantly different. (Green Plot = ***WAP-Cre; TgMFT***$_{121+/-}$, Yellow Plot= ***WAP-Cre; TgMFT***$_{121+/-}$ ***/p53*** $^{\Delta 2\text{-}10/+}$, Red Plot = ***WAP-Cre; TgMFT***$_{121+/-}$***/ p53*** $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$).

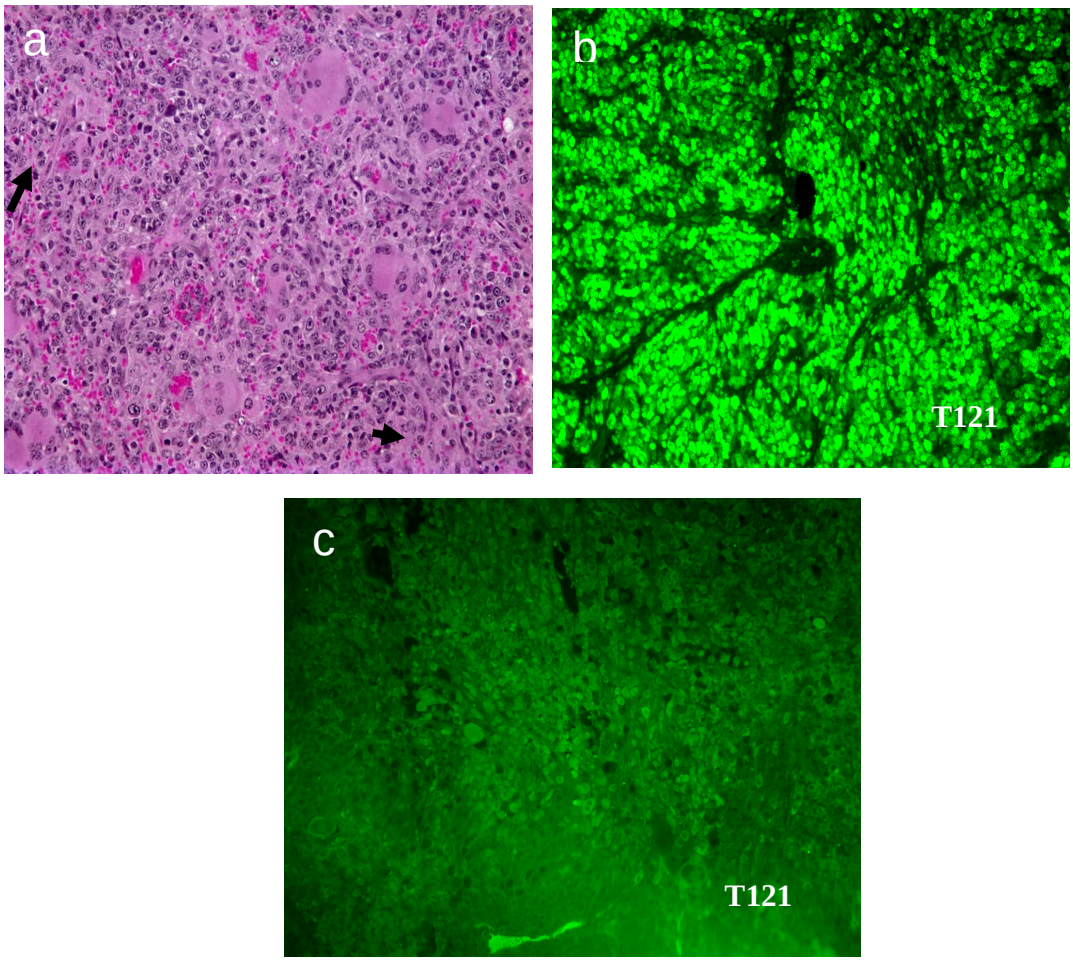


Figure 3.2, *WAP-Cre; TgMFT*$_{121+/-}$ */ p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice develop heterogeneous broad-spectrum mammary adenocarcinomas that stain positive for T_{121}. Mammary tumors develop in mice of this genotype with a hundred percent penetrance. Most tumors are high grade with pleomorphic nuclei and very high mitotic index (arrow, a). The mammary tumors

stain positive for T_{121} (b) with no staining with secondary antibody alone (no primary) (c). Magnification is 20X.

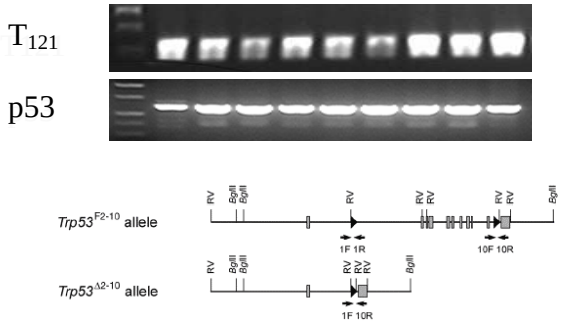


Figure 3.3 PCR shows expression of T_{121} in mammary tumors and Cre mediated deletion of *p53*. Representative tumors from eight mammary glands are shown (lane one is positive control). All tumors expressed T_{121} after Cre mediated deletion of floxed eGFP (upper lane). Lower lane represents Cre mediated deletion of *p53* in eight representative mammary tumors. Primers were designed as shown in figure to detect single Lox P site with 1F and 10R primers, if deletion of floxed allele had occurred. All mammary tumors tested showed this deletion event (Allele design taken from Jonkers et al. 2001).

3.3.2 Apoptosis and Proliferation remain unchanged with loss of *p53*

It was previously shown that Rb_f inactivation by T_{121} expression in the choroid plexus epithelial (CPE) cells (Lu et al. 2001) and mammary epithelial cells (Simin et al. 2004) induced both proliferation and apoptosis and in the mammary gland only apoptosis but not proliferation was *p53* dependent. To determine if in this somatic inactivation of Rb_f and *p53*, the role of *p53* remained the same we assessed proliferation and apoptosis using Ki67 staining in day 1 lactation mammary glands of *WAP-Cre;* $TgMFT_{121+/-}/p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice. Apoptosis was assayed by TUNEL staining in the mammary glands of all the above cohorts on day 1- lactation and compared with TUNEL staining in control $TgMFT_{121+/-}$ mice. *WAP-Cre;* $TgMFT_{121+/-}$ / $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice showed a slightly higher level of proliferation compared to the Cre-negative controls on day 1 lactation (**Figure 3.4, a**) but this was not a

significant difference (P=0.64205). The apoptosis levels of these mice (*WAP-Cre; $TgMFT_{121+/-}$ / $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$*) at day 1 were comparable to the *WAP-Cre; $TgMFT_{121+/-}$* mice (**Figure 3.4, b**). As a significant apoptotic response from P53 mediated pathways requires an oncogenic insult to be below a threshold level, we hypothesize that in the current model this does not occur on day one lactation due to low expression levels of T_{121} induced by a single copy transgene. We had observed before that using this model there was no significant apoptotic response by P53 upon loss of RB_f alone using T_{121} (results discussed in chapter 2). So not surprisingly we do not notice a significant decrease in apoptosis upon loss of P53. Our hypothesis is that since this model had low T_{121} levels due to the single transgene effect, levels of apoptosis were not significantly induced by expression of T_{121} alone. Hence loss of P53 did not have a great effect in lowering the already low levels of apoptosis.

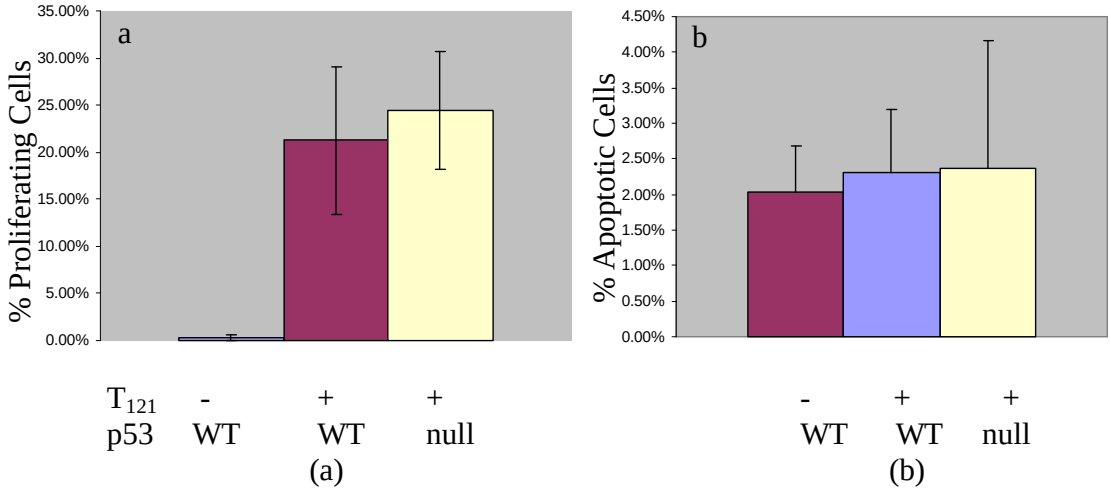


Figure 3.4 Proliferation and Apoptosis levels remain unaltered in *WAP-Cre; $TgMFT_{121+/-}$ /$p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* mice. (a) Proliferation levels (detected by Ki67 positive cells) did not change significantly with loss of P53 (P=0.64205). **(b)** Apoptosis levels remained the same upon loss of P53 in this model (P=0.85697). At least two animals were used for all apoptosis and proliferation studies. Five random sections from three slides of each animal were counted. The average cells counted in each slide were 200.

3.3.3 Time course mammary gland biopsies show accelerated mammary gland MIN and hyperplasia with complete loss of *p53*

One of the strengths of GEMs is the ability to study pre-cancereous lesions if any, and follow progressive changes in an organ as tumor formation occurs. The ability to do this provides avenues to explore the accumulation of stochastic genetic lesions that lead to palpable tumor formation. Also, marker studies in the pre-cancerous lesions can provide important information on the cell of origin of these tumors. We used this model to follow histopathological changes in the mammary gland post day 1-lactation. To do this we performed mammary gland biopsies on the inguinal mammary gland (#4) on day 1 lactation of a cohort of at least three mice for each genotype, *WAP-Cre; TgMFT*$_{121+/-}$ */ p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$, *WAP-Cre; TgMFT*$_{121+/-}$ and *TgMFT*$_{121+/-}$ (no Cre). Biopsy was performed after weaning the pups on day one lactation to also study the role of *Rb*$_f$ loss and *p53* on normal mammary gland involution. We observed that on day 1 lactation, the *WAP-Cre; TgMFT*$_{121+/-}$ */ p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mammary glands looked more hyperplastic with pleomorphic nuclei and higher mitotic index when compared to control mammary glands (*TgMFT*$_{121+/-}$) (**Figure 3.5, c**). These mammary glands also showed focal clusters of cells that had excessive luminal filling by epithelial cells, which was not seen in the controls.

Next we isolated the mice on the same mammary gland at 2 weeks post day 1 lactation. While the control mammary glands had undergone normal involution (**Figure 3.5, d & e**), the *WAP-Cre; TgMFT*$_{121+/-}$ */p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice mammary glands showed complete absence of involution (**Figure 3.5, f**). Besides the delay in involution there also were several clusters of small regions of mammary intra neoplastic (MIN) lesions (**Figure 3.5, f, arrow**).

Next we performed the final biopsy at 6 weeks post day 1-lactation on the same cohort of mice and looked at all the mammary glands of the experimental mice. This showed us conclusively that while the *WAP-Cre; TgMFT*$_{121+/-}$ and *TgMFT*$_{121+/-}$ showed complete involution with very sparse mammary glands in large areas of fat pad (**Figure 3.5, g & h**) (with the exception of some remnant large mammary gland structures observed in the double transgenic mice as described in chapter 2) the *WAP-Cre; TgMFT*$_{121+/-}$ / *p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mammary glands had started forming distinct but very small tumor nodules (**Figure 3.5, i**). Even though palpable tumors had not yet formed in these mice, histopathologically they looked pre-cancerous. Staining with keratin markers once again showed that the small tumor nodules were primarily keratin 8/18 positive and keratin 5 negative (**Figure 3.6, b**). This indicated that the tumor cell lineage was primarily mammary luminal epithelial type. This experiment allowed us to follow the stepwise progression of mammary lesions after layering on multiple genetic events.

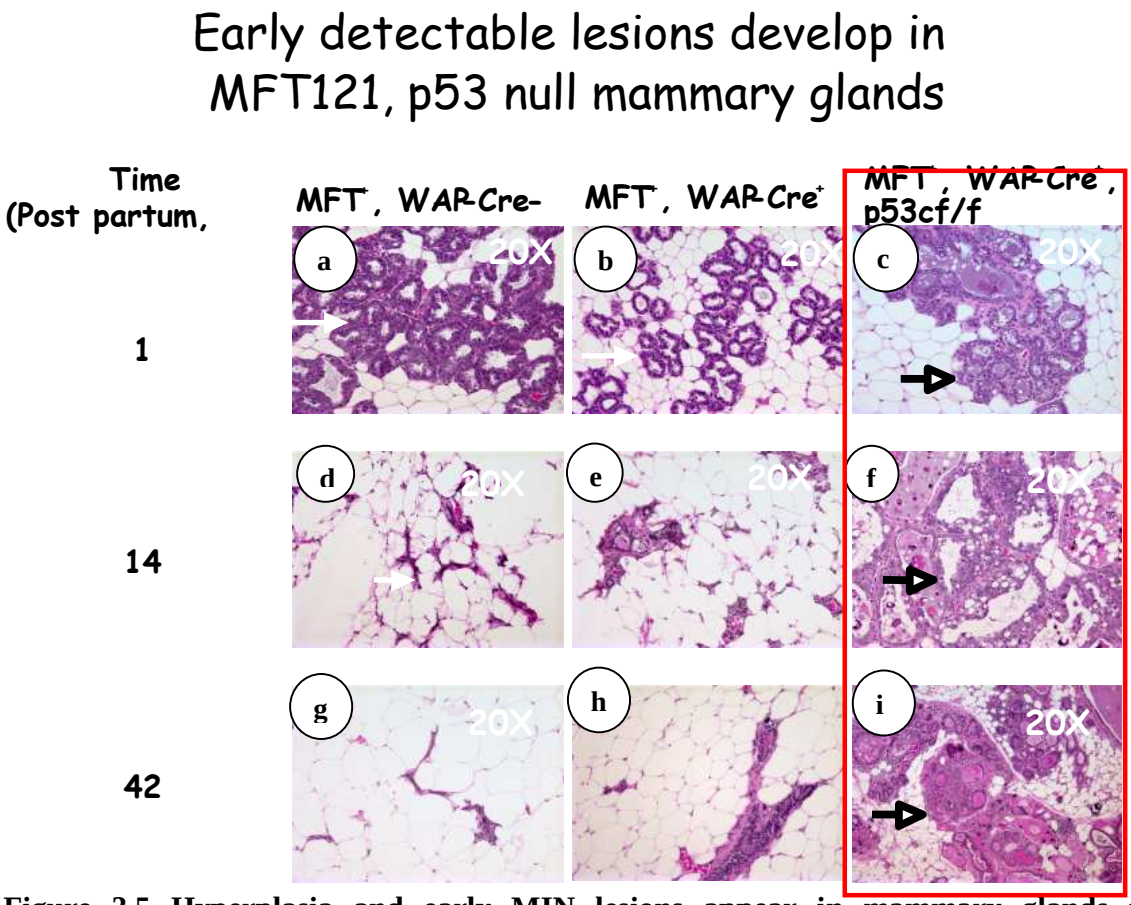
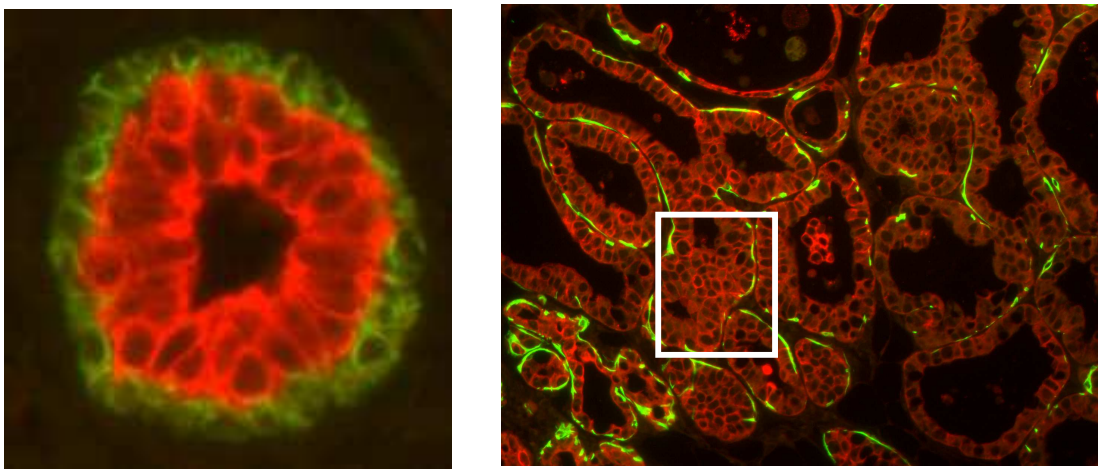


Figure 3.5 Hyperplasia and early MIN lesions appear in mammary glands not expressing *p53*. Representative mammary morphologies in H&E-stained sections of Day1 Lactation mammary glands are shown: **(a)** *$TgMFT_{121+/-}$ (Cre* negative*)* mammary glands with a normally thick layer of luminal (milk secreting) epithelial (black arrow) **(b)** *WAP-Cre; $TgMFT_{121+/-}$* and **(c)** *WAP-Cre; $TgMFT_{121+/-}$ / $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* mammary glands. The epithelial cells in *WAP-Cre; $TgMFT_{121+/-}$ / $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* Day 1 lactation mammary glands are hyperproliferative and grow in dense patterns piling on top of each other with ductal filling (**c**, arrow). Mammary duct hyperplasia and early MIN lesions develop in *WAP-Cre; $TgMFT_{121+/-}$ / $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* mice as early as 14 days post day 1 lactation **(arrow, f).** These mice fail to regress post day 1 lactation after removal of pups **(f)** At day 42 post day one the *p53* null develop small lesions that have the appearance of small tumor nodules **(arrow, i). n = 3 for each genotype.** The figures highlighted by red box indicate the new experimental data while all other figures (Figures a, b, d, e, g and h) have been shown and discussed in chapter 2.



a **b**

Figure 3.6, Analysis of mammary glands with antibodies specific for various cytokeratins in *WAP-Cre; TgMFT*$_{121+/-}$ / *p53*$^{\Delta\ 2\text{-}10/\Delta 2\text{-}10}$ mice show luminal filling by Keratin 8/18 positive cells. The mammary gland is composed of two types of epithelial cells, luminal epithelial cells that stain positive for Keratin 8/18 (**a, red**) and myoepithelial cells that stain positive for Keratin 5 (**a, green**) (Courtesy, Karl Simin). Keratin marker staining of the hyperplastic mammary glands (**b**) of *WAP-Cre; TgMFT*$_{121+/-}$ / *p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice show primarily luminal epithelial cells filling in the hollow lumen of the mammary glands (indicated by white box, b) and forming hyperplasia in day 14 post day one lactation.

3.3.4 Loss of *p53* heterozygosity contributed to mammary tumor heterogeneity

Identification of cell type lineage in human breast cancers is an important way to identify the more aggressive subtypes of cancer. It has been shown by molecular classification of breast cancers using microarray technologies, that the luminal sub type of human breast cancers (positive for Keratin 8/18) has a better prognosis compared to tumors that are Keratin 5 positive and have a more myoepithelial nature (Perou et al. 2000). Also breast cancers with a more myoepithelial signature indicated by increased expression of epithelial-to-mesenchymal-transition (EMT) markers have a worse prognosis. EMT is a process that allows epithelial cells to gain more fibroblast like properties thus making them more motile and aggressive. This is a reversible process and plays a role in distant metastasis, wherein tumor cells re-gain epithelial like properties when they find a distant metastatic site

to settle and grow in. Smooth muscle actin (SMA) and Vimentin are two markers for fibroblasts that have been implicated to be acquired by epithelial cells during the process of EMT. These tumors are frequently more aggressive and show lymph node metastasis.

To determine the cell types that contributed to mammary tumorigenesis in the *WAP-Cre; $TgMFT_{121+/-}$ /p53 $^{\Delta 2\text{-}10/+}$* and *WAP-Cre; $TgMFT_{121+/-}$ /$p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* mice we characterized each tumor by performing cell lineage marker staining. Tumors were stained for Keratin 8/18, a marker for mammary luminal epithelial cells and Keratin 5, a marker for mammary myoepithelial cells (basal cells) to identify cell lineage in these tumors. Also, as the *WAP-Cre; $TgMFT_{121+/-}$ /$p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* tumors showed a significant increase in the spindle shape morphology of cells, epithelial-mesenchymal transition (EMT) occurring in these tumors was a distinct possibility. To assess for possible EMT we stained the tumors of the different genotypes with SMA and Vimentin. The *WAP-Cre; $TgMFT_{121+/-}$ /p53 $^{\Delta 2\text{-}10/+}$* tumors appeared to be pre-dominantly solid adenocarcinomas (**Figure 3.7, a**) with some regions of angiogenesis (**Figure 3.7, a, arrow**). These tumors stained positive for Keratin8/18 and negative for Keratin 5 (**Figure 3.7, b**) indicating their pre-dominantly luminal epithelial nature. The tumors were also negative for the EMT markers SMA and Vimentin (**Figure 3.7, c**). In the *WAP-Cre; $TgMFT_{121+/-}$ /$p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* tumors (**Figure 3.7, lower panel**), a distinct increase in spindle shaped cells was observed (**Figure 3.7, d**). The tumors remained pre-dominantly Keratin 8/18 positive and Keratin 5 negative (**Figure 3.7, e**). The *WAP-Cre; $TgMFT_{121+/-}$ /$p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* tumors also showed an increase in SMA positive cells (**Figure 3.7, f, green staining**) but no significant Vimentin staining (**Figure 3.7, f, red staining**). The increase in SMA staining but not Vimentin indicated the occurrence of an incomplete EMT. This result corroborated the more aggressive nature of these tumors and the reduced latency.

Also the change in tumor spectrum along with the longer latency of *WAP-Cre; TgMFT*$_{121+/-}$ */p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice clearly indicates the selective pressures within the tumor for loss of the *p53* wild type allele. The various tumor phenotypes are tabulated in **Table 3.1**. The tumor latency and phenotype of each genotype is indicated graphically in **Figure 3.8**.

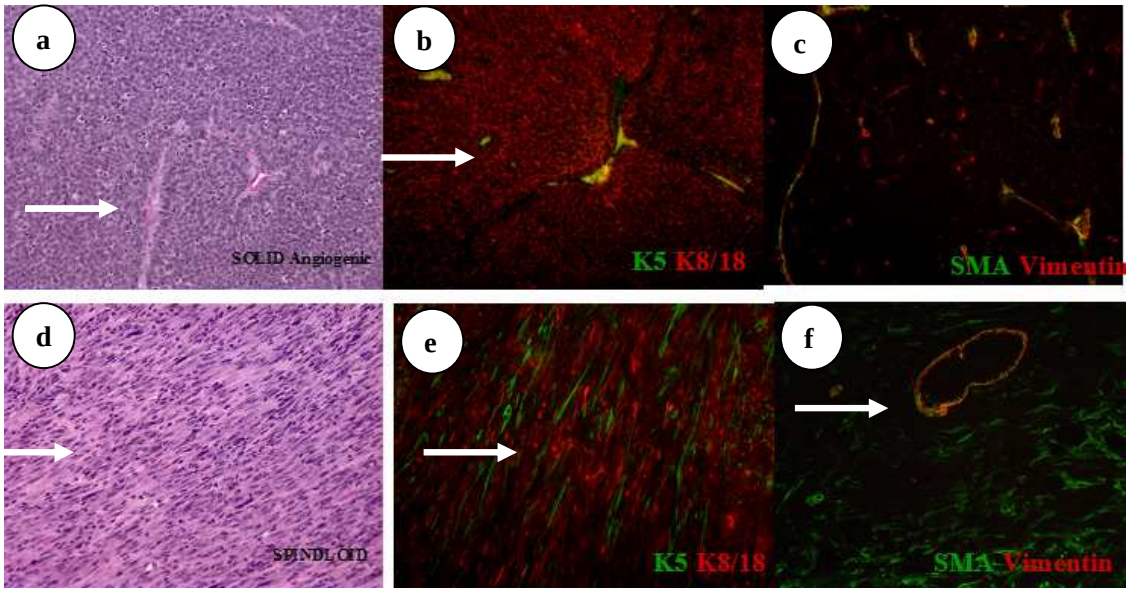


Figure 3.7 Histopathology and marker characterization of different mammary tumor sub-types from *WAP-Cre; TgMFT*$_{121+/-}$ */ p53*$^{\Delta 2\text{-}10/+}$ and *WAP-Cre; TgMFT*$_{121+/-}$ */ p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice. Serial sections from representative tumors were stained for Haemotoxylin and Eosin (left panel, **a & d**) or with antibodies against Cytokeratin 8/18 and Cytokeratin 5 (middle panel, **b and e**) and Vimentin and Smooth Muscle Actin that are known markers of Epithelial Mesenchymal Transition (**EMT**) (right panel, **c and f**). In (**a**), a representative poorly differentiated solid mammary gland carcinoma that arises in the *WAP-Cre; TgMFT*$_{121+/-}$ */ p53*$^{\Delta 2\text{-}10/+}$ mice in 250-300 days post day 1 lactation is shown. These tumors show strong positive staining for the luminal cell marker CK8/18 **(arrow, b)**. No Keratin 5 staining is detected in these tumors (**b**). These tumors are SMA and Vimentin negative **(c)**, indicating that they are not undergoing EMT. In (**d)** the histopathology of a representative mammary tumor arising in *WAP-Cre; TgMFT*$_{121+/-;}$ */ p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice within a median onset of 150 days of Day 1 Lactation is shown. A total of 14 mammary tumors of this genotype were assessed for all the markers shown. Complete loss of *p53* shows a shift in tumor spectrum to more a more spindloid morphology (arrow, **d, H&E**). These tumors typically are Keratin 8/18 positive and Keratin 5 negative (**e**). Only infiltrating myoepithelial cells in these tumors show positive Keratin 5 staining (**e**). Vimentin and SMA staining of these tumors indicate an increase in SMA but not Vimentin **(f)**. Vimentin in only seen in remnant mammary gland structures in the myoepithelial cells **(arrow, f)**.

T_{121}, Cre	n	Solid	Glandular	Pilar	Spindle	Angiogenic	Necrotic	Metastatic
$p53^{cf/+}$	10	71%	-	-	51%	50%	8%	-
$p53^{cf/f}$	14	55%	18%	13%	55%	27%	18%	-

Table 3.1 The pre-dominant histological features of each genotype of mouse is tabulated here. *p53* heterozygous mice have mostly solid adenocarcinomas with frequent presence of blood vessels (angiogenesis). Most of these tumors also have a significant spindloid component especially in the periphery of the tumors. Complete loss of *p53* shifts the tumor spectrum somewhat to a predominantly spindloid type. These tumors also have significant regions of solid adenocarcinoma and show infrequent presence of glandular and pilar type tumors. Both the *p53* heterozygous and *p53* nullizygous tumors show some angiogenesis and necrotic regions. As all tumors are heterogeneous in nature and are composed of many different cell types, the sum of all components in these tumors is greater than 100. Mammary gland histological classification was done by Robert Cardiff, Karl Simin and Malini Mukherjee. Dr. Cardiff is a medical pathologist and former Chairman of Medical Pathology at UC Davis, CA with expertise in mouse pathology and cancer.

3.4 Discussion

3.4.1 *Rb_f* and *p53* have a synergistic effect in mammary tumorigenesis.

The establishment of suitable mouse models to study the combined effect of loss of *Rb_f* and *p53* in mammary tumorigenesis has been hindered by (i) the embryonic lethality observed in *Rb_f* null mice and (ii) the high incidence of non-mammary tumors, mainly lymphomas and sarcomas in *p53* germline mutated mice that preclude the study of mammary tumor formation. Here we develop a conditional mammary tumor model to circumvent the above problems and report that while loss of *Rb_f* function in mammary epithelium predisposes to malignant adenocarcinoma by increased mammary cell proliferation and the concomitant loss of *p53* cooperates with *Rb_f* loss in promoting mammary adenocarcinoma. Using the Cre-Lox-P technology to our advantage we are able to get around the incidence of

non-mammary type tumors. Using WAP-Cre for the recombination event, we are able to restrict the mutation events to the mammary luminal epithelial cells. We see that loss of Rb_f alone results in a burst of increased proliferation that does not lead to tumorigenesis. We also observe a delayed involution in the mammary glands of Rb_f inactive mice, post day one lactation.

Upon the loss of a single allele of *p53* in this model, we observe a significant increase in mammary tumor formation. The mice develop mammary tumors with a median latency of 275 days. Complete loss of *p53* further lowers the latency to 150 days. Earlier mouse models with only *p53* inactivation in the mammary epithelium have shown that mammary tumors with a long latency of more than 11 months (330 days) (Lin et al. 2004b). This latency is significantly longer than the latency of mammary tumor appearance in the current model. This suggests that co-operative lesions have a synergistic effect in promoting mammary tumors. However, it is worth mentioning that genetic modifiers and mouse genetic strains play an important role in *p53* mutation susceptibility as well as susceptibility to the development of specific tumor types. For example, it has been shown that while mice of the C57BL/6 and 129sv strains are highly resistant to the development of mammary tumors, the BALB/c strain of mice are highly susceptible to mammary tumor formation (Medina 1974, Medina, Stockman & Griswold 1974, Ullrich et al. 1996). Significantly, BALB/c, p53+/- mice developed spontaneous mammary tumors with a very high frequency (55%) (Kupperwasser et al., 2000). This indicates that modifier genes in various strains of mice can greatly affect mammary tumor pre-disposition. In this study we have used a mixed genetic background of mice with significant proportions of the BDF1 and C57BL/6 and FVB strains.

However mouse strain specific effects reflected in the mammary tumor susceptibility of these mice cannot be entirely ruled out.

We have previously targeted *Rb$_f$* mutation in multiple cell types using T_{121} as the targeting molecule (Lu et al. 2001, Simin et al. 2004, Xiao et al. 2002). In each of the cell types targeted, despite differences in the cell type biology, *Rb$_f$* inactivation has lead to an increase in proliferation that may cause these cell types to be pre-disposed to future tumor formation upon the occurrence of additional genetic events. It is evident that further genetic events are required for tumor progression in the mammary gland, as we have shown before in this thesis that loss of *Rb$_f$* alone in the mammary gland can induce increased proliferation but not tumorigenesis. Tumorigenesis was reported in the mammary gland upon *Rb$_f$* loss alone in a distinct model reported by Simin et al., 2004, but tumors appeared with a very long latency, indicating again that further accumulation of genetic events were required for tumor progression. In the current model, low transgene copy numbers of T_{121} (perhaps resulting in incomplete sequestration of the RB family proteins) seem to have been insufficient to promote tumorigenesis. This result indicates that differences in gene dosage may alter cell type specific responses to the same oncogenic event (in this case loss of *Rb$_f$*). It was reported earlier that not all cell types are *p53* dependent for apoptotic response to *Rb* loss (Xiao et al., 2002, Hill et al., 2005). While the choroid plexus used P53 as its primary apoptosis regulator, the prostate epithelium used PTEN for its apoptotic response (Hill et al., 2005). Although gene dosage effects of T_{121} fail to show a robust apoptotic response in this model on day one lactation, the significance of P53's role is seen by the greatly reduced latency of tumor formation in the mice upon loss of *p53*. Also, insufficient decrease in apoptosis upon loss of *p53* in this model suggests redundant activities by P53 family proteins, P63 and P73 that

have earlier been implicated in playing important roles in mammary gland apoptosis (Flores et al. 2005). This model, like other models derived before, addresses the relative contributions of Rb_f and *p53* in tumorigenesis. This was not done by models using the wild type large T antigen (Li et al., 1996a, Husler et al., 1998, Green et al., 2000, Schulze-Garg et al., 2000) that blocked both pRB_f and P53. The mammary gland is also similar to the brain epithelium in showing a *p53* heterozygosity dependent decrease in tumor latency. It was seen in mouse models for breast cancer using v-H-Ras (Hundley et al., 1997) or Wnt-1 (Jones et al. 1997)that *p53* loss increased proliferation and did not affect apoptosis. This is different from what we see here. However, unlike those models that studied the terminal tumors, we have decided to look earlier at day one lactation not to have confounding factors like selection of tumors against apoptosis. All these models looked at apoptosis levels in the end stage tumor and not on day 1lactation as we did. As the end stage tumor may have selected against apoptosis, it cannot be concluded from these models that the loss of *p53* has contributed to both increased proliferation as well as reduced apoptosis. However in the $TgMFT_{121}$ model proliferation levels are not dependent on *p53*. This suggests that tumor suppressor mechanisms exerted by *p53* are context dependent and may also differ within the same cell type based on the initiating event.

In summary, this study provides some strong argument to support the notion that Rb_f and *p53* act synergistically in mammary tumorigenesis: (i) The median latency of mammary tumor formation in *WAP-Cre-;* $TgMFT_{121+/-;}$ / $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice is significantly reduced compared to *WAP-Cre;* $TgMFT_{121+/-;}$ mice (ii) Cre mediated excision of both alleles of *p53* is observed in all the mammary tumors tested in the *WAP-Cre-;* $TgMFT_{121+/-;}$ / $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice, indicating that complete loss of *p53* is a pre-requisite for tumor formation. Future

studies to test if LOH of *p53* occurred in the *WAP-Cre; TgMFT*$_{121+/-;}$ */ p53*$^{\Delta 2\text{-}10/+}$ mice can further strengthen this argument.

3.4.2 The complete loss of *p53* further enhances mammary tumor growth by reducing tumor latency.

Studying the complete loss of *p53* in the *TgWAP-T*$_{121}$ model (Simin et al. 2004) had been hindered by the fact that germline loss of *p53* using a knockout allele predisposed the mice to non-mammary tumorigenesis, mostly thymic lymphomas. So it was impossible to age these mice long enough to study mammary tumorigenesis. In the current model we overcame this difficulty by conditional and somatic inactivation of *p53* using the Cre-LOX P system. It was shown here that while loss of one allele of *p53* led to a 100% penetrant mammary tumor formation the latency for tumorigenesis was further reduced (shortened) by the complete loss of *p53*. Upon following post day 1 lactation regression in mammary glands by time-lapse biopsies, we found that while the *Rb*$_f$ inactivated mammary glands regressed normally, the addition of *p53* loss led to complete abrogation of mammary gland regression. Instead the mammary glands looked distinctly hyperplastic on day one lactation and progressively showed increased pro-tumorigenic events including formation of small tumor nodules in several areas of the gland. Curiously, *p53* conditional null mice alone do not develop mammary tumors even after prolonged aging (data not shown as the data for this cohort of mice was not collected systematically) in this model. It was shown before that also in the brain epithelium loss of p53 alone does not result in tumor formation though the combined loss of *Rb*$_f$ and *p53* results in tumors in the brain epithelia (Symonds et al. 1994a,

Symonds et al. 1994b). As the role of P53 is to maintain cell cycle checkpoint functions, it is conceivable that the loss of P53 results in a weakened genome that is highly susceptible to further oncogenic insults. An additional loss of a critical cell cycle regulator like pRB can then easily trigger tumor formation. In this model we find that while the loss of Rb_f promotes a measurable increase in cellular proliferation thus setting the stage for focal tumors to arise in the mammary gland, loss of *p53* alone seems to affect genetic stability of the mice in general, thus making them more tumor prone but not susceptible to mammary tumorigenesis per se.

3.4.3 A Model for Mammary Tumorigenesis Initiated by targeting the pRB Pathway

The $TgMFT_{121+/-}$ model is a clinically relevant model that can be used to further investigate the role of *pRb* and *p53* in mammary tumorigenesis. The *Rb* pathway is frequently found to be mutated in human breast cancers. Genomic studies have shown that sub classes of highly proliferative breast cancers that have a very poor clinical prognosis have a significant increase in E2F target gene expression, once again suggesting the importance of a deregulated *Rb* pathway in breast cancer (Fridlyand et al. 2006). But previous mouse models showed that mice heterozygous or nullizygous for $p16^{INK4a}$ (that acts upstream of RB and prevents RB inactivation by Cyclin D1) do not develop mammary tumors (Krimpenfort et al., 2001, Sharpless et al., 2001). Overexpression of Cyclin D1 (CCND1) that binds to and inactivates RB in the mammary gland is not highly oncogenic either (Wang et al., 1994). These results suggest that mutations in the *Rb* pathway are mutually exclusive and cell type specific. A mouse mammary tumor model using the C3Tag transgene (C3 promoter driving large T antigen that inactivates both pRB_f and P53) was able

to generate mammary tumors in mice by the combined inactivation of both RB_f and P53 (Green et al. 2000). However it was not possible using this model to understand whether either one of the two genetic lesions was required and sufficient to produce the effect, that is initiation and progression of mammary tumors. Also, new and yet unknown targets of the large T antigen used in the above model could contribute to tumorigenesis (Bocchetta et al., 2008). In the present model we were able to layer on two important genetic events and identify the specific contributions of each towards the promotion of mammary tumorigenesis. We have established here that Rb_f inactivation alone is not sufficient to promote mammary tumorigenesis but does result in abnormal mammary epithelium proliferation and delayed involution. This could set the stage for focal tumors to arise with a second genetic lesion. This occurs with the concomitant loss of *p53* resulting in high-grade mammary tumors that resemble human luminal type beast cancers. Further crosses of this model with breast cancer promoting genes like *Brca1*, *Brca2* and *Pten* will help provide useful insights into human breast cancer.

3.5 Materials and Methods

3.5.1 Transgenic breeding strategies

To study the effect of *p53* inactivation on mammary epithelium tumorigenesis *WAP-Cre; $TgMFT_{121}$* mice were mated to p53 $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$mice (obtained from MMHCC, FVB, Jonkers et al. 2001). To obtain *WAP-$Cre^{+/-}$; $TgMFT_{121}$ / p53 $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* mice *WAP-Cre; $TgMFT_{121}$ / p53 $^{\Delta 2\text{-}10/+}$* were generated first and then these mice were mated again to p53 $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice. We produced female mice with the genotypes *WAP-Cre; $TgMFT_{121}$/p53* $^{\Delta 2\text{-}10/+}$ and *WAP-Cre; $TgMFT_{121+/-}$ / p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$, and non-transgenic female littermates (p53 $^{\Delta 2\text{-}10/+}$

or $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$) served as controls. *TgMFT*$_{121\ +/-}$ mice were identified by PCR amplification of a 215-bp fragment using primers 5'-GCATCCAGAAGCCTCCAAAG -3' and 5'-GAATCTTTGCAGCTAATGGACC-3' complementary to the T_{121} sequence. In the *Wap-Cre; TgMFT*$_{121+/-}$, mice 5'-TGATGAGGTTCGCAAGAACC-3' and 5'-CCATGAGTGAACGAACCTGG-3' primers were used for the Cre sequence. For $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ detection of the deletion of Lox P sites was performed using primer sets 5'-CACAAAAACAGGTTAAACCCA-3' and 5'-GAAGACAGAAAAGGGGAGGG-3'. All PCR reactions were performed on DNA isolated from mouse toe clips and thermo cycling conditions were 30 cycles of 30 sec at 94°C, 30 sec at 58°C, and 50 sec at 72°C. All PCR reactions contained approximately 200 ng of toe DNA, 0.5 μM primers, 100 μM dNTPs, 2.5 units of *Taq*DNA polymerase, 2.5 mM $MgCl_2$, and 10x PCR buffer in a 20-μl total reaction volume.

3.5.2 Histopathology and Apoptosis Assays

Mammary gland tissue and tumor samples were dissected from *TgMFT*$_{121}$ transgenic or age matched littermate control animals. Part of each sample was snap frozen for RNA and DNA analysis and a portion was fixed overnight in 10% phosphate buffered formalin, transferred to 70% ethanol, and then embedded in paraffin. To analyze tumor histopathology mammary samples were sectioned for 10 successive layers. Sections at 4-um intervals were taken and stained with hematoxylin and eosin. Histopathological examination of the slides under light microscope was done as previously described. For detection of apoptosis levels in the samples staining of above sections using the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) kit as previously described (Symonds et

al. 1994a, Symonds et al. 1994b) was performed. The statistically significant differences in apoptosis levels between mice with varying genotypes was evaluated as described below ($p<.0.05$ considered of statistical significance).

3.5.3 Immunohistochemistry and Immunofluorescence

Immunohistochemical analysis for detection of specific markers in the mammary gland samples was performed on the formalin-fixed paraffin sections. For antigen retrieval the slides were boiled in citrate buffer (pH 6.0, Zymed, South San Francisco, CA) for 15 min. Endogenous peroxidase activity was quenched by incubating the slides for 10-min in 3% H2O2 in methanol. For IHC detection was done using the appropriate secondary antibody. The antibodies used were anti-K8/18 (Progen, 1:400), Anti-K5 (Covance, 1:5000), anti-Vimentin (RDI Technologies, 1:400), anti-SMA (SMA; 1:1,000, mouse A2537; Sigma, St. Louis, MO). Fluorescence detection for all antibodies was performed using Alexa Flour secondary antibodies.

3.5.4 Statistical Analysis

The nonparametric Wilcoxon rank-sum test (using Van der Waerden normal scores) was used for all pair-wise or two-group comparisons. This method tests for a differences, or shifts, in location between the two groups of interest, with a minimum of assumptions. This is in contrast to the parametric two-group t-test, which compares the means of the two groups, using assumptions that may not hold (i.e. normally distributed, with equal variances). All p-values reported are the nominal or unadjusted p-values. ('Unadjusted' meaning that they

have not been adjusted to account for multiple comparisons.) Adjustment was deemed not relevant due to the exploratory nature of this research project. All statistical analyses were performed with SAS statistical software, Versions 9.1, SAS Institute Inc., Cary, NC.

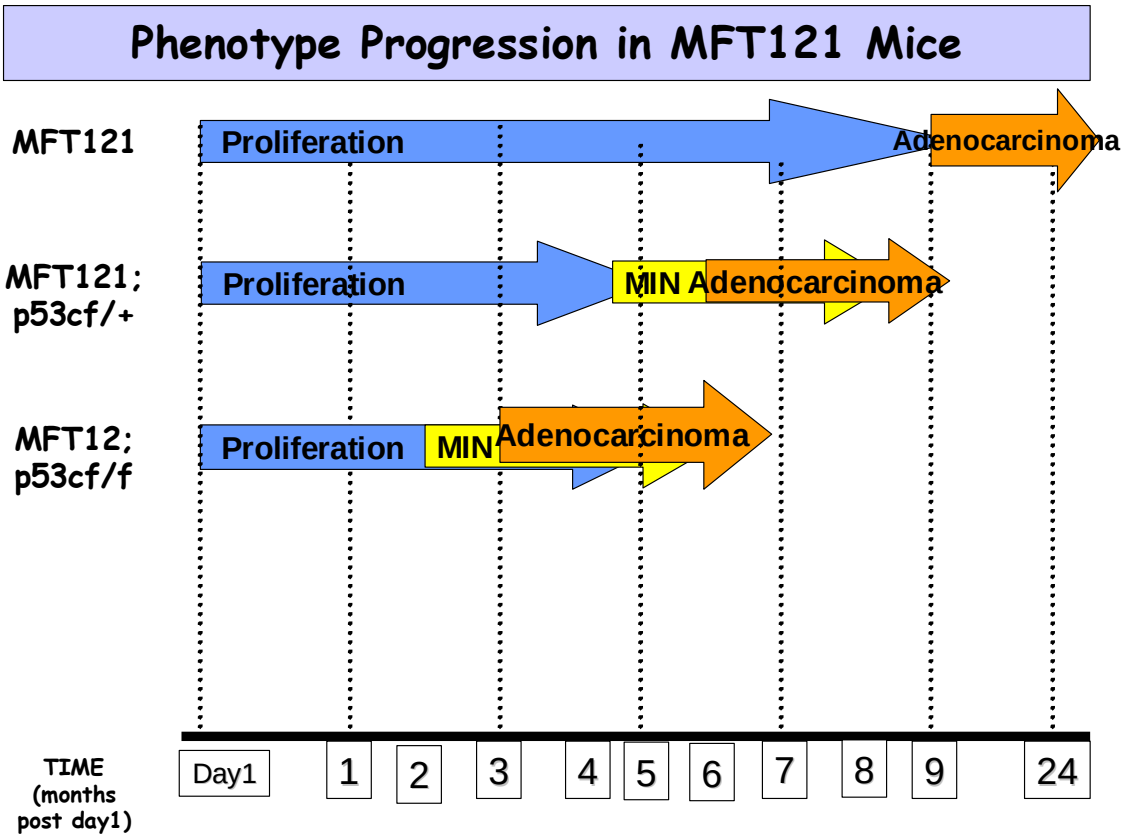


Figure 3.8, Progression of mammary tumors in *TgMFT*$_{121+/-}$ mice is accelerated and diversified by *p53* loss. The sequence of appearance of mammary lesions as well as the histopathological appearance of the lesion is graphed as a function of time based on the cumulative data presented in this chapter. For histopathological classification of the mammary tumors, H&E stained tumor sections were examined. Each tumor sample was scored for the presence of MIN lesions, atypia, nuclear pleomorphism, mitotic index and finally tumor cell lineage by Keratin stains. The earliest time examined for each genotype was on day 1 lactation. *TgMFT*$_{121+/-}$ mice do not show significant mammary tumor development on prolonged aging (> 365 days). *p53* haploinsufficiency accelerates mammary tumor formation in *TgMFT*$_{121+/-}$ mice. Finally, complete loss of *p53* further accelerates mammary tumorigenesis, and early tumor lesions appear as early as day 1 lactation in these mice.

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CHAPTER FOUR

Brca1 AND *p53* ACT SYNERGISTICALLY TO PROMOTE MAMMARY TUMORIGENESIS IN A Rb_f INACTIVATED MOUSE MAMMARY TUMOR MODEL

4.1 Abstract

Recent genomic analyses suggest that on an average a dozen cancer associated genes are mutated in each human breast tumor. Here we examine the effects of the combined loss of Rb_f (pRB_f) pathway signaling, *p53*, and *Brca1* in a conditional mouse model of breast cancer. Loss of *BRCA1* is often associated with *P53* loss in human tumors, and their collaboration in tumor suppression has been demonstrated in mouse models. The pRb pathway is frequently disrupted in all types of cancer, and previously we reported that diminished pRB_f activity in mouse mammary gland leads to increased cell proliferation and P53-dependent apoptosis and predisposes adenocarcinoma. We report here that the simultaneous loss of all three tumor suppressor activities drastically shortened tumor latency. Furthermore, the additional loss of *Brca1* in an Rb_f and *p53* inactivated background gives rise to distant metastasis to the lungs, indicating increased tumor malignancy. These studies establish a highly penetrant preclinical model for breast cancers associated with *Brca1* loss, and also provide insights into mechanisms by which breast cancer progression occurs including distant organ metastasis and increased genetic instability.

4.2 Introduction

Almost 50% of all familial breast cancers and about 90% of combined familial and ovarian cancers have germline mutations of the *BRCA1* gene (Hill et al. 1997, Miki et al. 1994, Moynahan 2002). There is currently no effective therapy for treating this class of mammary tumors. Their aggressive, metastatic nature and hormone and growth factor receptor status (usually ER, PR and HER2 negative) render them unresponsive to the more effective therapies that are currently available (Perou et al. 2000, Sorlie et al. 2001, Sorlie et al. 2003, van de Vijver et al. 2002). Unfortunately, the prognosis of these tumors is poor compared to all commonly occurring human mammary cancers due to their hormone non-responsiveness as well as frequent and early metastasis to distant organs, particularly to the lungs. *BRCA1* mutated familial breast cancers also have a basal like phenotype with the expression of mammary myoepithelial cell markers like Cytokeratin 5 (CK5) and Cytokeratin 14 (CK14). Some sporadic breast cancers also have similar characteristics (Perou et al. 2000, Sorlie et al. 2001). This suggests that sporadic cancers also have loss of *BRCA1* perhaps through promoter methylation pathways (Turner et al. 2004). The reason for their basal nature is not known though recent reports suggest that *BRCA1* plays a role in mammary stem cell differentiation. Thus loss of *BRCA1* may result in reduced stem cell differentiation to form mammary luminal epithelial cells, though the differentiation into myoepithelial cells remains unaffected. This may give explain the more basal like cancer patterns upon loss of *BRCA1* (Liu et al. 2008). Future work using mouse models is necessary to confirm this. A number of studies have examined *Brca1* function *in vivo* (Brodie et al. 2001, Brodie, Deng 2001, Deng, Brodie 2001). Both direct and conditional mutant mice of *Brca1* have been generated but none have recapitulated the nature of human familial breast cancers (Evers,

Jonkers 2006). *Brca1* germline knockout models resulted in embryonic lethality when bred to form homozygous mice, but the heterozygous *Brca1* knockout mice did not develop tumors on their own (Gowen et al. 1996, Hakem et al. 1996, Ludwig et al. 2001). Mice with conditional mutations of *Brca1* or with the hypomorphic allele of *Brca1* developed mammary tumors with long latencies but did not have the basal like tumor spectrum as seen in human cancers (Brodie et al. 2001, Ludwig et al. 2001, Xu et al. 2001). However the commonly used conditional allele of *Brca1* produces a splice variant of *Brca1* and therefore is not thought to produce a real "null" phenotype (Xu et al. 1999a). These results suggest that loss of *Brca1* alone is not sufficient to produce the mammary tumor phenotype seen in human breast cancers, but additional co-operating genetic lesions are required.

The combined role of *Brca1* and *p53* in mammary tumorigenesis (Brodie, Deng 2001, Liu et al. 2007, Moynahan 2002) has also been looked at using mouse models. The absence of P53 activity in these models greatly accelerates tumor onset and increases tumor incidence to from 25% to 100% (Brodie, Deng 2001, Marshall 1991), however, tumor formation is sporadic and stochastic, suggesting additional factors are required for tumorigenesis. Because pRb pathway loss is a common event in cancer, particularly cyclin D1 overexpression or p16 loss in breast cancer (Marshall 1991, Ortega, Malumbres & Barbacid 2002, Sherr 1996, Weinberg 1995), we examined the combined loss of *Rb*, *Brca1*, and *p53*.

In this study we have developed a highly penetrant and reproducible mouse model that mimics human *BRCA1* loss-mediated breast cancer. By layering on genetic mutations that occur very frequently in all human cancers, we have generated a mouse model that mimics several specific aspects of the human disease, including rapid tumor progression, distant metastasis, high mitotic index, large areas of necrosis within the tumors, as well as

high levels of angiogenesis. We have also attempted by specific genetic mutation combinations to provide insight into the disease etiology. For example, while Rb_f disruption alone does not lead to mammary tumor formation in the present transgenic model, loss of *p53* along with Rb_f results in a 100 % penetrant tumor formation. Also, as seen previously in other epithelial cancer models (Simin et al. 2004), loss of *p53* heterozygosity further reduces tumor latency and increases aggressiveness, although, loss of *p53* along with Rb_f did not result in metastasis, increased angiogenesis or necrosis. In contrast, layering on the loss of *Brca1* resulted in a more acute reduction in tumor latency along with distant metastasis to the lungs, large areas of necrosis and angiogenesis. Interestingly, loss of *Brca1* alone along with *Rb* pathway inactivation does not lead to reduced latency in mammary tumorigenesis, re-affirming the important role of *p53* loss in tumor progression in the mammary gland.

4.3 Results

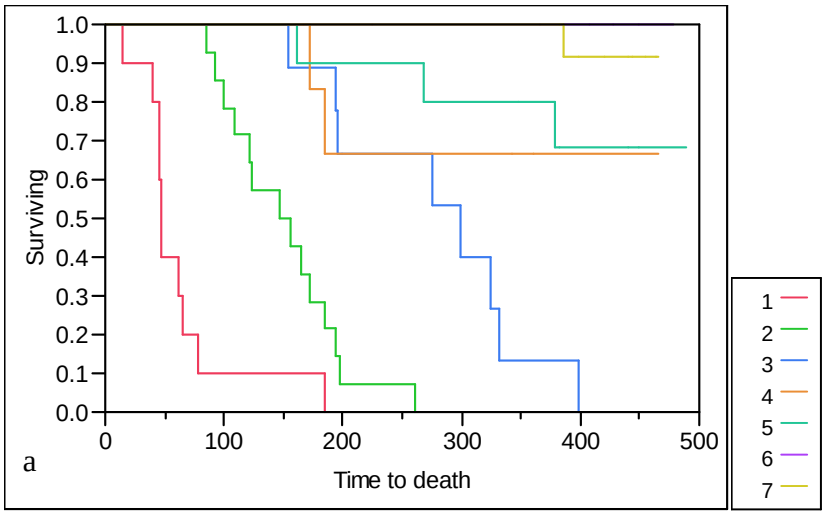
4.3.1 Somatic mutation of *p53* and *Brca1* in the $TgMFT_{121}$ mice causes rapid acceleration of mammary tumor formation

Loss of *BRCA1* and *P53* frequently occur together in human breast cancers. *BRCA1* mutated cancers also have a high proliferation signature with significant overexpression of E2F target genes. Since inactivation of the *RB* pathway can turn up E2F transcription factors, it is likely that *RB* pathway mutations (resulting in the loss of the growth inhibitory effect exerted by pRb) also occur in these breast cancers. A mammary tumor model to study the inactivation of all three genes (*RB1*, *P53* and *BRCA1*) has not been generated before. To this end we generated a conditional mouse model with mammary specific inactivation of *Rb* family, *p53* and *Brca1*. We crossed conditional $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ (FVB background, MMHCC,

Jonkers et al., 2001) and *Brca1*$^{F/F}$ mice (FVB background, MMHCC, Liu et al., 2007) to *WAP-Cre*; *TgMFT*$_{121+/-}$ mice described before. Cre recombinase expression is restricted to the mammary luminal epithelial cells only. We also generated *WAP-Cre; TgMFT*$_{121+/-}$ */ Brca1*$^{\Delta/\Delta}$ mice to study the effect of *Rb*$_f$ and *Brca1* inactivation together in the mammary gland, without the effect of *p53* inactivation. Female *WAP-Cre*; *TgMFT*$_{121+/-}$ */p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ */Brca1* $^{\Delta/\Delta}$ mice developed mammary tumors with a median latency of 50 days **(Figure 4.1, a, Survival Curve)** suggesting synergistic effect of the loss of the three tumor suppressors (*Rb1*, *p53* and *Brca1*) in mammary tumor formation. *WAP-Cre; TgMFT*$_{121+/-}$ */ Brca1* $^{\Delta/\Delta}$ mice developed mammary tumors with significantly longer latencies of more than 400 days. This indicated that *Rb1* and *Brca1* loss most likely did not co-operate in mammary tumorigenesis and other events are required for tumors to form. Ten mice from each cohort were maintained in the mouse colony and observed for tumors following at least one cycle of pregnancy and lactation. Mammary tumors in the *WAP-Cre; TgMFT*$_{121+/-}$ */p53*$^{cf/f}$ */Brca1*$^{cf/f}$ grew rapidly from a 100 cm^3 to 1500 mm^3 over a period of two to three days and appeared highly angiogenic. *WAP-Cre; TgMFT*$_{121+/-}$ */p53*$^{\Delta 2\text{-}10/+}$*/Brca1* $^{\Delta/+}$ mice also developed mammary tumors with a median latency of 382 days , indicating stochastic events like LOH of *p53* and *Brca1* occurring in these tumors. The *WAP-Cre; /p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ */Brca1* $^{\Delta/\Delta}$ mice (with wild type *Rb*$_f$) developed mammary tumors with a median latency of 178 days. This was significantly longer than the time taken by the triple mutant mice (*WAP-Cre; TgMFT*$_{121+/-}$ */p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ */Brca1*$^{\Delta/\Delta}$) to develop mammary tumors and once again confirming the synergistic effect of *Rb1*, *p53* and *Brca1* loss in mammary tumorigenesis.

The *WAP-Cre; TgMFT*$_{121+/-}$ */p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ */Brca1*$^{\Delta/\Delta}$ tumors (referred to as the triple mutant tumors) are highly proliferative (as observed by positive Ki67 staining, **Figure 4.1, b**)

and appeared highly angiogenic (**Figure 4.1, c**) and locally advanced. Distant metastasis was also observed frequently (in 60% of the mice) in the lungs of these mice. This was a significant finding, as distant organ metastasis was never observed in the *WAP-Cre; TgMFT*$_{121+/-}$ */p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice with wild type *Brca1*. This result indicated that metastasis was a direct outcome of *Brca1* loss in this model. PCR detected loss of *Brca1* and *p53* alleles in these tumors (**Figure 4.2**).



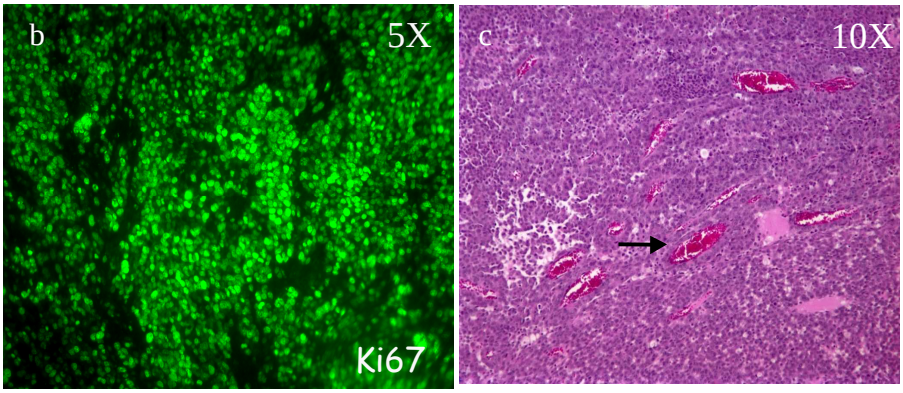


Figure 4.1, (a) Mammary Tumor Latency is shortened by *p53* reduction and further accelerated by combined loss of *p53* and *Brca1*. Kaplan Meir survival curves of *WAP-Cre; TgMFT*$_{121+/-}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ */ Brca1* $^{\Delta/\Delta}$ female mice (red curve, class 1, n=10, median latency 50 days), *WAP-Cre; TgMFT*$_{121+/-}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ female mice (green curve, class 2, n=15,

median latency 150 days), *TWAP-Cre; TgMFT$_{121+/-}$ / p53* $^{\Delta 2\text{-}10/+}$ female mice (blue curve, class 3, n=9, median latency 275 days), *WAP-Cre; p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ / *Brca1* $^{\Delta/\Delta}$ female mice (brown curve, class 4, n=8, median latency 178 days), *WAP-Cre; TgMFT$_{121+/-}$ / p53* $^{\Delta 2\text{-}10/+}$/ *Brca1* $^{\Delta/+}$ female mice (dark green, class 5, n=10, median latency 380 days) , *WAP-Cre; TgMFT$_{121+/-}$* female mice (violet curve, class 6, n=14, no mammary tumors) and *WAP-Cre; TgMFT$_{121+/-}$ / Brca1* $^{\Delta/\Delta}$ female mice (pale green, class 7, n=13, median latency 380 days). The difference in median latencies between all the curves were highly significant indicated by an over all P value of 0.00001 calculated using the Log Rank Test for survival. The mice were sacrificed when the mammary tumors reached a diameter of about 1 cm. Several mice developed multiple mammary tumors in multiple mammary glands and those were not counted separately but included in the overall mouse survival. **(b)** A representative image of Ki67 staining of Triple mutant tumors show high proliferation as indicated by positive Ki67 stain. **(c)** H&E staining of a representative triple mutant tumor shows multiple blood vessels **(arrow)**.

Classes of Tumors in Survival Curve: 1= *WAP-Cre; TgMFT$_{121+/-}$ / p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ */Brca1* $^{\Delta/\Delta}$, 2= *WAP-Cre; TgMFT$_{121+/-}$ / p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$, 3= *WAP-Cre; TgMFT$_{121+/-}$ / p53c* $^{\Delta 2\text{-}10/+}$, 4= *WAP-Cre; / p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ / *Brca1* $^{\Delta/\Delta}$, 5= *WAP-Cre; TgMFT$_{121+/-}$ / p53* $^{\Delta 2\text{-}1/+}$ / *Brca1* $^{\Delta/+}$, 6= *WAP-Cre; TgMFT$_{121+/-}$* *7= WAP-Cre; TgMFT$_{121+/-}$ / Brca1* $^{\Delta/\Delta}$

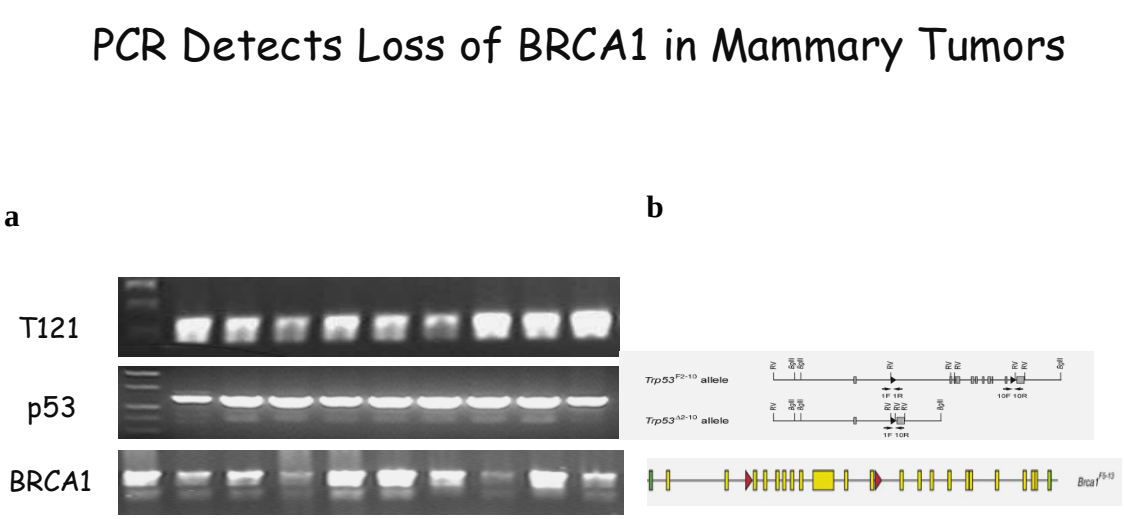


Figure 4.2 (a) PCR shows detection of T_{121} and loss of *p53* and *Brca1* in the mammary tumors. This indicates all three initiating events are occurring in the tumors efficiently. **(b)** *Brca1* floxed allele design is shown here. Allele design is taken from {{396 Evers,B. 2006}}.

4.3.2 Time point biopsies show earlier development of mammary lesions in triple mutant mice.

A major advantage to studying tumorigenesis in an *in vivo* model is the ability to track progressive changes over time. We sampled biopsies from the mammary glands of two cohorts of mice (*WAP-Cre; TgMFT*$_{121+/-}$ */ p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ */ Brca1* $^{\Delta/\Delta}$ and *WAP-Cre; TgMFT*$_{121}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$) from day1 lactation until tumorigenesis. Three mice were used from each cohort on day one lactation, two weeks post day one lactation and 6 weeks post day one lactation (see Methods). Pups were removed at day one lactation to control for the effects of mammary gland involution. Biopsies were also performed on three control Cre negative animals (*TgMFT*$_{121+/-,}$ no Cre) in parallel experiments. We also looked at biopsy mammary glands of *WAP-Cre; TgMFT*$_{121}$*+/- / Brca1* $^{\Delta/\Delta}$ to look for progressive changes in these glands that may predispose to tumorigenesis. The *WAP-Cre; TgMFT*$_{121\ +/-/}$ */ Brca1* $^{\Delta/\Delta}$ mouse mammary glands showed extensive apoptotic bodies in the mammary gland lumen (**Figure 4.3, c, arrow**) and a greater stromal response in the mammary fat pad **(Figure 4.3, c, star)** indicated by stromal cell hyper-proliferation. The *WAP-Cre; TgMFT*$_{121+/-}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$animals showed some abnormalities including dysplasia **(Figure 4.3, d)** and hyperplastic mammary glands at day1 lactation. The *WAP-Cre; TgMFT*$_{121+/-}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$*/Brca1* $^{\Delta/\Delta}$ showed a much more advanced phenotype on day 1 lactation, including apoptotic bodies in the mammary gland lumen, regions of mammary intraepithelial neoplasia (MIN) lesions and hyperproliferative regions within the mammary gland **(Figure 4.3, e)**. In biopsies performed two weeks post day one lactation the *WAP-Cre; TgMFT*$_{121+/-}$ */ Brca1* $^{\Delta/\Delta}$ mice show normal involution but with remnant large mammary structures that fail to involute (**Figure 4.3, h**). This is reminiscent of the incomplete involution observed in the *WAP-Cre; TgMFT*$_{121+/-}$ mice

(**Figure 4.3, g**). *WAP-Cre; TgMFT*$_{121+/-}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$mouse mammary glands showed a marked absence of involution and regions of advanced hyperplasia (mammary epithelial cells piling into the lumen, **Figure 4.3, i**). The *WAP-Cre; TgMFT*$_{121+/-}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$*/Brca1* $^{\Delta/\Delta}$ mice at this time point showed a dramatic advancement of phenotype with small tumor nodules beginning to form in some parts of the mammary gland and significant disruption of normal mammary gland alveolar structure along with complete lack of involution **(Figure 4.3, j)**. On the final biopsies performed on these mice at 6 weeks post day 1 lactation all five of the *WAP-Cre; TgMFT*$_{121+/-}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$*/ Brca1* $^{\Delta/\Delta}$ mice had already formed small but visible/palpable mammary tumors in multiple mammary glands **(Figure 4.3, o)**. Also notable was the fact that multiple mammary glands in the triple mutant mice developed mammary tumors with similar latencies, whereas the double mutants (*WAP-Cre; TgMFT*$_{121+/-}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$) only had tumors in one mammary gland typically. Also, the latency of tumor formation in all these genotypes is consistent with that of mice of the same genotype that did not have biopsies performed on them. In summary, the triple transgenic *WAP-Cre; TgMFT*$_{121+/-}$*/p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$*/ Brca1* $^{\Delta/\Delta}$ mice showed a very advanced phenotype in a very short time compared to the double transgenic counterparts. The layering of genetic events in these mice allows dissecting of the sequence of events necessary for advanced tumor phenotype and points out to the potentially critical combination of both *Brca1* and *p53* loss occurring in the context of Rb_f loss to give rise to an aggressive and rapidly growing mammary tumor phenotype.

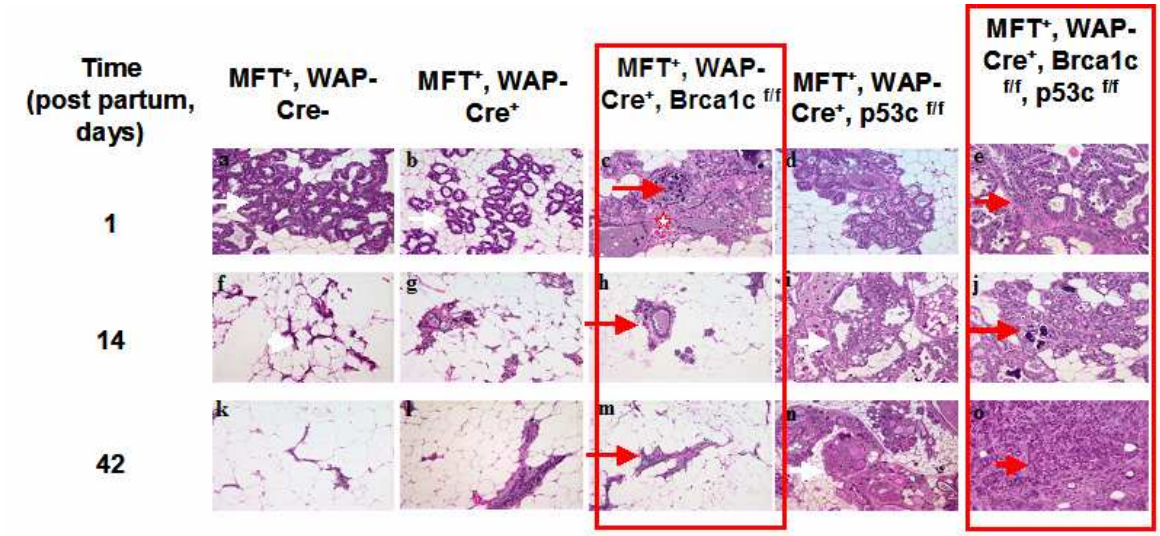


Figure 4.3 Histological characterization and progression of mammary tumorigenesis show faster tumor progression in triple mutant mice. Mammary morphologies in H&E-stained sections of Day1 Lactation mice are shown: (**a**) *TgMFT*$_{121+/-}$ (Cre negative littermate control*)* mice with a normally thick layer of luminal (milk secreting) epithelial **(white arrow) (b)** *WAP-Cre; TgMFT*$_{121+/-}$ **(c)** *WAP-Cre; TgMFT*$_{121+/-}$ */ Brca1* $^{\Delta/\Delta}$ **(d)** *WAP-Cre; TgMFT*$_{121+/-}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ and **(e)** *WAP-Cre ; TgMFT*$_{121+/-}$ */ Brca1* $^{\Delta/\Delta}$ */p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$. The epithelial cells in *WAP-Cre; TgMFT*$_{121+/-}$ Day 1 lactation mammary glands are normal looking with regular luminal epithelial ductal structure and a thin myoepithelial layer surrounding the luminal cells (**b**). *WAP-Cre; TgMFT*$_{121+/-}$ */ Brca1* $^{\Delta/\Delta}$ mammary glands contain an extensive hypercellular stroma **(c, star)** and no clear ductal pattern (**red arrow, c**). The epithelial cells in *WAP-Cre; TgMFT*$_{121+/-}$ / p53 $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ Day 1 lactation mammary glands grow in dense patterns piling on top of each other with ductal filling **(d)**. Similarly, *WAP-Cre; TgMFT*$_{121+/-}$ */ Brca1* $^{\Delta/\Delta}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ show dense epithelial cell clusters **(e, red arrow)** and thick stromal layers in Day 1 lactation. On day 14, *TgMFT*$_{121+/-}$ (*Cre* negative) and *WAP-Cre; TgMFT*$_{121+/-}$ mice show fairly normal regression of mammary glands with a large population of involuting mammary glands **(arrow, f, g)**. *WAP-Cre; TgMFT*$_{121+/-}$ */ Brca1* $^{\Delta/\Delta}$ mice show slightly delayed involution seen by large ducts that fail to involute **(red arrow, h)**. The *WAP-Cre; TgMFT*$_{121+/-}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice show significantly delayed mammary gland involution at Day 14 of lactation **(i, white arrow)**. Mammary duct hyperplasia and early tumor lesions develop in *WAP-Cre; TgMFT*$_{121+/-}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ */ Brca1* $^{\Delta/\Delta}$ mice as early as 14 days post day 1 lactation **(red arrow, j)**. At day 45 *TgMFT*$_{121+/-}$ (*Cre negative*) mice show complete involution **(k)** and *WAP-Cre; TgMFT*$_{121+/-}$ have some remnant gland structures in mammary fat pad **(l)**. *WAP-Cre; TgMFT*$_{121+/-}$ */ Brca1* $^{\Delta/\Delta}$ are similar to **(l)** and continue to show delayed involution pattern **(m)**. The *WAP-Cre; TgMFT*$_{121+/-}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice show significantly abnormal gland development with regions of tumor nodules **(white arrow, n)** but no full blown mammary tumor yet. The *WAP-Cre; TgMFT*$_{121+/-}$*/p53c* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ */ Brca1* $^{\Delta/\Delta}$ mice have a dramatic phenotype with palpable mammary tumor formation **(red arrow, o)** in all the biopsied mammary glands. (**n = 3 for each genotype)**

Keratin marker staining of the hyper-proliferating mammary glands of the triple negative mice showed an increase in the population of Keratin 18 positive cells in the mammary gland lumen **(Figure 4.4, b)**. Thus the luminal epithelial cells were seen to be the expanding population in this genotype, as observed before with the Rb_f, *p53* inactivated (double null) mice. However, in the triple null genotype, there were nests of Keratin 5 positive cells that had not been seen in the double null mice **(Figure 4.4, a, arrow)**. This is also a feature found in human basal like breast cancers.

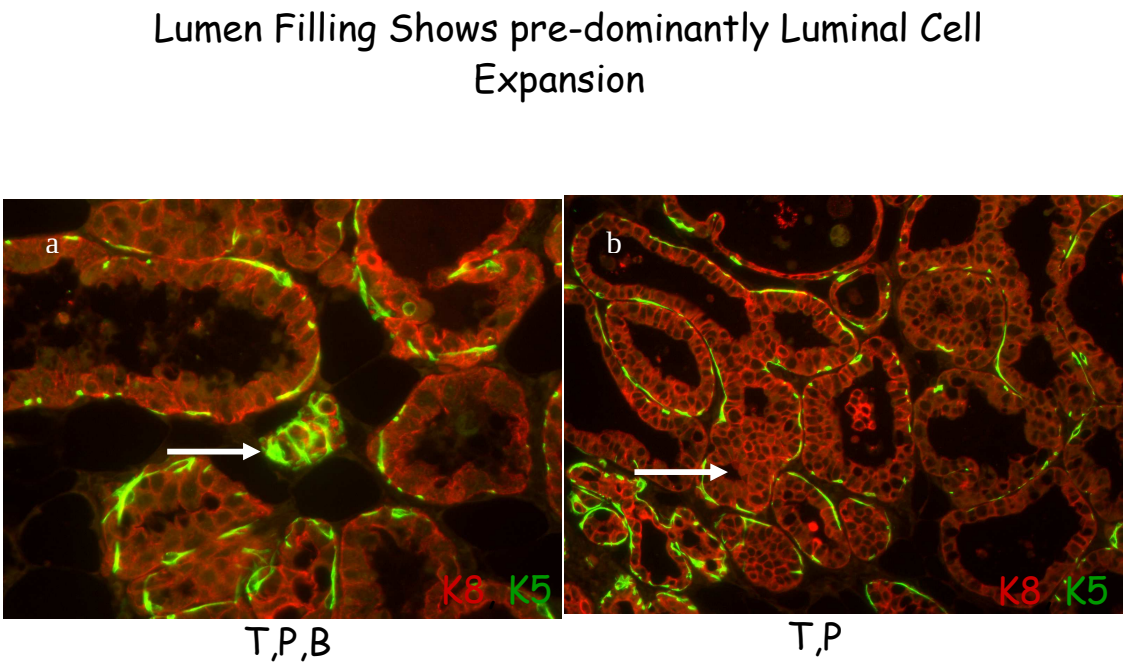


Figure 4.4 Keratin staining of progressively hyperplastic mammary glands from triple null and double mammary glands show increased luminal cell expansion. Mammary glands biopsied from *WAP-Cre; TgMFT*$_{121\ +/-}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ and *WAP-Cre; TgMFT*$_{121+/-}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ */ Brca1* $^{\Delta/\Delta}$ mice on day fourteen post day one lactation showed focal hyperplasia, increased lumen filling and absence of mammary gland involution. Staining with keratin markers to detect lineage of the cells expanding showed Keratin 8/18 positive cells forming the focal hyperplasias **(b, arrow)**. Keratin 5 stained clusters of cells were observed only in the triple null mammary glands, as early as day fourteen post day one lactation **(a, arrow)**. (T= T_{121} positive, P=*p53* mutant, B= *Brca1* mutant)

4.3.3 Role of *Brca1* and *p53* in apoptosis and proliferation

It was previously demonstrated that Rb_f inactivation by T_{121} expression in the choroid plexus epithelial (CPE) cells (Lu et al., 2001) and mammary epithelial cells (Simin et al., 2004) induced both proliferation and apoptosis and in the mammary gland only apoptosis but not proliferation was *p53* dependent. To determine the role of *Brca1* loss in mammary gland, proliferation and apoptosis proliferation were assessed using Ki67 staining in day one lactation mammary glands of *WAP-Cre; $TgMFT_{121+/-}$ /p53 $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$*, *WAP-Cre; $TgMFT_{121+/-}$ / Brca1 $^{\Delta/\Delta}$* and *WAP-Cre; $TgMFT_{121+/-}$ /p53 $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ / Brca1 $^{\Delta/\Delta}$* mice. Apoptosis was assayed by TUNEL staining in the mammary glands of all the above cohorts on day 1-lactation and compared with TUNEL staining in control *$TgMFT_{121+/-}$* mice. *WAP-Cre; $TgMFT_{121+/-}$* mice showed a higher level of proliferation compared to the Cre-negative controls on day 1 lactation (**Figure 4.5, a**). The apoptosis levels of these mice (*WAP-Cre; $TgMFT_{121+/-}$*) at day 1 were comparable to the non-transgenic littermates (**Figure 4.5, b**). The absence of tumor formation in these mice was attributed to the possible low level of transgene expression in these mice.

Both proliferation and apoptosis levels increased significantly with the combined loss of Rb_f and *Brca1* (P =0.0003 for proliferation and 0.00003 for apoptosis). This indicated that the combined loss of two major cell cycle checkpoint genes, *Rb1* and *Brca1* resulted in greatly increased proliferation as early as day one lactation in the mammary gland. However the combined increase in apoptosis indicated the role of a functional *p53* in these mice that balanced the high levels of proliferation and prevented early mammary tumor progression. While proliferation levels did not change significantly with the loss of *p53* in these mice (P values for each pairs listed in **Figure 4.5**) the apoptosis levels dropped significantly with the

loss of *p53* which indicated that most of the apoptosis in the mammary gland was *p53* regulated.

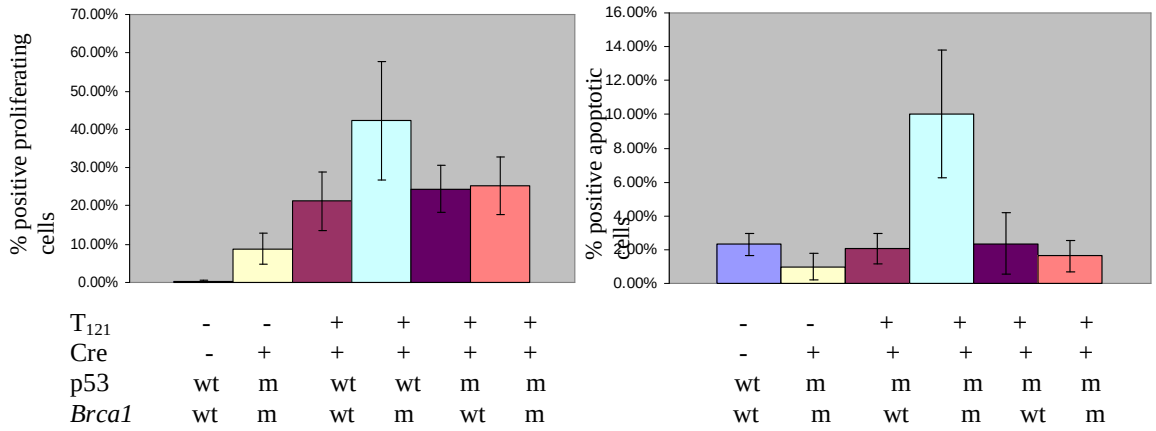


Figure 4.5, Loss of *Brca1* Activity Increases Proliferation and Apoptosis in Day One Lactation Mammary Glands. Upon loss of *Brca1* in a *p53* wild type background there is a significant increase in proliferation (compared to mice with loss of Rb_f alone, **a, plot # 3**) observed in mammary glands on day one lactation (P=0.00003, **a, plot # 4**). Loss of p53 either in combination with expression of T_{121} and wild type *Brca1* background (P= 0.76911, **a, plot # 5**) or in combination with loss of *Brca1* and expression of T_{121} (P= 0.87304, **a, plot # 6**) did not significantly alter the levels of proliferation. In plot b, representative apoptosis levels of each mouse genotype are indicated by percentage of TUNEL positive cells. The combined loss of *Brca1* with expression of T_{121} significantly increases the levels of apoptosis as indicated by plot # 4 in b (P=0.00003). Loss of *p53* either in combination with loss of Rb_f (by expression of T_{121} in a *Brca1* wild type background, P= 0.00002, **b, plot # 5**) or the combined loss of all three (pRb_f, *p53* and *Brca1*, P= 0.00008, **b, plot # 6**) significantly lowers the apoptosis levels bringing them down to apoptosis levels observed in normal ($MFT_{121,}$ **b, plot # 1**) day one lactation mammary glands. (m=mutant indicating floxed/floxed alleles after Cre expression). Two mice for the controls and three mice from the experimental animals were used. An average of 200 cells from four independent mammary gland sections from each mouse were counted.

4.3.4 Loss of *Brca1* results in persistent mitotic arrest

Cell culture experiments have suggested that loss of *Brca1* in mouse embryonic fibroblasts induces mitotic arrest by causing spindle checkpoint defects. Increased levels of P53 dependent apoptosis also accompany this mitotic arrest (Wang, Yu & Deng 2004). These

studies have found functional interactions of BRCA1 with spindle checkpoint protein MAD 2. Loss of BRCA1 was shown to significantly down regulate MAD 2 resulting in spindle checkpoint defects. In this study we noticed a marginal decrease in proliferation in day one lactation mammary glands of *WAP-Cre; TgMFT*$_{121+/-}$ */Brca1* $^{\Delta/\Delta}$ mice upon the concomitant loss of *p53*. All though this result was not significant it suggested a potential cell cycle arrest in the *WAP-Cre; TgMFT*$_{121+/-}$ */ Brca1* $^{\Delta/\Delta}$ mice that had a functional *p53*. We assessed the levels of mitosis in three cohorts of transgenic mice: *WAP-Cre; TgMFT*$_{121+/-}$ */ Brca1* $^{\Delta/\Delta}$ mice, *WAP-Cre; TgMFT*$_{121+/-}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice and *WAP-Cre; TgMFT*$_{121+/-}$ */ Brca1* $^{\Delta/\Delta}$*/p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice. Our results indicated that cells in the *WAP-Cre; TgMFT*$_{121+/-}$ */ Brca1* $^{\Delta/\Delta}$ mice undergo significant mitotic arrest that is also accompanied by *p53* associated apoptosis (as shown by **Figure 4.6**). This mitotic arrest persists through day 14 post day one lactation, as indicated by Phospho Histone H3 (PH3) staining of mammary glands from all three cohorts of mice on their day 14 (post day one lactation) biopsy samples (**Figure 4.6**). This gives further mechanistic insight into the co-operative events necessary for mammary tumorigenesis in this model. Upon the combined loss of Rb_f and *Brca1*, there is a significant increase in proliferation (measured by Ki67 staining cells in S phase). This is also accompanied by mitotic arrest of cells at the M phase of the cell cycle (measured by Phospho Histone H3 staining of cells) as well as P53-mediated apoptosis. So mammary tumors occur in these mice only after the stochastic loss of *p53* explaining their long latency (shown in survival curve, **Figure 4.1, a**). Upon the combined loss of Rb_f, *Brca1* and *p53* the tumor cells undergo uncontrolled proliferation with no apoptosis mediated by P53. However the small number of animals used in this study makes it difficult to make a definitive conclusion about mitotic arrest at least in this model. It suffices to say that the pRb_f, *Brca1* double mutant

animals show a trend towards mitotic arrest accompanied with increased *p53* mediated apoptosis on day one lactation. So loss of *p53* is required in these mice for mammary tumors to progress.

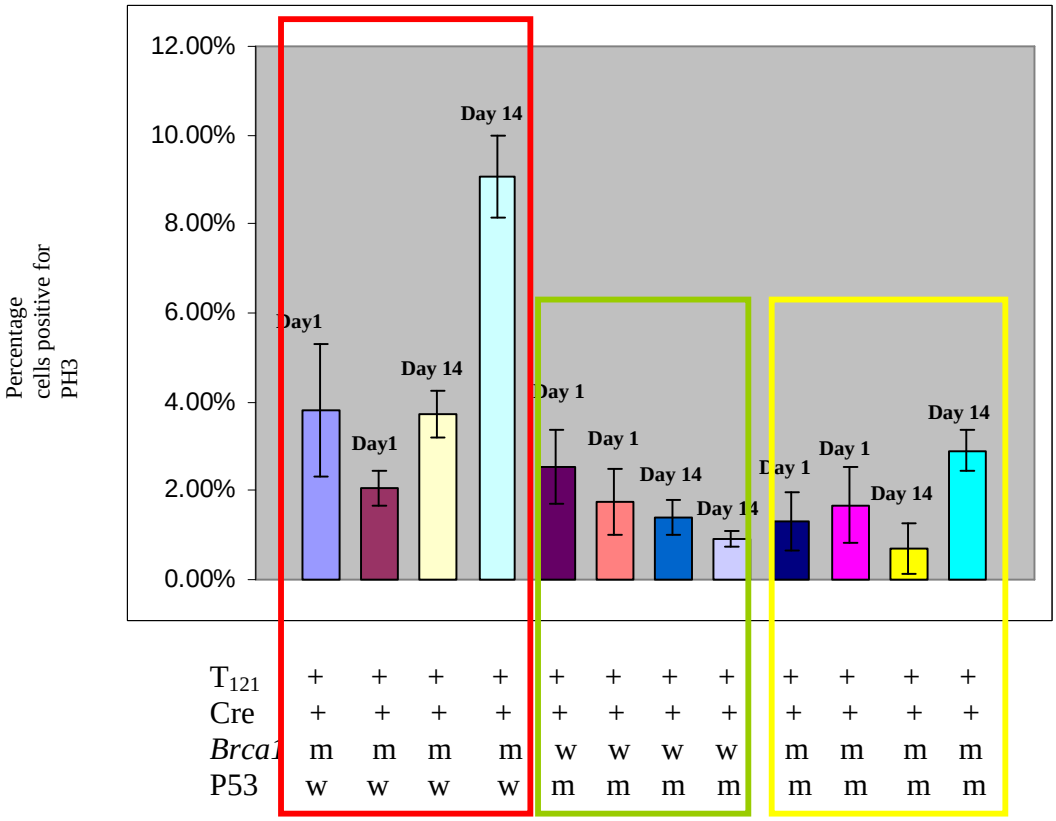


Figure 4.6 Loss of *Rb$_f$* and *Brca1* results in persistent mitotic arrest in day one and day fourteen (post day one) lactation mammary glands. Mammary glands from two animals of each genotype were stained for PH3 on day one and day 14 lactation. The *WAP-Cre; TgMFT$_{121+/-}$ / Brca1 $^{\Delta/\Delta}$* mice (indicated by red box) showed a greater percentage of cells in mitosis on day one as well as day fourteen lactation (P=.0.00269 with *WAP-Cre; TgMFT$_{121+/-}$ / p53 $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* mice, green box, and P=0.00261 with *WAP-Cre; Tg MFT$_{121\ +/-}$ / p53 $^{\Delta 2\text{-}10/\Delta\ 2\text{-}10}$/Brca1 $^{\Delta/\Delta}$* animals , yellow box). This mitotic arrest is significantly decreased on day fourteen in *WAP-Cre; TgMFT$_{121+/-}$ / p53 $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* (green box) and *WAP-Cre; Tg MFT$_{121\ +/-}$ / p53 $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ /Brca1 $^{\Delta/\Delta}$* animals (yellow box). There is no significant difference in mitotic cell number of the *WAP-Cre; TgMFT$_{121+/-}$ / p53 $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* and *WAP-Cre; Tg MFT$_{121\ +/-}$ / p53 $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ /Brca1 $^{\Delta/\Delta}$* animals either on day one or day fourteen post day one lactation (P=0.62025). Two mice from each cohort were used for this analysis. An average of 300 cells from 4 individual sections of each animal were counted.

4.3.5 Loss of *Brca1* induces a shift from heterogeneous, locally invasive mammary adenocarcinomas to more differentiated and metastatic mammary cancer

While *WAP-Cre; TgMFT*$_{121+/-}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice were diagnosed to have intermediate to high grade mammary adenocarcinomas with a fairly high mitotic index and absence of large necrotic areas or distant organ metastasis (summarized in **Table 4.1**) loss of *Brca1* significantly increased the percentage of solid differentiated adenocarcinomas (**Figure 4.7, a**) and glandular tumors (**Figure 4.7, b**). There was also a significant increase in the appearance of pilar like tumors (**Figure 4.7, c**) with keratin swirls and ghost cells. The solid and glandular tumors were frequently positive for Keratin 8 and 18 (**Figure 4.7, d, e**). The pilar tumors had an inverted phenotype with an inner Keratin 5 positive myoepithelial layer and outer Keratin 8 and 18 positive luminal epithelial cells (**Figure 4.7, f**). The aggressive nature of these tumors as well as distant metastasis suggested possible EMT occurring. When stained with Vimentin and SMA the solid tumors showed an increased positivity for Vimentin (**Figure 4.7, g**) The glandular tumors showed an increase in SMA positive infiltrating stromal cells but no substantial Vimentin staining (**Figure 4.7, h**). The pilar tumors displayed an inner layer of Vimentin positive cells and an outer layer of SMA positive cells (**Figure 4.7, i**). The triple null tumors also displayed large areas of necrosis (**Figure 4.8, a**) and showed positive CK5 and CK14 staining in clusters (**Figure 4.9, a, b**). Several of these clusters co-stained for CK8 and CK 14 (**Figure 4.9, a, b**). This was a very interesting finding and suggests the intriguing possibility that the *WAP-Cre; TgMFT*$_{121+/-}$ */p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ */ Brca1* $^{\Delta/\Delta}$ may have arisen from a progenitor cell niche within the mammary gland. All tumors were listed as high grade (grade III by the Nottingham Scale for grading

human cancers, listed in **Table 4.2**). The timing of appearance of the tumors in each genotype as well their histopathological appearance are listed in **Table 4.3** and shown graphically in **Figure 4.10**. The triple mutant tumors in these mice had large areas of necrosis and also metastasized very frequently to the lungs and formed visible tumor nodules in the lung (**Figure 4.8, a, b**).

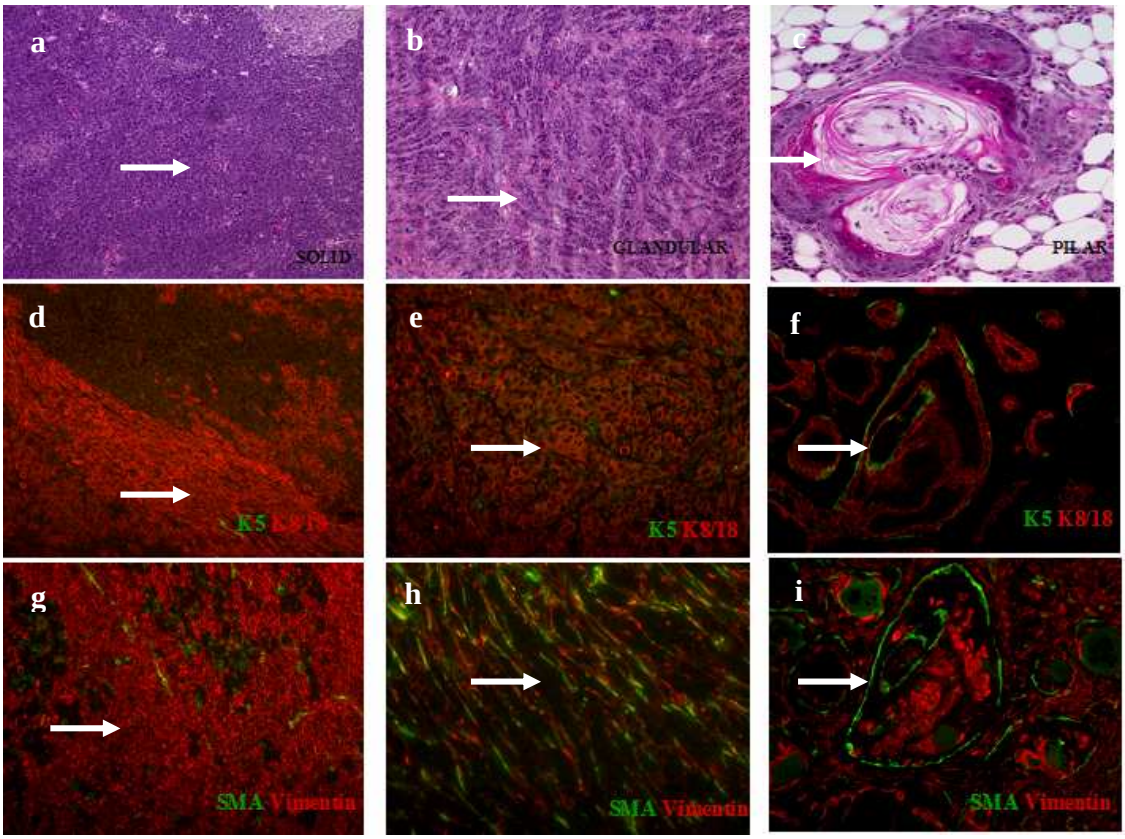


Figure 4.7, Marker characterization of *Brca1* mutant mammary tumors shows an altered cell lineage. Histopathology of representative mammary tumor types from *WAP-Cre; TgMFT*$_{121+/-}$ */ Brca1c* $^{\Delta/\Delta}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice show diverse and distinct tumor morphologies associated with each genotype. Serial sections from representative tumors were stained for Hematoxylin and Eosin **(Top Panel)** or with antibodies against Cytokeratin 8/18 and Cytokeratin 5 **(Middle Panel)** and Smooth Muscle Actin and Vimentin **(Bottom Panel)**. The top panel shows the three distinct types of tumors arising in the *WAP-Cre; TgMFT*$_{121\ +/-}$ */Brca1* $^{\Delta/\Delta}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice, solid undifferentiated **(a)**, glandular **(b)** and pilar **(c)**. The solid tumors and glandular tumors are frequently positive for Keratin 8/18 **(d, e)**. The pilar tumors have an inner layer of Keratin 5 positive and outer layer of Keratin 8 and 18 positive cells **(f)**. The solid tumors in the triple null mice are frequently Vimentin positive and SMA

negative **(g)**. The glandular tumors have infiltrating SMA positive cells and are usually Vimentin negative **(h)**. The Pilar tumors show an inner layer of Vimentin positive cells surrounded by a SMA positive layer **(i)**. The percentage of animals of each genotype showing the different phenotypes is summarized in **Table 4.1**. As each tumor has multiple different phenotypes the sum in each case is greater than a hundred percent.

Tumor Types in the different genotypes

Genotypes	n	Solid	Glandular	Pilar	Spindle	Angiogenic	Necrotic	Metastatic
MFT121+,Wap-Cre+,p53cf/+	10	71%	-	-	51%	50%	8%	-
MFT121+, WapCre+, p53cf/f	14	55%	18%	13%	55%	27%	18%	-
MFT121+,Wap-Cre+,p53cf/f, BRCA1cf/f	5	66%	88%	33%	20%	100%	100%	60%

Table 4.1 Tumor phenotypes in the different genotypes. Mammary tumor spectrum shifts from more solid and spindloid tumors in *p53* heterozygous and *p53* nullizygous animals to glandular and pilar tumors with loss of *Brca1*. Loss of *Brca1* also induces distant metastasis.

Brca1 Signature in Mammary Tumors : Geographic Necrosis & Metastasis

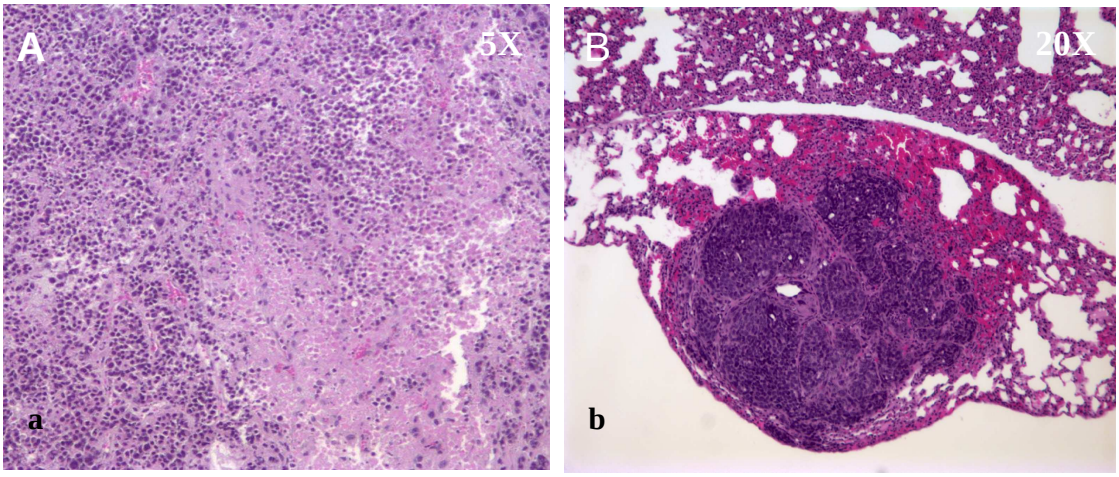


Figure 4.8, Morphologic features of *Brca1* tumors. (a) Foci of geographic tumor necrosis were a frequent finding in these tumors (5/5) **(b)** Metastasis to lungs was observed in 60% of the triple null tumors.

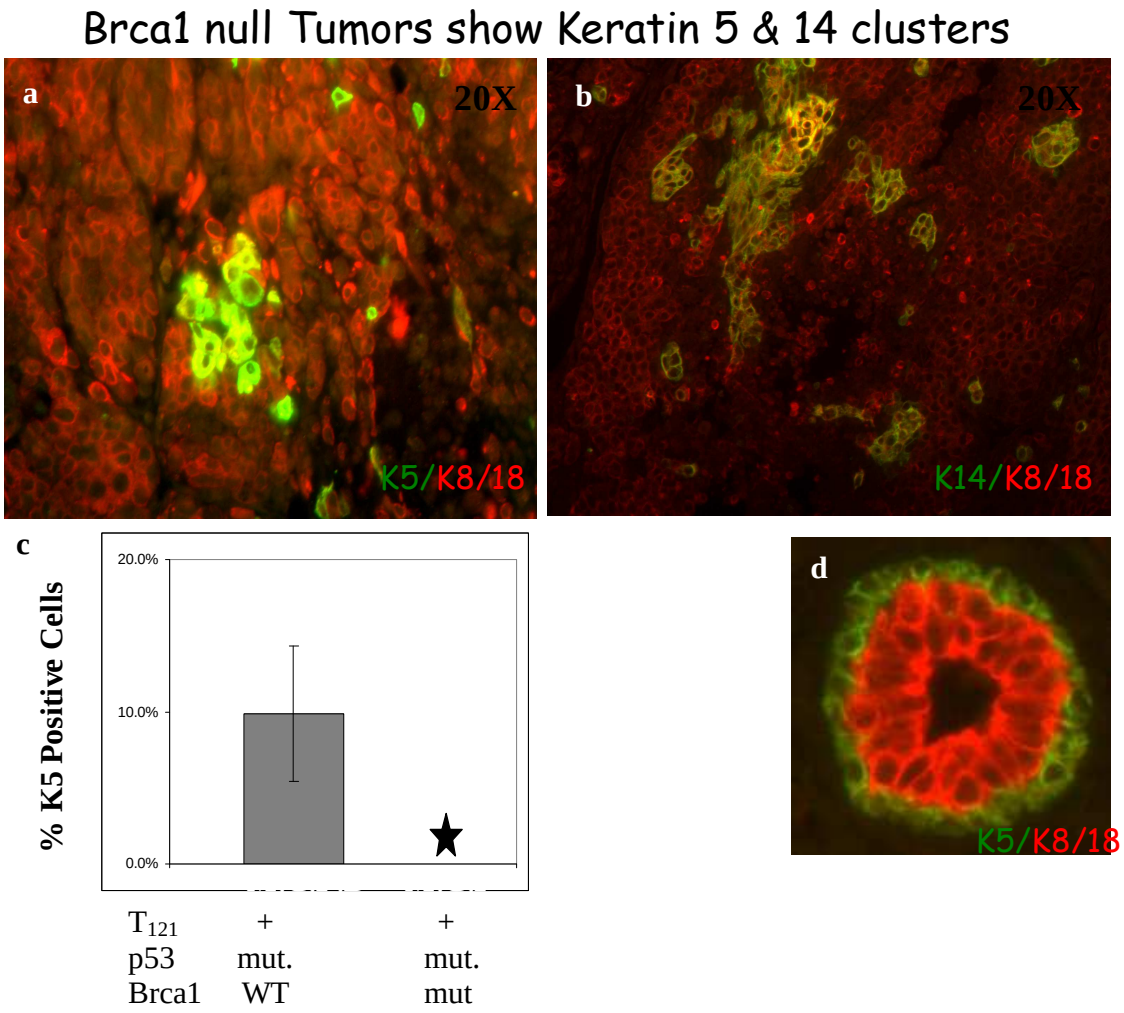


Figure 4.9, Distribution of Keratin 5 and Keratin 8/18 in normal mammary glands (a), Clusters of Keratin 5 positive cells among large areas of K8/18 positive cells (3/5) **(b)**, Clusters of K14 and K8/18 double positive cells (3/5) **(c)**. The percentage of Keratin 5 positive cells is quantitated in plot in **(d)**. It is noteworthy that they are completely absent in the *Brca1* wild type tumors. (P = 0.0005 using the unpaired t-test).

Tumor Trait	MFT+,WAP-Cre+p53cf/f	MFT+,WAP-Cre+,p53cf/+	MFT+,WAP-Cre+,p53cf/f,Brca1cf/f
N overall grade 3	11/14 (78%)	8/10 (80%)	5/5 (100%)
N overall grade 1 or 2	3/14 (21%)	2/10 (20%)	5/5 (100%)
Mitosis >25/10 high power field	11/14 (78%)	9/10 (90%)	5/5 (100%)
Geographic Necrosis	3/14 (18%)	1/10 (8%)	5/5 (100%)
Pushing Border	0/14 (0%)	0/10 (0%)	5/5 (100%)
Infiltrative Border	10/14 (71%)	0/10 (0%)	2/5 (40%)

Table 4.2, Analysis of mouse mammary tumors using human breast cancer classification criteria shows high grade tumors of Nottingham Score 3 for all genotypes. Striking features of *Brca1* null tumors are geographic necrosis and pushing borders. Grading the mammary tumors obtained in this study using human pathological criteria (done by Dr. Chad Livasy, MD, Associate Professor of Dept of Pathology & Lab Medicine, UNC School of Medicine) showed that all the mammary tumors from the different genotypes were high grade (mostly 3 on the Nottigham Scale) and had a very high mitotic index. *Brca1* null tumors however showed some distinguishing features that are also observed in human *Brca1* mutated breast cancers. These features include large regions of necrosis, termed "geographic necrosis" and a pushing but tightly defined border of the tumor mass, as opposed to an infiltrative border seen in the case of *Brca1* wild type tumors.

4.3.6 Micro RNA analysis reveals altered miRNA signature in triple mutant tumors

MicroRNAs are small non-coding RNAs (20-22 nucleotides) that have gained importance over the past few years because of their putative role as oncogenes and tumor suppressors. Specific expression of several miRNAs has been found to be associated with both tumors of the haematopoietic system and solid tumors using genomic techniques like microarray platforms or bead based flow cytometry (Hammond 2006a, Hammond 2006b, Thomson et al. 2006). Commonly dysregulated oncogenes in human cancer like c-Myc have

been shown to up regulate expression of certain microRNAs (O'Donnell et al. 2005). Gene expression profiling studies have also shown that several human cancers have a specific microRNA expression profile associated with them, that reflects the differentiation status of the tumor (Lu et al. 2005). We hypothesized that the triple mutant mouse mammary tumors may have an altered microRNA expression profile that could suggest pathways for their greatly reduced latency, increased metastasis and more differentiated (glandular) histopathological appearance. To investigate this possibility we took five *WAP-Cre; $TgMFT_{121+/-}$ / $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ /$Brca1^{\Delta/\Delta}$* and five *WAP-Cre; $TgMFT_{121+/-}$ / $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* mouse mammary tumors and performed custom miRNA microarray on them. Allowing a 0% False Discovery Rate we found that 14 miRNAs were differentially expressed in the *WAP-Cre; $TgMFT_{121+/-}$ / $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ /$Brca1^{\Delta/\Delta}$* tumors all within a 2-5 fold change. Intriguingly, while the *WAP-Cre ; $TgMFT_{121+/-}$ / $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* tumors expressed higher levels of stem cell like miRNAs (Houbaviy et al., 2003) like miR 291-5p and 292-3p the *WAP-Cre ; $TgMFT_{121+/-}$* / p53 $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ / *Brca1* $^{\Delta/\Delta}$ tumors had a more differentiated miRNA profile with high expression of miRNA 30 and the Let-7 family (discussed in chapter five) (Thomson et al. 2004). This reflected what we observed in the histopathology of these mouse tumors where the triple mutant tumors appeared more glandular in nature compared to the *Brca1* wild type tumors that had a more solid adenocarcinoma like appearance. We hypothesize that this difference could be related to the latency of these tumors. As the *Brca1* null tumors progressed very rapidly from the normal mammary gland stage to form highly aggressive mammary carcinomas, they still retained their well differentiated glandular structure, but the *p53* null tumors had a longer latency and were slow growing, leading to accumulated changes and loss of normal mammary gland characteristics. We also found a *Brca1* loss

associated up regulation of the 17-92 miRNA polycistronic cluster that has been associated with increased oncogenicity in a mouse Burkitts lymphoma model (Woods, Thomson & Hammond 2007). Also noteworthy was the significant up regulation of the miR 30 family (miR 30a-5p, 30b, 30c, 30d, 30e) in the *Brca1* null tumors. This study is the very first attempt to look for a *Brca1* associated miRNA profile and will be followed up with ongoing work on validation of the miRNA targets and mRNA expression profiles on the tumors and with functional studies to shed light on the role of the miRNAs in these tumor sets.

4.4 Discussion

4.4.1 *Brca1* loss promotes both primary tumor formation and metastatic disease

Though many claims have been made before of mouse models that reflect distinct features of the human basal like breast cancer , a good mouse model that truly reflects the biological and pathological aspects of this disease and has a reasonably short latency is absent. Several previous attempts have been made by others to generate mice with germline or mammary specific mutations of *Brca1*, both alone and in combination with mutations in *p53*. The results from these studies have been summarized in **Table 4.4**. Germline mutations in *Brca1* lead to an embryonic lethal phenotype indicating the critical role played by *Brca1* in development. Loss of *p53* rescued this lethal phenotype in some cases (Xu et al., 1999, 2001) by reducing apoptosis. The *Brca1* $^{co/ko}$/ *p53*$^{+/-}$ mice developed mammary tumors with a latency of about 6-8 months while the *Brca1* $^{co/ko}$ developed mammary tumors with a latency of about 10-13 months and incomplete penetrance (not all mice developed mammary tumors). This suggested synergy between loss of *p53* and *Brca1* in mammary tumorigenesis. However this model did not reflect a complete loss of *Brca1* as the mice produced a BRCA1-

Δ11 transcript which is a naturally occurring splice variant of *Brca1*. Also, previous studies indicated that genetic background of the mice played a role in determining a *Brca1* loss phenotype, perhaps due to the effect of modifier alleles. For instance, germline loss of *Brca1* in a 129 x C57BL/6 hybrid background resulted in very early embryonic lethality (EL 7.5), but loss of *Brca1* in a mixed genetic background with significant amounts of the DBA/2J strain prolonged survival of the embryos to about 13.5 days (listed in Table 4.4). Thus the effect of genetic modifiers in *Brca1* loss mediated mammary tumor latency and phenotype cannot be ruled out. To study the effect of complete loss of *Brca1* and *p53* in mammary tumorigenesis Liu et al. generated mice that had the exons 5-13 flanked by Lox P sites in a FVB/N strain. It is imperative to keep in mind that the FVB strain has been indicated by others to have a greater susceptibility to forming spontaneous mammary tumors compared to other strains of mice like the C57BL/6 strain (Davie et al., 2007). Upon mating with Keratin 14 Cre mice these mice lost *Brca1* in various epithelial tissues including the mammary epithelium. While the *Brca1* $^{\Delta/+}$ and *Brca1* $^{\Delta/\Delta}$ mice in this model did not develop mammary tumors even after 800 days, upon crossing to *p53*, the mice developed mammary tumors with varying latencies (listed in **Table 4.4**). This further confirmed the notion that loss of *Brca1* and *p53* co-operate in mammary tumor formation, though perhaps in a preferred genetic background. The high incidence of skin tumors associated with this model precluded a thorough study of the mammary phenotype. While some features of human basal mammary cancer were observed in these mice, progression to metastasis was not seen. Also the significantly long tumor latency (~7 months) suggested that other stochastic events may be necessary before mammary tumors form. We have created a mouse model with genetic lesions that frequently occur in the human familial and sporadic basal like breast cancers,

namely loss of *Brca1*, *p53* and *Rb1*. To restrict all events to the mammary gland we have used the Cre-Lox-P technology and WAP-Cre to target the mammary luminal epithelial cells only. To rule out the potential modifier effects of any one strain we have used a mixed genetic background of BDF1xFVB/N mice. The resulting model has generated mammary tumors with a very low latency and high penetrance as well as progression to metastasis, indicating that *Rb1*, *p53* and *Brca1* loss may cooperate in mammary tumorigenesis. The combined inactivation of all three, *Rb1*, *Brca1* and *p53*, seems to be prerequisite for advanced metastatic mammary tumor development. Reduction in *Brca1* gene dosage does not lead to reduced tumor latency in our model. (*WAP-Cre*; $TgMFT_{121+/-}$ /$p53^{\Delta 2\text{-}10\ /+}$/ $Brca1^{\Delta/+}$ animals have a comparable tumor latency to the *WAP-Cre*; $TgMFT_{121+/-}$ /$p53^{\Delta 2\text{-}10/+}$ animals, indicating that haploinsufficiency of *Brca1* does not provide any advantage to tumor growth). Consequently, mammary tumorigenesis in *WAP-Cre;* $TgMFT_{121+/-}$ / $Brca1^{\Delta/+}$/ $p53^{\Delta 2\text{-}10/+}$ mice may be dominated by *Brca1* and *p53* LOH in combination with other oncogenic mutations. It is noteworthy that synergy between *p53* loss and *Brca1* inactivation in this model is clearly demonstrated by the greatly reduced latency for tumor development in *WAP-Cre;* $TgMFT_{121+/-}$ / $Brca1^{\Delta/\Delta}$ / $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mice compared to *WAP-Cre;* $TgMFT_{121+/-}$ / $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ animals or *WAP-Cre;* $TgMFT_{121+/-}$ / $Brca1^{\Delta/\Delta}$ animals.

WAP-Cre-mediated conditional inactivation of *Brca1* alone in MFT_{121} mouse mammary epithelium predisposes to mammary tumors with a very long latency of more than 400 days. This is probably due to the fact that *Brca1* loss even in combination with pRb_f inactivation in mouse mammary epithelium induces cell cycle arrest along with rapid *p53* mediated apoptosis. In concordance with this, we detected very high levels of apoptosis in day one lactation mammary glands of *TgWAP-Cre;* $TgMFT_{121+/-}$ / $Brca1^{\Delta/\Delta}$ *mice* that was

reduced by loss of *p53*. Loss of *Brca1* in this model appears to be an early, tumor-initiating event, which in combination with loss of pRb is not sufficient to induce mammary tumorigenesis due to the presence of a functional *p53*.

Transformation of a normal cell to a cancer cell is a complex process and recruits multiple pathways as reviewed by (Hanahan, Weinberg 2000)). Loss of *Rb1* and *p53* results in loss of apoptotic responses and cell cycle checkpoint control. Even though there is clear synergy between losses of these two critical cell regulators in promoting mammary tumorigenesis (no mammary tumors form in *WAP-Cre* ; $TgMFT_{121+/-}$ mice), progression to metastasis as well as some key features of human basal breast cancer (like necrosis, angiogenesis) is not observed in these mice. Concomitant somatic loss of *Brca1* may provide key signals that promote tumor cell metastasis along with the pathological features typical of basal cancers. Our analysis of expression data (shown in chapter 5) show up regulation of several genes in the MMP and BMP pathways (for example, MMP-9, BMP-7) and also EMT promoter SNAIL in the *WAP-Cre*: $TgMFT_{121+/-}$ / $p53c^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ / $Brca1^{\Delta/\Delta}$. Several genes from these pathways have been reported to be responsible for increased vascularization and metastasis (Chantrain et al. 2006). The “triple mutant” tumors also appear more angiogenic indicated by increased PECAM staining. So somatic *Brca1* loss layered on to the already existing loss of *Rb1* and *p53* may be inducing increased angiogenic signals thus aiding in tumor cell survival and metastasis. Finally, these tumors have lost their *p53*-mediated apoptosis. This makes it easier for them to travel to distant sites and also overcome other possibly *p53*-independent pro-apoptotic signals. In summary, we conclude that, in our mouse mammary tumor model, loss of *Brca1* confers both early tumor growth advantage and late

stage metastasis, lending support to the theory that the initial genetic signature of a tumor can predict its final prognosis (van de Vijver et al. 2002).

4.4.2 *Brca1* loss induces a phenotypic change from highly heterogeneous and often spindle shaped adenocarcinoma to a more differentiated (glandular) and invasive carcinoma

It has been hypothesized by some (Althuis et al. 2005, Shackleton et al. 2006, Smalley, Ashworth 2003, Stingl et al. 2006) that the mammary gland develops from pluripotent "stem cells" that have the ability to form both luminal and myoepithelial cells and thus co-express CK5 (and/or CK14) and CK8/18 positive cells. All mammary carcinomas from the *WAP-Cre; TgMFT*$_{121+/-}$ */ Brca1* $^{\Delta/\Delta}$*; p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ model showed similar epithelial phenotypes, characterized by expression of the luminal marker CK8 and expression of nests of myoepithelial marker CK5 and CK14. These basal cell nests are always absent in the *WAP-Cre; TgMFT*$_{121+/-}$ */ p53* $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ derived tumors. Though the cell of origin for these tumors is not clear, this implies that somatic inactivation of *Brca1* results in a shift of tumor spectrum from a more luminal locally advanced tumor type to a more myoepithelial metastatic tumor type. This is similar to what is observed in human familial breast cancers with *BRCA1* mutation, that have a basal cytokeratin signature (Perou et al. 2000, Sorlie et al. 2001, Sorlie et al. 2003). The majority of *WAP-Cre; TgMFT*$_{121+/-}$ */ Brca1* $^{\Delta/\Delta}$ */p53* $^{\Delta 2\text{-}10/\Delta}$ 2-10 females developed invasive mammary tumors instead of heterogeneous and locally advanced carcinomas. This indicates that loss of *Brca1* alone causes the shift from locally advanced to metastatic cancer. *Brca1* is known to be important for genetic stability and maintenance of

normal spindle check point functions (Deng 2006, Moynahan 2002) and it is therefore conceivable that loss of these functions may lead to a far more aggressive disease with multiple cell signaling pathway changes. Our data gives us the tempting hypothesis that the "triple mutant" tumors may originate from mammary gland progenitor cells (CK14-CK8/18 positive). Cell sorting studies to be performed in the future coupled with mammary gland transplant experiments will help validate this hypothesis.

4.4.3 Metastatic mammary carcinoma in mice as a model for human breast cancer

Mouse models for tumorigenesis have been very useful in studying early tumor biology and progression but very few have recapitulated the later events like distant metastasis. One of the key features of human familial *BRCA1* mutated breast cancers is early progression to distant metastasis (Rakha et al. 2008). The sites for distant metastasis for this class of cancers are commonly the brain and lung and rarely the lymph nodes and bone which are the usual sites for breast cancer metastasis. Mouse metastatic mammary tumors in our model recapitulate several of the key features of human familial "basal" mammary tumors, including cellular morphology, pushing border, geographic necrosis, angiogenesis and sites of metastasis. Pathological analysis of these mouse tumors by a human pathologist using the Nottingham Scale of human mammary tumor classification identified several of the features present in human high grade basal like cancers in these mouse tumors (tabulated in **Table 4.2**). In summary, we have shown that the synergistic loss of *Brca1* and *p53* combined with Rb_f inactivation in mice induces mammary tumors with morphologic and histologic characteristics very similar to tumors arising in humans with familial *Brca1* mutations. Our

mammary tumor model may serve as a valuable pre-clinical model for further studies on human familial breast cancers. This may have a valuable impact in providing new diagnosis and treatments for breast cancer.

A caveat in this study is the possibility that the metastatic lesions observed in the lungs are not “true” metastatic nodules formed by invasion of cells from the primary mammary tumor, but “tumor emboli” that have been pinched off from the primary tumors and trapped in the lungs. The fact that some of the mouse models with metastasis to the lungs may be tumor emboli and not an example of human tumor metastasis have been suggested before (Sugino et al., 2002, Oshima et al., 2004). Further future observation of these mice to find if the nodules grew and invaded the surrounding lung parenchyma can confirm the nature of these nodules.

Inducible Staged Mammary Tumor Model

Mouse Genotype	Mammary neoplastic phenotype	Alive time post-day1 (mo)
MFT_{121},WapCre	none	24
$P53^{cf/f}$,WAPCre	none	12
$Brca1^{cf/f}$, WAPCre	none	12
$MFT_{121}^{+/-}$;$p53^{cf/+}$	Adenocarcinoma	9
MFT_{121}+/-,$p53^{cf/f}$	Adenocarcinoma, locally invasive	3-6
MFT_{121} $Brca1^{cf/f}$	Solid Adenocarcinoma	> 1year
$P53^{cf/f}$ $Brca1^{cf/f}$	Solid Adenocarcinoma	9
MFT_{121} p53 $^{cf/f}$,$Brca1^{cf/f}$	Glandular aggressive mammary tumor with metastasis	1.5

Table 4.3 Summary of timing of appearance post day one lactation and histopathology of mammary tumors in different genotypes.

Allele	Cre promoter	Phenotype	Tumor type	Mammary tumor percentage & latency						Metastasis	Strain	Reference	Notes
				Brca1 $^{+/-}$	*Brca1* $^{-/-}$	p53$^{+/-}$	p53$^{-/-}$	*Brca1* $^{+/-}$ */p53*$^{+/-}$	*Brca1* $^{-/-}$ */p53*$^{-/-}$				
Brca1 ex2	N/A	EL 7.5	None						↑ survival	N/A	C57BL/6	1	No *Brca1*
Brca1 5-6	N/A	EL 7.5-10.5	None						↑ survival	N/A	129x C57BL/6 hybrid	2	No *Brca1*
Brca1 11	N/A	EL 7.5-9.5	None						↑ survival	N/A	50% 129, 50% Black Swiss	3	*Brca1* Δ11
Brca1 Δ223-763	N/A	EL 10.5-13.5	None	None	EL	No MTs	Infrequent MTs	5.3 %, $T_{1/2}$ 18m	↑ survival	none	129,C57BL/6,DBA/2 Mixed strain	4,5,6	*Brca1* Δ11
Brca1 KO	N/A	EL 7.5-8.5	None	None					None	N/A	129x C57BL/6 hybrid	7	Part of *Brca1* Δ11
Brca1 ^{tr}	N/A	Partly viable	Lymphomas, sarcomas, adenomas, carcinomas	None	15 m			27 % MTs, $T_{1/2}$ 9m	Lymphomas in 3.5m	No	MF1	8	Part of *Brca1* Δ11
Brca1 1700T	N/A	EL 10.5	MTs on radiation	None	EL	N/A	N/A	None	None	No	129Ola, C57BL/6	9	Part of *Brca1* Δ11
Brca1^{fl} 5-6	*Lck-Cre*	↑apoptosis & chrom. abnormality & ↓ proliferation of thymocytes	No MTs	No MTs						N/A	129/C57BL/6	10	No *Brca1*
Brca1 co/ex 11	WAP / MMTV	EL 12.5, partial survival upon loss of p53	Solid, tubular & ductal MTs	30%, 10-13m	Not viable	Not done	Not done	6-8 m	Not done.	No	NIH Black Swiss	11	*Brca1* Δ11
Brca1 Δ5-13	K14 Cre	MTs and STs	MTs and STs	No Ts	No Ts.	Not done	63% mice, 9m $T_{1/2}$	Not done	7 m	No	FVB/N	12	No *Brca1* transcript

Table 4.4 Summary of previously generated mouse models with germline or conditional loss of *Brca1* and/or *p53* are listed here. Germline loss of *Brca1* resulted in embryonic lethality that could be partially rescued in some cases upon loss of *p53*. The combined loss of

Brca1 and *p53* resulted in significantly reduced mammary tumor latency. Genetic background of the mice had subtle effects on the lethal or tumor phenotypes observed in all these models.
(1=Ludwig et al. 1997, 2=Hakem et al. 1996, 3=Shen et al. 1998, 4=Gowen et al. 1996, 5=Cressman et al. 1999, 6=Donehower et al. 1992, 7=Liu et al. 1996, 8=Ludwig et al. 2001, 9=Hohenstein et al. 2001, 10=Mak et al. 2000, 11=Xu et al. 1999, Xu et al. 2001, 12=Liu et al. 2007)

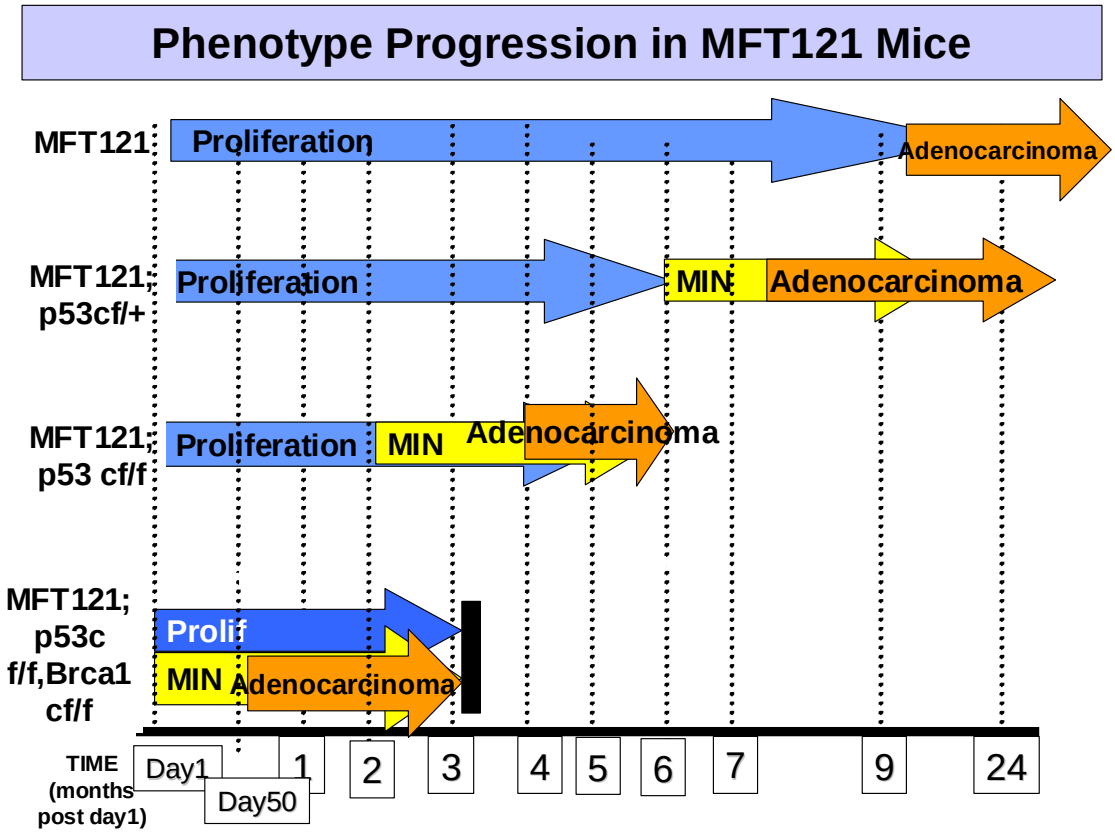


Figure 4.10, *pRb*$_f$, *p53* and *Brca1* act synergistically in initiation, progression and metastasis of mouse mammary tumors. The timing of appearance and progression to end stage mammary tumor in the different genotypes reported here is graphed here based on data presented in **Table 4.3**. Triple null tumors showed a dramatic reduction in tumor latency and developed mammary tumors as early as 30 days post day one lactation, with a mean latency for tumor development being 50 days. Tumor progression in these mice was followed by performing biopsies on mammary glands, the earliest time point being day one lactation. Even on day one lactation these mice showed significantly hyperplastic mammary glands and stromal infiltration. By day fourteen post day one lactation they had formed small but multiple focal tumor nodules. At six weeks post day one lactation all of these mice had formed full-blown mammary tumors. The progression studies allowed us to track the early

changes in the mammary glands of these genotypes and perform marker studies on pre-cancer lesions.

4.5 Materials and Methods

4.5.1 Derivation of MFT_{121} transgenic mice

Resulting and subsequent generation *MFT_{121}* transgenic mice were identified by PCR amplification of a 215-bp fragment using primers 5'-GCATCCAGAAGCCTCCAAAG -3' and 5'-GAATCTTTGCAGCTAATGGACC-3' complementary to the T_{121} sequence. In the *MFT_{121};Wap-Cre* mice 5'-TGATGAGGTTCGCAAGAACC-3' and 5'-CCATGAGTGAACGAACCTGG-3' primers were used for the Cre sequence. Cycling profile was 94°C for 2 minutes; 35 cycles of 94°C for 20 seconds, 62°C for 45 seconds, and 72°C for 45 seconds; and final incubation of 72°C for 2 minutes.

4.5.2 Transgenic breeding strategies

To study the effect of *p53* inactivation on mammary tumorigenesis, *WAP-Cre; $TgMFT_{121}$* mice were mated to *p53* conditional floxed over floxed mice (*$p53^{2\text{-}10/2\text{-}10}$*; FVB/N, Jonkers et al. 2001). *p53* genotypes were determined by PCR as described before. Cycling variables were as for the T_{121} reaction described above. We produced female mice with the genotypes *WAP-Cre; $TgMFT_{121}$/$p53^{\Delta 2\text{-}10/+}$* and *WAP-Cre; $TgMFT_{121}$ /$p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$*, and nontransgenic female littermates (*$p53^{\Delta 2\text{-}10/+}$* or *$p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$*,) served as controls. To study the effect of *Brca1* loss, *WAP-Cre; $TgMFT_{121}$* mice were mated to *$TgBrca1^{\Delta/\Delta}$ /$p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* mice and *$TgBrca1^{\Delta/\Delta}$* mice (obtained from MMHCC, FVB/N). PCR reactions to detect conditional *Brca1* alleles have been described before (Liu et al. 2007). We generated female

mice with the genotypes *WAP-Cre; $TgMFT_{121}$ / Brca1 $^{\Delta/+}$/p53 $^{\Delta 2\text{-}10/+}$* and *WAP-Cre; $TgMFT_{121}$/Brca1 $^{\Delta/\Delta}$ /p53 $^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* with nontransgenic (Cre negative) littermate controls for each cohort.

4.5.3 Histopathology and apoptosis assays

Mammary gland tissue and tumor samples were dissected from *$TgMFT_{121}$* transgenic or age matched littermate control animals. Part of each sample was snap frozen for RNA and DNA analysis and a portion was fixed overnight in 10% phosphate buffered formalin, transferred to 70% ethanol, and then embedded in paraffin. To analyze tumor histopathology mammary samples were sectioned for 10 successive layers. Sections at 4-um intervals were taken and stained with hematoxylin and eosin. Histopathological examination of the slides under light microscope was done as previously described. For detection of apoptosis levels in the samples staining of above sections using the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) kit as previously described (Symonds et al. 1994a, Symonds et al. 1994b) was performed. The statistically significant differences in apoptosis levels between mice with varying genotypes was evaluated as described below ($p<.0.05$ considered of statistical significance).

4.5.4 Immunohistochemistry and immunofluorescence

Immunohistochemical analysis for detection of specific markers in the mammary gland samples was performed on the formalin-fixed paraffin sections. For antigen retrieval the slides were boiled in citrate buffer (pH 6.0, Zymed, South San Francisco, CA) for 15 min. Endogenous peroxidase activity was quenched by incubating the slides for 10-min in 3%

H2O2 in methanol. For IHC detection was done using the appropriate secondary antibody. Antibodies were α-cytokeratins 8/18 (Ker8/18, 1:450 Progen, GP11), α-cytokeratin 5 (K5, 1:8000, Covance, PRB-160P), smooth muscle actin (SMA; 1:1,000, mouse A2537; Sigma, St. Louis, MO), anti–phosphorylated histone H3 (1:100, rabbit 06-570; Upstate, Waltham, MA) and SV40 (monoclonal Ab2, 1:100; Oncogene, Cambridge, MA). Detection for all antibodies was done using the Vector ABC Elite kit and a Vector 3,3'-Diaminobenzidine kit for substrate detection (Vector Laboratories, Burlingame, CA). All immunofluorescence reactions were done using AlexaFlour secondary antibodies (AlexaFluor 488 and 594, Molecular Probes). Slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted using Vector Hardset Mounting Media (Vector Laboratories).

4.5.5 Micro RNA extraction

Total RNA was collected from end-stage tumors of *WAP-Cre*; *TgMFT$_{121}$ / p53$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* and *Wap-Cre; TgMFT$_{121}$ / Brca1$^{\Delta/\Delta}$ /p53$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* mice. RNA was purified using the Trizol (Invitrogen) to preserve microRNAs as described before (Thomson et al. 2004, Thomson, Parker & Hammond 2007). Five micrograms of total RNA was labeled as described before (Thomson et al., 2004). Reference RNA consisted of total RNA harvested from equal numbers of C57BL/6J and 129 male and female Day1 pups (a gift from Dr. Cam Patterson, UNC). The Reference RNA was reverse transcribed, amplified, and labeled with Cy3. Hybridization was performed in disposable chambers from MJ Research (part number SLF-0601). Slides were washed once in 2 ×SSC, 0.025% SDS, three times in 0.8 ×SSC, and three times in 0.4 ×SSC, at 25 °C. Slides were scanned with a Genepix 4000B Scanner (Axon) and raw pixel intensities extracted with Axon software.

4.5.6 miRNA Data Analysis

Cy3 and Cy5 median pixel intensity values were background subtracted, and Cy3/Cy5 ratios were obtained. Data points were removed when Cy5 values did not exceed 200% background. All calculations derived from the database were done using the Winstat software for MS Excel. Cy3/Cy5 ratios were log-transformed (base 2) , median centered by arrays and genes, and clustered hierarchically (average linkage correlation metric) using the Cluster program from Stanford University (as described in Thomson et al., 2004). Expression maps and dendrograms were generated using Treeview from Stanford.

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CHAPTER FIVE

GENOMIC ANALYSIS OF *Brca1* INACTIVATED TUMORS SHOW A NOVEL mRNA, miRNA AND CGH SIGNATURE

5.1 Abstract

Breast cancer is a highly complex disease with many levels of biological pathways that are either deregulated or activated. Women with the inherited *BRCA1* mutation are pre-disposed to highly aggressive mammary tumors very early in life that have a poor prognosis. These mammary tumors are highly proliferative, metastatic and have increased angiogenesis. Microarray studies have identified an increased pattern of basal cytokeratin expression in these tumors, but the molecular reason for this expression pattern is not known. These tumors are also ER, PR and HER2 negative and frequently have mutation in *P53* as well as increased expression of E2F target genes. To study the role of combined loss of *Rb1, p53* and *Brca1* in breast cancer we have developed conditional mouse models in which all three genes are inactivated in the mammary gland. We have shown before that the somatic loss of *Rb1, p53* and *Brca1* results in the development of mammary tumors that are highly proliferative, metastatic and angiogenic (indicated by increased PECAM staining, data not shown). These tumors also display an increased expression pattern of the basal cytokeratins, CK5 and CK14. To further identify molecular pathways deregulated or overexpressed in these tumors we perform three different genomic analyses. We show here for the first time that the

mammary tumors which arise in the *Rb, p53* and *Brca1* negative mice have an increase in expression of genes involved in epithelial-mesenchymal-transition. Also the triple negative tumors show a dramatic increase in genetic instability with losses on chromosomes 4 and 10. Finally these tumors have an altered micro RNA signature that may indicate another level of complexity in the etiology of this disease. Thus we show that this mouse model has several of the hallmarks of human familial breast cancer both at the histological and molecular levels and can be used as a suitable pre-clinical model for future drug targeting studies.

Part I

Microarray Analysis

5.2 Introduction

Familial *BRCA1* mutations confer a 40-50% increased risk of breast cancer and also an increased lifetime risk of ovarian, colon and prostate cancer (Cannistra 2004, Scully, Livingston 2000, Thompson, Easton & Breast Cancer Linkage Consortium 2002, Venkitaraman 2002, Welcsh, King 2001). *BRCA1* is a multifunctional protein with roles in maintenance of genomic integrity (Kinzler, Vogelstein 1997), DNA repair (Scully et al. 1996), chromatin remodeling (Bochar et al. 2000, Pao et al. 2000, Welcsh, Owens & King 2000, Yarden, Brody 1999) and transcriptional regulation (Scully et al. 1997, Somasundaram 2003). The mechanism by which familial mutation of *BRCA1* causes breast cancer is yet unknown. Studies looking at *BRCA1* functions at a cellular level have shown that *BRCA1* mutated cells have proliferation and survival defects (Gowen et al. 1996, Hakem et al. 1996, Ludwig et al. 1997, Shen et al. 1998) increased sensitivity to radiation (Shen et al. 1998,

Welcsh, Owens & King 2000), increased genetic instability and CIN (chromosomal instability) (Mak et al. 2000, Xu et al. 1999a) , loss in the G2/M cell cycle checkpoint control (Xu et al. 1999a) and also defective homologous recombination (Moynahan 2002).

Classification of breast cancer patients for optimal treatment of their specific cancer sub type is largely based on histological characterization of their cancer from biopsy specimen. However histopathology of breast cancer is often inconclusive due to great heterogeneity of the disease and the presence of many different cell types in a single tumor. This often results in the mis-classification of patients leading to failure or moderate success of treatment methods selected. An example of this is the use of the HER2 receptor inhibitor, Imatinib (Trastuzumab). When this drug was used in an un-classified population of breast cancer patients, it had a very low success rate. But identification of a sub-class of patients who had significant overexpression of the HER2 receptor in their cancer, greatly increased the specificity of using this drug and had a dramatic success rate for those patients. Genome wide analysis, like using microarray technology to classify breast cancer into subtypes based on their molecular signature can generate markers for identifying specific types of cancers. This can help tailor the treatment of the patient towards their individual needs and hence provide better outcome.

Several genome wide studies using microarray analysis have been used to identify potential molecular markers in the familial breast cancer resulting from loss of *BRCA1* in the human mammary gland. Array analysis classified the *BRCA1* mutated human cancers as “basal like” with a predominantly proliferative signature and overexpression of the basal cytokeratins 5 and 14 (Perou et al. 2000). This signature in this class of cancers has been confirmed by many groups since then (Carey et al. 2006, Hu et al. 2006, Kapp et al. 2006,

Sorlie et al. 2003, Sotiriou et al. 2003). As much as this work has resulted in the identification of new prognostic markers like CK5 and CK14, good mouse models for this class of breast cancer are needed to identify the pathways that result in the specific basal phenotype. Recent work identified mouse models of breast cancer that have a basal molecular profile through microarray classification, similar to the human basal like cancer (Herschkowitz et al. 2007). But these mouse models did not replicate all aspects of the human disease, for instance, distant metastasis did not occur (Liu et al. 2007). This suggested that additional events were necessary to better model the human disease. We have developed a mouse model with the combined loss of *Rb$_f$*, *p53* and *Brca1*, all of which are implicated to be lost in human basal breast cancers (Crook et al. 1998, Phillips 1999, Phillips et al. 1999, Phillips, Andrulis & Goodwin 1999a). This mouse model has several of the pathological features found in human basal cancers, like large areas of necrosis, metastasis to lungs, increased angiogenesis and nests of CK5 and CK14 positive cells. We use microarray technology to identify new classifiers that can provide clues to the molecular pathways deregulated in this class of cancers. We find that the triple mutant cancers (*Rb$_f$*, *p53* and *Brca1* inactivated) have a significant overexpression of genes involved in metastasis and also some basal cytokeratins like CK14. The similarities to human basal like breast cancer both by histology and microarray profile make this a good model for pre-clinical studies in the future.

5.3 Results

5.3.1 Expression Array Analysis of Murine Tumors

We performed microarray analyses using mRNA harvested from twenty tumors and directly compared these results with a published survey of tumors from thirteen mouse models (Herschkowitz et al. 2007) to gain insight into the global gene expression patterns of these tumors in the context of other mouse models. We then performed a supervised analysis of identify sets of genes that show significant gene expression differences between the triple and double mutant tumors.

We used oligonucleotide microarrays (Agilent) and a common reference experimental design (Perou et al. 2000). mRNA was harvested from ten tumors of each genotype, *WAP-Cre; TgMFT*$_{121+/-}$*/p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ and *WAP-Cre; TgMFT*$_{121+/-}$*/p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ */ Brca1*$^{\Delta/\Delta}$

We focused our analysis on the published set of 866 genes (so called "intrinsic genes") that were sufficient to segregate mouse tumors equally well as using all genes represented on the arrays. It has been reported before that mouse tumors can be clustered into ten groups and four main categories (Herschkowitz et al. 2007). The groups as depicted in **Figure 5.1** are "normal mammary gland samples (group I)", mesenchymal tumors (group II), basal/myoepithelial like tumors (groups III-V), luminal like tumors (groups VI-VIII) and tumors with mixed properties (groups IX and X). We performed hierarchical clustering of our mouse tumors combined with the published data set. Upon clustering our mouse tumors with this intrinsic data set we found that the triple mutant tumors (*WAP-Cre; TgMFT*$_{121+/-}$*/ p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ */ Brca1*$^{\Delta/\Delta}$) clustered in a tight group on the extreme right alongside the "Mixed" tumors (Group IX-X) (**Figure 5.1, Triple**). The *Rb*$_f$ and *p53* inactivated tumors alone were

more spread out but all fell within the luminal tumor cluster on the left within the C3 Tag tumors (Groups VI-VIII) (**Figure 5.1, Double**). One of the *Brca1* null tumors (sample # 133574) fell in the squamous cluster along with the DMBA tumors. This tumor when observed histopathologically (by H&E) showed several keratin swirls, squamous differentiation and "pilar" like features confirming its similarity to the other squamous tumors in the array. The "triple mutant" tumors from our mouse model that did not have *Brca1* expression did not cluster with the other mouse tumor cluster with loss of expression of *Brca1* as reported by Hershkowitz et al., 2007. But the triple mutant tumors show increased expression of metastasis genes as well some basal like genes like Cytokeratin 14. We hypothesize that as the genetic alterations in the *WAP-Cre; TgMFT*$_{121+/-}$*/ p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$*/ Brca1*$^{\Delta/\Delta}$ mice were targeted to the luminal epithelial cells by the WAP promoter, the tumors that developed had significant luminal characteristics. It can be hypothesized that the combined loss of *Rb*$_{f}$, *p53* and *Brca1* in the myoepithelial cells in the mammary gland may give rise to the true "basal" like tumors that are a hallmark of human familial (*BRCA1* mutated) breast cancers.

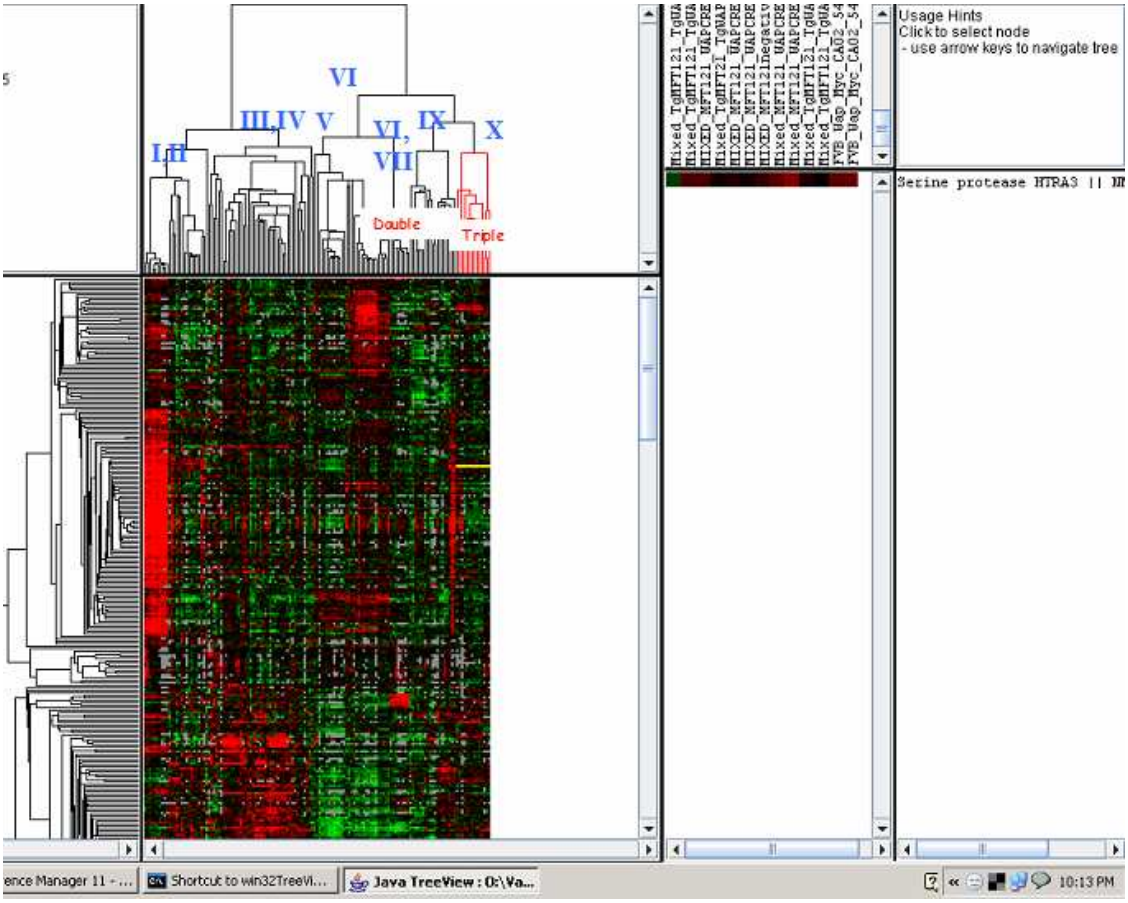


Figure 5.1. Gene expression patterns of mouse mammary tumors analyzed by hierarchical-clustering using an "intrinsic" gene set. The tumors divided into four broad classes. The classes as indicated by roman numbers on top of the heat map are: I=Normal breast cluster, II= Mesenchymal gene cluster, III-V=Basal/myoepithelial gene cluster, VI-VIII=Luminal gene cluster, IX-X=mixed gene cluster. The double mutant (*WAP-Cre; TgMFT*$_{121}$*/p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$) tumors fell within the luminal gene cluster and the triple mutant (*WAP-Cre; TgMFT*$_{121}$*/p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ */Brca1*$^{\Delta/\Delta}$) tumors fell within the mixed cluster of tumors that included tumors with mesenchymal as well as luminal properties.

5.3.2 Significance Analysis of Microarray

To test the hypothesis that the triple mutant tumors (*Rb*$_f$*, p53* and *Brca1*) have gene expression profile distinct from double mutant (*Rb*$_f$ and *p53*), we used SAM (Storey, Tibshirani 2003, Taylor, Tibshirani 2006, Tibshirani 2006) to select genes that show

significant gene expression differences between the two genotypes. Given the differences between these classes of tumors (for example the altered cell lineage profile indicated by keratin 5 and 14 staining in the triple mutant tumors and their distant metastatic profile) we expected this analysis to identify genes that may indicate the molecular mechanisms that underlie these biological differences, such as genes associated with higher mitotic rates, cell lineage markers genes and basal like cytokeratins based on the "basal" clusters seen in the triple mutant tumors (K5 and/or K14 positive) and genes involved in metastasis (based on distant lung metastasis seen in these tumors). SAM confirmed the significant activation of several specific subsets of genes in the triple mutant tumors (*WAP-Cre; TgMFT*$_{121+/-}$*/p53*$^{cf/f}$*/Brca1*$^{cf/f}$) that included genes involved in metastasis (*TWIST-1, SNAIL homologue 1*), chemokine receptors (Chemokine-receptor-1) and cell cycle proteins (MAD2). SAM also showed down regulation of a small set of genes in these tumors compared to the *Brca1* wild type tumors. A full list of SAM genes can be found in supplementary table **(S1)**.

5.3.3 CXCR4 and SDF1 were expressed only in *Brca1* mutant tumors

SAM showed up regulation of the chemokine receptor C-X-C-ligand 4. As CXCR4 has been implicated before in playing a role in breast cancer cell metastasis (Orimo, Weinberg 2006), we looked for expression patterns of this chemokine receptor and its ligand (SDF1) in our tumor samples. We saw strong expression of CXCR4 and SDF1 in the lung metastases from the triple mutant tumors (**Figure 5.2, a**). The primary tumors only expressed CXCR4 (no SDF1) if the mice had lost BRCA1 expression (**Figure 5.2, b**). Previous studies have identified CXCR4 expression in highly aggressive and metastatic mammary but none

have shown a correlation of *Brca1* mutation with activation of this chemokine pathway. Here we show a direct correlation of CXCR4 expression to loss of *Brca1*. Even though the *WAP Cre; TgMFT*$_{121+/-}$*/Brca1*$^{cf/f}$ mice (double mutant) developed mammary tumors that were slow growing and had a long latency (> 300 days), and these did not metastasize to the lungs, they still expressed CXCR4 (data not shown). Expression of CXCR4 seemed to be in a gradient with most robust expression in the "normal" like mammary gland regions that surrounded the central mammary tumor (**Figure 5.2, e**). This is in keeping with the hypothesis that the CXCR4 –SDF1 axis act as "homing" molecule, drawing tumor cells outward, away from their primary site to distant organs. The role of CXCR4 in these non-metastasizing tumors needs to be better studied but it indicates a change in the primary tumor gene expression with loss of *Brca1* that may stamp the tumor for future metastasis. This study has important implications for the treatment of human breast cancers that arise from familial *Brca1* mutations, with drugs that target and block the expression of CXCR4 and may then prevent the tumors from metastasizing.

Target Validation : Triple null tumors & mets in lungs express CXCR4 / SDF1

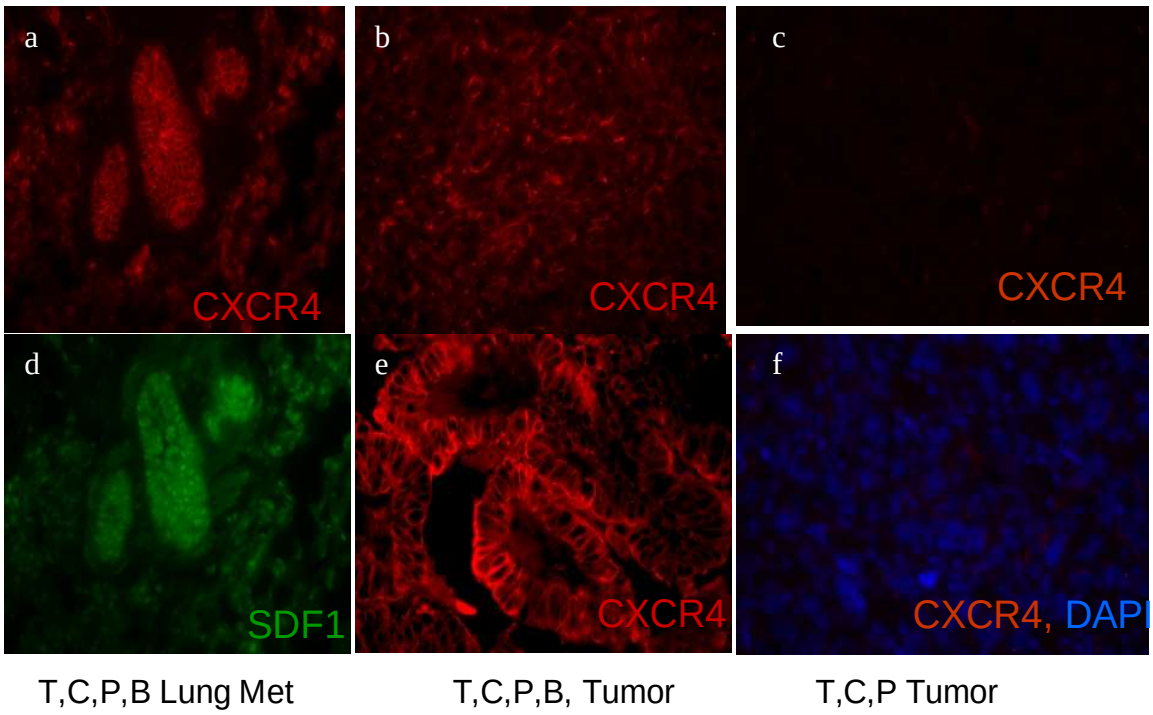


Figure 5.2, CXCR4-SDF1 expression is altered in triple mutant tumors. (a) CXCR4 is strongly expressed in lung metastasis nodules from "triple mutant" (*WAP-Cre; TgMFT*$_{121+/-}$*/p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ */Brca1*$^{\Delta/\Delta}$) tumors **(b)** CXCR4 expression in "triple mutant" (*WAP-Cre; TgMFT*$_{121+/-}$*/p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ */Brca1*$^{\Delta/\Delta}$) primary mammary tumor. Arrow points to cytoplasmic location of CXCR4 which indicates active form (G protein coupled) of the receptor **(c)** Double null (*WAP-Cre; TgMFT*$_{121+/-}$*/p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$) primary tumors stained with CXCR4 show no expression **(d)** Lung metastasis nodules of triple null tumors show strong nuclear (arrow) expression of SDF1 **(e)** Gradient expression of CXCR4 is seen in triple null positive tumors. The "normal like" mammary gland regions surrounding primary tumor have stronger CXCR4 staining than the primary tumor itself. (**f**) DAPI staining of a representative *WAP-Cre; TgMFT*$_{121+/-}$*/ p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ tumors shows absence of CXCR4 staining.

5.4 Discussion

5.4.1 Triple mutant tumors have a unique metastasis signature

Array analysis of mouse tumors and their comparison to human tumors is a powerful tool that can serve many different purposes. First it can classify mouse tumors from varying genetic backgrounds into specific categories and help narrow down the key features of each class of mouse models (e.g., luminal tumors, highly proliferating tumors, etc.). Classification can help identifying pathways that may be relevant to human mammary tumor biology. Second, array analysis can help compare the mouse tumors to human tumors using their gene expression "signature". As one of the main goals of making a mouse model is to be able to use it to study human cancer, it is important to draw parallels between murine and human tumors. As has been shown by Hesrchkowitz et al., 2007, many mouse tumors actually reflect quite accurately a single class of human breast cancer. Especially interesting was that human basal like mammary tumors that are known to be highly proliferative clustered with the *Rb* pathway mutated, highly proliferative mouse mammary tumors. Also human and mouse *BRCA1* mutated tumors shared common marker patterns indicating a common cell lineage. This suggests that array analysis can be used to identify novel diagnostic markers for specific classes of breast cancers. Important and currently insurmountable differences exist between mouse and human mammary gland biology (like expression of ER), but even so, the mouse models can be used for many important drug-targeting studies. It was our goal to first identify whether there existed a gene expression signature that could distinguish the *Brca1* inactivated tumors from the *Brca1* wild type tumors in our model. The second goal was to compare and contrast our mouse models to existing murine mammary tumor models to identify classifiers of human breast cancer. It is our hypothesis that functional loss of *Brca1*

in luminal mammary epithelial cells along with loss of Rb_f and *p53* will lead to mouse mammary tumors that are highly reflective of human *BRCA1* mutated mammary tumors, as most human familial breast cancers with *BRCA1* mutations also show mutations in *RB1* and *P53* (Perou et al. 2000). We find that even though the triple mutant tumors do not cluster along with the other murine *Brca1* mutated tumors that have been proposed to be "basal like" tumors (the *MMTV-Cre;* $Brca1_{cf/f}$ mouse tumors as reported by Herschkowitz et al., 2007), the triple mutant tumors in this report show a robust metastasis signature. Several genes that have been implicated in playing a role in breast cancer metastasis like MMP1 and 2 (Kim et al. 2007), SNAIL (Moody et al. 2005) and CXCR4 (Orimo, Weinberg 2006) are upregulated in the *BRCA1* null mammary tumors. The validation of expression of at least one of them (CXCR4) corroborates the fact that these results are significant and explain the decreased latency and frequent lung metastasis in the "triple mutant" tumors. Further validation of the "signature" genes will be required to establish the *Brca1* signature in this model. This is the first time *Brca1* mutant murine mammary tumors have shown progression from primary aggressive mammary tumor to distant metastasis. That coupled with other features of this tumor like the clusters of Keratin 5 and Keratin 14 seen by immunofluorescence suggest that this is a good model for clinical evaluation of drugs to be used for treatment of *BRCA1* mutated familial cancers.

5.4.2 Mutation of Brca1 in the "right" cell of origin determines the cell lineage of the resulting mammary tumor

The finding that *Brca1* loss (coincident with *p53* mutation) even in predominantly luminal mammary epithelial cells in mice gives rise to tumors with a basal-like phenotype is

notable because it addresses a matter of debate. Human data shows that people carrying *BRCA1* germline mutations tend to develop basal-like tumors (Foulkes et al. 2003, Sorlie et al. 2003) and that human *BRCA1* mutant tumors tend to be *P53* deficient (Crook et al. 1998, Phillips 1999, Phillips, Andrulis & Goodwin 1999a, Phillips, Andrulis & Goodwin 1999b). However it is not clear whether the *BRCA1* mutations that tend to give the "basal-ness" to these tumors arise in luminal epithelial cells or in myoepithelial cells which then expand to form the tumor mass. Our results suggest that the *Brca1* mutation itself is not sufficient to give rise to completely "basal like" tumors even though there is a shift in tumor spectrum that is caused by loss of *Brca1*, as seen by the K5 and/or K14 positive clusters. Most likely the cell of origin of these tumors is also important. So the most likely hypothesis is that combined loss of Rb_f, *p53* and *Brca1* in myoepithelial cells will give rise to human basal like (*BRCA1* familial) tumors. Future studies will test this hypothesis by targeting the same set of genetic events as described in this study in the myoepithelial cells by using a K 5 or K14 Cre.

These results confirm the notion that there is not a single murine model that perfectly represents a human breast cancer subtype, however, the mouse models do show shared features with specific human subtypes and it is these commonalties that should be the focus of future work.

5.4.3 Up regulation of the CXCR4-SDF1 axis is required for *Brca1* null mammary tumor metastasis

The CXCR4-SDF1 axis has been implicated to play an important role in breast cancer metastasis. Increased expression of CXCR4 in primary breast tumors is associated with poor prognosis (Balkwill 2004, Staller et al. 2003). Tumor associated fibroblasts in breast cancer

(referred to as carcinoma associated fibroblasts, CAFs) have increased expression of SDF-1 that attracts CXCR4 expressing tumor cells to distant organs like the lungs, liver, brain and spleen (Orimo, Weinberg 2006). More recently it has been shown that HER2 neu positive breast cancers show increased expression of CXCR4 and a feedback loop mechanism exists between HER2 expression and expression of SDF-1 that predicts distant metastasis of these tumors. Interestingly, the HER2 CXCR4 connection appears to be tissue specific (Pils et al. 2007) and while Her2 positive breast cancers have increased CXCR4 expression, HER2 positive ovarian cancers do not. Also, recent evidence has emerged that ER, PR negative human breast tumors have an elevated expression of CXCR4 and this predicts poor prognosis for them (Woo et al. 2008). These reports suggest that while CXCR4 is a commonly elevated marker for metastatic cancers there are specific underlying genetic mutations that are required in a particular cellular context for it's over expression. In our studies we report elevated CXCR4 expression associated with loss of *Brca1* in mammary tumors. As commonly human *Brca1* mutated breast cancers are ER, PR negative, this corroborates the earlier result seen with human tissues. Mechanistic insights into how the chemokine pathway signaling occurs in response to *Brca1* mutation will help shed light on this phenomenon that we observed. A possible but untested hypothesis is the activation of CXCR4-SDF1 axis by VEGF that is a known activator of CXCR4. Specifically in breast cancer cells it has been shown that VEGF acts in an autocrine fashion to up regulate CXCR4 expression (Bachelder, Wendt & Mercurio 2002). Increased VEGF secretion has been correlated to loss of *BRCA1* expression in ovarian cancers (Ozols et al. 2004). We have seen from our microarray data that the triple null tumors show an increased expression of VEGF. Histology supports this data as the triple null tumors appear more angiogenic and also show increased CD31 staining

(staining for blood vessels, data not shown). So it is conceivable that loss of *Brca1* results in signaling to VEGF regulated pathways which in turn up regulate CXCR4 expression. This hypothesis if verified can provide a powerful model for *BRCA1* mutated breast cancer metastasis and provide a valuable pre-clinical model to study the effect of anti-VEGF drugs for this class of breast cancers. While debate is ongoing about whether a primary tumor is stamped with a metastatic signature from its initiation or acquires this signature later as it progresses, our model suggests that it is the former. We see that even tumors that are Rb_f, *Brca1* mutant (double mutant) and do not physically metastasize have an increased CXCR4 expression. It is tempting to hypothesize that allowed to live longer these mice would eventually have metastatic nodules in their lungs similar to the triple negative tumors. Finally, Hanahan and Weinberg have referred to increased genetic instability as being "an enabling characteristic" (Hanahan, Weinberg 2000) that allows tumors to invade and metastasize with greater ease. We find our model to support this notion and provide a platform for further mechanistic studies on yet unexplored areas like tumor invasion and metastasis.

5.5 Methods

5.5.1 Murine and human tumors

The murine tumor samples were obtained from multiple mice of the *WAP-Cre;* $TgMFT_{121+/-}$ / $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ and *WAP-Cre;* $TgMFT_{121+/-}$ / $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ / $Brca1^{\Delta/\Delta}$ genotypes that had been harvested and stored at -80 F.

5.5.2 Microarray experiments

Microarray experiments were performed as described before (Herschkowitz et al. 2007). Briefly, total RNA was isolated from murine tumors and wild type mammary glands of FVB and BALB/c inbred strains. RNA was purification was done using the Qiagen RNeasy Mini Kit according to the manufacturer's protocol using 20-30 mg tissue. RNA quality was assessed using the RNA 6000 Nano LabChip kit followed by analysis using a Agilent Bioanalyzer. Reverse transcription of two micrograms of total RNA was performed and the RNA was then amplified and labeled with Cy5 using a Low RNA Input Amplification kit (Agilent). The common reference RNA used for these experiments consisted of total RNA harvested from equal numbers of C57Bl6/J and 129 male and female Day1 pups (a gift from Dr. Cam Patterson, UNC). The Reference RNA was treated the same way as the experimental RNA except that it was labeled with Cy3. The amplified sample and reference were co-hybridized overnight to Agilent Mouse Oligo Microarrays (G4121A). The hybridized slides were washed and scanned the next morning, using an Axon GenePix 4000B scanner, analyzed using GenePix 4.1 software and uploaded into our database where Lowess normalization is automatically performed.

5.5.3 Microarray data analysis

Data analysis was performed as reported by Herschkowitz et al., 2007. Briefly, filtered genes that had a log2 ratio value of Cy5/Cy3 in more than 70% samples were hierarchically clustered using Cluster v2.12 (Eisen et al. 1998). For the unsupervised cluster analysis only genes that had a three fold or more variation were used in at least 3 or more samples. Average linkage clustering was performed on genes and arrays and cluster viewing

and display was performed using JavaTreeview v1.0.8 (Saldanha 2004). (These methods are taken from Herschkowitz et al., 2007).

Part II

CGH Analysis

5.6 Introduction

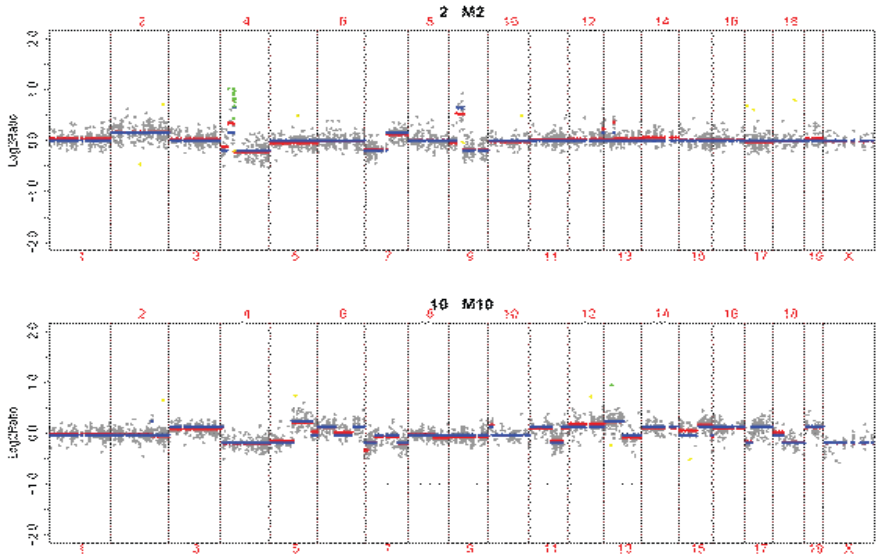
Genomic DNA copy number aberrations occur frequently in solid tumors (Albertson 2003). The many different chromosomal losses and gains associated with various cancers suggest that the tumors take multiple routes to escape cellular mechanisms that try to keep them in check. While some gene defects common in human cancer are known to give rise to increased genetic aberrations (like *RB1*, *P53*), there are many others not known. It is a likely hypothesis that mutations in genes that are important in maintaining normal cell cycle regulations and mitosis or play a role in DNA damage repair may give rise to CIN (Cahill et al. 1998, Lengauer, Wang 2004). RB pathway mutation leading to increased E2F target gene expression and aberrant proliferation is one suggested route for increased genetic instability (Hernando et al. 2004). A recent study has shown that increased expression of E2F target genes is associated with increased genomic instability in a class of human breast tumors. While the ER positive breast cancers appeared to have the best prognosis and also had very little genetic instability, tumors resulting from familial or somatic *BRCA1* mutations had a genome wide increase in deletions and amplifications. These tumors also had poor prognosis. Genes that played a role in mitosis, cell cycle regulation and DNA damage response were more frequently involved in the copy number changes (Fridlyand et al. 2006). *P53* mutations

were far more frequent in familial *BRCA1* mutated cancers than in grade matched sporadic cancers. It was earlier shown that *BRCA1* mutated tumors also showed a highly proliferative signature characterized by increased expression of E2F target genes. We have earlier shown that in a Rb_f inactivated mammary tumor model in mice, LOH of *p53* can lead to low-level genetic instability (Simin et al. 2004). It was also shown recently that mammary tumors in mice that have a combined loss of *p53* and *Brca1* have large-scale genetic instability (Liu et al. 2007). We compared proliferation and apoptosis levels in the triple mutant tumors versus double mutant tumors as an explanation for the dramatic differences in tumor latency and the progression to distant metastasis. Also intriguing were nests of tumor cells that displayed basal-like or metaplastic cell lineage markers, among the triple mutant tumors, both features of human tumors bearing *Brca1* mutations. We hypothesized that *Brca1* loss would cause genomic instability, as many others have shown, and thereby accelerate tumor evolution through dysregulation of tumor suppressors or activation of oncogenes. Our rationale was that mouse tumors tend to show less genomic instability than human tumors, perhaps in part, due to the added protection of longer telomeres (Artandi et al. 2000, Artandi et al. 2002, Chang, Khoo & DePinho 2001) and increased genomic instability would better emulate the changes observed among more aggressive human breast cancers (Fridlyand et al. 2006). As the combined loss of the three genes (*Rb1*, *p53* and *Brca1*) most likely occur in human familial breast cancers, we hypothesized that the dramatic reduction in tumor latency in our triple negative mice as well as progression to metastasis was partly due to increased tumor aneuploidy caused by loss of *Brca1* function.

5.7 Results

Rb_f, p53 and *Brca1* loss of function have been associated with genomic instability. To assess the contribution of each tumor suppressor gene to tumor aneuploidy we performed array-based comparative genomic hybridization (CGH) analysis on mammary tumors from *WAP-Cre; $TgMFT_{121+/-}$/$p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* and *WAP-Cre; $TgMFT_{121+/-}$/$p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ / $Brca1^{\Delta/\Delta}$* females. DNA copy number alterations (CNAs) of tumor DNA compared to normal mammary gland from a wild type FVB female was measured using a genome wide mouse BAC arrays (Hodgson et al. 2001, Pinkel et al. 1998, Snijders et al. 2001). While somatic inactivation of *pRb* and *p53* combined resulted in an appreciable amount of instability as measured by CNAs, the additional loss of *Brca1* resulted in a great increase in instability (**Figure 5.3 and Table 5.1**). Besides overall low levels losses and gains on all chromosomes, which were more subtle changes (summed up in table), there were large portions of Chromosome 4 and 10 lost in the triple negative tumors. **(Figures 5.4, a and 5.5, a)**. This indicated that the loss of *Brca1* is a major determinant for tumor aneuploidy. It is our hypothesis that loss of important tumor suppressor and apoptotic genes on Chromosomes 4 and 10 (FGFR, Gad45, Fas-Ligand, **Figures 5.4, b and 5.5, b**) plays an important role in the tumor progression of these mice. Even though these tumors are have lost *p53* the loss of other apoptotic genes that function in a *p53* independent manner, like Fas ligand and Tumor Necrosis Factor Receptor Family 1 b and 4, can lead to a further weakened genome and an easy target for uninhibited proliferation and distant metastasis, leading to cancer. We are currently testing this hypothesis by combining CGH and microarray data to look for decreased expression of genes present on chromosomes 4 and 10 in our expression results.

BRCA1 null mammary tumors have increased genetic instability



Sample	type	Mad	numChromGain	numChromLoss	numChrom	numChromAmp	numcopy no. transitions	fgaGain	fgaLoss	fga	chrom&transitions*
M1	0	0.15	0	0	0	0	0	0	0	0	0
M2	0	0.12	0	0	0	1	9	0.15	0.12	0.27	9
M3	0	0.12	1	0	1	0	4	0.38	0	0.38	5
M4	0	0.1	4	1	5	1	7	0.2	0.5	0.7	12
M5	0	0.11	1	1	2	2	7	0.08	0.05	0.13	9
M6	1	0.11	3	2	5	0	4	0.36	0	0.36	9
M7	1	0.13	2	0	2	1	9	0.27	0.54	0.81	11
M8	1	0.13	0	0	0	0	27	0.08	0.27	0.35	27
M9	1	0.13	0	1	1	2	16	0.06	0.18	0.24	17
M10	1	0.11	1	1	2	1	17	0.4	0.22	0.62	19

*p < 0.0356

Figure 5.3 Genome plots of two representative samples. M2 (top) represents a double null (*Rb*$_f$, *p53* mutant) tumor and M10 (bottom) represents a triple null (*Rb*$_f$, *p53*, *Brca1* mutant) tumor. The log2ratio data are shown for each BAC clone plotted against the chromosome location (x axis). The red lines show the CBS assignment of copy number levels and the blue lines the Merge Level procedure. Green dots indicate clones that were scored as amplified. Yellow dots indicate outliers. CGH was performed by Huey Bing, UCSF.

Table 5.1. **Summary of Instability Metrics Table.** Counts of different types of aberrations, e.g. whole chromosome changes or amplifications. We score low level copy number changes by the transition from one copy number level to another and call these positions "copy number transitions." There is a trend towards more copy number transitions in the *Brca1*-deficient tumors, but there are too few samples to reach statistical significance; however, the sum of whole chromosome changes and transitions is significantly higher in *Brca1*-deficient animals ($p<0.0356$, t-test). Statistical analysis was performed by Jane Fridlyand, USCF.

Abbreviations:
Type 0= *Brca1*+/+, Type 1= *Brca1* deficient

numChromGain	Number of whole chromosomes gained
numChromLoss	Number of whole chromosomes lost
numChrom	Number of whole chromosomes lost and gained
numChromAmp	Number of chromosomes with at least one amplified region
numcopy no. transitions	Number of copy number transitions ~ number of low level gains and losses
FGA	fraction of the genome = proportion of the genome at abnormal copy number
fgaGain	fraction of the genome gained
fgaLoss	fraction of the genome lost
chrom&transitions	Sum of numChrom and numcopy no. transitions

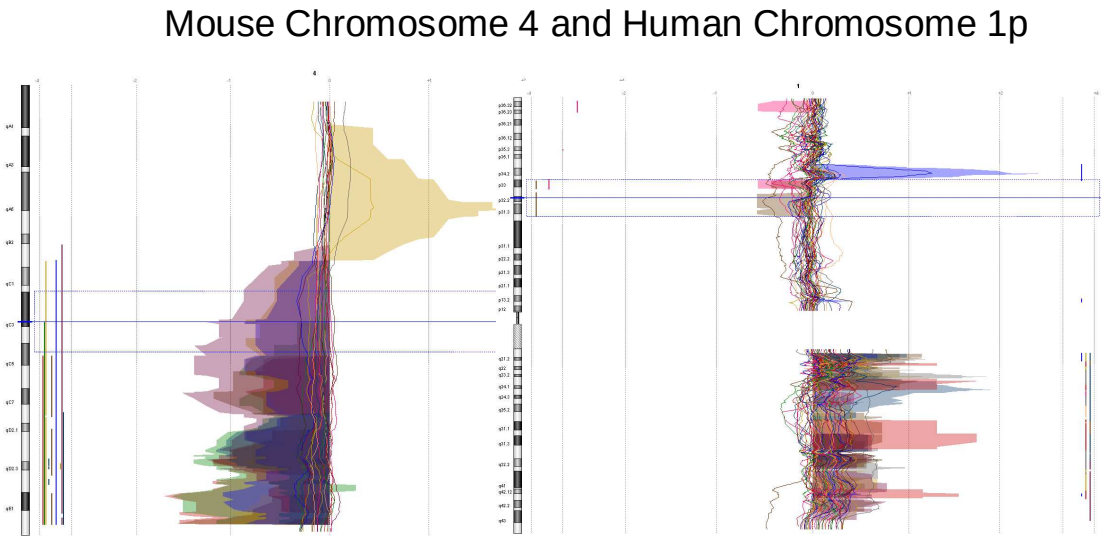


Figure 5.4, a. CGH analysis showing loss of a large region on Chromosome 4 is shown here. The corresponding conserved homologous region on human chromosome 1 p is indicated, showing loss of multiple genes on 1p in human breast cancers.

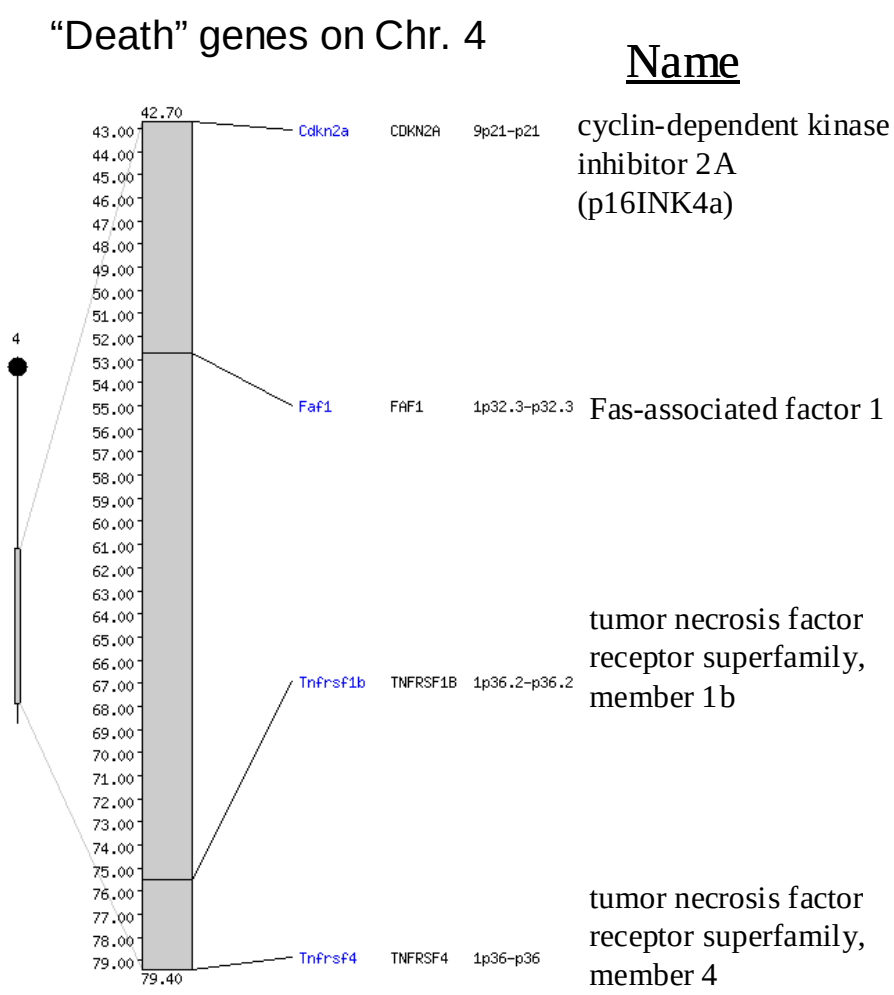


Figure 5.4, b. Genes lost on Chr 4 in the triple mutant tumors are shown here. Genes that are lost on Chromosome 4 include tumor suppressor p16 INK4a (a negative regulator of cell cycle), Fas associated factor 1 which plays an important role in the apoptosis pathway, and two members of the Tumor Necrosis Factor family which are also important players in regulating cell proliferation and apoptosis in a p53 independent manner.

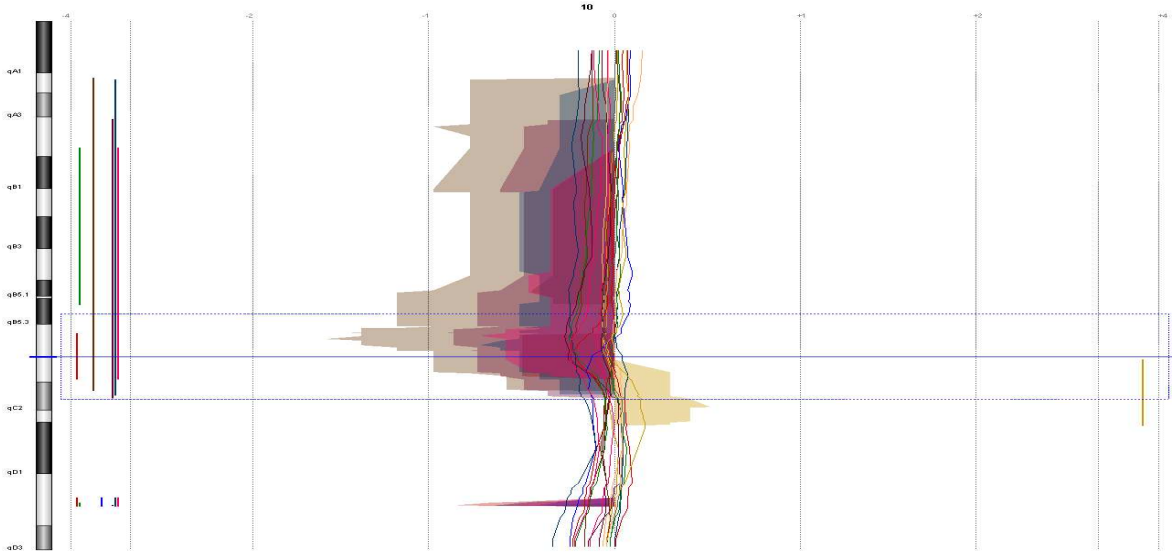


Figure 5.5, a. Triple negative mouse mammary tumors show loss of a region of Chromosome 10 that is shown here.

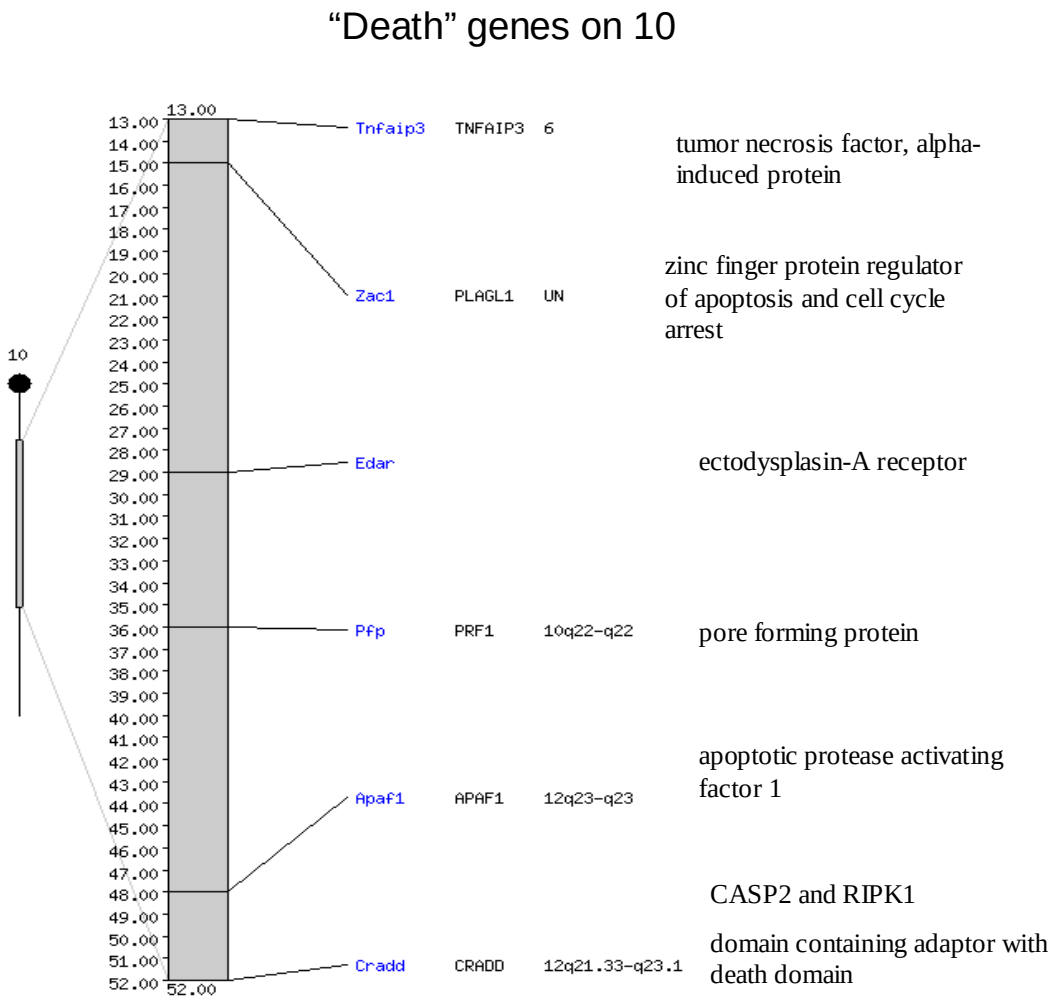


Figure 5.5, b. Significant portions of Chromosome 10 are lost in the triple mutant mice. The region of Chromosome 10 that is lost in triple null tumors includes several pro-apoptotic genes that are shown here. This indicates that loss of *p53* independent apoptotic pathways result in a further weakened genome in the triple mutant mice.

5.8 Discussion

Genomic instability is a hallmark of many human solid tumors. Both *P53* and *BRCA1* have been thought to play important roles in genome surveillance, and loss of both is thought to predispose to increased genetic instability. Studies in mouse models have shown that in the brain epithelium loss of *p53* does not give rise to increased genetic instability even though

tumors arise in the brain with increased frequency (Lu et al. 2001). In the mammary gland mammary tumors that arise in Rb_f null, *p53* null mice have limited genetic instability (Simin et al. 2004). Identification of some recurrent genetic changes in the mammary tumors however suggests that certain low level genetic aberrations may play a role in mammary tumorigenesis in that model. Here we show that additional loss of *Brca1* significantly increases both low-level copy number changes as well as larger regions of deletions in chromosomes 4 and 10. *Brca1* loss results in defective S phase, G_2/M (Xu et al. 1999b) and spindle checkpoints. The combined loss of cell cycle checkpoint, DNA damage repair along with abnormal centrosome duplication resulting from *BRCA1* loss have been hypothesized to cause genetic instability (Brodie et al. 2001, Brodie, Deng 2001, Shen et al. 1998, Weaver et al. 2002). Interestingly similar low level copy number alterations were also reported in familial *BRCA1* mutated human tumors (Chin et al. 2006a, Chin et al. 2006b, Fridlyand et al. 2006). These human tumors were classified as the "complex" that did not have large amplicons but displayed overall instability. While the low-level copy number alterations did not confer an immediate growth advantage they seemed to alter metabolism and provide an early proliferative/survival advantage to the tumor cells. Our results support this human scenario and provide a mouse model for further testing of the effects of these CINs.

An in vivo model showing the synergistic effect of loss of *Rb1*, *p53* and *Brca1* in mammary tumorigenesis, with *Brca1* dependent chromosomal instabilities similar to those seen in human *BRCA1* mutated cancers, has not been generated before. We show in our model, that *Brca1* plays a vital role in the mammary gland in maintaining DNA damage checkpoints and hence leading to delayed tumorigenesis, even in the absence of important tumor suppressors like *Rb1* and *p53*. Loss of *Brca1* leads to an overall "crumbling" of the

genome, with reduced cellular defenses, including lowering of apoptosis, increased proliferation and also an increase in multiple metastatic pathways. All these combined lead to generation of highly aggressive and metastatic mammary tumors. Blocking specific pathways that are activated by *Brca1* mutations by targeted drug delivery will be an important step towards treatment of patients with familial *BRCA1* mutation triggered breast cancers. Our model suggests activation of multiple metastasis genes, including *Snail homologue 1*, *Twist*, *Forkhead Box C2*, *TGF beta receptor*, *MMP1*, *MMP2* and chemokines, *CXCR4* and *SDF1*. Testing our hypothesis that they in fact regulate distant lung metastasis would require knocking out these genes in cell cultures derived from the primary tumors and transplanting them into nude mice. Future experiments have been designed that will address these issues. In itself, this is a powerful model to study *Brca1* loss mediated mammary tumor metastasis.

5.9 Method for Array CGH

Samples were analyzed using Scanning and OncoBAC arrays as described before (Chin et al. 2006b). Scanning arrays consisted of 2464 BACs that were picked along the genome at megabase intervals as described previously (Hodgson et al. 2001, Snijders et al. 2001). DNA sample labeling for array CGH was done as described before. Briefly, 500 ng each of murine tumor and normal (FVB wild type) mammary gland genomic DNA sample was labeled using random priming with CY3- and CY5-dUTP, respectively. The labeled DNA was then denatured and hybridized to CGH arrays. Post hybridization, the slides were washed and imaged using a 16-bit CCD camera through CY3, CY5, and DAPI filters (Chin et al. 2006a, Pinkel et al. 1998).

Part III

Micro RNA Analysis

5.10 Introduction

MicroRNAs are small non-coding RNAs (20-22 nucleotides) that have gained importance over the past few years because of their putative role as oncogenes and tumor suppressors. Specific expression of several miRNAs has been found to be associated with both tumors of the haematopoietic system and solid tumors using genomic techniques like microarray platforms or bead based flow cytometry (Hammond 2006b, Thomson et al. 2006). Commonly dysregulated oncogenes in human cancer like c-Myc have been shown to up regulate expression of certain microRNAs (O'Donnell et al. 2005). Gene expression profiling studies have also shown that several human cancers have a specific microRNA expression profile associated with them that reflects the differentiation status of the tumor (Lu et al. 2005). We hypothesized that the *Brca1* null mouse mammary tumors may have an altered microRNA expression profile that could suggest pathways for their greatly reduced latency, increased metastasis and more differentiated (glandular) histopathologic appearance.

5.11 Results

5.11.1 miRNA expression in triple null versus double null mouse mammary tumors

To investigate this possibility we took five *WAP-Cre*; $TgMFT_{121+/-}$ / $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$, $Brca1^{\Delta/\Delta}$and five *WAP-Cre;* $TgMFT_{121+/-}$ / $p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ mouse mammary tumors and performed custom miRNA microarray on them. Allowing a 0% False Discovery Rate (FDR) SAM was performed. We found that 47 miRNAs were differentially expressed in the *WAP-*

Cre; TgMFT$_{121+/-}$*/p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ */Brca1*$^{\Delta/\Delta}$ tumors. Of the 47 distinguished miRNAs, 36genes were "up" in the triple negative tumors and 11 genes were "down" in them. Of the 36 genes up, some, like miR 467 showed a greater than 5 fold change. Most genes showed a fold change of 1-2 while some of them were at a lower fold change (0.1-0.6). A heat map based on cluster analysis along with the SAM list of genes illustrates the differentiated expression levels of these probes (**Figure 5.6**). Intriguingly, while the *WAP-Cre; TgMFT*$_{121+/-}$ */ p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ tumors expressed higher levels of stem cell like miRNAs like miR 291-5p and 292-3p the *WAP-Cre; TgMFT*$_{121+/-}$ */ p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ */ Brca1*$^{\Delta/\Delta}$ tumors had a more differentiated miRNA profile with high expression of miRNA 30 and the Let-7 family (Let-7a, f) (**Figure 5.6**) (Thomson et al. 2004). This reflected accurately what we observed in the histopathology of these mouse tumors where the *Brca1* tumors appeared more glandular and differentiated in nature while the *Brca1* wild type tumors had a much more undifferentiated, solid adenocarcinoma like appearance. We hypothesize that this difference could be related to the latency of these tumors. As the triple mutant tumors (*Rb*$_f$, *p53* and *Brca1* inactivated) progressed very rapidly from the normal mammary gland stage to form highly aggressive mammary carcinomas, they still retained their well differentiated glandular structure, but the double mutant tumors (*Rb*$_f$ and *p53*) had a longer latency and were slow growing, leading to accumulated changes and loss of normal mammary gland characteristics. We also found a *Brca1* loss associated up regulation of the 17-92 miRNA polycistronic cluster. This cluster has been associated with increased oncogenicity in a mouse Burkitts lymphoma model (Woods, Thomson & Hammond 2007) as well as increasing proliferation and reducing differentiation of lung cells thus pre-disposing to oncogenesis (Lu et al. 2007). Also interesting was the significant up regulation of the miR 30 family (miR 30a-5p, 30b, 30c, 30d

and 30e) in the *Brca1* null tumors. This study is the very first attempt to look for a Brca1 associated miRNA profile and will be followed up with ongoing work on validation of the miRNA targets and mRNA expression profiles on the tumors and with functional studies to shed light on the role of the miRNAs in these tumor sets.

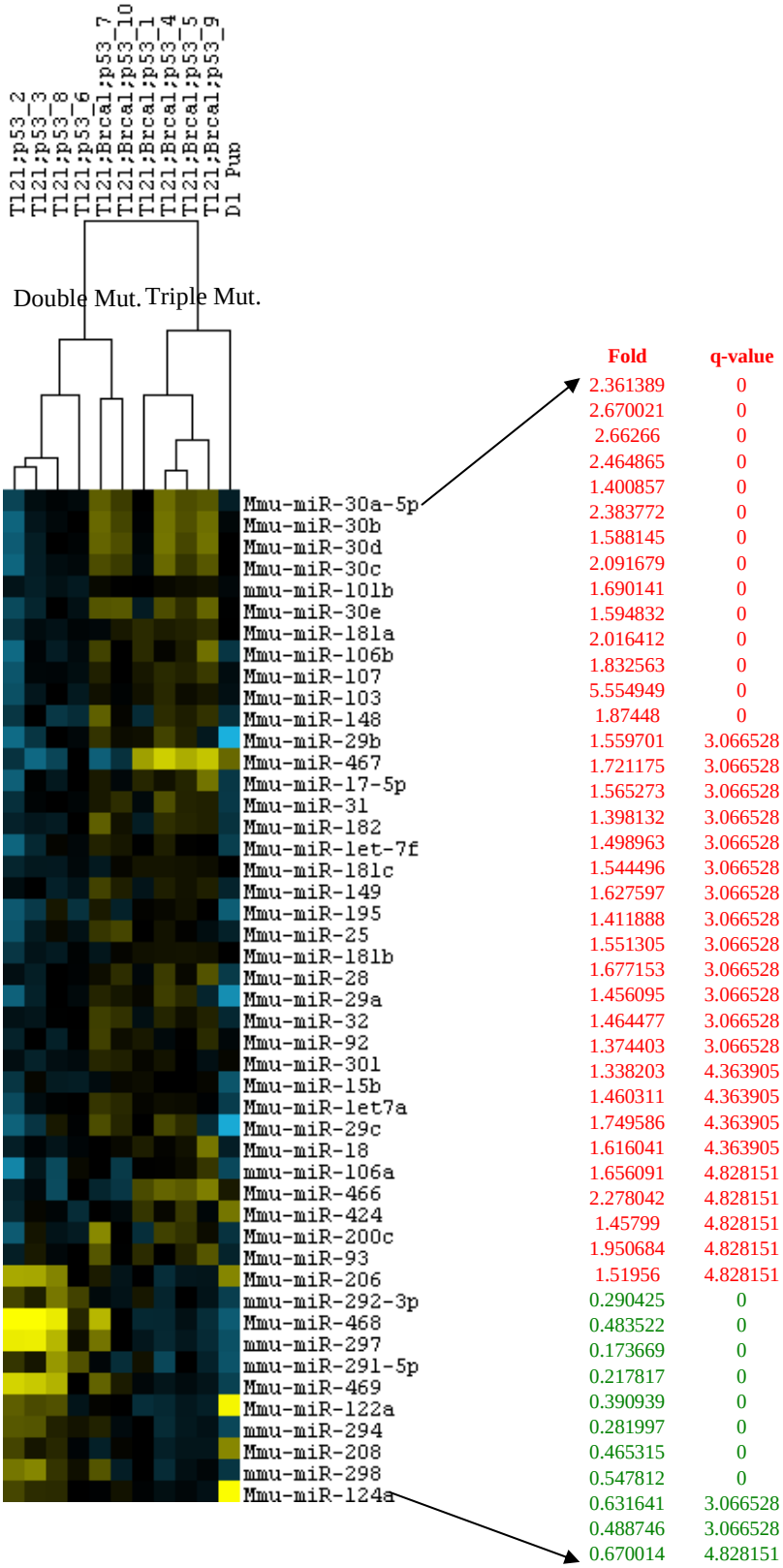
5.12 Discussion

Recent data revealed that breast cancer sub types have unique micro RNA profiles (Blenkiron et al. 2007). Breast cancer subtypes that were identified using expression arrays (luminal A, B, basal, ER positive and ER negative) also show significant differences in their miRNA signature. Whether these profiles indicate real differences in the pathways that these tumors take in their initiation and progression is the matter of intense current research. Some recent mechanistic studies have identified specific microRNA targets like CD44 that may play a role in promoting breast cancer invasion and metastasis (Huang et al. 2008). Recent work has also shown that a group of micro RNAs are regulated by *P53* and play tumor suppressor roles including G1 arrest, apoptosis and senescence (Bommer et al. 2007, Chang et al. 2007, Corney et al. 2007, He et al. 2007a, He et al. 2007b, He, He & Hannon 2007, Raver-Shapira et al. 2007, Tarasov et al. 2007, Tazawa et al. 2007). Also it has been suggested that miRNAs exist on regions of the genome that are frequently altered in cancer, thus representing another layer of complexity to the potential causes for cancer (Calin et al. 2004, Calin, Croce 2007, Sevignani et al. 2007). A *BRCA1* mediated regulation of micro RNA s has not been shown before. It is conceivable that the multi-functional role of *BRCA1* in multiple aspects of maintaining a stable genome is aided by microRNAs, so loss of *BRCA1* may show a corresponding change in some miRNA expression. Whether these miRNAs act

as potential tumor suppressors or oncogenes or what role they play in *BRCA1* loss mediated cancer progression are the questions for further research. . We sought out, as a first pass, to assess the difference in miRNA profiles between *Brca1* positive (wild type) and *Brca1* negative mouse mammary tumors. Several interesting points are readily apparent in the expression map (**Figure 5.6**). The *Brca1* inactivated tumor tissues separate on the dendrogram from the *Brca1* wild type tumors. The data is complicated by the fact that the tumors are largely heterogeneous in nature, specially the *Brca1* wild type tumors. Nevertheless, a large group of microRNAs are highly expressed in the *Brca1* wild type tumors but are not detectably expressed in the *Brca1* inactivated samples. This includes the *mir-292-3p, 291-5p, 468, 469, 297 and 206.* Similarly the miRNA 30 family goes up significantly in the *Brca1* mutant families, as do the Let-7 family of miRs and the 17-5p polycistron. The *let-7* family is induced at embryonic day (E) 17 and increases in adult tissues. *C. elegans let-7* is a well-characterized developmental regulator. Their expression pattern in the *Brca1* null tumors suggests this family has a role in the differentiation pattern of these tumors. Our follow-up experiments in the future are designed to generate hypotheses about possible mechanisms that could explain the altered distribution of miRNAs reported in this study. Also we will look at differences in microRNA signature between the primary and metastatic tumors to uncover potential mechanisms for invasion and metastasis in breast cancer. We speculate that these motifs might represent real differences in these two genotypes and point to regulation of target transcripts. Further study now underway is required to test the hypothesis that altered miRNA expression pattern is involved in the etiopathology of the synergistic role of pRb_f, *p53* and *Brca1* in mammary tumorigenesis.

5.13 Methods

Total RNA was isolated form mouse tumors and normal moue mammary gland (FVB) using Trizol reagent (Invitrogen, Carlsbad, CA) and resuspended in DEPC treated water to a concentration of about 1ug/ul. Labeling and hybridization are performed exactly according to methods described in (Thomson, Parker & Hammond 2007).



Fold	q-value
2.361389	0
2.670021	0
2.66266	0
2.464865	0
1.400857	0
2.383772	0
1.588145	0
2.091679	0
1.690141	0
1.594832	0
2.016412	0
1.832563	0
5.554949	0
1.87448	0
1.559701	3.066528
1.721175	3.066528
1.565273	3.066528
1.398132	3.066528
1.498963	3.066528
1.544496	3.066528
1.627597	3.066528
1.411888	3.066528
1.551305	3.066528
1.677153	3.066528
1.456095	3.066528
1.464477	3.066528
1.374403	3.066528
1.338203	4.363905
1.460311	4.363905
1.749586	4.363905
1.616041	4.363905
1.656091	4.828151
2.278042	4.828151
1.45799	4.828151
1.950684	4.828151
1.51956	4.828151
0.290425	0
0.483522	0
0.173669	0
0.217817	0
0.390939	0
0.281997	0
0.465315	0
0.547812	0
0.631641	3.066528
0.488746	3.066528
0.670014	4.828151

Figure 5.6, Global expression pattern of micro RNA is altered in *Brca1* inactivated tumors when compared to the expression of *Brca1* wild type tumors. Heat map shows triple mutant tumors on the right node and double mutant tumors on the left node. The fold change in expression of each of the genes that are altered (as generated by SAM) are listed in table and correspond to the genes showed in heat map.

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CHAPTER SIX

ESTABLISHMENT OF A 3-D EPITHELIAL CULTURE SYSTEM USING PRIMARY MAMMARY EPITHELIAL CELLS

6.1 Abstract

While mouse models are powerful tools for studying co-operating genetic lesions, a significant amount of time and resources are involved in making a mouse model of cancer. So cell culture systems have long been used as an alternative system to study genetic interactions in vitro. Traditional cell cultures grown in a two dimensional cell culture plate do not represent the cell's natural environment in vivo. This can cause the cells to undergo phenotypic and genotypic changes to enable them to survive in an artificial surrounding. These accumulated mutations in cells that have been grown and maintained over many years can seriously affect any cell culture experiment and create several unknown variables affecting the experimental read out. To avoid this three-dimensional cell culture systems have been established that better represent the cell's in vivo environment. But currently established systems using the three-dimensional culture system have employed human cell lines that have been passaged for decades, and therefore have been subjected to the selective pressures for growth under culture conditions. This can introduce confounding variables in any genetic experiment conducted using these cells. To overcome this problem and in an effort to rapidly screen potential genetic interactions prior to making a mouse model we have established a

primary three-dimensional mammary cell culture system derived from strains of mice established before. The long-term goal is to use the three-dimensional epithelial culture system to investigate mechanisms of pRb family inactivated mammary tumorigenesis in parallel with mouse genetic studies described before. By adopting established cell culturing techniques we have successfully isolated primary mammary epithelial cells from the previously reported *TgWAP-T_{121}* mouse mammary tumor model (Simin et al., 2004). We have grown the primary cells both in short-term two-dimensional collagen gel cultures using standard cell culture techniques, and in three-dimensional culture using the matrigel "on-top" method reported before. By using freshly isolated primary mammary epithelial cells we have avoided the accumulation of random mutations in these cells. We believe the use of primary cells reported here is an innovation that may offer distinct advantages.

6.2 Introduction

The advent of three-dimensional cell culture systems has revolutionized research in the mammary gland cell culture field. Many limitations of standard, two-dimensional culture have been overcome using the three dimensional system. In particular, two-dimensional culture forces cells to grow on a flat surface, resulting in the disruption of cell-microenvironment interactions. In contrast, three-dimensional cell culture provides an artificial extracellular matrix that more closely mimics the natural microenvironment.

Matrigel was first isolated from mouse tumors originally thought to be chondrosarcomas with large amounts of extracellular matrix (Orkinet al., 1977). These tumors were named the "EHS" tumors after J. Engelbreth-Holm and Richard Swarm, the

investigators who first characterized them. EHS tumors were a source of basement membrane, rich in components such as Laminin, Type IV Collagen, Heparan Sulfate Proteoglycan, several proteases, including matrix metalloproteases (MMPs, e.g. MMP-2, 9), as well as many growth factors, such as EGF, TGFβ, PDGF and FGF. The unique characteristics of the EHS tumors prompted these investigators to harvest and purify the basement membrane components, which was later commercialized as the product Matrigel (BD Technologies). For a number of cell types, growth in matrigel evokes cellular phenotypes more reminiscent of cells grown in their native, *in vivo* environment. For example, melanocytes showed rapid pigmentation (Kleinman et al., 1986) and endothelial cells formed characteristic tube-like structures (Kubota et al., 1988). Therefore, among certain cell types matrigel provides additional cell differentiation cues. In addition, some cell lines and primary cells grown in matrigel demonstrate reduced proliferation and increased differentiation when grown in this culture medium, and the cells grown in matrigel resembled the morphology formed *in vivo*. Furthermore, tumor cells grown in matrigel also displayed invasive properties by forming "tunnels" that invaded through the matrigel. This was another reflection of "*in vivo*" behavior by cells in matrigel (Kramer et al., 1986).

Bissell and colleagues showed for the first time that primary mouse mammary epithelial cells when grown in matrigel formed duct-like structures similar to mammary ducts *in vivo* and also developed a hollow lumen similar to mammary gland acini. Most significantly, the addition of specific hormones in the cell culture media resulted in secretion of beta-casein, an enzyme found in milk, into the mammary gland lumen in culture (Li et al., 1987), a further reflection of the ability of these cultures to mimic *in*

vivo biology. Taken together, these studies show that 3-D culture of mammary epithelial cells in an artificial matrix might better resemble the cell's natural environment, and provide additional avenues for investigating mammary gland development in a cell culture-based system. The three-dimensional cell culture system has more recently been adopted for breast cancer studies, specifically in investigations of tumor-microenvironment and signaling pathway interactions. Mammary tumor cell lines grown in 3D display several characteristics of mammary glands in vivo. These include forming hollow spheroid like structures that are reminiscent of mammary acini and maintaining apicobasal polarity that is key for mammary gland architecture in vivo (Petersenet al., 1992, Streuli et al., 1990). Matrigel can be manipulated to reduce secretion of its inherent growth factors (by using reduced growth factor matrigel) and using this several hitherto unknown paracrine cell signaling pathways were identified that resulted in increased proliferation of mammary epithelial cells and disruption of the hollow lumen. Examples of this are the pioneering work done by Brugge et al. using the human mammary epithelial cell line MCF-10A that identified Bim as the proapoptotic factor leading to hollow lumen formation in vitro by apoptosis of cells inside the lumen. It was shown that anoikis (loss of contact of cells with basement membrane) might disrupt integrin mediated signaling pathways and provide the cue for Bim regulated apoptosis (Reginato et al., 2003). Parallel mouse models validated these results by showing that knocking out Bim delayed apoptosis in mammary terminal end buds and thus resulted in delayed lumen filling (Mailleux et al., 2007). This result had important significance in mammary tumorigenesis, as one of the initial hallmarks of human breast cancer is lumen filling, possibly by decreased apoptosis. Brugge et al have used the MCF-10A cell line for

further studies on possible pathways for mammary tumorigenesis and metastasis. Another area of seminal research conducted using the three dimensional cell culture systems was to establish a human immortalized cell line series that progressively showed normal to more malignant properties in matrigel. These cell lines derived from the HMT-3522 human cell line have been used to study the role of β1-integrin and EGF receptor signaling pathways in the disruption of normal mammary gland architecture (Wang et al., 1998, Weaver et al., 1997).

This system has also been useful for studying potential factors responsible for mammary cell metastasis. Invasive phenotypes can be easily observed using light microscopy and compared to non-invasive controls. Metastasis can also be studied in a more quantitative manner by using invasion matrigel chambers (Gunawardane et al., 2005). In summary, the three dimensional culture of mammary epithelial cell lines has proved to be a powerful tool to study mechanisms involved in maintenance of normal mammary gland architecture and signaling pathways involved in disrupting this normal architecture and possibly promoting tumorigenesis.

Given the history of successful use of the three dimensional cell culture system in understanding many aspects of mammary gland development and tumorigenesis, it is clear that the use of primary cells that have not undergone random mutations and accumulated changes over time is the next step. The use of primary mammary epithelial cells in 3D culture for studying the cooperative role of gain or loss of functions of genes known to be disrupted in human breast cancer have been hindered by the great heterogeneity of the system, that makes growing and working with them technically challenging. Most labs have used immortalized, non-transformed cell lines like the MCF-

10A that are easier to grow and handle and have a homogenous population of cells all behaving the same way. These cell lines, while recapitulating certain features of human mammary epithelial cells, also have significant differences. For instance, while the MCF-10A cells form a single layered mammary acinar structure in matrigel, human mammary acini are formed of a double layer of cells, an inner luminal layer and an outer myoepithelial layer. MCF-10A cells show molecular markers characteristic of both luminal and myoepithelial cells, but have mainly myoepithelial characteristics (Perou et al., 2000). This is a caveat in using these cell lines especially to study the role of *BRCA1* in breast tumorigenesis. *BRCA1* mutated familial breast cancers have often shown a basal keratin marker profile (e.g. Keratin 5/14) and it is currently not known why these tumors have this distinguishing marker characteristic. Using the MCF-10A cells to study changes brought about by loss or gain of *BRCA1* would be confounding as these cells have an already existing mixed marker profile. It has also been shown before that co-culture of mammary luminal epithelial cells with myoepithelial cells help polarization of the luminal epithelial cells by their secretion of Laminin 1. In tumors the secretion of Laminin 1 is often disrupted which also disrupts polarization of the mammary gland (Gudjonsson et al., 2002). These and other studies indicate the importance of having the separate myoepithelial and luminal cell layers in the mammary gland structure.

Knowing the advantages of the 3D cell culture system as discussed above it was our long-term goal to utilize this system to investigate mechanisms of T_{121}-initiated mammary tumorigenesis in parallel with genetic studies described in the previous chapters. We wanted to establish a cell culture system that recapitulated the in vivo situation as closely as possible. As others have avoided working with primary

mammary epithelial cells due to their great heterogeneity, we contemplated using cell sorting techniques using specific cell surface markers (like MUC1) to derive pure populations of primary mammary luminal and myoepithelial cells. But highly pure populations of either luminal or myoepithelial cells would not reflect the heterogeneity of the mouse mammary gland and would not ideally reflect mammary tumorigenesis, which includes interactions between the myoepithelial and luminal epithelial cells, as well as cues from the surrounding fibroblasts and mammary stroma. So we used established techniques to culture and grow unsorted primary mouse mammary epithelial cells in matrigel. We hypothesized that expression of T_{121} would boost survival of the cells both in 2 D and 3 D culture by increasing proliferation as seen in animal models developed before. To initially establish this system we used ME cells from wild type (BALB/c) mice and from the more established *TgWAP*T_{121} mouse line (Simin et al., 2004). An important goal was to establish the morphological features of the control and transgenic cultures in 3D and use immunohistochemistry/immunofluorescence to look for T_{121} expression in cell culture. The WAP promoter is induced by lactogenic hormones in vivo, and can be induced in a mouse mammary cell line in 2D culture by a combination of insulin, prolactin and dexamethasone (Wagner et al., 1997). To establish that the 3D system recapitulates effects seen in vivo, we decided to investigate T_{121} expression, cell proliferation and apoptosis. Confocal microscopy was utilized to evaluate acinar structure and cellular properties. T_{121} expression was to be detected by immunostaining; proliferation to be detected by immunofluorescence staining of Ki-67 while apoptosis to be detected by ethidium bromide fluorescence as described (Debnath et al., 2002, Debnath et al., 2003, Debnath et al., 2005, Reginato et al., 2003). We also wanted to

determine if apoptosis is p53-dependent in the 3D system, as seen in our in vivo mammary gland models, by performing parallel experiments with ME from *TgWAPT$_{121}$ / p53$^{-/-}$* animals. Thus, our studies of these initial responses in vivo would guide the adaptation of accurate representation in vitro. In addition, use of the *TgMFT$_{121}$/Cre*-inducible system would facilitate studies in the progression from normal to tumorigenic epithelium in an isogenic cell set. For the *TgMFT$_{121}$* system we would use the expression of eGFP as the initial read out for MMTV promoter activity in cell culture and then use Cre viral infection to express T_{121}.

To further evaluate genetic pathways and cooperating lesions in T_{121} oncogenesis, in the future we would combine T_{121} expression with dominant expression or suppression of additional genes. *TgWAPT$_{121}$* mammary cells would be infected with retroviruses that express potential cooperating oncogenes or repress TSGs using short hairpin (sh) RNAs for RNA interference. We would initially use RNAi directed at *p53* as a positive control for this system (Hemann et al., 2003). Additionally, ME would be harvested from compound mutant mice described in earlier experiments (e.g. *TgWAPT$_{121}$* with *Pten$^{+/-}$*, *Brca1$^{+/-}$* or *Brca2$^{+/-}$*) to determine the effects of these lesions on proliferation, apoptosis and genomic instability (using CGH) in vitro. Cre retroviruses would be used in cases of ME derived from *TgWAPT$_{121}$* mice harboring conditional alleles of TSG's such as PTEN. Finally, candidate cooperating oncogenes or TSGs from array experiments proposed above would be tested to determine if cooperative effects are seen in vitro. In cases where cooperative interactions are observed, such candidates would be further evaluated in GEMMs.

6.3 Results

6.3.1 Wild type primary mammary epithelial cells form tight spheroids in culture and develop a hollow sphere by day 10.

Primary mammary epithelial cells form wild type (BALB/c) late stage pregnant mice were grown in matrigel for ten days and then stained with DAPI. When observed under confocal microscope these cells were seen to have formed hollow spheroids. Z section images were taken across the cell cross-section using confocal microscope. The serial sections revealed gradual formation of hollow lumen in multiple spheres. This indicated that primary mouse MECs behaved like the immortalized but non-transformed cell lines in matrigel **(Figure 6.1)**.

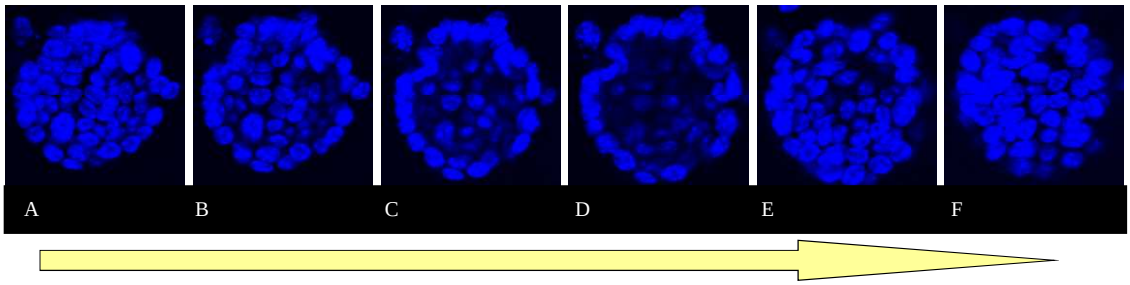

Figure 6.1, Wild type primary ME cells form spheroids with hollow lumen in 3D culture. Wild type (BALB/c) primary ME cells were cultured in matrigel for 10 days. Representative confocal images of Z sections taken through the cell are shown. The cell nuclei have been stained with DAPI. The cells form spheroids with a central hollow lumen as shown in D.

6.3.2 $WAPT_{121}$ primary mammary epithelial cells form delayed and incomplete hollow lumens in culture

Primary ME cells isolated form *TgWAPT*$_{121}$ mice were stained with DAPI on day 10 in 3D culture. These cells formed larger and more loosely held spheroids compared to the wild type BALB/c cells. When observed under the confocal microscope at day 10 these cells were commonly seen to form multi acinar structures within one spheroid and also had smaller lumens **(Figure 6.2)**. The hollow lumen grew larger when observed at day 13 but there was also a corresponding increase cellular structure break down and cell death at this stage.

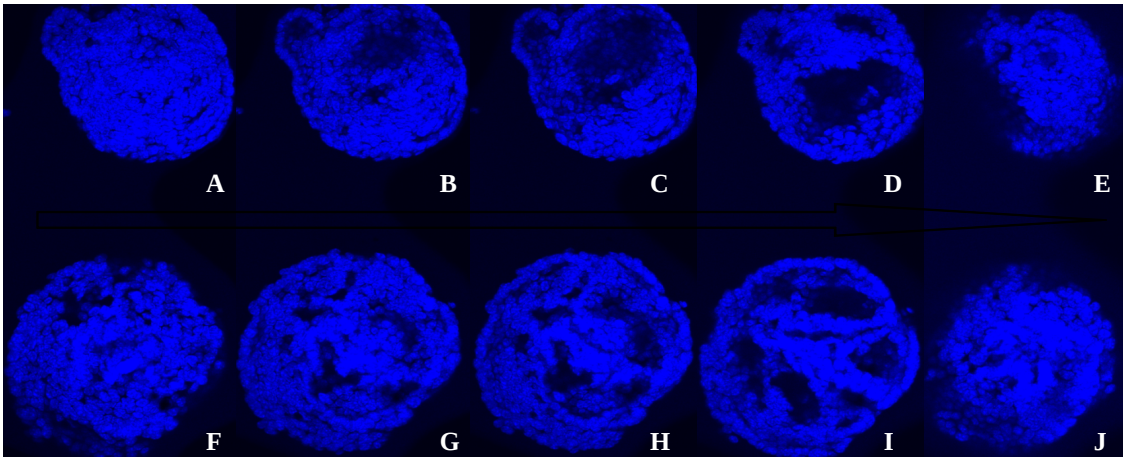

Figure 6.2, T_{121} expressing primary ME cells show delayed and incomplete lumen formation in 3D culture. *TgWAPT*$_{121}$ primary mammary epithelial cells were harvested from late pregnant female mice and grown in matrigel. Representative confocal images of Z sections taken through the cells isolated from two independent *TgWAPT*$_{121}$ mice are shown in the upper and lower panels respectively. The cell nuclei have been stained with DAPI. Hollow lumen formation is incomplete as seen in C and D. Concentric acinar structures are seen within the main spheroid (H, I). The spheroids are larger in diameter than wild type cells and more loosely attached.

6.3.3 Sporadic T_{121} expression is detected in primary *TgWAPT_{121}* mouse mammary epithelial cells grown in 3-D by immunohistochemistry

Whey acidic protein (WAP) is a hormone regulated milk protein that is expressed in high levels during late stage pregnancy and lactation in the mouse mammary gland. The promoter sequence of this protein has been commonly used to target gene expression in a mammary specific manner in transgenic mice. However, one of the problems of using the WAP promoter has been the need for multiple (at least two) cycles of pregnancy and lactation to express the promoter and hence drive the target gene expression in the mammary gland. We have successfully used the WAP promoter to drive T_{121} expression in the mouse mammary gland. The *TgWAP-T_{121}* mice have developed mammary tumors with a hundred percent penetrance with a long latency of 16 months (Simin et al., 2004). It was shown before by other labs that WAP expression is turned off in cell culture conditions (Brown et al., 1986, Lee et al., 1984, Lee et al., 1985) even though expressing some other milk proteins like β-casein, α-lactalbumin, lactoferrin and transferrin in culture has been relatively easier (Blum et al., 1987, Chen et al., 1987, Emerman et al., 1977, Li et al., 1987, Medina et al., 1987, Suard et al., 1983, Wicha et al., 1982, Wilde et al., 1984). This could be due to the tight regulation of WAP protein secretion that is dependent on a fine balance of hormones and the stage of mammary gland development. It was shown for the first time by Chen and Bissell in 1989 that WAP secretion occurred when primary mouse mammary epithelial cells were grown in matrigel and formed acinar structures that resembled mammary gland alveoli in vivo. So it was concluded that WAP expression was regulated by specific culture conditions and was also dependent on the ability of the cells to form alveolar structures. Regulation was

through diffusible factors from cell-extracellular matrix paracrine signaling pathways. In the right culture conditions WAP mRNA levels could be detected but the protein was undetectable (Chen et al., 1987). As our goal was to establish the expression of T_{121} in three-dimensional cultures, we were dependant on WAP and/or MMTV expression in our culture system. We harvested mammary epithelial cells from late stage pregnant WAP-T_{121} mice and used established methods to grow these cells both in cell culture plates and in matrigel. We observed that in matrigel these cells developed spheroids in about 7 days. Using confocal microscopy we determined that the spheroids formed a semi hollow lumen at about day 10. If observed earlier the cells showed significant lumen filling though spheroid acinar structures had already formed and these structures appeared to have reached their maximum size. We did immunohistochemistry by embedding the cells at day seven in paraffin and then making sections from them. Upon staining with antibodies specific for T_{121} expression we were able to detect T_{121} expression in some of these samples as shown in **Figure 6.3, A, B**. This was validation that WAP was being expressed in our culture conditions and transgene expression could be detected. We were unable to maintain longer term cultures for follow up observations with the primary mammary epithelial cells as these cells showed signs of cell death after a period of about fifteen days.

It is worth mentioning here that the system had poor reproducibility perhaps due to great heterogeneity that made each culture very different from another. A quantitative study would require more rigorous establishment of culture conditions that allow high reproducibility. This required time and technical expertise that we decided not to pursue.

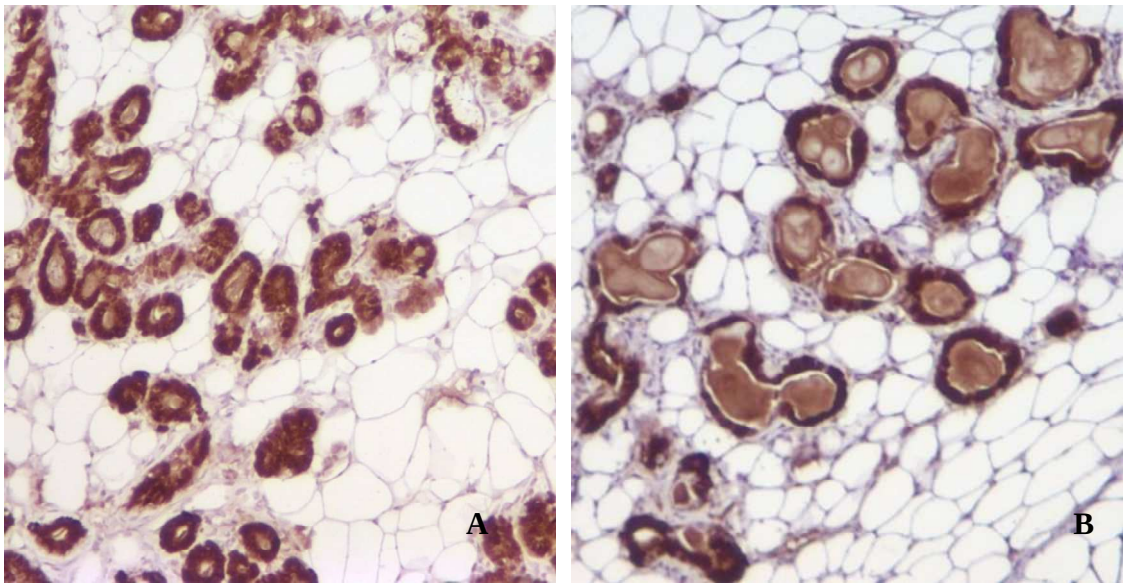

Figure 6.3, Primary ME cells isolated from late pregnant *TgWAPT$_{121}$* mice were grown in matrigel using total embedment method. Cells at day 10 were formalin fixed and paraffin embedded and 4-micron sections were stained for T_{121}. T_{121} expression is observed in the luminal layer of the ME cells indicated by brown staining in A and B.

6.3.4 Expression of eGFP by primary *TgMFT$_{121}$* cells in three-dimensional cultures is sporadic.

It was our goal to establish the primary mammary epithelial cell culture of the *TgMFT$_{121}$* mouse line as this conditional system would allow us to progressively study the effects of *pRb* loss, *p53* loss and other genetic interactions in culture. The hope was to be able to use eGFP expression in the *TgMFT$_{121}$* cells as an initial read out for cell viability and to be able to establish the normal (nontransgenic) ME cells in culture first. Then we would use retroviral Cre infection to turn on T_{121} expression and hopefully be able to observe functional changes in the cells, like increased proliferation (using Ki67 staining). With this goal in mind, we harvested and established primary mouse ME cells from late stage pregnant *TgMFT$_{121}$* mice. Expression of eGFP was observed in culture only sporadically. The cells grew and formed tight spheroids and also developed a hollow

lumen (as seen by DAPI staining of the cell nuclei using a confocal microscope). However we were unable to get robust eGFP expression in this system. Weak and sporadic eGFP expression was observed sometimes, but this was not a consistent result. Tweaking the culture conditions with varying levels of hormones (like hydrocortisone and prolactin) did not improve the expression of eGFP.

6.3.5 Complete loss of p53 increases invasiveness in 3D culture and in Boyden chamber assays.

One of the powerful features of the matrigel culture system is its use in studying tumor cell invasiveness. While normal, non-malignant cells grow to form tight spheroids in matrigel, invasive malignant cells do not form these tight structures, instead they form tunnels through the matrigel and form a network. Chemoinvasion assays using the Boyden Chamber have been used to identify several metalloproteinases (MMPs) (Yu et al., 1996), cytokines (Giavazzi, 1996, Levine et al., 1995) and serine proteinases (Andreasen et al., 1997) that play an important role in tumor metastasis. The three dimensional culture of mammary epithelial cell lines in matrigel have been used to study the cooperative effects of ErbB2 and TGFβ in promoting breast cancer metastasis (Seton-Rogers et al., 2004).

We had observed from the tumors developed in our transgenic mouse models that the *WAP Cre; $TgMFT_{121}$ /$p53^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* mice developed very aggressive tumors that were frequently attached to the peritoneum. This was unlike the less aggressive, more localized tumors formed in the *WAP Cre; $TgMFT_{121}$ /$p53^{\Delta 2\text{-}10/+}$* mice. We wanted to test if this difference in metastatic potential was reflected using the three dimensional culture of the

tumor derived cells in matrigel. We found that the three dimensional culture systems accurately reflected what we had observed in vivo. While the *WAP Cre; TgMFT$_{121}$ / p53$^{\Delta 2\text{-}10/+}$* tumor cells formed localized spherical colonies in matrigel **(Figure 6.4, D)**, the *WAP Cre; TgMFT$_{121}$ / p53$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* tumor cells invaded through the matrigel to form an elaborate network **(Figure 6.4, A)**. This was strong evidence that the system could be used as a tool to study the effect of potential pro-metastatic candidates in vitro and could also be used to study suppressors of metastasis. When grown in 2D the tumor cells from the *WAP Cre; TgMFT$_{121}$ / p53$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* mice grew rapidly covering the cell culture plates in about 5 days **(Figure 6.4, B)**. These cells also had a spindle shaped morphology that is reminiscent of cells undergoing epithelial to mesenchymal transition (EMT). The *WAP Cre; TgMFT$_{121}$ / p53$^{\Delta 2\text{-}10/+}$* tumor cells grew slowly in 2D and formed clusters of cells with more epithelial features like cuboidal morphology **(Figure 6.4, E)**. We also performed a separate Boyden Chamber invasion assay that reflected that same result, that is, while the *WAP Cre; TgMFT$_{121}$ / p53$^{\Delta 2\text{-}10/+}$* cells remained primarily on the top of the chamber the *WAP Cre; TgMFT$_{121}$ / p53$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$* cells invaded through the matrigel and coated the bottom of the chamber **(Figure 6.4, F)**.

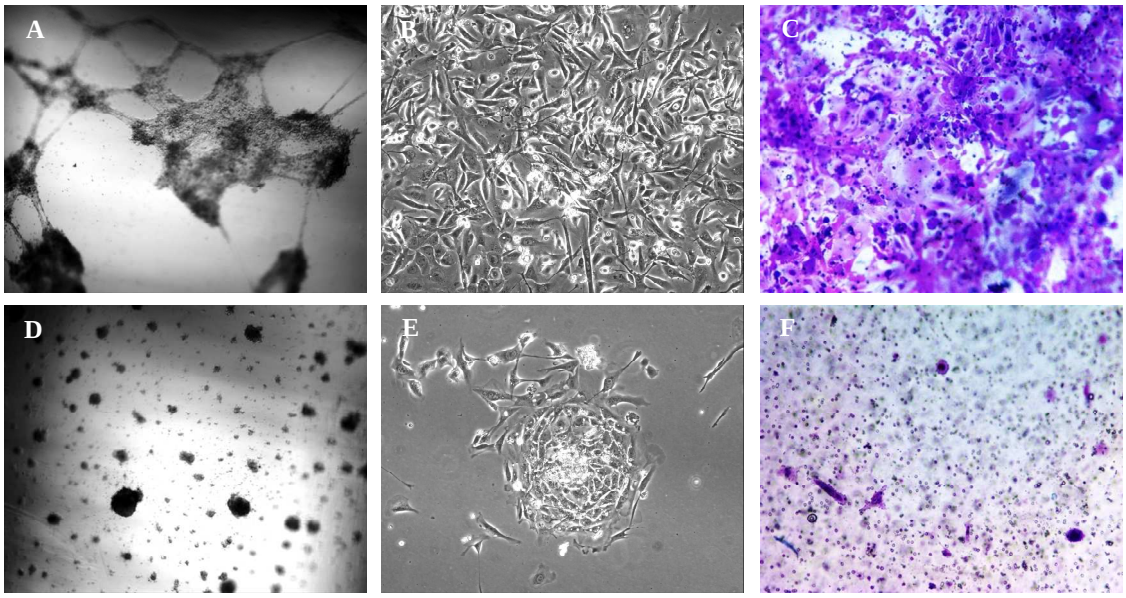

Figure 6.4, Complete loss of p53 in primary ME cells caused increased metastatic potential in vitro. Tumor cells were harvested from *WAP-Cre; TgMFT*$_{121}$ */p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ and *WAP-Cre; TgMFT*$_{121}$ */ p53*$^{\Delta 2\text{-}10/+}$ mice mammary glands and grown in 2D **(B, E)** and 3D **(A, D)** respectively. Representative images of the cells in 2D and 3D observed under light microscope are shown here. After 5 days in 3D culture *WAP-Cre; TgMFT*$_{121}$ */ p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ cells formed elaborate networks **(A)** through the matrigel that indicated their invasive potential. The *WAP-Cre; TgMFT*$_{121}$ */ p53*$^{\Delta 2\text{-}10/+}$ cells at the same time point remained as small spheroids in matrigel **(D)**. In 2D the *WAP-Cre; TgMFT*$_{121}$ */ p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ tumor cells grew rapidly and formed a monolayer of epithelial cells that looked remarkably fibroblast like due to their spindle shaped morphology that was unlike the cobblestone nature of epithelial cells **(B)**. At the same time point, after about five days in culture the *WAP-Cre; TgMFT*$_{121}$ */ p53*$^{\Delta 2\text{-}10/+}$ tumor cells remained as sparse local clusters with a more epithelial like cuboidal morphology **(E)**. In the Boyden chamber invasion assay the *WAP-Cre; TgMFT*$_{121}$ */p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ tumor cells invaded the matrigel and formed a dense layer of cells on the bottom chamber surface **(C)** in 24 hours. The *WAP-Cre; TgMFT*$_{121}$ */ p53*$^{\Delta 2\text{-}10/+}$ cells remained on top of the matrigel and did not invade **(F)**.

6.4 Discussion

6.4.1 Primary mammary epithelial cells form hollow spheres in three-dimensional cultures and can be used to study relevant cancer gene influences on mammary cell architecture.

We have established primary mouse mammary epithelial cells in 2 D and 3 D culture and shown that wild type (non transgenic) mammary epithelial cells grow in matrigel for about two weeks and form a hollow spheroidal structure at 10 days. This is identical to what has been shown in immortalized but non-transformed mammary epithelial cell lines. We have also seen WAP expression on three-dimensional culture and the ME cells from the late pregnant *TgWAPT*$_{121}$ mice have formed spheroids with slightly delayed formation of hollow lumens. This delay observed in short term cultures is in keeping with studies done in the MCF-10A cell line where retroviral infection with the oncoprotein HPV16 E7 resulted in an initial lumen filling but this was later abrogated by increased apoptosis and formation of a hollow lumen (Debnath et al., 2002). It was shown using the MCF-10A cell line that suppression of the Rb family proteins and p21 by E7 was enough to promote initial proliferation resulting in lumen filling. But this was not enough to overcome apoptosis later by the Caspase pathway and that resulted in clearing of the lumen. Only cells that were co-infected with pro-proliferation factors (like E7) and anti-apoptotic factors (like Bcl-2) could maintain increased proliferation and did not form a hollow lumen (Debnath et al., 2002). As T_{121} binds with and inactivates the RB family proteins (pRB, p107 and p130), it is very likely that there was an increased proliferation followed by a P53-induced apoptosis that resulted in the late stage hollow lumen formation.

Our preliminary results using the three-dimensional cell culture system in matrigel gave us encouraging results and we believe that further fine-tuning of this system will result in a powerful tool for screening cooperating lesions. Such lesions should result in increased proliferation, decreased apoptosis, increased genomic instability or disruption of acinar structures. The increased accessibility of an in vitro system will also allow more careful examination of T_{121}-induced phenotypes, possibly providing additional clues about the mechanism of tumorigenesis. For example, the position of apoptotic cells within acinar structures may suggest the nature of the apoptotic signal. A recent study using immortalized human ME demonstrated that cells in the lumen of acini underwent apoptosis, suggesting the need for survival signals provided by matrix attachment. A combination of increased proliferation and apoptosis suppression was required for cells to fill the lumen (Debnath et al., 2002). Our in vivo results support the need for apoptosis suppression in combination with T_{121}-induced proliferation for tumorigenesis, suggesting that a variety of anti-apoptotic lesions may cooperate with T_{121} in this assay. If so, additional lesions in the same apoptotic pathway will be tested to elucidate how the pathway is initiated in the system. Finally we also expect to extend the 3-D culture study to other organ systems including prostate and ovary (for which we have already established mouse lines) using strategies described above for mammary gland**.**

6.4.2 The 3D culture system can be used to screen potential metastatic genes in vitro

Metastasis is often the major cause of death in cancer as the disease becomes incurable at that point. In human breast cancer metastasis commonly occurs to the bones, brain, lungs and liver. The primary tumor histopathology and morphology has been commonly used to predict the metastatic potential of a tumor. But these parameters are

often misleading and not good predictors of distant metastasis. The more recent advances in gene expression profiling have revealed distinct metastasis signatures in tumor subtypes that predict both the possibility for distant metastasis (van de Vijver et al., 2002, Wang et al., 2005, Wang et al., 2007) and also the most likely site for metastasis (Kang et al., 2003, Minn et al., 2005). Mouse models have been designed to study human breast cancer metastasis. But biological differences exist between mice and humans that makes mouse mammary tumors commonly non metastatic or metastatic only to the lungs (Derksen et al., Lin et al., 2004, Muraoka et al., 2003). Metastasis is a very complex multi-step process involving detachment from and lysis of extracellular matrix, surviving proapoptotic signals in cell microenvironment and in the lymph nodes and blood stream, seeding at distant sites and finally proliferating at those sites to form tumor nodules. The many steps in this process are impossible to model in mice or to study in human tissues. Therefore cell culture systems are most useful in the study of metastasis. Most cell cultures done in a two dimensional setting have severe limitations. The non-in-vivo like environment make the cells generate survival signals or apoptotic signals that lead to abnormal cell behavior. The cells are unable to maintain their normal architecture in this setting and this makes it impossible to study cell-extracellular matrix interactions involved in the process of metastasis. The three dimensional culture of cell lines has been used as a more tractable tool to study cell-extracellular matrix interactions and several important studies have been performed on cancer metastasis (Lauffenburger and Horwitz, 1996, Montesano et al., 1991). But even there studies have been restricted by the use of cell lines that do not correctly reflect the in vivo situation. Cell lines have cumulative changes that have occurred over many years that may play unpredictable roles

in cell behavior. The ultimate goal is to develop a better cell culture model by using short-term cultures of primary epithelial cells that have not accumulated random changes over time. Also important is to model the heterogeneity of cancer and preserve cell-stroma and cell-fibroblast interactions to study non-cell autonomous effects and the effects of paracrine signaling pathways in the induction of metastasis. Keeping these in mind, in this study we have established two important points. First, primary mammary epithelial cells can be gown in 3-D culture to form spheroids as has been shown by others using cell lines, and can also be manipulated to express transgenes in culture. Second, cell invasion and metastasis can be visualized easily using the 3-D culture system. In our system the *WAP-Cre; TgMFT*$_{121}$ */ p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ tumor cells formed branching networks in matrigel, indicating their invasive properties. Cells from *WAP-Cre; TgMFT*$_{121}$ */p53*$^{\Delta 2\text{-}10/+}$ mice did not form these invasive structures, but rather grew as loose clumps of spheroids in matrigel. So we were able to distinguish loss haploinsufficiency of p53 by corresponding changes in tumor behavior in cell culture. These results were corroborated by mouse model studies that indicated the *WAP-Cre; TgMFT*$_{121}$ */ p53*$^{\Delta 2\text{-}10/\Delta 2\text{-}10}$ were of a far more aggressive nature and often attached to the peritoneum.

So this system can be used for the relatively high throughput screening of possible genes that either promote or suppress metastasis. Mechanistic studies involving the precise role of these genes in the process of metastasis such as induction of EMT, breakdown of ECM and cell-cell junction protein breakdown can be done with further technical improvements in maintaining and handling the primary cells in culture as well

as by using hormone independent promoters to drive gene expression in culture unlike MMTV Cre (like Keratin promoters).

6.5 Materials and Methods

6.5.1 Method of harvesting primary mouse mammary epithelial cells

Mouse mammary glands were isolated from late stage pregnant transgenic or control mice. After isolation of the #3 and #4 mammary glands of the mouse they were washed in PBS and minced into very small pieces using pre-sterilized forceps and razor blade and while working inside a cell culture hood to maintain sterile conditions. The glands were then placed in a pre-warmed digestion media (Collagenase Solution), and placed in a 37 deg C water bath with slow shaking (100 rpm) for 2 hours to allow complete digestion. After complete digestion the digested glands were spun in 50 ml falcon tubes at 1500 rpm for 10 minutes. The top layer that separated was the fatty layer from mammary gland fat pad. This layer also contained mammary organoids that have not settled to the bottom. So 10ml of the top layer was taken in a 15ml falcon tube and pipetted up and down several times to break free the organoids. The tube was spun again and this time the top fat layer was thrown away and the organoid clump of epithelial cells was resuspended in 10ml of 0% DMEM F12 (no FCS added). The pellet in the 50 ml falcon tube was also resuspended in similar manner and the two suspensions were combined. They were then spun at 1500 rpm for 10 minutes. The pellet was resuspended in 5ml of 0% DMEM/F12 and 40ul of DNase (2U/ml). The tube was shaken for 2-5 minutes vigorously by hand to break up all the clumps. Then the tube was spun at 1500 rpm for 10 minutes to remove DNase. The pellet was resuspended in 10ml 0%

DMEM/F12. The falcon tube was then pulsed in a rotor for one second only, by letting the rotor speed reach 1500 rpm and then cutting the breaks immediately. This resulted in settling of the more heavy epithelial cells and floating of the supernatant fibroblasts that could be either grown separately to discarded at this time. The pulse spinning was repeated 8-10 times to achieve complete separation of the epithelial cells. After each pulse spin the supernatant was discarded and the pellet was resuspended in fresh 0%DMEM/F12. Also after each pulse spin and resuspension, about 10µl of cells were observed under the microscope to look for enrichment of organoids. When a large population of organoid structures and very few fibroblasts were observed under the microscope the pellet of epithelial cells were resuspended at high density in cell culture media. Media composition was done exactly as reported by Debnath et al, Methods, 2003. Cells were plated on cell culture plats coated with a thin layer of Collagen I (Vitrogen) or on plastic directly. Cells were passaged after 3 days or when they become confluent at a 1:1 or 1:2 ratios.

6.5.2 Method of 3-D culture of primary mouse mammary epithelial cells in matrigel

Two methods have been reported for growing mammary epithelial cells in matrigel three-dimensional cultures. One of them is the total embedment method where the cells are embedded completely in matrigel and the second is the overlay or “on top” method where the cells are embedded in matrigel on three sides. Instead of total embedment, here a cell culture solution with diluted matrigel is used to cover the cells from the top. Both these culture methods have been published in (Lee et al., 2007) and (Debnath et al., 2003) respectively. Briefly, in the “on top” method matrigel is thawed

overnight at 4°C and a thin layer (40µl) of matrigel is coated on pre-chilled chamber slides (BD). This is done on ice to prevent matrigel from solidifying while coating the plates. The coated chambers are then allowed to sit at 37°C in an incubator for 15 minutes to solidify but not allowed to overdry. During the drying of the matrigel coated plates, the cells are removed from the plate using Dispase, suspended in cell culture media and spun down for 3 minutes in a rotor at 1500 rpm. The pellet is washed twice using media each time and the final pellet is resuspended in cell culture media at the concentration required obtained after cell counting. A concentration of about 25000 cells/ml of media is desirable. A separate media solution is made by adding 4% matrigel to the regular cell growth media. Finally the cells are mixed with the matrigel containing media at a 1:1 ratio and 400µl of the mixture is plated in each chamber slide on top of the coated matrigel. This gives about 5000-cells/ ml in each well, embedded in 2% matrigel solution. The cells are allowed to grow in a 5% CO_2 humidified chamber and fed every 4 days with 2% matrigel containing media. The cells usually start forming spheroids in 4-5 days and hollow centers could be observed at around day 10 in culture.

In the total embedment method the cells are resuspended in matrigel alone and plated on an already coated chamber slide. The cells embedded in the matrigel are then allowed to solidify at 37°C before adding cell culture media from the top.

6.5.3 Immunostaining of cells in 3-D culture

Two methods for immunostaining were used in our system. One was the direct immunostaining of the cells in the chamber slides as reported by Debnath et al., 2003. Briefly in this method, the cell culture media was aspirated and the cells structures were

immediately fixed using 2% paraformaldehyde in PBS, pH 7.4. After fixing the cells were permeabilised with 0.5% Triton X-100 in PBS for 10 minutes at 4°C. Then after performing a series of rinses with PBS/glycine the cells were blocked with the primary blocking solution for 1.5 hours at room temperature. After primary blocking secondary blocking was performed using IF buffer with 10% goat serum and 20μg/ml goat anti-mouse F $(ab')_2$ for 40 minutes. Then overnight incubation was done with primary antibody at 4°C. The next day primary antibody was rinsed with IF buffer and secondary antibody incubation was performed at room temperature for 40 minutes using Alexa Flour secondary antibodies for immunofluorescence. A 1:200 dilution was typically used for secondary antibody. Primary antibody concentration was determined by manufacturer recommendations and by trial and error methods. Finally rinsing of secondary antibody was done followed by counterstaining with DAPI (4,6-diaminino-2-phenylindole) for 5 minutes at room temperature. The slides were then washed in PBS and mounted using Prolong Antifade Reagent obtained from Molecular Probes.

In the second method using the embedded cells, the matrigel embedded cells were removed from the chamber slides using gentle dislodgement with razor blades. The cells in matrigel were then fixed in formalin (Sigma) at room temperature for 20 minutes and then washed in PBS and paraffin embedded. Slides of about 4 μm thickness were then made like regular tissue slides and staining was performed using staining protocol used in chapter 2.

6.5.4 Boyden chamber assay

BD Biocoat Matrigel chambers were used for this assay. Assay was performed according to manufacturer's instructions. Briefly warmed cell culture media is first used to rehydrate matrigel in the bottom of the cell culture wells and the inserts. Media containing chemoattractants like growth hormones is added to the wells of the BD Companion Plate. After this sterile forceps are used to transfer the control and test inserts into the chamber wells, making sure no bubbles arise. Immediately, approximately 1.5 x 10^5 cells/ml are suspended in cell culture media and plated on cell culture inserts. The chambers are then incubated at 37°C, 5% CO_2 humidifying chambers for 24 hours. After 24 hours the non-invading cells are removed from the top pf the chambers by scrubbing twice using cotton swab. The cells that have invaded through the matrigel and settled on the bottom of the chamber are stained using a Diff-Quik kit. The cell nucleus staining is purple and the cytoplasm staining is pink. Cell counting is done by removing the membrane from the insert and placing on a microscope slide. Multiple fields of the slide are then photographed under a light microscope for cell counting.

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CHAPTER SEVEN

CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Introduction

Recent advances in diagnosis and treatment of breast cancer have made it possible to manage the disease for many years and allow patients a good quality of life. However certain subtypes of breast cancer, particularly ones that are metastatic still have a very poor prognosis. This dissertation focuses on the roles played by the RB pathway, *P53* and *BRCA1* mutations in the development and progression of breast cancers. Both RB pathway mutations and *P53* mutations are common events in human sporadic breast cancers (Bosco, Knudsen 2007, Greenblatt et al. 1994, Knudson 1993, Lehman et al. 1994) while *BRCA1* mutations (often coupled with *P53* mutations) are the single most common reason for human familial breast cancer development (Moynahan 2002). The role of mutations in these genes have been studied singly or in co-operation with other genetic lesions before, but the potential co-operation of all three have not been studied in detail before. Recent evidence suggests that human breast cancer is a highly heterogeneous disease with multiple co-operating genetic lesions deciding the pathway and outcome of each class of cancer. Also, mutations in *P53* and *BRCA1* have been found to occur frequently together in the basal subtype of human cancer (either familial *BRCA1* mutated breast cancers or sporadic basal like cancers), while these cancers also show an

increased proliferation profile indicating deregulation in the RB pathway (Fridlyand et al. 2006). Therefore an understanding of the role of the RB pathway, *P53* and *BRCA1*, alone and in co-operation with each other in defining the etiology of human breast cancer in a suitable animal model is of utmost importance in the understanding of the human disease and the identification of possible drug targets. Particularly, the early onset of *BRCA1* mutated familial breast cancers and their aggressive nature has made *BRCA1* mutations important to study. Combined with the complex pathological features of this type of cancer (ER, PR, Her 2 negative) is their propensity to metastasize to the lungs and brain leading to early morbidity (Rakha et al. 2008). Currently there is a dearth of mouse models that accurately model this subtype of human breast cancer. One of the goals of this dissertation was to model some specific genetic and histopathological properties of this disease that could result in a suitable pre-clinical model for drug development. Some of the important results of this work and the future directions they can take are summarized below.

7.2 Rb_f Inactivation Does Not Promote Mammary Tumorigenesis in This Model

It was shown before using a WAP promoter to specifically target the expression of T_{121} in the mouse mammary gland that Rb_f loss in the mammary epithelium results in mammary adenocarcinoma, albeit with a long latency (Simin et al. 2004). In that model mouse lines with high levels of T_{121} expression (as detected by western blot) were selected for further studies. It is expected that these mice carry multiple copies of the transgene, thus resulting in robust expression of T_{121}. It is therefore likely that there is virtually complete loss of function of RB and RB related proteins in these mice. In

addition the adenocarcinomas noted in this line, loss of Rb_f resulted in increased proliferation in the mammary glands as well as increased apoptosis that is *p53* dependant. In the studies reported in this thesis, we do not observe identical results. In the $TgMFT_{121}$ model the mouse mammary glands exhibit delayed involution but not tumorigenesis upon Rb_f inactivation. There are multiple possibilities for this altered result. First, the Cre recombinase excises the head to tail array of transgenes reducing transgene expression to single copy only. It is likely that the single copy of T_{121} in the $TgMFT_{121}$ model did not completely sequester the RB_f proteins resulting in incomplete loss of the Rb_f expression. A gene dosage effect of *Rb* may thus be playing a role in the reduced mammary phenotype seen here. However, the phenotype seen in these animals still suggests that substantial sequestration of RB has occurred. In addition, a number of features of this transgenic line make it ideal of the studies reported here as well as future studies examining interaction of members of the RB family with other oncogenic proteins.

The $TgMFT_{121}$ model has several advantages over the $TgWAP\text{-}T_{121}$ model. First, loss of Rb_f alone has rarely been found to be the single cause for human breast cancer progression and in that sense the $TgMFT_{121}$ mouse model may better represent the human disease where there is a quantitative change in the activity of this pathway. In the $TgMFT_{121}$ mouse the loss of Rb_f function alone is not enough to drive breast cancer, but it is sufficient to cause a burst of proliferation on day one lactation. This increase in proliferation may eventually result in the generation of a pre-cancerous condition in the mammary gland. Alternatively it is possible that the delayed involution reflects alterations not only in proliferation but also in important pathways for ensuring the apoptotic loss of proliferative cells after nursing has ceased. It is easy to imagine that the

failure to remove cells from the gland at the end of lactation could increase the probability that some cells could accumulate additional changes that would allow them proliferate independent hormonal regulation.

Second and perhaps most importantly, the design of the *TgMFT*$_{121}$ transgenes allow the expression of the gene and therefore the inhibition of the RB family to be regulated by the expression of a cre recombinase. The cre recombinase can be placed under the control of a tissue specific or temporally regulated promoter. This will be important in the future for experiments in which the importance of the timing of loss of RB relative to the acquisition of mutations in TP53 and BRCA1 are examined. This an ideal model for combining multiple genetic mutations that seem to play an important role in breast cancer progression, like mutations in *p53* and *Brca1* and turning on the gain or loss of function of all genes of interest by using a single Cre recombination event. The study of genetic mutations both in combination and alone can be used to answer specific questions regarding the sequence and timing of each mutation and the role it may play in the final outcome of the disease. This is a specific advantage over the *TgWAP-T*$_{121}$ model as being a germline model, the *TgWAP T*$_{121}$*/p53* mutant mice succumbed to non-mammary tumors (lymphomas and sarcomas).

7.3 *"Layering on"* of Loss of *p53* Results in the Formation of Mammary Adenocarcinomas with No Distant Metastasis.

P53 is the most commonly lost gene in any human cancer and is lost in more than 50% human sporadic breast cancers (Greenblatt et al. 1994, Lehman et al. 1994). Familial breast cancers resulting from the loss of *BRCA1* have an even higher frequency of *P53*

mutation (Lakhani et al. 2005, Narod and Foulkes 2004). Earlier mouse models designed to study the loss of *p53* in the germline resulted in a high frequency of tumors of the lymph nodes and skin (Donehower et al. 1992) that precluded the study of mammary tumors. Mammary specific loss of *p53* resulted in mammary tumors with a long latency of about 10 months (longer depending on the genetic background) indicating that other co-operating genetic lesions may be required for mammary tumor initiation in these models (Lin et al. 2004). In this dissertation, by using a conditional T_{121} to specifically block the *Rb* pathway it is possible to study the effect of loss of the *Rb* pathway in the mouse mammary gland. Also, using the Cre-Lox-P technology it is possible combine other genetic mutations like the loss of *p53*. The concomitant loss of *p53* in this model results in the formation of mammary adenocarcinomas with a latency of one hundred and fifty days. A haploinsufficient effect of *p53* in mammary tumor progression is observed, as the loss of a single allele of *p53* results in mammary adenocarcinoma formation with a median latency of two hundred and seventy five days. By generation of mice in which the mammary epithelial cells are engineered to be deficient in two genes, genes that are frequently deregulated in human breast cancer it is possible to have a better understanding of the role played by each gene in cancer initiation and progression. Studies of the $TgMFT_{121}$ disease model suggest that while loss of the *Rb* pathway results in the creating a suitable pre-cancer environment in the mammary gland, the combined loss of *p53* removes the important cell cycle checkpoint and allows the cells to progress to form local mammary adenocarcinomas. However the combined loss of Rb_f and *p53* is still not enough for progression to distant metastasis and acquisition of mutations in additional loci clearly play a part in tumorigenesis. .

7.4 Acquisition of Mutations in *Brca1*, in Addition to Loss of *p53* and *Rb* Function, Decreases Tumor Latency and Increases the Frequency of Tumor Metastasis.

BRCA1 mutations are responsible for 80% familial human breast cancers (Moynahan 2002). *BRCA1* mutated breast cancers are clinically often referred to as "triple negative" as they are ER, PR and Her2 negative and respond poorly to hormonal therapy (Dent, Warner 2007, Liedtke et al. 2008, Perou et al. 2000). These cancers also frequently have a basal histopathological profile marked by an increase in Keratin 5 positive mammary epithelial cells (Hicks et al. 2006). *P53* mutations are more common in these hereditary breast cancers than in the sporadic breast cancers and may contribute to their highly aggressive nature. This class of cancers also has a highly proliferative signature marked by the increased expression of E2F target genes that are responsible for the progression of cell cycle from G1 to S phase (Fridlyand et al. 2006). A suitable mouse model for this sub class of breast cancer is currently absent though many efforts have been made to model this disease (Liu et al. 2007). The two aspects of this disease that have been difficult to model in mice in the past have been the clusters of Keratin 5 positive cells observed in the human mammary tumors and the metastasis pattern of these tumors, primarily to the lungs and brain (Luck et al. 2008).

Using the $TgMFT_{121}$ model in this thesis it was possible to examine the impact of combined loss of *Brca1 and RB and/or p53*. The studies of these animals suggests that while the combined loss of any two of these genes, that is, *Rb* and *Brca1*, *Rb* and *p53* or *p53* and *Brca1* are unable to produce features of the human familial breast cancers, the combined loss of all three (*Rb, p53* and *Brca1*) resulted in the formation of tumors with pathological characteristics common in some classes of human tumors.

Mouse mammary tumors arise in the triple mutant mice with a greatly reduced median latency of only fifty days. The tumors are locally invasive (as indicated by frequent attachment to the peritoneum) and about 60% of them metastasize to the lungs. Perhaps most interesting was our observation that these tumors also contain nests of Keratin 5 positive cells. As Keratin 5 is a marker for human "basal" like tumors that frequently occur in patients with familial *BRCA1* mutations, this suggests that the loss of *Brca1* has an effect on myoepithelial cell proliferation. Taken together our studies support a model in which *Rb, p53* and *Brca1* have a synergistic role in the initiation and progression of a specific sub class of human breast cancer. Array analysis of the mammary tumors arising in mice deficient in *Rb*, *Brca1*, and *p53* function show a significant overexpression of metastasis promoting genes that also have a putative role in EMT (like *SNAIL, TWIST* and *Vimentin*). We expect that further analysis of the tumors arising in these animals will identify novel biological pathways that are involved in the familial *BRCA1* mutated (as well as sporadic basal like) sub type of human breast cancer. The very short latency of the mammary tumors arising in the triple mutant mice as well as their genetic profile that mimics some aspects of the human familial breast cancers makes this a very suitable candidate for future testing of drugs for this sub class of human breast cancers.

7.5 Loss of Rb_f and *p53* Does Not Promote Genetic Instability in The Mammary Gland But Synergistic Loss of *Brca1* Leads to Increased Genetic Instability.

P53 is mutated in about 50% human cancers (Levine, Hu & Feng 2006, Varley 2003). Loss of function of *P53* results in loss of its tumor suppressor activities. There has also been evidence of some gain of function mutations in *P53* that give it oncogenic

potential. Inheritance of a mutant *P53* allele pre-dispose these individuals to Li-Fraumeni syndrome where they are highly susceptible to developing a wide variety of cancers including breast and prostate adenocarcinomas and lymphomas (Hainaut, Hollstein 2000, Hollstein et al. 1991, Petitjean et al. 2007). It is generally believed that loss of *P53* results in genetic instability resulting from loss of cell cycle checkpoint and DNA repair pathways as well as cell death pathways through apoptosis (Livingstone et al. 1992). Support for this notion came primarily from the study of hereditary forms of human cancer like Ataxia–Telangiectasia and Werner syndrome. These cancers primarily arose from mutations in genes that are responsible for repairing damaged DNA within the cells and thus preventing the accumulation of genetic instability. The high frequency of *P53* mutations in these cancers suggested that loss of *P53* allowed genetic instability to progress and accumulate in human cells, ultimately fueling cancer (Kinzler, Vogelstein 1997). Early mouse models with mutations in genes that were responsible for maintaining genetic stability (like mTERC mice with a missing RNA component of telomerase) showed a rescue of tumor phenotype upon loss of *p53* (Chin et al. 1999, Lee et al. 1998). Also mouse models with germline mutations in p*53* developed the human counterpart of Li-Fraumeni syndrome where they were susceptible to a wide variety of tumors in their life time (Donehower et al. 1992, Donehower et al. 1996). These and other similar studies seemed to support the idea that loss of *p53* alone could result in genetic instability that ultimately increased the probability of the cells acquiring additional mutations beneficial to its growth and eventually its ability to metastasize to other organs. . However some early studies done using human cell lines challenged this paradigm and showed that disruption of *p53* alone in human cells does not result in aneuploidy (Bunz et al. 2002).

Supporting this contrary notion, that *p53* mutations alone may not contribute to genetic instability were mouse models that showed in the brain epithelium the loss of *p53* alone or in combination with the loss of the Rb_f resulted in very limited genetic instability (Lu et al. 2001). This was also the case when the impact of the loss of these two genes was examined in mammary epithelium (Simin et al. 2004). In the current model, using CGH analysis to look for genetic instability it is seen that the combined loss of Rb_f and *p53* in the mammary epithelium results in limited genetic instability. However the combined loss of Rb_f, *p53* and *Brca1* results in genome wide instability. Curiously the loss of Rb_f and *Brca1* combined does not result in quick progression of mammary cancers and is associated with high levels of *p53* mediated apoptosis. So taken together our studies suggest that while *p53* alone may not be responsible for promoting genetic instability, the loss of *p53* may play a rate limiting step in the process of accumulation of instability that promotes cancer progression. Whether any of these models accurately reflect human cancer is debatable. However, it is unlikely that the entire burden of maintaining human genome stability would fall upon *p53* alone. It is a much more likely scenario that the synergistic loss or mutation of multiple important checkpoint proteins would be necessary to trigger human cancer.

Whether the synergistic role of the loss Rb_f, *P53* and *BRCA1* in promoting cancer is conserved across different cell types is another intriguing question. The human tumor spectrum resulting from *BRCA1* mutations are limited to the breast and ovary. So it is a likely hypothesis that even though *BRCA1* is expressed in all cell types, it has special tumor suppressor functions only in these two cell types. Future studies with mouse

models designed to study the synergistic loss of *Rb_f*, *p53* and *Brca1* in other cell types can answer this question.

7.6 What Comes First? Genetic Instability or Cancer?

This is an important and rather complex question to answer. Data from our lab has shown that cancer in mice, in multiple cells types, can occur without the induction of overall genetic instability. Mutations in a few important genes, like *Rb_f*, *p53* and or *Pten* can result in aggressive epithelial cancers with varying latencies. It is not clear if this is also the case with human cancers. Clinical data from human tumors suggest that most human solid tumors have widespread aneuploidy and mutations of multiple major tumor suppressor genes (Cahill et al. 1999, Pihan et al. 2003, Pihan, Doxsey 2003). The lack of "pre-cancer samples" or early lesions from human studies makes it difficult to answer questions like the sequence of the mutations, the precise timing of aneuploidy, etc. However studies in human colon polyps that can later form colon cancer if untreated has shown that in that particular cell type, genetic instability and aneuploidy occur very early in the initiation process (Lengauer, Kinzler & Vogelstein 1997, Thibodeau, Bren & Schaid 1993). This coupled with the fact that the human genome is more resistant than mouse to random mutations (Cahill et al. 1999) suggests that in human sporadic cancers at least, genetic instability may occur before mutations in major tumor suppressors.

7.7 Loss of *Brca1* Induces Metastasis.

While treatment for breast cancers that have either ER, PR or HER2 is significantly improved now with the advent of new drugs (like Trastuzumab for HER 2

positive cancers), there is still no good treatment option for the familial *BRCA1* mutated cancers (Carey et al. 2006, Livasy et al. 2006, Tutt et al. 2005). These cancers are ER, PR and HER2 negative and rapidly metastasize to distant organs like the lungs and liver. Recent molecular profiling studies have shown that these cancers co-cluster with sporadic breast cancers that have a "myoepithelial/basal" signature to them (Perou et al. 2000). The reason for their "basalness" is frequent expression of the breast myoepithelial/basal cell markers Keratin 5 and Keratin14. The function of *BRCA1* in DNA double strand break repair and cell cycle checkpoint have been well elucidated by many studies both *in vitro* and in mouse models. However few of these studies have yielded potential drug targets for treating *BRCA1* mutated breast cancers. Some of the obvious classes of drugs for treating *BRCA1* mutated cancers are DNA damaging agents like Carboplatin and drugs that cause "synthetic lethality" like the PARP inhibitors (Farmer et al. 2005, McCabe et al. 2006, Turner, Tutt & Ashworth 2005, Tutt et al. 2005). These drugs are currently in clinical trials for treating this type of cancer. But as much of the morbidity of these cancers arise from their distant metastasis, it is very important to model metastatic pathways that are upregulated as a result of *BRCA1* mutations. In this dissertation we have established and characterized a mouse model with combined mutations of Rb_{f}, *p53* and *Brca1*. All three mutations occur frequently together in human breast cancer. Using genomic technologies we have identified specific metastatic pathways that may be overexpressed in these cancers. Our results indicate that the CXCR4-SDF1 axis is significantly overexpressed in *Brca1* mutated breast cancers. This raises the possibility that this receptor could provide a potential drug target for this group of cancers. Also we have shown that tumors developing in the mice with mutations

in *Rb_f, p53* and *Brca1* also have high levels of the Epithelial-to-Mesenchymal-Transition program as indicated by the significant overexpression of the transcription factors *Snail*, *Twist* and *Foxc2*. Examination of the genes regulated by these factors might identify additional proteins for which drug agonist/antagonist could be developed in future studies.

7.8 Micro RNA Signature - What More To It?

Micro RNAs and their potential role in tumorigenesis is currently one of the most fascinating areas of research. Several recent papers have attempted to classify breast cancers by their miRNA signature using similar approaches to those used to classify tumors based on their mRNA signature (Blenkiron et al. 2007, Sempere et al. 2007). The results from these studies indicate that miRNA distribution among breast cancers is subtype specific and in fact differential expression of miRNA distinguishes ER positive, ER negative and HER 2 positive tumors. Also, miRNAs distribution was different between "luminal" type and "basal" type breast cancers."

We report here the first time a study examining the impact of loss of *Brca1* (in the mammary tumors developed in *WAP-Cre; $TgMFT_{121}$/p53/Brca1* mice) on the miRNA signature of mammary epithelial cells derived tumors. We show that loss of *Brca1* alone in the mammary gland results in an altered spectrum of mammary tumors and that these tumors have a unique mi RNA expression pattern. Several mi RNA s of interest are significantly overexpressed in the triple null tumors, for example the 17-5-p polycistron and the Let 7 mi RNAs and miR-29 a, b. The 17-5-p mi RNAs have been shown to play an oncogenic role in Burkitts lymphoma and is an established pro-proliferative target of

c-Myc (O'Donnell et al. 2005). An attractive hypothesis is that this class of micro RNAs has an oncogenic role in mammary tumorigenesis. The overexpression of the Let 7 family of mi RNAs is intriguing, as published studies report that Let 7 mi RNA s are overexpressed in more differentiated and less embryonic stem cell like tissues. Thus Let 7s have been shown to have a tumor suppressor function in multiple cell types (Kent, Mendell 2006, Kumar et al. 2008). However the role of Let 7 overexpression in the triple mutant (*WAP-Cre; $TgMFT_{121}$/p53/Brca1* mice) mouse mammary tumors, if any, is unclear and requires future work. Also our mRNA data shows the significant overexpression of RAB7, a member of the Ras oncogene family. The Let 7 family has been well characterized in both *in vitro* (Yu et al. 2007) and *in vivo* (Kumar et al. 2008) studies to suppress the Ras family of oncogenes and thus inhibit Ras mediated proliferation. In the triple null model, increase in Let-7 expression could be a possible pathway for the cells self defense mechanisms being turned on with the increased over all genetic instability. The miR29 family has recently been implicated to be regulated in a cell cycle dependent manner (Hwang, Mendell 2007). The loss of *Brca1* and *p53* results in loss of two important cell cycle checkpoint controls and it is likely that this turns on other genetic mechanisms for cell cycle arrest, for example, miR-29b.

The study of miRNA in tumorigenesis is a rapidly growing field. Current work is under way to confirm the presence of the changes in micro RNA expression that we observed in the tumors various mammary tumors using quantitative PCR and in situ hybridization on paraffin embedded sections. Ultimately functional studies using siRNA and viral vectors to alter specific mi RNA expression in the mammary glands will result in new mechanistic views of breast cancer regulation.

7.9 Three Dimensional Culture of Primary ME Cells

In this dissertation we have established and characterized several mouse models generated for the study of the role of *Rb_f*, *p53* and *Brca1* in breast cancer. Considerable time and resources is required for the development of the appropriated lines and intercross of these individual transgenic mice to generate the triple transgenic populations of animals. It is therefore of interest to establish alternative approach which can provide information concerning the interaction of various gene in the transformation of mammary epithelial cells. Towards this end efforts were made to establish and characterize a primary mammary epithelial cell culture system. Some limited success was achieved and a number of the hurdles which will require investigation if this approach is to succeed were identified. The great heterogeneity of primary mouse mammary epithelial cells hampered our efforts and limited our ability to draw conclusion based on differences between cultures established from different animals. However we were able to observe T_{121} expression in culture thus establishing that WAP expression can be controlled *in vitro*. The formation of hollow mammary spheres by the primary cells recapitulating what had been shown before using cell lines. This was very encouraging. Recent work using human primary mammary tumor cells in a three dimensional culture has reinforced the importance of this technique (Becker, Blanchard 2007).

7.10 Location is Important - Targeting the Mammary Myoepithelial Cells

The reason for the basal nature of *Brca1* mutated human breast cancers is incompletely understood. Studies with human tissues have shown increased expression of Cytokeratin 5 and 14 in some regions of these tumors (Rakha, El-Sayed, et al, 2008).

This may indicate that these tumors arise from the mammary myoepithelial cells and thus have a more myoepithelial/basal profile. Alternatively it may indicate that the tumors arise in a progenitor mammary epithelial cell that has features of both luminal and myoepithelial cells. Earlier work has shown that the combined loss of *p53* and *Brca1* in a conditional mammary gland model using a Keratin 14 promoter gives rise to a wide spectrum of mammary tumors (Liu et al. 2007). Some of these mammary tumors have an increased basal profile, indicated by their microarray signature. However, these tumors lacked many other key features of human familial breast cancer, including progression to distant metastasis. Our data suggests that the combined loss of Rb_f, *p53* and *Brca1* is necessary for tumor progression. We also observe nests of Keratin 5 and Keratin 14 positive cells in the tumors arising in the triple mutant (*WAP-Cre; $TgMFT_{121}$/p53/Brca1*) mice. As the use of WAP-Cre in the MFT_{121} model limits the loss of Rb_f, *p53* and *Brca1* to the mammary luminal epithelial cells known to express the WAP promoter and the events putatively do not occur in the mammary myoepithelial cells, this result is intriguing. Future experiments designed to target the same genetic combination shown here (in the *WAP-Cre; $TgMFT_{121}$/p53/Brca1* mice) in the mammary myoepithelial cells using a MMTV or K14 Cre can be used to specifically answer questions like does the combined loss of *Rb*, *p53* and *Brca1* result in an increased basal keratin expression profile (compared to that seen in the current model) if occurring in the mammary myoepithelial cells or not. The ideal mouse model for the familial *BRCA1* mutated breast cancer in humans could be one that targets a specific combination of genetic mutations to the right cell type within the mammary gland. A hypothetical model for familial breast cancer progression as seen in the *$TgMFT_{121}$* mouse model is depicted in **Figure 7.1**.

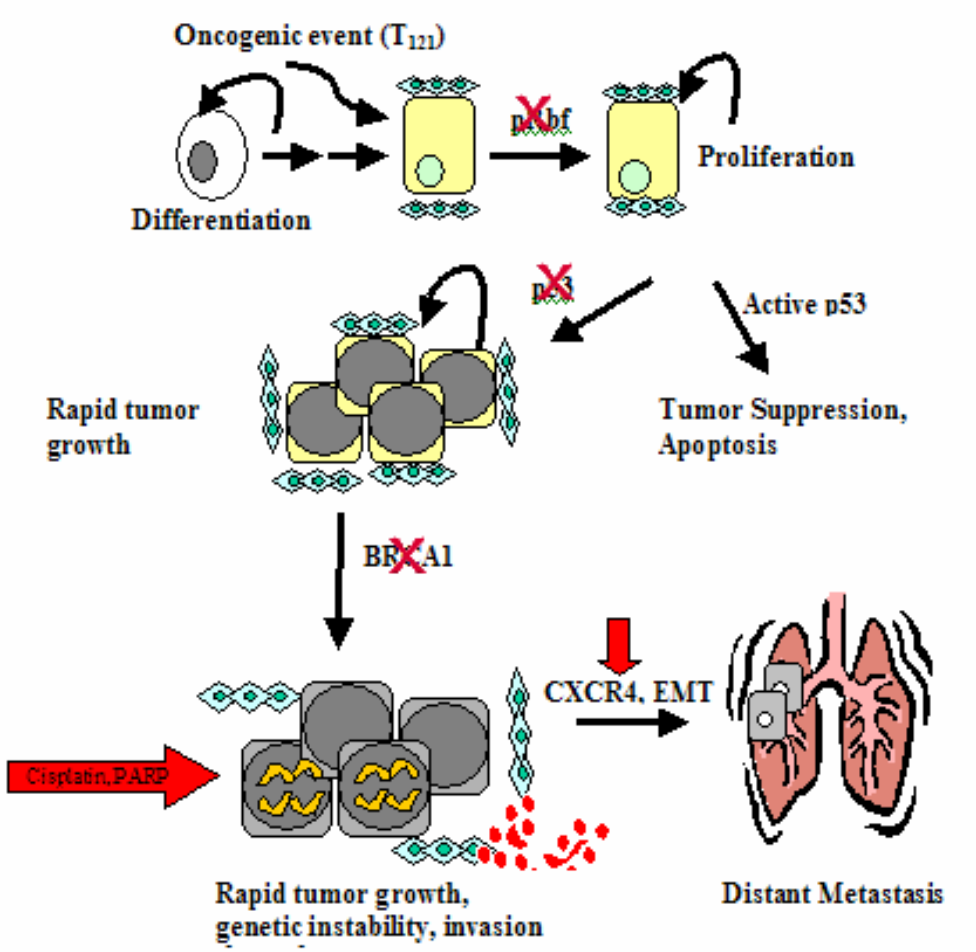


Figure 7.1 Hypothetical model for the role of *Rb_f*, *p53* and *Brca1* in mammary tumor progression is shown here. Abnormal proliferation is induced in the mammary glands by loss of *Rb_f*. This is balanced by *p53* dependant and independent apoptosis. Loss of *p53* results in increased uncontrolled proliferation and tumor growth. Loss of *Brca1* further removes DNA damage repair pathways and results in increased genetic instability, invasion and metastasis. Current therapeutic trials target the DNA damage defects of *Brca1* with drugs like Cisplatin and PARP-inhibitors. New strategies can be developed using this model to target the metastatic pathways **(red arrow)**.

SUPPLEMENTARY MATERIAL 1

Genes overexpressed in Triple Mutant Tumors

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)	
9664	Cartilage oligomeric matrix protein || NM_016685 ||	0.536668003	7.921977459		0
5004	|| NM_001033177 ||	0.245413752	5.320638869		0
9198	Forkhead box A3 || NM_008260 ||	0.360569866	4.927615523		0
8216	RIKEN cDNA D630045J12 gene || XM_485743 ||	0.266862402	3.363934539		0
12533	Lymphoid enhancer binding factor 1 || AK083328 ||	0.453784706	4.658576404		0
7916	Fetuin beta || NM_021564 ||	0.438399259	4.789250287		0
12300	Sulfotransferase family 1E, member 1 || NM_023135 ||	0.330553484	3.683145017		0
9525	RIKEN cDNA 1500015O10 gene || NM_024283 ||	0.601697694	6.069637592		0
12938	|| NM_027283 ||	0.244125634	2.932279134		0
4352	Myotubularin related protein 9 || NM_177594 ||	0.199022212	2.368096647		0
9710	Heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1 || NM_018805 ||	0.415353618	5.875007096		0
12503	N-myc downstream regulated gene 4 || NM_145602 ||	0.367015492	3.430210936		0
9557	LIM and cysteine-rich domains 1 || NM_144799 ||	0.400049361	4.301963346		0
12765	Vitelliform macular dystrophy 2 homolog (human) || NM_011913 ||	0.203364604	2.427688622		0
6701	Cell adhesion molecule with homology to L1CAM || NM_007697 ||	0.307932744	3.231189442		0
6203	Growth arrest and DNA-damage-inducible 45 alpha || NM_007836 ||	0.338993038	3.362870629		0
1657	Microsomal glutathione S-transferase 2 || NM_174995 ||	0.520704766	4.649406198		0
1807	Progressive ankylosis || NM_020332 ||	0.459594059	4.492396324		0
8875	PDZ domain containing 3 || AK020883 ||	0.311088719	3.006411508		0
7033	Antigen p97 (melanoma associated) identified by monoclonal antibodies 133.2 and 96.5 || NM_013900 ||	0.541821289	5.061580495		0
12807	RIKEN cDNA 6030413G23 gene || NM_178664 ||	0.321144723	3.067857212		0
1734	Hypothetical protein D030056L22 || NM_177640 ||	0.20114765	2.056452253		0
12185	Sprouty homolog 1 (Drosophila) || NM_011896 ||	0.27046233	2.567960535		0
3284	|| NM_029309 ||	0.215732155	2.275683589		0
11273	Aggrecan 1 || NM_007424 ||	0.357870978	3.129192694		0
8281	Heparan sulfate (glucosamine) 3-O-sulfotransferase 1 || NM_010474 ||	0.465946505	3.747112322		0
1843	Cytokine receptor-like factor 1 || NM_018827 ||	0.353449782	3.009628449		0
7413	LSM5 homolog, U6 small nuclear RNA associated (S. cerevisiae) || NM_025520 ||	0.289187714	2.715908393		0

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)	
6342	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5 || NM_033149 ||	0.49021635	3.32594558		0
12511	SRY-box containing gene 5 || NM_011444 ||	0.345437295	2.73196056		0
4875	Glypican 4 || NM_008150 ||	0.259471126	2.293923725		0
5327	Serine (or cysteine) proteinase inhibitor, clade E, member 2 || NM_009255 ||	0.425099624	2.88321157		0
6425	|| NM_029588 ||	0.3620214	3.004446326		0
6437	|| BC100404 ||	0.51367068	3.623514294		0
8983	CDNA sequence BC011209 || NM_145447 ||	0.530612336	2.634347996		0
9179	RIKEN cDNA 4930443F05 gene || BC063103 ||	0.570503911	4.689463027		0
5784	KiSS-1 metastasis-suppressor || NM_178260 ||	0.240289998	2.325426917		0
3500	Meteorin, glial cell differentiation regulator || NM_133719 ||	0.282806905	2.339098919		0
12833	|| AK021164 ||	0.49792483	4.009813322		0
6400	RIKEN cDNA 1700113O17 gene || NM_026627 ||	0.374189385	2.798587973		0
1321	Peptidoglycan recognition protein 1 || NM_009402 ||	0.417129365	2.970986474		0
9777	Mast cell protease 8 || NM_008572 ||	0.296922598	2.506179774		0
2608	Diphtheria toxin receptor || NM_010415 ||	0.483968826	3.103358293		0
6596	Integrin alpha 8 || NM_001001309 ||	0.445015225	2.943843351		0
920	CDNA sequence AJ430384 || NM_130880 ||	0.362761303	3.978254529		0
5420	Twist gene homolog 1 (Drosophila) || M63650 ||	0.454045987	2.928165505		0
6289	|| NM_146937 ||	0.336085915	2.858312334		0
10187	Matrix gamma-carboxyglutamate (gla) protein || NM_008597 ||	0.487563505	3.538896919		0
10633	Abhydrolase domain containing 2 || NM_018811 ||	0.310159631	2.479668162		0
8155	RIKEN cDNA 4933425F03 gene || NM_028903 ||	0.346741685	2.824640912		0
983	RIKEN cDNA E030003N15 gene || XM_128129 ||	0.271649985	2.289310227		0
2900	Secernin 1 || AK129084 ||	0.37848524	2.673395336		0
1879	Glutamate receptor, ionotropic, NMDA2D (epsilon 4) || NM_008172 ||	0.40833941	3.29445065		0
5313	Interleukin 11 || NM_008350 ||	0.256804431	2.868850015		0
223	TruB pseudouridine (psi) synthase homolog 2 (E. coli) || NM_145520 ||	0.194454156	1.836871282		0
6713	Melatonin receptor 1A || NM_008639 ||	0.418247918	3.405046336		0
3942	ATP-binding cassette, sub-family G (WHITE), member 1 || NM_009593 ||	0.297836648	2.206802734		0
12834	Chondroitin sulfate GalNAcT-2 || NM_030165 ||	0.254062666	2.040461623		0
6509	CDNA sequence BC056474 || NM_001001493 ||	0.215371465	1.94005677		0
12178	8-oxoguanine DNA-glycosylase 1 || NM_010957 ||	0.237858731	1.977614296		0
10560	Replication protein A3 || NM_026632 ||	0.263227738	2.127687912		0

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)	
3100	Forkhead box C2 || NM_013519 ||	0.333925411	3.700430527		0
1808	RIKEN cDNA 4930488P06 gene || AK019676 ||	0.233587348	1.996510779		0
11875	Forkhead box Q1 || NM_008239 ||	0.340091424	2.82742315		0
5251	Chloride channel calcium activated 2 || NM_030601 ||	0.56288736	3.03074327		0
5331	Acyl-CoA synthetase long-chain family member 4 || NM_019477 ||	0.47392392	2.743192129		0
106	Hypothetical gene supported by BC030430 || XM_358706 ||	0.371780324	2.298079062		0
8076	Secreted phosphoprotein 1 || NM_009263 ||	0.669430741	3.509867686		0
8937	Mitochondrial ribosomal protein L53 || NM_026744 ||	0.1974104	1.848162507		0
8390	RIKEN cDNA 3110032G18 gene || NM_028443 ||	0.421903031	3.138105586		0
12893	Nicolin 1 || NM_025449 ||	0.483206062	3.403503705		0
12038	DiGeorge syndrome critical region gene 2 || NM_010048 ||	0.247836804	2.050474922		0
12071	Phosphoglucomutase 2-like 1 || NM_030677 ||	0.561781757	2.928838416		0
549	Scavenger receptor class A, member 3 || NM_172604 ||	0.309514511	2.363090141		0
9860	RIKEN cDNA 4930597O21 gene || NM_025278 ||	0.24319821	1.928130166		0
6430	RIKEN cDNA 1200016B17 gene || NM_026267 ||	0.198450817	1.692998768		0
490	Solute carrier family 26, member 11 || NM_178743 ||	0.217523625	1.758079479		0
8864	RIKEN cDNA 3110079O15 gene || AK014261 ||	0.745467007	2.850757982		0
5218	Epsin 2 || NM_010148 ||	0.301341329	2.235316846		0
9376	RIKEN cDNA 2010319C14 gene || NM_024464 ||	0.244181971	1.929100373		0
11044	T-cell leukemia, homeobox 2 || NM_009392 ||	0.374392507	2.826967042		0
9139	Sterol O-acyltransferase 1 || NM_009230 ||	0.324110179	2.275204364		0
11189	Procollagen, type XI, alpha 1 || NM_007729 ||	0.470852412	2.664341063		0
6920	CD9 antigen || NM_007657 ||	0.382196071	2.544849102		0
2756	Matrix metalloproteinase 1a (interstitial collagenase) || NM_032006 ||	0.263654694	2.881299796		0
10208	Gene trap ROSA 26, Philippe Soriano || U83174 ||	0.239291099	1.870271176		0
6339	Hypothetical protein D830035I06 || NM_007507 ||	0.242554756	1.94504227		0
5880	Immediate early response 3 || NM_133662 ||	0.413374769	2.58271886		0
2628	Down syndrome critical region homolog 5 (human) || NM_019543 ||	0.258545373	1.945426845		0
2883	Cyclin-dependent kinase 5, regulatory subunit 2 (p39) || NM_009872 ||	0.481958886	3.595722586		0
9463	Voltage-dependent calcium channel gamma subunit-like protein || NM_019432 ||	0.421435184	2.395953672		0
3603	Protein tyrosine phosphatase, non-receptor type 2 || NM_008977 ||	0.248278552	1.854647194		0
12564	RIKEN cDNA 1810009J06 gene || NM_023707 ||	0.183810354	1.668206623		0
3941	Expressed sequence AI987986 || NM_025311 ||	0.256529677	1.940397281		0

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)	
1014	Parathyroid hormone-like peptide || NM_008970 ||	0.487305387	2.762992103		0
4942	RIKEN cDNA 4930563E22 gene || NM_026068 ||	0.196410157	1.718394373		0
8711	RIKEN cDNA 0610012H03 gene || NM_028747 ||	0.17385504	1.661848228		0
461	Urokinase plasminogen activator receptor || NM_011113 ||	0.272772985	1.92069021		0
13135	Breast cancer anti-estrogen resistance 3 || NM_013867 ||	0.325731402	2.245122639		0
1436	RIKEN cDNA 1300002C08 gene || NM_026182 ||	0.235587561	1.802421074		0
13131	Selenoprotein K || NM_019979 ||	0.207505606	1.704734621		0
8178	Chitinase 3-like 1 || NM_007695 ||	0.439608817	2.854187484		0
6497	Actin related protein 2/3 complex, subunit 4 || NM_026552 ||	0.22224022	1.758353462		0
9914	Villin-like || NM_011700 ||	0.342231676	2.160223288		0
12536	Sphingosine kinase 1 || NM_025367 ||	0.354768368	2.137913981		0
7725	RIKEN cDNA 2610209M04 gene || NM_025665 ||	0.17363756	1.575301976		0
8425	Protein phosphatase 2, regulatory subunit B (B56), epsilon isoform || AK014554 ||	0.461380131	3.153488368		0
11598	RIKEN cDNA 1200004M23 gene || NM_026169 ||	0.205147252	1.703977757		0
4196	Ets variant gene 4 (E1A enhancer binding protein, E1AF) || NM_008815 ||	0.311980537	2.234708838		0
8942	Inhibitor of DNA binding 1 || NM_010495 ||	0.458535289	2.971264989		0
8851	Embigin || NM_010330 ||	0.563583902	2.900644558		0
6982	|| NM_147083 ||	0.189773266	1.685258364		0
7527	|| NM_030256 ||	0.260268418	1.893155859		0
10184	Natriuretic peptide precursor type B || NM_008726 ||	0.27878435	1.955484818		0
6766	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4) || NM_007670 ||	0.303548358	2.218771847		0
1418	Fibronectin leucine rich transmembrane protein 3 || NM_178382 ||	0.509762008	2.410113011		0
5371	Transient receptor potential cation channel, subfamily V, member 4 || NM_022017 ||	0.348816547	2.161238068		0
6068	RIKEN cDNA D030074K08 gene || NM_024189 ||	0.225692446	1.7342264		0
6743	|| NM_153168 ||	0.594856992	4.617915548		0
3400	Otoraplin || NM_020595 ||	0.335292687	2.062666941		0
10836	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma) || NM_013737 ||	0.361560913	2.167147011		0
6865	Synaptotagmin 10 || NM_018803 ||	0.373344756	3.02442638		0
11182	Glutathione S-transferase kappa 1 || NM_029555 ||	0.320607106	2.097926315		0
6534	Ets variant gene 1 || NM_007960 ||	0.493486075	2.243639894		0
356	Centrin 4 || NM_145825 ||	0.244255971	1.891549281		0
1147	Transmembrane, prostate androgen induced RNA || NM_022995 ||	0.248072781	1.83185404		0
4636	Retinitis pigmentosa GTPase regulator interacting protein 1 || NM_023879 ||	0.353410509	2.087270791		0

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)	
4076	DNA segment, Chr 11, ERATO Doi 99, expressed || NM_026618 ||	0.195691071	1.688929205		0
2120	Snail homolog 1 (Drosophila) || NM_011427 ||	0.325174954	2.090159517		0
1248	RIKEN cDNA 1700011J10 gene || NM_183265 ||	0.212970776	1.664110615		0
7257	Tribbles homolog 3 (Drosophila) || NM_144554 ||	0.397501648	2.214546443		0
10198	Nucleolar protein family A, member 3 || NM_025403 ||	0.291812123	2.017002847		0
8677	Steroid sulfatase || NM_009293 ||	0.461627614	3.379323305		0
7814	Glycoprotein galactosyltransferase alpha 1, 3 || NM_010283 ||	0.286659946	1.86072378		0
11664	RIKEN cDNA A330103N21 gene || AK087668 ||	0.376234269	2.476888337		0
1550	Mesothelin || NM_018857 ||	0.672374899	2.250531585		0
10860	Myosin VIIa || NM_008663 ||	0.164064166	1.497586215		0
1506	RIKEN cDNA 2610018G03 gene || NM_133729 ||	0.289483477	2.107583133		0
12844	POU domain, class 3, transcription factor 1 || NM_011141 ||	0.351992576	2.47987192		0
4662	CCAAT/enhancer binding protein (C/EBP), beta || NM_009883 ||	0.345117146	2.136099165		0
9370	|| BI151098 ||	0.321273016	2.045838712		0
5256	Expressed sequence R74740 || BU851393 ||	0.718185568	3.438844355		0
8073	Olfactory receptor 1221 || NM_146902 ||	0.497310915	2.720893056		0
1941	Interleukin-1 receptor-associated kinase 2 || NM_172161 ||	0.293994562	1.963634396		0
9894	RIKEN cDNA 1810059G22 gene || NM_026325 ||	0.164502582	1.535774248		0
4495	Transmembrane 4 superfamily member 1 || NM_008536 ||	0.483840172	2.349228645		0
9504	MAD homolog 7 (Drosophila) || NM_008543 ||	0.290677865	2.192609216		0
10470	|| AK048085 ||	0.356767624	2.095766432		0
8079	RIKEN cDNA 2600001B17 gene || AK081685 ||	0.324256115	2.341769053		0
3220	Small nuclear ribonucleoprotein polypeptide G || NM_026506 ||	0.236814932	1.798988248		0
767	Melanoma antigen, family D, 2 || NM_030700 ||	0.322772924	1.940457835		0
7650	|| AK051922 ||	0.561539217	3.45900902		0
576	Leucine-rich repeats and immunoglobulin-like domains 1 || NM_008377 ||	0.264265729	1.938094252		0
11862	RAD18 homolog (S. cerevisiae) || NM_021385 ||	0.217503016	1.744508358		0
12882	Tissue inhibitor of metalloproteinase 1 || NM_011593 ||	0.415896706	2.125793674		0
3363	SEC61, gamma subunit || NM_011343 ||	0.26057417	1.803495958		0
3958	Zinc finger protein 286 || NM_138949 ||	0.290253856	1.852328501		0
4227	Ectonucleotide pyrophosphatase/phosphodiesterase 1 || NM_008813 ||	0.360407608	2.05384808		0
9389	Cornichon homolog (Drosophila) || NM_009919 ||	0.213613257	1.611667052		0
2244	Hypothetical protein A330042H22 || NM_173014 ||	0.317196028	1.881978217		0

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)	
7206	RIKEN cDNA 2010208K18 gene || NM_028095 ||	0.238451697	1.696244268		0
7479	|| NM_133779 ||	0.561684744	6.371696868		0
1623	DEAH (Asp-Glu-Ala-His) box polypeptide 36 || NM_028136 ||	0.204670923	1.590493779		0
9245	|| NM_001025612 ||	0.203179239	1.625419338		0
4683	Pre B-cell leukemia transcription factor 2 || NM_017463 ||	0.223791769	1.694912325		0
2130	Mitochondrial ribosomal protein L33 || NM_025796 ||	0.229222562	1.735696454		0
2720	Matrix metalloproteinase 13 || NM_008607 ||	0.64628674	1.358078442		0
11226	|| BU851290 ||	0.457563055	2.607316131		0
8794	PHD finger protein 15 || NM_199299 ||	0.258211566	1.841836459		0
5983	Selenoprotein K || NM_019979 ||	0.210844168	1.610638475		0
2751	DEAH (Asp-Glu-Ala-His) box polypeptide 35 || NM_145742 ||	0.19030307	1.582410666		0
10799	RIKEN cDNA 1110019C08 gene || NM_171826 ||	0.277238963	2.02149401		0
13051	Dual specificity phosphatase 4 || NM_176933 ||	0.235935218	1.776356465		0
5301	CDC42 effector protein (Rho GTPase binding) 3 || NM_026514 ||	0.314099597	1.915725008		0
7280	DNA segment, Chr 7, ERATO Doi 743, expressed || NM_153525 ||	0.202730941	1.560532268		0
10949	Annexin A9 || NM_023628 ||	0.273067846	1.737542503		0
8260	Synaptobrevin like 1 || NM_011515 ||	0.300708089	1.974683178		0
9667	Inositol hexaphosphate kinase 1 || AK006216 ||	0.286034381	1.945708458		0
5707	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9 || NM_025358 ||	0.211961452	1.623254435		0
3889	|| TC1571425 ||	0.208139731	1.638356122		0
4836	BTB (POZ) domain containing 3 || NM_145534 ||	0.217178069	1.607450159		0
4328	CXXC finger 5 || NM_133687 ||	0.291160523	1.90041147		0
4596	Src family associated phosphoprotein 2 || NM_018773 ||	0.341848127	1.914304982		0
2254	ADP-ribosylation factor-like 5 || NM_182994 ||	0.199670842	1.580072786		0
3293	RIKEN cDNA 5031439A09 gene || NM_026582 ||	0.30369135	1.795572944		0
7010	Angiotensinogen || NM_007428 ||	0.413063265	2.291517997		0
11674	Von Hippel-Lindau binding protein 1 || NM_011692 ||	0.254529253	1.744407113		0
4664	Carboxypeptidase E || NM_013494 ||	0.483690037	2.361524419		0
4640	Aquaporin 3 || NM_016689 ||	0.510479743	2.513221297		0
10759	Nuclear DNA binding protein || NM_020558 ||	0.248214251	1.713149028		0
2211	RIKEN cDNA A730010A20 gene || NM_025446 ||	0.254962113	1.664130282		0
9568	RIKEN cDNA 2310008M10 gene || NM_025509 ||	0.242764891	1.698198102		0
7374	RIKEN cDNA 1810055G02 gene || NM_028077 ||	0.206215776	1.554757246		0

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)	
8192	Immediate early response 5-like || NM_030244 ||	0.295320413	1.837899811		0
4991	S100 calcium binding protein A8 (calgranulin A) || NM_013650 ||	0.668041342	1.864093153		0
7911	|| AK170453 ||	0.59893372	2.659523482		0
6391	Deleted in bladder cancer chromosome region candidate 1 (human) || NM_019967 ||	0.677407948	2.727389167		0
5730	Transmembrane 4 superfamily member 9 || NM_019571 ||	0.254293151	1.662108075		0
10083	Flotillin 1 || NM_008027 ||	0.212835659	1.601553571		0
4898	|| NM_027186 ||	0.217770466	1.613579154		0
10442	Hepsin || NM_008281 ||	0.405892927	1.971825073		0
2360	CD14 antigen || NM_009841 ||	0.384545095	2.36381963		0
10477	Nudix (nucleoside diphosphate linked moiety X)-type motif 1 || NM_008637 ||	0.206309758	1.583811333		0
5973	Solute carrier family 29 (nucleoside transporters), member 1 || NM_022880 ||	0.348620583	1.986562659		0
9517	V-raf-1 leukemia viral oncogene 1 || NM_029780 ||	0.216299003	1.570260854		0
7688	Protein kinase, interferon inducible double stranded RNA dependent activator || NM_011871 ||	0.240404266	1.667960516		0
8552	RIKEN cDNA E030034P13 gene || AK034869 ||	0.640124039	2.470778275		0
4964	T-cell lymphoma invasion and metastasis 2 || NM_011878 ||	0.311679772	1.909149298		0
6662	AP2 associated kinase 1 || BC028270 ||	0.282021522	2.04669979		0
12724	|| XM_992800 ||	0.220923764	1.777180281		0
1412	Stress 70 protein chaperone, microsome-associated, human homolog || NM_030201 ||	0.204309252	1.56933197		0
5943	CDNA sequence BC006965 || BC006965 ||	0.437694129	2.405374355		0
6586	RIKEN cDNA 1600012F09 gene || NM_025904 ||	0.25012552	1.714904079		0
4226	Immunoglobulin superfamily, member 3 || AK019524 ||	0.318989359	1.843001724		0
9324	Translocase of inner mitochondrial membrane 8 homolog b (yeast) || NM_013897 ||	0.2610067	1.697839624		0
5698	RIKEN cDNA 1810027O10 gene || AK014035 ||	0.228649718	1.756189832		0
7412	AE binding protein 1 || NM_009636 ||	0.310291578	1.771913269		0
12563	|| NP382146 ||	0.340145738	1.777733563		0
6634	RIKEN cDNA 2410003K15 gene || AK031878 ||	0.206786689	1.632144851		0
8182	Fibrinogen, gamma polypeptide || NM_133862 ||	0.409057938	2.35867738		0
5071	RIKEN cDNA 1110061O04 gene || NM_026849 ||	0.1691858	1.455784111		0
3071	RIKEN cDNA 3010021M21 gene || NM_180600 ||	0.290261096	1.880075583		0
12395	Programmed cell death 10 || NM_019745 ||	0.311005072	2.013046437		0
12205	|| AK137548 ||	0.297224698	1.816421288		0
7416	ATPase, H+ transporting, V0 subunit || NM_025272 ||	0.228193563	1.597265051		0
13067	Gene rich cluster, C2f gene || NM_013536 ||	0.200035628	1.522388137		0

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)	
4209	MAD2 (mitotic arrest deficient, homolog)-like 1 (yeast) || U83902 ||	0.329505799	1.86986767		0
6188	Expressed sequence AI413582 || NM_001002895 ||	0.22602977	1.59164146		0
8914	Major intrinsic protein of eye lens fiber || NM_008600 ||	0.232888459	1.671267544		0
6591	Demilune cell and parotid protein || NM_019910 ||	0.535839802	0.784890065		0
4408	Guanine nucleotide binding protein (G protein), gamma 5 subunit || NM_010318 ||	0.24571369	1.756340672		0
5118	Arginine vasopressin-induced 1 || NM_027106 ||	0.304306203	1.702477042		0
1133	|| AV030849 ||	0.283094326	1.665326406		0
9999	Regulator of G-protein signaling 2 || NM_009061 ||	0.360286203	1.924872188		0
8656	Beta-site APP-cleaving enzyme 2 || NM_019517 ||	0.410191357	2.073702579		0
5192	Olfactory receptor 1280 || NM_146908 ||	0.543025168	2.60776068		0
10695	Immunoglobulin superfamily, member 8 || NM_080419 ||	0.281657184	1.691755655		0
9927	RIKEN cDNA 1500026D16 gene || NM_026616 ||	0.222232802	1.59368316		0
6516	Retinoblastoma binding protein 4 || NM_009030 ||	0.252493842	1.68574499		0
5512	RIKEN cDNA 1500034J20 gene || BC008259 ||	0.272105575	1.774791205		0
7602	Melanoma inhibitory activity 1 || NM_019394 ||	0.263863821	1.735253342		0
3061	CDNA sequence BC035537 || NM_153535 ||	0.301557601	1.74252582		0
1887	RIKEN cDNA 1810006K23 gene || AY523601 ||	0.325845468	1.808569763		0
12087	Slit-like 2 (Drosophila) || NM_139307 ||	0.327309327	1.905630768		0
5645	|| NM_021713 ||	0.221505614	1.591493503		0
12055	Protein kinase, lysine deficient 4 || NM_175638 ||	0.4298373	1.737886249		0
3660	Protein phosphatase 3, catalytic subunit, alpha isoform || NM_008913 ||	0.307672918	1.75054513		0
2296	SKI-like || NM_011386 ||	0.285492829	1.666929624		0
3176	|| NM_001012765 ||	0.152531009	1.438583709		0
1349	CDNA sequence BC014699 || NM_145570 ||	0.412518739	2.100033288		0
1922	Numb-like || NM_010950 ||	0.370860629	1.863039023		0
1334	Molybdenum cofactor sulfurase || XM_484710 ||	0.363159025	1.806522806		0
10978	|| NG_002016 ||	0.142274648	1.355603544		0
8082	WW domain binding protein 2 || NM_016852 ||	0.232039043	1.609814372		0
7131	Immunoglobulin superfamily, member 3 || NM_207205 ||	0.339063626	1.948763001		0
10459	|| NM_029557 ||	0.226066235	1.583622977		0
11931	Leptin receptor overlapping transcript-like 1 || NM_026609 ||	0.273167862	1.692985516		0
4232	Zinc finger protein 239 || NM_001001792 ||	0.347369455	2.133314214		0
7169	RIKEN cDNA 8430432M10 gene || NM_176831 ||	0.172682645	1.475284438		0

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)
5245	RNA binding motif protein 18 || NM_026434 ||	0.165134542	1.450572155	0
11529	PDZ and LIM domain 4 || NM_019417 ||	0.445502959	1.942532641	0
12792	RIKEN cDNA D130042I01 gene || NM_053082 ||	0.292012827	1.71884755	0
3614	RIKEN cDNA 5133401H06 gene || NM_028943 ||	0.216500726	1.774356941	0
5766	Glutamic acid decarboxylase 1 || NM_008077 ||	0.366480661	1.745245596	0
6761	RIKEN cDNA 5033428A16 gene || BC018498 ||	0.250440299	1.596794703	0
7618	Solute carrier family 14 (urea transporter), member 1 || NM_028122 ||	0.38790847	2.022532349	0
11673	Ubiquitin carboxy-terminal hydrolase L1 || NM_011670 ||	0.299412603	1.759585154	0
8451	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2 || NM_139272 ||	0.256111889	1.671106847	0
2591	|| ENSMUST00000021282 ||	0.632590232	2.615907297	0
5108	RIKEN cDNA 4933407C03 gene || AK016712 ||	0.323048749	1.876009277	0
6624	Homeo box A5 || NM_010453 ||	0.379816512	2.853219951	0
3960	Dedicator of cytokinesis 5 || BC016533 ||	0.210388402	1.509204063	0
11495	RIKEN cDNA 2410007P03 gene || NM_025475 ||	0.187976368	1.553664073	0
7398	DNA segment, Chr 19, ERATO Doi 737, expressed || NM_029648 ||	0.214756085	1.517788478	0.1722319
2618	RIKEN cDNA 4930488E11 gene || NM_207267 ||	0.413218878	2.018316227	0.1722319
8843	Caspase 3, apoptosis related cysteine protease || NM_009810 ||	0.265670231	1.917672793	0.1722319
10800	Epithelial membrane protein 3 || NM_010129 ||	0.437033011	1.720893936	0.1722319
259	Tumor necrosis factor receptor superfamily, member 1a || NM_011609 ||	0.205678353	1.551147312	0.1722319
4027	RIKEN cDNA 4930456L15 gene || AK015480 ||	0.390227045	2.044598178	0.1722319
2447	Glutamine fructose-6-phosphate transaminase 1 || NM_013528 ||	0.27624309	1.703867685	0.1722319
6004	RIKEN cDNA A430091O22 gene || AK040404 ||	0.286821439	1.7653516	0.1722319
12122	C1q and tumor necrosis factor related protein 4 || AK038464 ||	0.293435886	1.975919771	0.1722319
9983	RIKEN cDNA 9130211I03 gene || NM_030060 ||	0.402952391	1.759946819	0.1722319
4705	RIKEN cDNA A430019L02 gene || AF084575 ||	0.234799222	1.573002333	0.1722319
5571	Docking protein 1 || NM_010070 ||	0.305758577	1.783502089	0.1722319
4098	Ribonuclease P/MRP 30 subunit (human) || NM_019428 ||	0.228177479	1.569751844	0.1722319
9019	U2 small nuclear ribonucleoprotein B || NM_021335 ||	0.237894006	1.599975514	0.1722319
4759	BMP and activin membrane-bound inhibitor, homolog (Xenopus laevis) || NM_026505 ||	0.375567441	1.736183829	0.1722319
4487	Zinc finger protein 422 || NM_026057 ||	0.258427157	1.634365463	0.1722319
9111	Transforming growth factor, beta 1 || NM_011577 ||	0.207034488	1.571942386	0.1722319
435	Frataxin pseudogene, complete sequence. || AF223568 ||	0.195683005	1.505830441	0.1722319
8163	V-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian) || NM_010658 ||	0.412531719	1.936851229	0.1722319

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)
4048	Transmembrane protein 5 || AK043098 ||	0.261552816	1.671293842	0.1722319
12796	Immunoglobulin heavy chain (J558 family) || BC004786 ||	0.393132413	2.49910035	0.1722319
5515	Capping protein (actin filament), gelsolin-like || NM_007599 ||	0.310097488	1.691570565	0.1722319
5503	RIKEN cDNA 1700013H19 gene || NM_027954 ||	0.326547105	1.721049881	0.1722319
8215	Chemokine (C-X-C motif) ligand 1 || NM_008176 ||	0.440556937	1.940021412	0.1722319
708	Phosphodiesterase 2A, cGMP-stimulated || BC057029 ||	0.320413241	1.712042227	0.1722319
12555	Cytochrome P450, family 26, subfamily b, polypeptide 1 || NM_175475 ||	0.415380451	1.813983393	0.1722319
13065	|| NM_026767 ||	0.218105422	1.582485417	0.1722319
8419	|| NM_146868 ||	0.592588674	2.805691952	0.1722319
9654	Solute carrier family 20, member 1 || NM_015747 ||	0.304082753	1.770173557	0.1722319
1972	Chromobox homolog 3 (Drosophila HP1 gamma) || NM_007624 ||	0.247629524	1.624868237	0.1722319
3382	PTPRF interacting protein, binding protein 1 (liprin beta 1) || NM_026221 ||	0.341072456	1.547050804	0.1722319
10098	Amphiphysin || NM_175007 ||	0.666452692	2.363968393	0.1722319
11829	Sialyltransferase 7 ((alpha-N-acetylneuraminyl 2,3-betagalactosyl-1,3)-N-acetyl galactosaminide alpha-2,6-sialyltransferase) C || NM_011372 ||	0.17443719	1.409717448	0.1722319
6923	Ceroid-lipofuscinosis, neuronal 5 || AK085741 ||	0.234423645	1.556540943	0.1722319
8686	Mitogen activated protein kinase kinase kinase 12 || NM_009582 ||	0.252881792	1.566833972	0.1722319
2592	Protection of telomeres 1 || NM_133931 ||	0.225015065	1.610716845	0.1722319
8913	Synaptotagmin 12 || NM_134164 ||	0.230854559	1.613719964	0.1722319
1420	Inositol polyphosphate phosphatase-like 1 || NM_010567 ||	0.313537667	1.852759522	0.1722319
8333	Apolipoprotein D || NM_007470 ||	0.451007072	2.056632637	0.1722319
12018	Damage specific DNA binding protein 2 || BC058589 ||	0.400485387	2.068644448	0.1722319
11606	RIKEN cDNA 2810003C17 gene || NM_145144 ||	0.402058132	1.992713546	0.1722319
8367	Calumenin || NM_007594 ||	0.364921154	1.818933586	0.1722319
8083	Mitogen activated protein kinase 7 || NM_011841 ||	0.220485289	1.532826096	0.1722319
1167	RIKEN cDNA 2310036D22 gene || NM_027992 ||	0.3068909	1.683013489	0.1722319
2361	Transcription elongation factor B (SIII), polypeptide 1 || NM_026456 ||	0.242012435	1.543220829	0.1722319
7570	Ubiquitin-activating enzyme E1C || NM_011666 ||	0.255327569	1.615947777	0.1722319
9304	SRB7 (supressor of RNA polymerase B) homolog (S. cerevisiae) || NM_025315 ||	0.276949536	1.593599439	0.1722319
5477	Basic helix-loop-helix domain containing, class B2 || NM_011498 ||	0.285949941	1.634273512	0.1722319
5813	ADP-ribosylation factor-like 6 interacting protein 6 || NM_022989 ||	0.256070774	1.604221099	0.1722319
10764	RIKEN cDNA 2410066E13 gene || NM_026629 ||	0.172899929	1.444200029	0.1722319
12083	CDNA sequence BC028440 || NM_172148 ||	0.270570404	1.61714742	0.1722319

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)
1864	RIKEN cDNA 2010002N04 gene || NM_134133 ||	0.404034802	1.919330113	0.1722319
2201	Translocator of inner mitochondrial membrane 17a || NM_011590 ||	0.228085876	1.693854057	0.1722319
8703	Replication factor C (activator 1) 4 || NM_145480 ||	0.293776184	1.631374642	0.1722319
1516	Lin 7 homolog c (C. elegans) || NM_011699 ||	0.251813691	1.597111932	0.1722319
12752	Down syndrome critical region gene 3 || NM_007834 ||	0.190600441	1.442356859	0.1722319
4470	Nuclear receptor subfamily 4, group A, member 1 || NM_010444 ||	0.346924551	1.741353388	0.1722319
4043	DnaJ (Hsp40) homolog, subfamily C, member 10 || AK128945 ||	0.230761032	1.504842834	0.1722319
12810	Lipocalin 2 || NM_008491 ||	0.516842186	2.539988673	0.1722319
1906	|| NM_029930 ||	0.28311998	1.636445408	0.1722319
7303	RIKEN cDNA 1110057H19 gene || NM_025409 ||	0.239692118	1.640114331	0.1722319
3955	Formin-like 2 || AK080318 ||	0.296637178	1.696080849	0.1722319
1205	Guanine nucleotide binding protein (G protein), gamma 11 || NM_025331 ||	0.369447249	1.709202177	0.1722319
5770	RIKEN cDNA 6330407D12 gene || NM_175098 ||	0.269064105	1.597940116	0.1722319
758	Anaphase promoting complex subunit 11 homolog (yeast) || NM_025389 ||	0.207458619	1.467356141	0.1722319
4057	RIKEN cDNA 2600010E01 gene || NM_175181 ||	0.277961161	1.591217099	0.1722319
11938	|| XM_986727 ||	0.319176473	1.824333898	0.1722319
7788	SEC14 and spectrin domains 1 || NM_175465 ||	0.227430289	1.529138335	0.1722319
2808	RIKEN cDNA 1500015L24 gene || NM_183277 ||	0.439024698	2.564207354	0.1722319
7391	Interleukin enhancer binding factor 2 || NM_026374 ||	0.523775458	2.582909184	0.1722319
652	Serine carboxypeptidase 1 || NM_029023 ||	0.275180635	1.616537363	0.1722319
4906	Expressed in non-metastatic cells 1, protein || NM_008704 ||	0.271803312	1.647296383	0.1722319
2329	RIKEN cDNA 1700001E18 gene || NM_026503 ||	0.214139926	1.516932063	0.1722319
1917	Minichromosome maintenance deficient 2 mitotin (S. cerevisiae) || NM_008564 ||	0.338963705	1.706445255	0.1722319
12517	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 12 || NM_025894 ||	0.204486737	1.493093249	0.1722319
852	Naked cuticle 1 homolog (Drosophila) || NM_027280 ||	0.493453727	1.807422099	0.1722319
3460	Aquaporin 5 || NM_009701 ||	0.573060867	2.04081315	0.1722319
4366	ADP-ribosylation factor 4-like || NM_031160 ||	0.454976646	2.766308552	0.1722319
10280	Retinitis pigmentosa 2 homolog (human) || NM_133669 ||	0.228205959	1.526911475	0.1722319
4916	Dynein, cytoplasmic, light chain 2A || NM_025947 ||	0.216312577	1.49924203	0.302436
5507	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5 || NM_011404 ||	0.437768765	2.038853451	0.302436
8483	Inducible T-cell co-stimulator || NM_017480 ||	0.495546511	2.405404429	0.302436
4486	|| XM_898828 ||	0.218243113	1.488189281	0.302436
6681	Potassium inwardly-rectifying channel, subfamily J, member 8 || NM_008428 ||	0.403113522	2.061291034	0.302436

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)
11958	Sialyltransferase 9 (CMP-NeuAc:lactosylceramide alpha-2,3-sialyltransferase) || NM_011375 ||	0.386962411	1.642299207	0.302436
12520	Deoxycytidine kinase || NM_007832 ||	0.273305999	1.719246532	0.302436
7025	RAS protein-specific guanine nucleotide-releasing factor 1 || NM_011245 ||	0.26167889	1.866196542	0.302436
11245	Cytochrome P450, family 7, subfamily b, polypeptide 1 || NM_007825 ||	0.330136887	1.730025077	0.302436
1673	RIKEN cDNA 5830427H10 gene || NM_026365 ||	0.239448897	1.548212457	0.302436
5097	Glutathione S-transferase, mu 5 || NM_010360 ||	0.303844294	1.725946124	0.302436
9491	S100 calcium binding protein A9 (calgranulin B) || NM_009114 ||	0.660418514	1.381514151	0.302436
7217	Paraspeckle protein 1 || NM_025682 ||	0.223307424	1.475304137	0.302436
10547	Tubby-like protein 3 || NM_011657 ||	0.205211088	1.495463778	0.302436
8103	Olfactory receptor 449 || NM_147064 ||	0.147194398	1.347389019	0.302436
12786	RIKEN cDNA A930014D08 gene || NM_138758 ||	0.248858374	1.526535886	0.302436
2184	Chitinase 3-like 3 || NM_009892 ||	0.825724935	2.286020051	0.302436
11577	Lurcher transcript 1 || NM_144861 ||	0.283060659	1.679834853	0.302436
10900	Src-like-adaptor 2 || NM_026124 ||	0.236110339	1.502704089	0.302436
2088	ADAM-like, decysin 1 || NM_021475 ||	0.474935628	2.972090703	0.302436
769	|| XM_001001886 ||	0.20221064	1.458351087	0.302436
4691	Dolichyl pyrophosphate phosphatase 1 || NM_020329 ||	0.199550851	1.440766381	0.302436
11102	Claudin 10 || NM_021386 ||	0.65643027	1.85010245	0.302436
4517	Glial cell line derived neurotrophic factor family receptor alpha 4 || NM_020014 ||	0.15879019	1.413248011	0.302436
12583	Dual specificity phosphatase 6 || NM_026268 ||	0.450859743	1.820348968	0.302436
1227	RAB7, member RAS oncogene family || NM_009005 ||	0.186778294	1.415733563	0.302436
12767	Retinol binding protein 7, cellular || NM_022020 ||	0.422949793	2.356363759	0.302436
6038	RIKEN cDNA 2310016M24 gene || NM_183256 ||	0.205093002	1.531331176	0.302436
8634	G protein coupled receptor 24 || NM_145132 ||	0.409424213	2.077563271	0.302436
6194	Origin recognition complex, subunit 6-like (S. cerevisiae) || NM_019716 ||	0.258780765	1.570977416	0.302436
2421	Expressed sequence AL117801 || NM_026063 ||	0.218600649	1.491821415	0.302436
1853	Lymphotoxin B receptor || NM_010736 ||	0.241646965	1.584845797	0.302436
5429	RIKEN cDNA 5630401J11 gene || NM_134114 ||	0.297809269	1.770906569	0.302436
1019	Somatostatin receptor 4 || NM_009219 ||	0.181654214	1.422354359	0.302436
8888	CDNA sequence BC020108 || BC020108 ||	0.235258255	1.505145928	0.302436
8088	Transmembrane protein 9 || NM_025439 ||	0.237568728	1.499241021	0.302436
6278	RIKEN cDNA 6720456B07 gene || NM_133937 ||	0.194332011	1.416625981	0.302436
8650	RIKEN cDNA 1110034G24 gene || AK004090 ||	0.412779137	1.98164599	0.302436

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)
2818	Immunoglobulin heavy chain (J558 family) || BC004786 ||	0.545053601	2.498208237	0.302436
8275	|| NM_029529 ||	0.357374602	1.97548398	0.302436
4416	Eukaryotic translation initiation factor 1A, Y-linked || NM_025437 ||	0.21921274	1.48527149	0.302436
2632	RIKEN cDNA 2610209A20 gene || NM_026010 ||	0.176428571	1.424079817	0.302436
3191	Esterase D/formylglutathione hydrolase || NM_016903 ||	0.297349924	1.524155238	0.302436
12863	Caspase 4, apoptosis-related cysteine protease || NM_007609 ||	0.441775496	1.477977337	0.302436
11596	H2A histone family, member V || BC028539 ||	0.268284499	1.572258193	0.302436
5547	Integrin alpha 2b || NM_010575 ||	0.313487876	1.844315403	0.302436
12223	Casein alpha || NM_007784 ||	0.792337087	2.386488063	0.302436
12949	RIKEN cDNA 5730458M16 gene || NM_025422 ||	0.404851954	1.671253181	0.302436
11611	|| TC1536721 ||	0.337909651	1.713994206	0.302436
12449	Deafness, autosomal dominant 5 homolog (human) || NM_018769 ||	0.288196896	1.644320981	0.302436
7568	RIKEN cDNA D430022A14 gene || NM_009366 ||	0.344182015	1.636595047	0.302436
1564	RIKEN cDNA 1700030B17 gene || NM_027407 ||	0.757122176	2.11640126	0.302436
841	Zinc finger protein 637 || NM_177684 ||	0.221981666	1.519541216	0.302436
7328	Procollagen, type XXV, alpha 1 || NM_029838 ||	0.14984838	1.320779263	0.302436
3846	Dynamin 1 || NM_010065 ||	0.387761159	1.862038846	0.3901798
6864	RIKEN cDNA B230205O20 gene || NM_153555 ||	0.18838975	1.408582625	0.3901798
6455	|| ENSMUST00000025293 ||	0.274736945	1.623273975	0.3901798
10562	Rho GTPase activating protein 22 || NM_153800 ||	0.388797417	1.786019064	0.3901798
9811	Prominin 1 || NM_008935 ||	0.381360636	1.860113109	0.3901798
12088	|| NM_001039534 ||	0.230334308	1.517594627	0.3901798
1658	DNA segment, Chr 18, Wayne State University 98, expressed || NM_025299 ||	0.263086495	1.600214996	0.3901798
12136	|| NM_030131 ||	0.251477219	1.561338592	0.3901798
12836	CDNA sequence BC003236 || NM_030249 ||	0.227086918	1.489913059	0.3901798
10485	RIKEN cDNA 1700027N10 gene || NM_029338 ||	0.297531753	1.620257899	0.3901798
8799	FXYD domain-containing ion transport regulator 5 || NM_008761 ||	0.365745871	1.588465751	0.3901798
934	|| NM_053219 ||	0.344415973	2.071935431	0.3901798
1776	DNA segment, Chr 1, Brigham & Women's Genetics 1363 expressed || NM_001001566 ||	0.249932509	1.572620413	0.3901798
5639	RIKEN cDNA 1700049J03 gene || AK006745 ||	0.348156377	1.767273276	0.3901798
12700	Aldehyde dehydrogenase 3 family, member B1 || NM_026316 ||	0.272645468	1.545193475	0.3901798
11481	Microfibrillar-associated protein 3 || NM_145426 ||	0.196729351	1.549262199	0.3901798
2881	Proviral integration site 3 || NM_145478 ||	0.306058965	1.618939586	0.3901798

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)
5483	TPA regulated locus || AK007181 ||	0.464917621	2.305277058	0.3901798
3503	Expressed sequence AI461788 || AK030894 ||	0.224853415	1.498440023	0.3901798
6105	FK506 binding protein 1b || NM_016863 ||	0.226885694	1.469980672	0.3901798
6781	RIKEN cDNA C330012H03 gene || NM_183029 ||	0.449097302	1.880324505	0.3901798
11532	RIKEN cDNA 1700019B03 gene || AK006106 ||	0.145474007	1.313610374	0.3901798
9321	Solute carrier family 16 (monocarboxylic acid transporters), member 1 || NM_009196 ||	0.35713228	1.74362706	0.3901798
4582	Proteolipid protein 2 || NM_019755 ||	0.238446222	1.484346454	0.3901798
6822	Chemokine-like factor super family 2A || NM_027022 ||	0.223236598	1.52738375	0.3901798
4388	Demilune cell and parotid protein || NM_145386 ||	0.536283449	0.417034043	0.3901798
4216	Procollagen, type IX, alpha 1 || NM_007740 ||	0.558410119	1.827105441	0.3901798
11566	Sialyltransferase 8 (alpha-2, 8-sialyltransferase) F || NM_145838 ||	0.502769934	1.851319421	0.3901798
7324	SUMO1/sentrin specific protease 7 || NM_001003972 ||	0.196779548	1.393914088	0.3901798
8230	Small nuclear ribonucleoprotein E || NM_009227 ||	0.23642069	1.640966909	0.3901798
5381	BCL2/adenovirus E1B 19kDa-interacting protein 3-like || NM_009761 ||	0.216850873	1.450407207	0.3901798
11983	RIKEN cDNA 5730427C23 gene || NM_153777 ||	0.281842844	1.706897751	0.3901798
9253	Chemokine-like factor || NM_029295 ||	0.231713316	1.531552554	0.3901798
5756	Arsenic (+3 oxidation state) methyltransferase || NM_020577 ||	0.271178907	1.556453591	0.3901798
4290	RIKEN cDNA 0610010O12 gene || BC028765 ||	0.386604563	1.737513797	0.3901798
350	COP9 (constitutive photomorphogenic) homolog, subunit 7a (Arabidopsis thaliana) || NM_012003 ||	0.167142504	1.384152118	0.3901798
12698	DNA segment, Chr 11, ERATO Doi 497, expressed || NM_029976 ||	0.199730374	1.429651091	0.3901798
4307	|| AK157817 ||	0.207698341	1.537788202	0.3901798
6271	RIKEN cDNA 1110032D12 gene || NM_019770 ||	0.273561153	1.563325614	0.3901798
12592	|| AK033738 ||	0.383607856	1.84666499	0.3901798
7573	CEA-related cell adhesion molecule 10 || NM_007675 ||	0.399618662	1.874221763	0.3901798
12931	Similar to anti-poly(dC) monoclonal antibody heavy chain || BF134160 ||	0.247854238	1.497473681	0.3901798
7583	A kinase (PRKA) anchor protein (gravin) 12 || NM_031185 ||	0.441408234	1.593234366	0.3901798
8348	Proteasome (prosome, macropain) subunit, beta type 1 || NM_011185 ||	0.169925217	1.367289441	0.3901798
10364	Expressed sequence AI850995 || AK036079 ||	0.329755529	1.777200313	0.3901798
11509	RIKEN cDNA 1700020A23 gene || BC049710 ||	0.14285838	1.297304239	0.3901798
3173	Similar to hypothetical protein FLJ10706 || NM_201364 ||	0.340379053	1.701929239	0.3901798
4773	Regulator of G-protein signaling 16 || NM_011267 ||	0.343505609	1.785269261	0.3901798
7561	Matrix metalloproteinase 2 || NM_008610 ||	0.607358088	2.049998333	0.3901798
7702	1-acylglycerol-3-phosphate O-acyltransferase 1 (lysophosphatidic acid acyltransferase, delta) || NM_026644 ||	0.407802267	1.678784004	0.3901798

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)
1013	Preimplantation protein 3 || NM_025283 ||	0.258189051	1.493909769	0.3901798
8210	ADP-ribosylation factor GTPase activating protein 3 || NM_025445 ||	0.205109305	1.453603666	0.3901798
8526	RIKEN cDNA B130066H01 gene || NM_145533 ||	0.293181091	1.586782028	0.3901798
4122	DNA segment, Chr 2, Brigham & Women's Genetics 1356 expressed || BC054558 ||	0.232653976	1.641686744	0.3901798
6159	RIKEN cDNA 2010209O12 gene || BC047275 ||	0.273192875	1.601145804	0.3901798
411	Solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1 || NM_009199 ||	0.307812773	1.593108642	0.3901798
9784	Crystallin, zeta (quinone reductase)-like 1 || NM_133679 ||	0.203447633	1.41186178	0.3901798
7525	B-cell translocation gene 3 || NM_009770 ||	0.301016524	1.523770155	0.3901798
5803	Solute carrier family 6 (neurotransmitter transporter, taurine), member 6 || AF020194 ||	0.301266806	1.706258787	0.3901798
7661	RIKEN cDNA 2610040E16 gene || NM_024194 ||	0.215586689	1.434171384	0.3901798
2537	TEA domain family member 4 || D87966 ||	0.221236779	1.46497069	0.3901798
2600	Upregulated during skeletal muscle growth 5 || NM_023211 ||	0.251904332	1.533125135	0.3901798
1311	Eph receptor B4 || U06834 ||	0.192018439	1.423588668	0.453112
1259	UDP-glucose dehydrogenase || NM_009466 ||	0.358291475	1.606656196	0.453112
7738	Dual specificity phosphatase 4 || AK012530 ||	0.340938519	1.616699314	0.453112
7856	Mitochondrial ribosomal protein L35 || NM_025430 ||	0.231882487	1.756796445	0.453112
6132	RIKEN cDNA 2310044D20 gene || NM_026321 ||	0.245758496	1.523379591	0.453112
2800	RIKEN cDNA 2010012C16 gene || NM_025564 ||	0.236987683	1.526319085	0.453112
5052	Protease, serine, 18 || NM_011177 ||	0.462272828	1.891596564	0.453112
3560	AP2 associated kinase 1 || AK011997 ||	0.184513635	1.415481773	0.453112
10930	RIKEN cDNA 1110036O03 gene || AK029271 ||	0.298753203	1.530519839	0.453112
6836	Netrin 3 || NM_010947 ||	0.331264392	1.926390233	0.453112
3448	RIKEN cDNA 1110008P14 gene || NM_198001 ||	0.278266697	1.691961344	0.453112
196	RIKEN cDNA 2010007H12 gene || NM_027242 ||	0.231206393	1.469070643	0.453112
2240	RIKEN cDNA 4930428J16 gene || AK015225 ||	0.178769751	1.380888901	0.453112
11167	Ribosomal protein L24 || NM_024218 ||	0.257483426	1.640152699	0.453112
8822	Leucine-rich repeat-containing G protein-coupled receptor 8 || AK018844 ||	0.198314255	1.427475863	0.453112
3565	SPARC-like 1 (mast9, hevin) || NM_010097 ||	0.452258014	2.211962396	0.453112
9339	Solute carrier family 9 (sodium/hydrogen exchanger), member 2 || AK077026 ||	0.424790071	3.033241093	0.453112
332	|| NM_001024700 ||	0.529117367	2.025378501	0.453112
603	Rap guanine nucleotide exchange factor (GEF) 3 || NM_144850 ||	0.396895574	1.57993896	0.453112

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)
4097	CDC28 protein kinase 1b || NM_016904 ||	0.296668412	1.622473692	0.453112
8420	Ubiquitin domain containing 1 || NM_145500 ||	0.269785234	1.485029882	0.453112
5684	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 11 || BC059729 ||	0.291530798	1.670195868	0.453112
4500	RIKEN cDNA 2610307O08 gene || BC046640 ||	0.296303432	1.604392489	0.453112
3522	Transcriptional adaptor 3 (NGG1 homolog, yeast)-like || NM_133932 ||	0.194150486	1.405486715	0.453112
5869	RIKEN cDNA 5033430I15 gene || AK017206 ||	0.292381125	1.636128563	0.453112
10728	|| A_51_P443920 ||	0.366562375	1.871027041	0.453112
2390	Nuclear interacting partner of anaplastic lymphoma kinase (Alk) || NM_172735 ||	0.197171703	1.405755677	0.453112
3980	|| ENSMUST00000043610 ||	0.224931754	1.469848186	0.453112
12435	Hairy and enhancer of split 1 (Drosophila) || NM_008235 ||	0.190040854	1.505699125	0.453112
11733	Neogenin || NM_008684 ||	0.265914432	1.488312113	0.453112
3705	Zinc finger protein 521 || NM_145492 ||	0.335403565	1.60547903	0.453112
9369	Insulin-like growth factor binding protein 4 || NM_010517 ||	0.239606051	1.500301798	0.453112
4970	Protein phosphatase 6, catalytic subunit || NM_024209 ||	0.185239905	1.481355801	0.453112
11939	High mobility group AT-hook 1 || NM_016660 ||	0.434261574	2.090937157	0.453112
7969	|| AK004585 ||	0.172034161	1.38898973	0.453112
4175	RIKEN cDNA 2610304G08 gene || NM_027434 ||	0.308609024	1.702411169	0.453112
5350	Fibulin 2 || NM_007992 ||	0.463254717	1.682384169	0.453112
3908	RIKEN cDNA 1810013H02 gene || NM_145465 ||	0.214406174	1.457391895	0.453112
11833	|| NM_028894 ||	0.333780511	1.636851831	0.453112
3995	Thymidine kinase 1 || NM_009387 ||	0.320229462	1.59768234	0.453112
12137	Bone morphogenetic protein 1 || NM_009755 ||	0.314292558	1.67992328	0.453112
3235	|| AK158756 ||	0.298476982	1.610678939	0.453112
11916	Unc-51 like kinase 1 (C. elegans) || NM_009469 ||	0.609586999	2.098960779	0.453112
7373	Scinderin || NM_009132 ||	0.395046744	1.94279341	0.453112
9258	RIKEN cDNA 9030611O19 gene || NM_027828 ||	0.223541608	1.544636298	0.453112
6325	Syntaxin binding protein 1 || NM_009295 ||	0.288317228	1.584131744	0.453112
8250	Low density lipoprotein receptor-related protein 5 || NM_008513 ||	0.353106721	1.754816316	0.453112
12546	UDP-glucuronate decarboxylase 1 || NM_026430 ||	0.293974006	1.530751497	0.453112
8810	Origin recognition complex, subunit 4-like (S. cerevisiae) || NM_011958 ||	0.264993976	1.583673951	0.453112
12529	NIMA (never in mitosis gene a)-related expressed kinase 2 || NM_010892 ||	0.327187113	1.634594073	0.7696697
9131	AT rich interactive domain 5B (Mrf1 like) || NM_023598 ||	0.345498668	1.8433934	0.7696697
12890	Synaptogyrin 4 || NM_021482 ||	0.157457803	1.335171354	0.7696697

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)
10307	Transmembrane 4 superfamily member 2 || NM_019634 ||	0.314369384	1.637480094	0.7696697
8089	RIKEN cDNA 1810029G24 gene || NM_025468 ||	0.330178653	1.625865522	0.7696697
7074	|| NM_008381 ||	0.448447226	1.732293733	0.7696697
9936	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 2 (p85 beta) || NM_008841 ||	0.231836974	1.438347464	0.7696697
9236	RIKEN cDNA 2410042D21 gene || NM_024254 ||	0.25914956	1.569366107	0.7696697
896	Insulin-like growth factor 2 receptor || NM_010515 ||	0.277316449	1.563821754	0.7696697
10089	RIKEN cDNA 3110023G01 gene || AK014074 ||	0.275735263	1.626731434	0.7696697
10550	HS1 binding protein || NM_011826 ||	0.188299322	1.410363858	0.7696697
2625	|| XM_978251 ||	0.230282438	2.238786402	0.7696697
8402	Transducin-like enhancer of split 6, homolog of Drosophila E(spl) || NM_053254 ||	0.29060286	1.575250879	0.7696697
9804	GA repeat binding protein, beta 1 || NM_010249 ||	0.226301516	1.453553633	0.7696697
2456	Signal-regulatory protein beta || AK054545 ||	0.429868413	1.953458799	0.7696697
2665	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 2 || NM_138651 ||	0.198562781	1.400153315	0.7696697
4436	RIKEN cDNA A230105L22 gene || NM_024444 ||	0.230233993	1.421272825	0.7696697
11578	RIKEN cDNA 2010317E24 gene || BC070477 ||	0.356151918	1.620164604	0.7696697
2005	Sestrin 2 || NM_144907 ||	0.191059679	1.384826058	0.7696697
6927	Vitamin K epoxide reductase complex, subunit 1-like 1 || NM_027121 ||	0.177045682	1.346599922	0.7696697
9070	|| NM_026988 ||	0.399657162	1.911419783	0.7696697
12195	RIKEN cDNA 9030607L02 gene || NM_025423 ||	0.236727564	1.504229673	0.7696697
105	CDNA sequence BC034054 || NM_178049 ||	0.290910641	1.539716317	0.7696697
6904	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 || NM_011122 ||	0.205485498	1.588302611	0.7696697
999	Aldo-keto reductase family 1, member B8 || NM_008012 ||	0.385252335	1.547644989	0.7696697
4681	Calcium/calmodulin-dependent protein kinase kinase 2, beta || NM_145358 ||	0.279183261	1.622554809	0.7696697
478	RIKEN cDNA 2610528M18 gene || NM_031863 ||	0.24757281	1.461592418	0.7696697
9442	Procollagen, type IX, alpha 2 || NM_007741 ||	0.396052107	1.575010313	0.7696697
5517	Centromere autoantigen A || NM_007681 ||	0.301931653	1.527013306	0.7696697
6223	|| NM_029153 ||	0.197062509	1.424306558	0.7696697
5031	Proteasome (prosome, macropain) subunit, alpha type 5 || NM_011967 ||	0.194560891	1.441979686	0.7696697
5641	RIKEN cDNA 2700050P07 gene || NM_133927 ||	0.166765394	1.330619553	0.7696697
11796	ADP-ribosyltransferase 1 || AK033068 ||	0.597442743	2.12667171	0.7696697
9318	StAR-related lipid transfer (START) domain containing 6 || NM_029019 ||	0.197584745	1.43008366	0.7696697
5334	RIKEN cDNA 2010004A03 gene || NM_029646 ||	0.294199459	1.468779252	0.7696697
2369	Golgi reassembly stacking protein 2 || NM_027352 ||	0.214896584	1.422679158	0.7696697

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)
3077	NIMA (never in mitosis gene a)-related expressed kinase 8 || BC057995 ||	0.18041659	1.390335055	0.7696697
7743	TGFB inducible early growth response 1 || NM_013692 ||	0.288213386	1.569850211	0.7696697
11720	RIKEN cDNA C330023D02 gene || BC057368 ||	0.230369617	1.454825134	0.7696697
970	RIKEN cDNA 2700088M22 gene || NM_026025 ||	0.233453524	1.484530279	0.7696697
2896	|| NM_027347 ||	0.315829228	1.710743964	0.7696697
1977	Bridging integrator 3 || NM_021328 ||	0.198395063	1.407631889	0.7696697
7606	RIKEN cDNA 0610042E07 gene || NM_026158 ||	0.304298103	1.518597775	0.7696697
5886	Mus musculus, clone IMAGE:1244192, mRNA || BC028808 ||	0.562759173	2.232051241	0.7696697
5070	FUN14 domain containing 2 || NM_026126 ||	0.231537216	1.694343704	0.7696697
2649	Non-catalytic region of tyrosine kinase adaptor protein 1 || NM_010878 ||	0.264656414	1.542888665	0.7696697
5438	Interleukin 1 receptor, type I || NM_008362 ||	0.339705599	1.499754293	0.7696697
9088	Kringle containing transmembrane protein 1 || NM_032396 ||	0.387792992	1.88934512	0.7696697
5194	Phosphatidylinositol glycan, class F || NM_008838 ||	0.227250212	1.449386805	0.7696697
10213	|| AK147270 ||	0.231111035	1.429625889	0.7696697
10393	Ataxin 7-like 3 || AK008158 ||	0.184295272	1.354050035	0.7696697
12663	RIKEN cDNA 0610037H22 gene || NM_025332 ||	0.205642547	1.443135063	0.7696697
1352	Dermatan 4 sulfotransferase 1 || NM_028117 ||	0.252364683	1.47155863	0.7696697
1253	|| AV065344 ||	0.376710596	1.544985114	0.7696697
8102	Zinc finger like protein 1 || NM_024231 ||	0.179734889	1.337546051	0.7696697
8309	RIKEN cDNA 1110014K08 gene || NM_176902 ||	0.304638968	1.564670131	0.7696697
877	Poliovirus receptor-related 3 || NM_021495 ||	0.34508374	1.627962022	0.7696697
11328	Carbohydrate sulfotransferase 12 || NM_021528 ||	0.259400474	1.446497041	0.7696697
4895	RIKEN cDNA 1700008O03 gene || XM_133454 ||	0.245364442	1.473422598	0.7696697
4081	Myb protein P42POP || NM_145579 ||	0.15534933	1.294888748	0.7696697
7784	LSM3 homolog, U6 small nuclear RNA associated (S. cerevisiae) || NM_026309 ||	0.230748819	1.520937475	0.7696697
1263	Ring finger protein 13 || NM_011883 ||	0.204406302	1.391586239	0.7696697
10608	Regulator of G-protein signaling 19 interacting protein 1 || NM_018771 ||	0.21980398	1.446776044	0.7696697
1655	RIKEN cDNA D230019K24 gene || NM_172511 ||	0.211042776	1.367422373	0.7696697
927	Brain protein I3 || NM_018772 ||	0.201874363	1.409344306	0.7696697
324	G-protein signalling modulator 2 (AGS3-like, C. elegans) || NM_029522 ||	0.279501238	1.507424006	0.7696697
1678	Ropporin 1-like || NM_145852 ||	0.257368322	1.508998289	0.7696697
4983	Limb region 1 || BC016110 ||	0.224576447	1.422074928	0.7696697
2804	Mitochondrial ribosomal protein S33 || NM_010270 ||	0.214002401	1.417442427	0.7696697

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)
10452	RIKEN cDNA 6820402I19 gene || NM_144839 ||	0.246566802	1.450790024	0.7696697
11478	Expressed sequence AI839550 || NM_198027 ||	0.21450217	1.406081838	0.7696697
6557	|| NM_146759 ||	0.148770365	1.283457474	0.9796563
2009	|| NM_028492 ||	0.174019678	1.375506299	0.9796563
9829	RIKEN cDNA 9130011J15 gene || NM_172396 ||	0.19516251	1.36406944	0.9796563
6898	Gene rich cluster, C10 gene || NM_013535 ||	0.217767454	1.447499687	0.9796563
9105	Cappuccino || NM_133724 ||	0.167931896	1.316778379	0.9796563
3641	Psoriasis susceptibility 1 candidate 2 (human) || NM_020576 ||	0.358163644	1.828921165	0.9796563
12722	RIKEN cDNA 2310022A10 gene || NM_175107 ||	0.190281105	1.368915316	0.9796563
10487	Tumor necrosis factor, alpha-induced protein 8 || NM_134131 ||	0.319163519	1.546961099	0.9796563
5324	Mannoside acetylglucosaminyltransferase 4, isoenzyme B || BC026638 ||	0.331588194	1.51954853	0.9796563
528	RIKEN cDNA 1110005A03 gene || NM_028865 ||	0.242747593	1.571281846	0.9796563
1943	Potassium inwardly-rectifying channel, subfamily J, member 15 || AF085696 ||	0.178931392	1.392108478	0.9796563
13099	|| NM_001017959 ||	0.287830138	1.584887224	0.9796563
6576	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B || NM_009153 ||	0.782024436	1.141296548	0.9796563
6945	Hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase) || NM_173371 ||	0.321790278	1.606281007	0.9796563
3322	Casein kinase II, alpha 2, polypeptide || NM_009974 ||	0.190917682	1.360891033	0.9796563
2507	Trophinin associated protein || AK050381 ||	0.30252868	1.53519626	0.9796563
1468	RIKEN cDNA 2500002L14 gene || NM_025607 ||	0.208946703	1.371833035	0.9796563
8916	Stefin A1 || NM_001001332 ||	0.595339572	0.894288688	0.9796563
8138	EH-domain containing 3 || NM_020578 ||	0.439607353	1.669281301	0.9796563
13012	RIKEN cDNA 5530601I19 gene || NM_027797 ||	0.209644652	1.618203976	0.9796563
2389	Olfactory receptor 935 || NM_146746 ||	0.600974227	2.047735397	0.9796563
1978	Epidermal growth factor-containing fibulin-like extracellular matrix protein 2 || NM_021474 ||	0.382002282	1.572003589	0.9796563
12609	Phosphatidic acid phosphatase type 2B || NM_080555 ||	0.401450267	1.616996631	0.9796563
11631	G protein-coupled receptor 171 || NM_173398 ||	0.339524114	1.716450176	0.9796563
3184	|| XM_895899 ||	0.274232606	1.494602545	0.9796563

Genes underexpressed in triple mutant tumors

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)
5015	TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor || NM_027427 ||	0.362767437	0.286261918	0
7372	RIKEN cDNA 1700007H16 gene || NM_027945 ||	0.237423553	0.49770807	0
3087	Ribosomal protein S15 || NM_009091 ||	0.259298709	0.448588336	0
136	Leukotriene A4 hydrolase || NM_008517 ||	0.226951913	0.487989117	0
4181	Parkinson disease (autosomal recessive, early onset) 7 || NM_020569 ||	0.20086799	0.556649764	0
2039	|| BC056356 ||	0.293113473	0.378460859	0
3931	Heterogeneous nuclear ribonucleoprotein A3 || NM_198090 ||	0.239498573	0.485125112	0
8238	Minichromosome maintenance deficient 3 (S. cerevisiae) associated protein || NM_019434 ||	0.182111875	0.579857031	0
9089	Citrate synthase || NM_026444 ||	0.26703468	0.450325487	0
9029	Peptidylprolyl isomerase (cyclophilin)-like 4 || NM_026141 ||	0.211503225	0.558025118	0
7448	Aldehyde dehydrogenase 4 family, member A1 || NM_175438 ||	0.388418935	0.272098788	0
9183	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent), methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthase || NM_138745 ||	0.245153385	0.49017932	0
4553	Dual specificity phosphatase 16 || NM_130447 ||	0.320879127	0.440338871	0
8243	Chaperonin subunit 2 (beta) || NM_007636 ||	0.223479577	0.524733737	0
8513	Mitochondrial ribosomal protein L54 || NM_025317 ||	0.221465387	0.552730804	0
3273	|| NM_001037846 ||	0.203192056	0.567709755	0
2075	Talin 2 || AK029828 ||	0.304057442	0.408656956	0
4272	RIKEN cDNA 1190005F20 gene || XM_355244 ||	0.237469019	0.547059908	0
12356	Mitogen activated protein kinase kinase kinase 4 || NM_011948 ||	0.204826022	0.570808117	0
7655	Methionine aminopeptidase 2 || NM_019648 ||	0.235881866	0.513555311	0
10717	Pyrophosphatase || NM_026438 ||	0.267528569	0.483148262	0.1915428
11238	PDZ and LIM domain 1 (elfin) || NM_016861 ||	0.246388146	0.519083905	0.1915428
5585	CDNA sequence BC059842 || NM_198170 ||	0.211233451	0.604785879	0.1915428
495	GTPase activating RANGAP domain-like 1 || NM_019994 ||	0.180657954	0.641825746	0.1915428
9170	THUMP domain containing 1 || NM_145585 ||	0.200921707	0.589857904	0.1915428
7768	Signaling intermediate in Toll pathway-evolutionarily conserved || NM_012029 ||	0.230942354	0.52867453	0.1915428
9685	|| AK156076 ||	0.184709041	0.622510437	0.1915428
11956	|| BC100509 ||	0.448993421	0.215017463	0.1915428

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)
11869	Glyoxalase 1 || NM_025374 ||	0.232328604	0.537950171	0.1915428
10527	RIKEN cDNA 2310001H13 gene || BC058971 ||	0.291853571	0.473462738	0.302436
2212	|| AK007939 ||	0.260692368	0.524262924	0.302436
11954	Ribonuclease H1 || NM_011275 ||	0.198338369	0.611926342	0.302436
2724	Transformation related protein 53 || NM_011640 ||	0.270826951	0.476181038	0.302436
8635	Mediator of RNA polymerase II transcription, subunit 8 homolog (yeast) || NM_173719 ||	0.226566039	0.586542156	0.302436
3462	ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit || NM_016774 ||	0.314205604	0.432665347	0.302436
9246	Zinc finger protein 609 || NM_172536 ||	0.297525844	0.494526184	0.302436
11828	CDP-diacylglycerol synthase 1 || NM_173370 ||	0.278037222	0.481399545	0.302436
4316	Receptor tyrosine kinase-like orphan receptor 2 || NM_013846 ||	0.215178605	0.615892621	0.302436
856	Prostate cancer associated protein 6 || NM_145977 ||	0.256337352	0.525241366	0.302436
2377	AHA1, activator of heat shock 90kDa protein ATPase homolog 1 (yeast) || NM_146036 ||	0.185059313	0.649778304	0.302436
6810	RIKEN cDNA 2810012H18 gene || AK012728 ||	0.257634315	0.497682117	0.302436
8582	Angiopoietin-like 2 || CA490698 ||	0.287984127	0.496983068	0.3901798
11131	Dipeptidylpeptidase 8 || AK009140 ||	0.197055493	0.623883201	0.3901798
6364	Tubulin, delta 1 || NM_019756 ||	0.301783509	0.475230403	0.3901798
4781	Hypothetical protein 9530028C05 || AK035387 ||	0.295107188	0.469656435	0.3901798
12243	|| XM_902491 ||	0.197680627	0.617781263	0.3901798
1706	RIKEN cDNA 4732497O03 gene || NM_144826 ||	0.191157936	0.640852729	0.3901798
1372	DEAD (Asp-Glu-Ala-Asp) box polypeptide 50 || NM_053183 ||	0.216168708	0.58097795	0.3901798
2607	WD repeat domain 9 || NM_145125 ||	0.220776921	0.578237977	0.3901798
8798	PDZ domain containing 11 || NM_028303 ||	0.182311703	0.67305326	0.3901798
2274	CDNA sequence AF155546 || AK076287 ||	0.22572038	0.597573591	0.3901798
11179	RIKEN cDNA A130042E20 gene || NM_172550 ||	0.222730853	0.588479345	0.3901798
11082	RIKEN cDNA 4930467M19 gene || AK014551 ||	0.321524232	0.432251097	0.3901798
9210	WD repeat domain 19 || AK052303 ||	0.258606728	0.552348667	0.3901798
12710	Nucleosome assembly protein 1-like 1 || NM_015781 ||	0.247975595	0.540771635	0.3901798
3988	DNA segment, Chr 10, Wayne State University 52, expressed || NM_145422 ||	0.190967084	0.630403667	0.3901798
9413	Rhesus blood group-associated B glycoprotein || NM_021375 ||	0.234433403	0.562817488	0.453112
2197	Serine/threonine kinase 11 || NM_011492 ||	0.191825499	0.635629461	0.453112
6318	Solute carrier family 30 (zinc transporter), member 1 || NM_009579 ||	0.217605679	0.585632971	0.453112
8388	Expressed sequence AU020206 || AK049415 ||	0.320378777	0.450211338	0.453112
9464	RIKEN cDNA 2300002M23 gene || NM_175148 ||	0.255207267	0.550897113	0.453112

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)
13133	SRY-box containing gene 13 || NM_011439 ||	0.367305797	0.374440366	0.453112
4783	RIKEN cDNA 6530406A20 gene || AK087211 ||	0.176184848	0.662503223	0.453112
11166	RIKEN cDNA 2410011G03 gene || NM_025551 ||	0.215025077	0.592572611	0.453112
2622	A kinase (PRKA) anchor protein (yotiao) 9 || NM_194462 ||	0.200561964	0.629129296	0.453112
3754	RIKEN cDNA 1700023O11 gene || NM_029339 ||	0.209548803	0.604020868	0.453112
2491	Unc-13 homolog B (C. elegans) || AK122422 ||	0.414012458	0.310842963	0.453112
1563	RIKEN cDNA 9430037G07 gene || AK034782 ||	0.36380687	0.376965848	0.453112
2793	Fibroblast growth factor 2 || AK076764 ||	0.338896419	0.411988337	0.453112
10567	Eukaryotic translation initiation factor 4 gamma, 3 || NM_172703 ||	0.201344258	0.639735015	0.453112
7572	Transmembrane protein 4 || NM_019953 ||	0.207765172	0.620548632	0.453112
7215	SUMO1/sentrin specific protease 7 || NM_025483 ||	0.208805607	0.621682923	0.453112
9470	Ubiquitin specific protease 52 || NM_133992 ||	0.208985054	0.617427079	0.453112
13015	Trypsin domain containing 1 || AK005069 ||	0.230537359	0.570735109	0.453112
5403	RIKEN cDNA 4921537D05 gene || NM_029852 ||	0.198837712	0.633459392	0.453112
10904	RIKEN cDNA 2010013E08 gene || NM_175152 ||	0.205465145	0.642951285	0.453112
6009	Eukaryotic translation elongation factor 2 || NM_007907 ||	0.24744648	0.558753618	0.453112
11147	|| NM_001039552 ||	0.19397557	0.647969375	0.453112
1970	Ankyrin repeat domain 5 || NM_019416 ||	0.279613992	0.538042582	0.453112
4358	Ts translation elongation factor, mitochondrial || NM_025537 ||	0.230149969	0.607667444	0.453112
10107	RIKEN cDNA 2310044P18 gene || NM_144829 ||	0.234119826	0.59586446	0.7696697
4589	Ribosomal protein S8 || NM_009098 ||	0.222079709	0.62846877	0.7696697
7421	|| AK160524 ||	0.218617282	0.591460891	0.7696697
8278	|| XM_619639 ||	0.311856455	0.448521929	0.7696697
6387	Ribosomal protein S26 || NM_013765 ||	0.254047422	0.576064919	0.7696697
10396	Trafficking protein particle complex 3 || NM_013718 ||	0.281869788	0.528439538	0.7696697
12697	Histidine-rich glycoprotein || NM_053176 ||	0.378901237	0.40043619	0.7696697
6602	Neutral sphingomyelinase (N-SMase) activation associated factor || NM_010945 ||	0.234775443	0.584345478	0.7696697
3160	RIKEN cDNA 2210411K19 gene || NM_018860 ||	0.239056607	0.620596304	0.7696697
5894	|| NM_029671 ||	0.474234394	0.279403251	0.7696697
8956	Nuclear receptor binding factor 1 || NM_025297 ||	0.23891955	0.579897307	0.7696697
8353	RIKEN cDNA 2410006N06 gene || NM_028019 ||	0.180910366	0.679112096	0.7696697
3587	Nudix (nucleoside diphosphate linked moiety X)-type motif 2 || NM_025539 ||	0.226419678	0.60855926	0.7696697
3558	|| NM_172952 ||	0.205831732	0.633894947	0.7696697

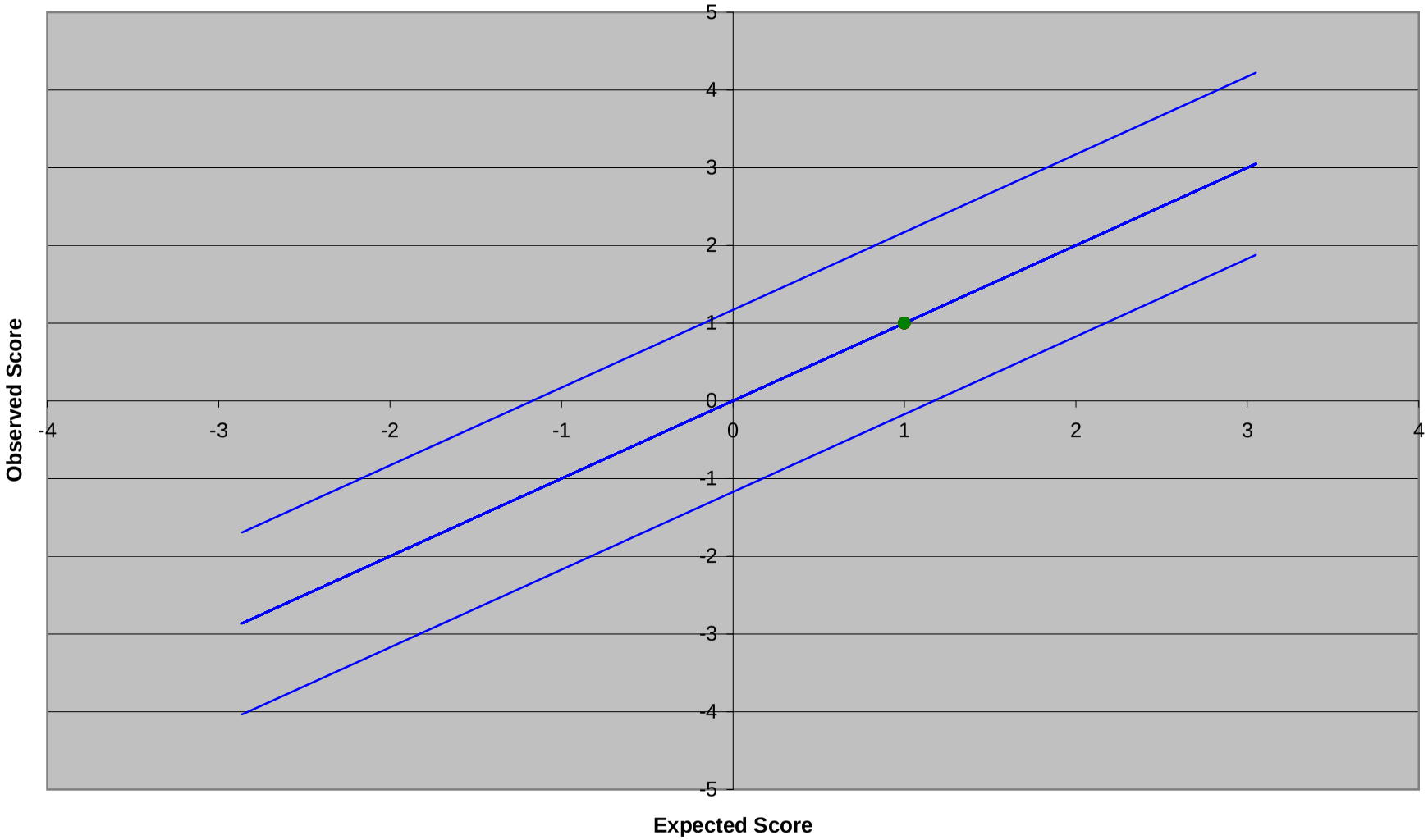
Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)
13008	Rap guanine nucleotide exchange factor (GEF) 5 || AK122234 ||	0.255723273	0.557338337	0.7696697
7306	Mitochondrial ribosomal protein S15 || NM_025544 ||	0.204060192	0.661284669	0.7696697
1656	Per-pentamer repeat gene || NM_012022 ||	0.354401873	0.431062179	0.7696697
2440	RIKEN cDNA A030012M09 gene || BC019193 ||	0.241444821	0.591312969	0.7696697
10797	RIKEN cDNA 1810074P20 gene || NM_026194 ||	0.205981199	0.657566495	0.7696697
3254	RIKEN cDNA C230071I02 gene || AK082631 ||	0.368167352	0.375313453	0.7696697
9934	|| NM_001042558 ||	0.234061498	0.583419234	0.7696697
12448	RIKEN cDNA 1700012A03 gene || NM_029587 ||	0.352072343	0.430844056	0.7696697
8912	Feline sarcoma oncogene || AK004587 ||	0.256712463	0.577270572	0.7696697
376	Single-stranded DNA binding protein 3 || NM_023672 ||	0.243584297	0.566963623	0.7696697
11463	Polymerase (DNA directed), gamma || NM_017462 ||	0.215800415	0.692606807	0.7696697
2484	Holocarboxylase synthetase (biotin- [propriony-Coenzyme A-carboxylase (ATP-hydrolysing)] ligase) || NM_139145 ||	0.205576062	0.629382259	0.7696697
11393	RIKEN cDNA 1190005P17 gene || NM_025431 ||	0.264701773	0.5409465	0.7696697
11022	RIKEN cDNA 2410166I05 gene || NM_029759 ||	0.214501521	0.626306586	0.7696697
5267	RIKEN cDNA 4933421G18 gene || AK014915 ||	0.215684745	0.63134139	0.7696697
2449	|| NM_001033190 ||	0.168883998	0.71542871	0.7696697
4159	RIKEN cDNA 4930418P06 gene || NM_029777 ||	0.531646569	0.2734699	0.7696697
9801	Adaptor-related protein complex 3, delta 1 subunit || NM_007460 ||	0.187568811	0.663156353	0.7696697
10346	RIKEN cDNA 1700008D07 gene || BC025128 ||	0.241525639	0.592241206	0.9796563
876	Expressed sequence AW538196 || NM_133952 ||	0.224001039	0.601196044	0.9796563
4980	RIKEN cDNA 2010003O18 gene || AK047064 ||	0.192160672	0.666097685	0.9796563
5290	Fas apoptotic inhibitory molecule || NM_011810 ||	0.219824836	0.592981891	0.9796563
12599	Tetratricopeptide repeat domain 10 || NM_009376 ||	0.192073924	0.68564226	0.9796563
9522	A kinase (PRKA) anchor protein 2 || NM_009649 ||	0.256779912	0.59672713	0.9796563
1444	Leucine zipper protein 1 || NM_024452 ||	0.215743778	0.627395347	0.9796563
3484	|| NM_146289 ||	0.451465423	0.297515031	0.9796563
9141	Dickkopf-like 1 || NM_015789 ||	0.503280175	0.230049333	0.9796563
5279	Mitofusin 2 || NM_133201 ||	0.257548243	0.567980048	0.9796563
3106	Tripartite motif protein 2 || NM_030706 ||	0.334529551	0.446736948	0.9796563
8187	Transmembrane protease, serine 3 || NM_080727 ||	0.33091013	0.492054447	0.9796563
6064	Exportin, tRNA (nuclear export receptor for tRNAs) || XM_125902 ||	0.285162849	0.532063622	0.9796563

Row	Gene ID	Denominator (s+s0)	Fold Change	q-value (%)
3509	Stanniocalcin 2 || NM_011491 ||	0.369995329	0.378353031	0.9796563
12934	Erythrocyte protein band 4.1-like 4a || NM_013512 ||	0.36856255	0.450960767	0.9796563
9428	Heterogeneous nuclear ribonucleoprotein A1 || NM_010447 ||	0.296011085	0.510549989	0.9796563
336	Adrenergic receptor kinase, beta 2 || NM_177078 ||	0.35967025	0.442427192	0.9796563

Significant: 742
Median number of false positives: 7.27
False Discovery Rate (%): 0.98

SAM Plotsheet

Tail strength (%): 49.2
Standard Error (%): 12.1



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