Identification of the Rassf3 Gene as a Potential Tumor Suppressor Responsible for the Resistance to Mammary Tumor Development in MMTV/neu Transgenic Mice

Isabelle Jacquemart
Clemson University, isajacquemart@hotmail.com

Follow this and additional works at: https://tigerprints.clemson.edu/all_dissertations

Part of the Microbiology Commons

Recommended Citation
https://tigerprints.clemson.edu/all_dissertations/26

This Dissertation is brought to you for free and open access by the Dissertations at TigerPrints. It has been accepted for inclusion in All Dissertations by an authorized administrator of TigerPrints. For more information, please contact kokeefe@clemson.edu.
IDENTIFICATION OF THE Rassf3 GENE AS A POTENTIAL TUMOR SUPPRESSOR RESPONSIBLE FOR THE RESISTANCE TO MAMMARY TUMOR DEVELOPMENT IN MMTV/neu TRANSGENIC MICE

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Microbiology

by
Isabelle C. Jacquemart
December 2006

Accepted by:
Dr. Wen Y. Chen, Committee Chair
Dr. Charles D. Rice
Dr. Lyndon L. Larcom
Dr. Lesly Temesvari
ABSTRACT

The MMTV/neu transgenic mouse line is a well-documented animal model for studying HER2/neu-related breast cancer. It has been reported that a small percentage, approximately 20%, of the virgin female MMTV/neu mice seems resistant to the development of mammary gland adenoma, despite the overexpression of the neu oncogene.

To identify the factors that are responsible for the tumor resistance in these MMTV/neu female transgenic mice, comparative genetic profiling was used to screen the alterations in gene expression in the mammary gland. A novel gene named the RAS Association domain (RalGDS/AF-6) Family 3 (Rassf3), which belongs to a family of RAS effectors and tumor suppressor genes was identified in this study. Data presented in this dissertation show: 1) that the Rassf3 gene is overexpressed in the mammary gland of the tumor-resistant MMTV/neu mice compared to their tumor-susceptible MMTV/neu transgenic littermates or age-matched non-transgenic FVB mice, and 2) that the Rassf3 gene is significantly up-regulated in neu-specific mouse mammary tumors compared to adjacent normal tissues.

To further confirm the role of the Rassf3 gene in mammary carcinogenesis, a series of in vitro and in vivo experiments were explored. The results show that overexpression of RASSF3 inhibits cell proliferation in HER2 positive human and mouse breast cancer cell lines. The inhibitory effect of RASSF3 seems to be through induction
of apoptosis. In addition, co-transfection of the Rassf3 gene with the activated H-RAS gene in SKBR3 human breast cancer cells decreased H-RAS protein level, suggesting that RASSF3 protein can indirectly interact with H-RAS protein. A novel MMTV/Rassf3-neu bi-transgenic mouse line, overexpressing both Rassf3 and neu genes in mammary glands, was also established. The mammary tumor incidence in virgin female bi-transgenic mice was delayed compared to their MMTV/neu +/- littermates. Together, these data suggest that Rassf3, a RAS effector, is a candidate gene that may influence the mammary tumor incidence in virgin female MMTV/neu transgenic mice.
ACKNOWLEDGEMENTS

Several individuals have played a major role in the development of this dissertation. My advisor, Dr. Wen Y. Chen, has been of great assistance and guidance throughout my four years of study at Clemson. His depth of knowledge and experience in the field of Molecular Biology has sparked many conversations and ideas that are shown through my work. I am very grateful for the role he has played in my education.

I would also like to recognize the support received from my friends and colleagues, Jang P. Park, John F. Langenhe im and Seth Tomblyn at the Molecular Biology Lab of the Oncology Research Institute in Greenville Hospital System. Special thanks go to my friends and colleagues Michele L. Scotti for her encouragement and friendship throughout my program and Alison E. Spring for her support and assistance in the preparation of this dissertation.

In addition, I would like to thank Ms. Margaret Nicholson, the Executive Director of the Fulbright Program in Belgium and Dr. Calvin L. Schoulties, the Dean of the College of Agriculture, Forestry and Life Sciences at Clemson University for their sponsor and support throughout my program at Clemson University.

Finally, I would like to thank my family. There have been countless conversations of motivation that have kept my morale up and pushed me to complete this work. Their love, understanding and encouragement are what brought me to Clemson and helped me achieved my goals during these four years of study.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>xii</td>
</tr>
</tbody>
</table>

## CHAPTER

1. INTRODUCTION .................................................................................................................. 1
   1.1. Cancer .................................................................................................................. 1
   1.2. Oncogenes, Tumor Suppressors and Stability Genes .......................................... 3
   1.3. Hallmarks of Cancer .......................................................................................... 6
   1.4. Breast Cancer ..................................................................................................... 10
   1.5. HER2/neu Oncogene .......................................................................................... 23
   1.6. RAS Oncogene .................................................................................................... 36
   1.7. The Use of Transgenic Mouse Technology in Cancer Research ............................... 47
   1.8. The Use of Gene Expression Profiling in Cancer Research .................................. 53

2. HYPOTHESIS AND OBJECTIVES ................................................................. 57

3. MATERIAL AND METHODS ....................................................................................... 58
   3.1. Animal Model ...................................................................................................... 58
   3.2. RNA Isolation .................................................................................................... 58
   3.3. Microarray Analysis .......................................................................................... 59
   3.4. Reverse Transcription-PCR (RT-PCR) ............................................................... 62
   3.5. Cloning and Plasmid Construction ................................................................... 65
Table of Contents (Continued)

3.6. RASSF3 Protein Production ...................................................... 68
3.7. Custom Antibody Design and Production ................................. 69
3.8. Cell Culture and Reagents ......................................................... 72
3.9. Protein Isolation and Western Blot Analysis ............................. 73
3.10. Transient Transfection ............................................................. 76
3.11. Cell Proliferation MTS/PMS Assay ......................................... 76
3.12. Apoptosis Assay ....................................................................... 77
3.13. Generation and Study of Transgenic Mice .............................. 78
3.15. Statistical Analyses ................................................................. 81

4. RESULTS ............................................................................................... 82

4.1. Comparison of the Mammary Gland Morphology of the MMTV/neu Transgenic Mice ................................................................. 82
4.2. Identification of Differentially Expressed Genes by cDNA Microarray Analysis ................................................................. 84
4.3. Analysis of the Differentially Expressed Candidate Genes ............................................................................................................. 90
4.5. Generation and Analysis of Novel Transgenic Mouse Lines: The MMTV/Rassf3 Transgenic Mice and MMTV/Rassf3-neu Bi-Transgenic Mice ................................................................ 114

5. DISCUSSION ......................................................................................... 125

5.1. HER2/neu Human Breast Cancer and its Mouse Models ................................................................. 125
5.2. Identification of the Genes Responsible for the Tumor Resistant Phenotype in MMTV/neu Transgenic Mice by cDNA Microarray Analysis ........................................................................... 129
5.3. Analysis of the Candidate Genes ............................................... 135
5.4. Functional Studies of the Rassf3 Gene in HER2/neu-positive Lines ................................................................. 139
5.5. Functional Study of the Rassf3 Gene in Transgenic Mice ............................................................................................................. 144
<table>
<thead>
<tr>
<th>Table of Contents (Continued)</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6. CONCLUSION ....................................................................... 149</td>
<td></td>
</tr>
<tr>
<td>BIBLIOGRAPHY ....................................................................... 152</td>
<td></td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Pairs of primers used for RT-PCR and PCR analyses with mouse (m) or human (h) tissues and cell lines and corresponding PCR annealing temperature</td>
<td>64</td>
</tr>
<tr>
<td>2. List of genes that are overexpressed in the mammary glands of tumor-resistant MMTV/neu female transgenic mice compared to tumor-susceptible MMTV/neu littermates, obtained by cDNA microarray analysis</td>
<td>87</td>
</tr>
<tr>
<td>3. List of genes that are overexpressed in the mammary glands of both female tumor-resistant MMTV/neu transgenic mice and tumor-susceptible MMTV/neu transgenic mice, compared to the mammary gland of non-transgenic mice</td>
<td>90</td>
</tr>
<tr>
<td>4. Mammary tumor recurrence and secondary mammary tumor development in female MMTV/Rassf3-neu bi-transgenic mice compared to their MMTV/neu(^{+/}) littermates</td>
<td>123</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1.</td>
<td>Schematic representation of the Receptor Tyrosine Kinase (RTK) signaling pathway</td>
</tr>
<tr>
<td>2.</td>
<td>Schematic representation of the RAS activation of multiple effector-mediated signaling pathways</td>
</tr>
<tr>
<td>3.</td>
<td>Nomenclature of the different groups of mice used in the study and flow chart of the experimental design</td>
</tr>
<tr>
<td>4.</td>
<td>Maps of the plasmids used in the study</td>
</tr>
<tr>
<td>5.</td>
<td>Elisa testing of the anti-mouse RASSF3 polyclonal antibodies and Western blot analysis of RASSF3-Nt (S-1228-1) antibody</td>
</tr>
<tr>
<td>6.</td>
<td>Images of the mammary gland whole mounts of MMTV/neu transgenic mice (R) and non-transgenic FVB mice (C)</td>
</tr>
<tr>
<td>7.</td>
<td>Scatter-plot representation of the comparative analysis of samples analyzed by microarray technology</td>
</tr>
<tr>
<td>8.</td>
<td>Confirmation of the microarray data for the four selected candidate genes by RT-PCR analysis</td>
</tr>
<tr>
<td>9.</td>
<td>Comparison of gene expression in mammary tumors and adjacent normal tissues by RT-PCR analysis</td>
</tr>
<tr>
<td>10.</td>
<td>Comparison of the gene expression patterns in the epithelial vs. fibroblast cells derived from a HER2/neu mammary tumor by RT-PCR analysis</td>
</tr>
<tr>
<td>11.</td>
<td>Comparison of the gene expression patterns of the Ras Association domain Family genes (Rassf-1, -2, -3, -4, -5) in mouse mammary tissues by RT-PCR analysis</td>
</tr>
</tbody>
</table>
List of Figures (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.</td>
<td>Correlation between HER2/p-HER2 protein and RASSF3, SOCS2 and ETV1 mRNA levels in multiple human breast cancer cell lines by Western blot and RT-PCR analyses</td>
<td>99</td>
</tr>
<tr>
<td>13.</td>
<td>RT-PCR analysis of RASSF3 expression in tumors vs. adjacent normal tissues of various human organs</td>
<td>101</td>
</tr>
<tr>
<td>14.</td>
<td>Schematic comparison of the protein domains and amino acid sequences of human RASSF1 (isoforms A and C), human RASSF3 and mouse RASSF3 proteins</td>
<td>103</td>
</tr>
<tr>
<td>15.</td>
<td>The effects of the Rassf3 gene expression on SKBR3 human breast cancer cell proliferation</td>
<td>105</td>
</tr>
<tr>
<td>16.</td>
<td>The effects of the Rassf3 gene expression on cell proliferation of various cell lines</td>
<td>107</td>
</tr>
<tr>
<td>17.</td>
<td>The effects of the Rassf3 gene expression on apoptosis in SKBR3 human breast cancer cell line</td>
<td>108</td>
</tr>
<tr>
<td>18.</td>
<td>Western blot analysis of the effect of Rassf3 gene expression on intracellular signal transduction pathways in SKBR3 human breast cancer cell line</td>
<td>110</td>
</tr>
<tr>
<td>19.</td>
<td>Western blot analysis of the interaction of RASSF3 protein with constitutively activated H-RAS in SKBR3 human breast cancer cell line</td>
<td>112</td>
</tr>
<tr>
<td>20.</td>
<td>The effects of co-transfection of Rassf3 with activated H-RAS or dominant negative H-RAS on cell proliferation in SKBR3 human breast cancer cell line</td>
<td>113</td>
</tr>
<tr>
<td>21.</td>
<td>Representative PCR analysis of the genomic DNA from MMTV/Rassf3 transgenic mice, MMTV/Rassf3-neu bi-transgenic mice and their littermates</td>
<td>115</td>
</tr>
<tr>
<td>22.</td>
<td>RT-PCR analysis of the total RNA from various tissues of MMTV/Rassf3 transgenic mice and their littermates</td>
<td>115</td>
</tr>
</tbody>
</table>
List of Figures (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>23.</td>
<td>Western blot and RT-PCR analyses of RASSF3 expression in mammary gland and brain tissues of the transgenic mice</td>
<td>117</td>
</tr>
<tr>
<td>24.</td>
<td>Images of the mammary gland whole mounts of MMTV/Rassf3 transgenic mice, FVB non-transgenic mice, MMTV/Rassf3-neu bi-transgenic mice and MMTV/neu +/- littermates</td>
<td>119</td>
</tr>
<tr>
<td>25.</td>
<td>Comparison of the mammary tumor incidence in the MMTV/Rassf3-neu bi-transgenic mice and the MMTV/neu +/- littermates</td>
<td>121</td>
</tr>
<tr>
<td>26.</td>
<td>Western blot analysis of RASSF3 expression in mammary tumors of the MMTV/Rassf3-neu bi-transgenic mice and their littermates</td>
<td>122</td>
</tr>
<tr>
<td>27.</td>
<td>Analysis of the recurrence of mammary tumors in MMTV/Rassf3-neu bi-transgenic mice after surgical removal of the primary tumor</td>
<td>124</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>AI</td>
<td>aromatase inhibitor</td>
<td></td>
</tr>
<tr>
<td>APC</td>
<td>adenoma polyposis coli</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>non-transgenic FVB mice</td>
<td></td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependant kinase</td>
<td></td>
</tr>
<tr>
<td>CMV</td>
<td>human cytomegalovirus immediate-early</td>
<td></td>
</tr>
<tr>
<td>CRD</td>
<td>cysteine-rich domain</td>
<td></td>
</tr>
<tr>
<td>DCIS</td>
<td>ductal carcinoma In Situ</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
<td></td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
<td></td>
</tr>
<tr>
<td>EGF-R</td>
<td>epidermal growth factor receptor</td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
<td></td>
</tr>
<tr>
<td>ES</td>
<td>estrogen</td>
<td></td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
<td></td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
<td></td>
</tr>
<tr>
<td>FTI</td>
<td>farnesyltransferase inhibitor</td>
<td></td>
</tr>
<tr>
<td>g</td>
<td>gravity</td>
<td></td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
<td></td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diposphate</td>
<td></td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide-exchange factor</td>
<td></td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
<td></td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
<td></td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
<td></td>
</tr>
<tr>
<td>h</td>
<td>human</td>
<td></td>
</tr>
<tr>
<td>HB-EGF</td>
<td>heparin binding-epidermal growth factor</td>
<td></td>
</tr>
<tr>
<td>HER2/ErbB2</td>
<td>human epidermal growth factor receptor-2</td>
<td></td>
</tr>
</tbody>
</table>
Abbreviations (Continued)

HRG, heregulin
IGF-1, insulin-like growth factor-1
JAK, janus kinase
JNK, jun-amino-terminal kinase
LCIS, lobular carcinoma In Situ
LOH, loss of heterozygosity
m, mouse
MAPK, mitogen-activating protein kinase
MG, mammary gland
MMTV LTR, mouse mammary tumor virus long terminal repeat
mo, month
mRNA, messenger RNA
NGF, nerve growth factor
NRG, neuregulin
NSCLC, non-small-cell lung cancers
PBS, phosphate-buffered saline
PCR, polymerase chain reaction
PDGF, platelet-derived growth factor
PI3K, phosphatidylinositol 3-kinase
PIP2, phosphatidylinositol 4,5-bisphosphate
PIP3, phosphatidylinositol 3,4,5-trisphosphate
PLC, phospholipase C
PMSF, phenylmethylsulphonyl fluoride
PR, progesterone receptor
PRL, prolactin
PTB, phosphotyrosine-binding domain
R, tumor-resistant MMTV/neu transgenic mice
RASSF, Ras Association domain (RalGDS/AF-6) Family
Rb, retinoblastoma
Abbreviations (Continued)

RBD,  RAS binding domain
R-MG,  tumor-resistant mammary gland from tumor-susceptible MMTV/neu mice
RTK,  receptor tyrosine kinase
RT-PCR,  reverse transcription-polymerase chain reaction
S,  tumor-susceptible MMTV/neu mice
SAPK,  stress-activated protein kinase
SDS-PAGE,  sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM,  standard error of the mean
SERM,  selective estrogen receptor modulators
SH,  Src homology
SOCS,  suppressor of cytokine signaling
STAT,  signal transducers and activators of transcription
TBS-T,  tris-buffer saline-Tween 20
TGF-α,  transforming growth factor alpha
TGF-β,  transforming growth factor beta
TK,  tyrosine kinase
TNF,  tumor necrosis factor
VEGF,  vascular endothelial growth factor
1. INTRODUCTION

1.1. Cancer

Cancer can be defined as a collection of diseases characterized by unregulated cell growth leading to invasion of surrounding tissues and spread to other part of the body, which are life-threatening. The process by which cancers are generated is called carcinogenesis and is a multi-step mechanism resulting from the accumulation of errors (altered DNA bases or mutations) in vital regulatory pathways. It is initiated in a single cell (clonal origin) which then multiplies and acquires additional changes that give it a survival advantage over its neighbors (Huang D. et al., 1997). The altered cells must be amplified to generate billions of cells that constitute a tumor. The age distribution of several cancers indicates that cancer risk increases with age and cancer is often defined as a disease of old age (King R., 2000). However, there are exceptions in that some cancers are characterized by onset in childhood, such as cancers of the eye and certain leukemias. In addition, certain cancers are prevalent in different countries, suggesting that environment, lifestyle and diet have an impact on cancer development. For example, breast and prostate cancers are common in Western countries while cancer of the cervix and stomach are more prevalent in Asia and notably in China (King R., 2000). Also, men and women have different cancer patterns, which indicate the major role of sex hormones. For instance, breast cancer represents approximately 32% of all cancers in women but less than 1% in men in Western countries. Some tissues are also more prone
to develop cancer than others. The four most common cancers (breast, lung, colon and prostate cancers) in both sexes are of epithelial origin; however, most cell types are susceptible to develop cancer.

Carcinogenesis is often divided into initiation and promotion stages and is followed by progression, often leading to invasion and metastasis. The initiation stage corresponds to genetic alterations caused by an initiating agent (i.e. carcinogenic chemicals) which then requires cell proliferation, triggered by the promoting agent (i.e. hormones, viral infection), to transform a single potential cancer cell into a multicellular tumor. As they progress, cancers continue to change their behavior and acquire a higher degree of autonomy, which reflects the genetic instability of the cells involved. The concept of initiation has generated an industry devoted to the identification of agents that cause it. There are some clear examples for which the initiating agents have been identified such as the lung cancer (tobacco smoke), bladder cancer and leukemia (ionizing radiation) (King R., 2000). However, in some cancers such as colon cancer, leukemia unrelated to radiation or hormone-related breast and prostate cancers, it remains difficult to define the initiating agents. Considerable data indicate that the progression from pre-malignancy to malignancy is a slow process, which is consistent with a natural selection model. Evidences suggest that cancer development follows a “multi-hit” model, implying that multiple mutations or errors in the cellular machinery are required for cancer to develop and that damaging and proliferative influences must be continuous throughout cancer progression in order to reach full metastatic potential (Huang D. et al. 1997).
Mutations in genes can occur in single somatic cells, resulting in sporadic tumors or in the germline, resulting in hereditary predispositions to cancer. The great majority of cancers are sporadic. Hereditary or familial cancers represent a minority of cancers (10% of total cancer) and tend to occur at a younger age than if generated in a somatic cell. The offspring are most often heterozygous, carrying one defective allele from the affected parent and one normal allele. The human retinoblastoma (Rb) gene was the first gene identified as a defective gene in familial retinoblastoma cancer; however, mutations of the Rb gene are also observed in sporadic cells. Similarly, mutation in the adenoma polyposis coli (APC) gene is responsible for familial colon cancer but also for some sporadic forms of colon cancer. Germline mutations in BRCA1 or BRCA2 genes are linked to familial breast cancers; however, mutations in these two genes have not been found in sporadic cases, but BRCA1 gene expression has been found to be reduced in some sporadic cancers, implying that the gene regulation and not only the gene function can influence the disease.

1.2. **Oncogenes, Tumor Suppressors and Stability Genes**

In the last decade, many important genes responsible for the development of various cancers have been discovered, their mutations precisely identified, and the pathways through which they act characterized. Three groups of genes have been identified, in which alterations can contribute to tumorigenesis: oncogenes, tumor suppressor genes and caretaker or stability genes (Vogelstein B. and Kinzler K., 2004).

Oncogenes are normal regulatory genes whose activity is increased as a consequence of genetic alteration in either their coding region or regulatory sequences. This mutation results in a gain of function. The activation of oncogenes can result from
chromosomal rearrangement (e.g. acute and chronic myelogenous leukemia), gene amplification (e.g. \textit{ERBB2}/\textit{Her2}) or from intragenic mutation affecting crucial residues that regulate the activity of the gene product (e.g. \textit{B-RAF}). An activating mutation in one allele of an oncogene is generally sufficient to confer a selective growth advantage to the cell.

Tumor suppressor genes encode for inhibitory proteins whose function is to inhibit cell functions by complexing with other effector proteins and blocking their actions. A mutation in a tumor suppressor gene results in a loss of function. Such inactivation can arise from misense mutations at residues that are essential for its activity, from mutations that result in a truncated protein, from deletions or insertions of various sizes, or from epigenetic silencing. The mutation acts in a recessive manner, meaning that a mutation in both alleles is required to block the gene function. For tumor suppressor-related cancers, individuals usually inherit one mutant copy of the tumor suppressor gene and the second mutation occurs sometime during life in the target cell. However, recent data have shown that for some tumor suppressor genes (e.g. \textit{p53}), mutation and inactivation of one allele is sufficient to block the full gene function (haploinsufficiency) (Largaespada D., 2001; Paige A., 2003). Epigenetic silencing results in a decrease in gene expression through modifications that doesn’t affect the DNA sequence. Silencing can be achieved by different mechanisms including DNA methylation, histone acetylation or deacetylation, histone methylation and poly(ADP-ribosylation) (Tycko B., 2000; Eberharter A. and Becker P., 2002). It has been demonstrated that numerous tumor-suppressor genes (e.g. \textit{Rb}, \textit{APC}, \textit{BRCA1}, and \textit{RASSF1A}) are silenced by DNA methylation in different types of cancers. DNA
methylation of regions rich in cytosine-guanine doublets, called CpG (phosphodiester-linked cytosine and guanine pairs) islands, in the promoter region of a gene is a common mechanism of epigenetic silencing. Cancer cells often display aberrant patterns of DNA methylation, with hypomethylation throughout most of the genome and site-specific hypermethylation in CpG islands. It has been proposed that promoter hypermethylation is a frequent pathway alternative to loss of heterozygosity (LOH) for the somatic “second hit” in tumorigenesis, since, in many cases, the heterozygote tumors have lost the expression of the non-mutated allele via promoter hypermethylation.

Thus, the mutations in oncogenes and tumor suppressor genes operate in a similar way at the physiologic level by conferring a selective growth advantage to the cell by stimulating cell proliferation or inhibiting cell death or cell-cycle arrest.

In comparison, caretaker or stability genes promote tumorigenesis in a completely different way when mutated or epigenetically silenced. This group includes the mismatch repair (MMR), the nucleotide-excision repair (NER) and the base-excision repair (BER) genes which encode for enzymes that are responsible for sensing and repairing subtle mistakes in DNA made during normal DNA replication or induced by exposure to mutagens. It also includes other stability genes which control processes involving large portions of chromosomes, such as those responsible for mitotic recombination and chromosomal segregation. The role of these stability genes is to correct genetic alterations to preserve the genetic integrity. Thus, when these genes are inactivated, mutations in other genes such as oncogenes and tumor suppressor genes will occur at a higher rate. A common example is the Lynch syndrome (also called hereditary
non-polyposis colorectal cancer, HNPCC), which results from the germline mutation in two mismatch repair genes (\textit{MSH2} and \textit{MLH1}) (Viel A. \textit{et al.}, 1997).

1.3. \textbf{Hallmarks of Cancer}

There are multiple pathways by which cells can acquire biological capabilities that promote the development of cancer. These capabilities are defined as the hallmarks of cancer (Hanahan D. and Weinberg R., 2000). The first hallmark consists of the self-sufficiency in growth signals of cancer cells. Indeed, tumor cells have a greatly reduced dependence on exogenous growth stimulation. This autonomy can be achieved by three molecular strategies. First, they can show altered extracellular growth signals. Some cells can synthesize many of their own growth signals to which they are responsive (autocrine stimulation), therefore reducing their dependence on stimulation from their normal tissue microenvironment. Second, tumor cells can show altered trans-cellular transducer of those signals, such as deregulated growth factor receptors on their surface. Many growth factor receptors (e.g. EGFR and HER2) are found to be overexpressed in many cancers, which render cells hyper-responsive to ambient levels of growth factors. Third, they can have altered intracellular signaling pathway that translate those growth signals into action. For instance, the SOS-RAS-RAF-MAPK cascade has been shown to be structurally altered in about 25% of cancers and to enable tumor cells to release a flux of mitogenic signals into the cells (Medema R. and Bos J., 1993).

The insensitivity of tumor cells to antigrowth signals constitutes the second hallmark. These anti-growth signals include soluble growth inhibitors and immobilized inhibitors embedded in the extracellular matrix and on the surface of nearby cells. They can stop cell proliferation by either blocking cells into the quiescent state (G0 phase) of
the cell cycle, or inducing cells to enter into post-mitotic states associated with acquisition of specific differentiation traits. The anti-proliferative signals (i.e. TGFβ) are almost all funneled through the retinoblastoma protein (pRb) and have been shown to be disrupted in a majority of human cancers.

The third hallmark is the evasion of apoptosis or programmed cell death. This evasion occurs by alteration in sensors and effectors of the apoptotic machinery. The sensors include cell surface receptors that bind survival signals (e.g. IGF1/2, IL-3) or death signals (FAS ligand, TNFα) whose signaling will consequently regulate the effectors of apoptotic death. Abrogation of pro-apoptotic effectors (e.g., Bax, Bid, Bak and p53) can provide cells with resistance to apoptosis. The tumor suppressor p53 is the most common pro-apoptotic regulator found to be abrogated in tumors. This has important implications since signals evoked by DNA damage, hypoxia and oncogene expression are all funneled via p53 to the apoptotic machinery (Levine A., 1997). As a consequence, inactivation of p53 is one crucial way to allow cells to evade apoptosis.

Tumor cells appear to be immortalized and to have acquired a limitless replicative potential during tumor progression. This aspect is referred to as the fourth hallmark. Evidences suggest that telomerase enzymes play a role in cell immortalization and their expression is up-regulated in 85-90% of all tumors (Harley C. and Kim N., 1996; Shay J. and Bacchetti S., 1997). In fact, their activity allows maintaining telomeres at a length above a critical threshold which in turn permits unlimited multiplication of descendant cells. Conversely, shortened telomeres activate cell senescence.

A prerequisite to the rapid clonal expansion associated with the formation of macroscopic tumors, is the supply of nutrients by the generation of new blood vessels or
angiogenesis. It is documented that tumor cells acquire the ability to induce and sustain the growth of new blood vessels during tumor development via an “angiogenic switch” from vasculature quiescence (Folkman J., 2002). This characteristic is referred as the fifth hallmark. The induction of angiogenesis is an early to mid-stage event in many human cancers and it is realized by a change in the balance of angiogenesis inducers (e.g. VEGF, FGF) and countervailing inhibitors (e.g. Thrombospondin-1, β-interferon) through a change in gene expression. In addition, this switch seems to be also controlled by integrin signaling, cell-cell adhesion and extracellular proteases which demonstrate a complex homeostatic regulation of normal tissue angiogenesis and vascular integrity, and to differ at the molecular mechanism level depending on the tumor cell type.

The sixth hallmark is defined by the capability of cancer cells to escape the primary tumor mass, invade adjacent tissues and colonize new terrain in the body to form distant settlements of tumor cells, called metastases. This process involves changes in the physical interactions of cells to their microenvironment and activation of extracellular proteases. The function of cell-cell adhesion molecules such as E-cadherin has been found to be lost in most epithelial cancers (Christofori G. and Semb H., 1999). E-cadherin acts as a suppressor of invasion by epithelial cells and its functional elimination represents a key step in the acquisition of this capability. Cell-matrix interactions also play an important role in invasiveness. Indeed, successful colonization of new sites by tumor cells has been shown to be possible through a shift in integrin expression (Varner J. and Cheresh D., 1996). Matrix-degrading proteases genes are also up-regulated and proteases inhibitors genes are down-regulated in tumors, which facilitate invasion of cancer cells into the stroma, across the blood vessel walls and through normal epithelial
layers. Of these different acquired abilities, invasion and metastasis of cancer cells are the major causes of cancer-related deaths and yet, the least well understood at the molecular level.

The six previous capabilities are directly or indirectly acquired through mutations in the genome of cancer cells. This increased mutability implies a malfunction of specific components of the genomic “caretaker” system. It has been found that the caretaker genes are often lost in different cancers, resulting in genomic instability. This phenomenon is referred as the seventh hallmark of cancer.

In addition, some tumor cells seem to have acquired the capacity to initiate new, full-fledged tumors. These cells differ from the other tumor cells in that their behavior is similar to that of normal stem cells, which have the unique ability to differentiate into any number of cell types, and, importantly, possess an unlimited capacity for self-renewal. These rare cancer-initiating cells are named cancer stem cells. They can originate either from mutation of normal stem cells or from differentiated cells or restricted progenitors that acquired stem cells characteristics (Reya T. et al., 2001). The acquisition of stem cell characteristics by tumor cells can be considered as the eighth hallmark of cancer.

Finally, tumor cells have an altered metabolism, referred to as the ninth hallmark, compared to normal cells. They live in an oxygen-poor (hypoxic) and highly acidic environment. They have adapted to this environment and they survive by obtaining energy through a much less efficient anaerobic glycolysis which requires much higher levels of glucose. This altered metabolism is due to a change in gene expression profile of genes involved in metabolism. It is characterized by a higher rate of glycolysis, an increased rate of glucose transport, an increased gluconeogenesis and lactic acid
production, a reduced fatty acid oxidation and a modified amino acid metabolism. Mitochondria which play a role in the oxidative metabolism are involved either directly or indirectly in many aspects of the altered metabolism, and demonstrate several notables differences at the genetic, molecular and biochemical levels in normal and in cancer cells (Warburg O., 1956; Zanssen S. and Schon E., 2005).

1.4. Breast Cancer

1.4.1. Incidence and Risk Factors

Breast cancer is the most common form of cancer in females, affecting approximately one out of eight women who reach age ninety in the United States (http://www.cancer.gov/cancertopics/factsheet/Detection/probability-breast-cancer). It is, after lung cancer, the second most fatal cancer in women. On the other hand, the lifetime risk for men to get breast cancer is about one hundred time less than for women. There are marked geographical differences in breast cancer incidence which reflect the difference in cancer causes. The rate of increase of breast cancer incidence slowed in the 1990s even though, at the same time, the incidence of ductal in situ breast lesions increased. The mortality rate also significantly decreased in the early 1990s, probably due to early diagnosis and continuing new therapeutic approaches. Nevertheless, the high death rate of women with invasive form of breast cancer remains a sobering fact and indicates the need to understand this disease in greater depth and to develop new interventions, both preventive and therapeutic.

Epidemiological observations have demonstrated that the risk factors associated with breast cancer are the age of first pregnancy (lower risk if under 30), total number of pregnancies, early menarche, late menopause, family history or genetics, breast feeding
(lower risk in premenopausal women) and diet (reviewed by Kelsey J. et al., 1993; reviewed by Medina D., 2005). These risk factors imply a strong association between breast cancer risk and hormone exposure during a lifetime. It has been estimated that breast cancer risk is reduced 10-20% for each year of delay in the onset of menarche and it has been shown that the high calorie Western-style diet is responsible for the predisposition of children to an earlier puberty. The early age of menarche translates into earlier hormone exposure (estrogen and progesterone) and breast epithelial cell growth. On the other hand, early age of first full-term pregnancy is a strong protective factor, especially observed in postmenopausal women, corresponding to the peak of incidence (reviewed by Russo J. et al., 2005b). Studies have estimated that every year that first birth childbearing is delayed results in a 3.5% increase in lifetime breast cancer risk. In addition, epidemiological literature on the history of breast-feeding in humans shows that prolonged breast-feeding is additionally protective against breast cancer among premenopausal women (Perez-Escamilla R. and Guerrero M., 2004).

These observations led to a rush to further understand the molecular basis for hormone-mediated breast cancer protection or protective effect of pregnancy and led to the establishment of two different experimental systems that demonstrate parity/hormone-induced protection. The first model, the pre-treatment model is characterized by hormonal stimulation followed by carcinogen administration, whereas the second model is characterized by carcinogen assault followed by hormone treatment for a specified time period (Medina D., 2005). Numerous studies performed in rats or mice showed that pregnancy had a preventive effect since it reduced mammary
carcinogenesis incidence by more than 75% and prolong the latency in a pre-treatment model (Russo I. et al., 1991; Thordarson G. et al., 1995; Medina D. and Smith G., 1999).

During pregnancy, there is a dramatic increase in the level of several circulating hormones such as estrogens, progesterone, prolactin, growth hormone and placental lactogens, which cause proliferation, development and differentiation of the mammary gland in preparation of lactation. At the end of pregnancy, the mammary gland under the influence of lactogenic hormones becomes fully lactational. After weaning of the offspring, the highly differentiated lobuloalveolar structures subsequently involute as a result of the decrease in lactogenic hormones. It has been shown that hormone treatments to mimic pregnancy using either estrogen and progesterone or chorionic gonadotropin are also effective in reducing mammary tumor incidence in rodents (Russo I. et al., 1991; Guzman R. et al., 1999; Sivaraman L. et al., 1998). In the case of estrogen and progesterone, the use of single hormone treatment showed that both hormones are necessary for the protective effect in a pre-treatment model whereas, single hormone treatment could be useful in post-treatment models (Rajkumar L. et al., 2004). Prolactin (PRL) is another hormone intimately involved in the regulation of normal breast growth, development and differentiation. The study of its role in human breast cancer has been controversial and some evidences show a positive association between prolactin and breast cancer risk in post-menopausal women (Kelly P. et al., 2002; Tworoger S. and Hankinson S., 2006).

It is believed that hormone stimulation of mammary gland development can induce sufficient changes in cell type and/or regulatory pathways to create a significantly
resistant cellular phenotype, which persists in cells and progeny (Medina D., 2005). Russo J. et al. (2005b) hypothesized that the mechanisms of protection was due to the differentiation of the mammary gland, induced by the hormonal milieu of pregnancy, which resulted in the permanent removal of a population of cancer-susceptible cells. The search for the identification of the type, origin and localization in mammary gland structures of those cancer-susceptible cells have led to the hypothesis of breast cancer stem cells (Kordon E. and Smith G., 1998; Russo J. et al., 2005a; Russo J. et al., 2005b). The same group showed experimentally that pregnancy induces the expression of a specific genomic signature in the breast and that the same signature was also induced by treatment with chorionic gonadotropin (Russo J. et al., 2005a). In addition, Thordarson G. et al. (1995) emphasized that the resistance is caused by persistent changes in the mammary gland hormonal environment since decreased levels of circulating PRL, growth hormone (GH), estrogen receptor (ER) and epidermal growth factor receptor (EGF-R) were detected in parous rats compared to age-matched virgin rats. Also, Schedin P. et al. (2004) reported that the reproductive state does persistently alter the composition and function of the mammary stroma extracellular matrix.

Another hypothesis was introduced, the “cell fate hypothesis”, suggesting that each time a woman undergoes a pregnancy, a portion of the undifferentiated stem-cell-like cells are induced to differentiate into cells that are no longer susceptible to neoplastic transformation. While both pregnancy and menstrual cycle contribute to the risk of transforming the undifferentiated cells to a neoplastic state, the probability of this occurring decreases with each pregnancy because of the progressive depletion of the pool of undifferentiated susceptible cells. Thus, in the absence of pregnancy, the size of the
population of undifferentiated cells, which are susceptible to transformation, remains undiminished, and the cumulative risk of breast cancer increases steadily until menopause (Russo J. et al., 2005a; Russo J. et al., 2005b; Medina D., 2005; Weinberg R., 2006).

This discovery that differentiation may be a powerful inhibitor of cancer initiation has some major consequences in prevention of human breast cancer because it provides the basis for the strategy using endogenous hormones. However, some major challenges still remains such as the translation of the basic concepts developed in animal models to the human population and identifying the optimal age-window at which young women are the most sensitive to hormone-mediated prevention to cancer.

1.4.2. Subtypes

Due to its numerous causes, breast cancer is considered as a collection of complex, heterogeneous diseases. Epidemiological evidences suggest three possible groups of genetic, endocrine and exogenous factors responsible for breast cancer development. Many efforts have been made to distinctly classify breast tumors. The multiple changes in gene structure and gene function identified in breast tumors have been used to classify breast cancers into either inherited or sporadic forms of cancer. Inherited gene changes in germ cells have been reported in three tumor suppressor genes, the BRCA1, BRCA2 and p53 (Li-Fraumni Syndrome) genes and are responsible for the rare familial forms of breast cancer which represent approximately 5% of all breast cancer cases. Sporadic forms of breast cancer, resulting from changes in somatic cells, have been classified based on the presence of oncogene (HER2/neu) and on hormone receptor status (estrogen receptor (ER), progesterone receptor (PR)). Numerous studies
have shown an intriguing interrelationship between ER and HER2, and, to a lesser extent, PR.

Comprehensive gene expression profiles, obtained using DNA microarrays and hierarchical clustering, have revealed new ways to classify this heterogeneous disease into subtypes of breast cancer. The different subtypes identified are the basal-like (ER negative, PR negative and HER2 negative), the HER2 positive, luminal A (ER positive) and luminal B (ER positive) types of breast cancer (Sorlie T. et al., 2001). It has been shown that these subtypes are characterized by specific biological behaviors and that they show different relationships with age, pregnancy, genetic history, postmenopausal hormone use and body mass index after menopause. The ER negative breast cancer type represents 30-40% of all breast cancer in U.S. women. The expression of these markers (ER, PR and HER2) and the following categorization of breast tumors are of clinical significance as they directly influence therapeutic choices. Unfortunately, the etiology and growth-promoting pathways of these cancers are not fully elucidated, impeding development of new successful therapies.

The histology of the breast of a normally cycling woman is classically composed of 15 to 20 lobes composed of smaller structures called lobules linked by ducts. The lobular structure is generally classified into three identifiable types of lobules: the undifferentiated type (Lobule 1), the intermediate or more developed type (Lobule 2) and the differentiated type (Lobule 3) (Russo J. et al., 2005a). The histopathological pattern is also used to classify breast cancers into different subtypes. The subtypes are: Ductal Carcinoma In Situ (DCIS), Lobular Carcinoma In Situ (LCIS), invasive ductal carcinoma, invasive lobular carcinoma, inflammatory breast cancer and Paget’s disease.
Approximately 80% of all breast cancers are of ductal origin and only 10% are of lobular epithelium origin. DCIS, which is confined to the ducts, corresponds to the most rapidly growing subgroup of breast cancer. The invasive ductal cancer is the most common of all breast cancers and makes up about 70 to 80 percent of all newly diagnosed cases. The invasive lobular carcinoma is a form of breast cancer which occurs at the ends of the ducts or in the lobules and accounts for 5 to 10% of cases. The invasive Paget's disease is a rare form (only 1%) which develops in the ducts beneath the nipple. The inflammatory type is an especially aggressive type of breast cancer (1-3% of all cases) that occurs in women of any age. It is unique because it often does not present a lump and therefore is often not detected by mammography or ultrasound. It presents the signs and symptoms of infection of the breast skin which is swollen due to blockade of lymph vessels by cancer.

### 1.4.3. Therapeutic Strategies

The choice of therapy to treat breast cancer depends on a number of factors such as the stage and type of breast cancer, the estrogen receptor and progesterone receptor levels in the tumor tissues, the women’s age and menopausal status and whether it is primary or recurrent. The prognosis is also determined by these factors. Four types of standard treatments used today are surgery, radiation therapy, chemotherapy and hormone therapy. Most of the patients with breast cancer have surgery to remove the cancer from the breast. Often patients who underwent surgery will receive another type of treatment, termed adjuvant therapy, following surgery to try to prolong survival.

Radiation therapy is a cancer treatment that uses high-energy X-rays or other types of radiation to kill cancer cells. There are two types of radiation therapy: external radiation therapy which uses an apparatus outside the body to send radiation towards the
cancer, and the internal one which uses a radioactive substance sealed in needles, seeds, wires or catheters that are placed directly into or near the cancer.

Chemotherapy is a treatment that uses non-specific cytotoxic drugs to stop the growth of cancer cells, either by killing cells or blocking cell division. It is distinguished into a systemic chemotherapy in which the drugs taken by the mouth or injected into a vein or muscle enter the bloodstream and reach cancer cells throughout the body, and a regional chemotherapy in which the drugs are placed directly into the spinal column, an organ or a body cavity, so that the drugs mainly affect cancer cells in those areas. Among the most common chemotherapy drugs used in breast cancer, there are the anti-metabolites 5-fluorouracil and methotrexane, the anti-neoplastic antibiotic doxorubicin, the alkylating agent cyclophosphamid and the mitotic inhibitor paclitaxel. Even though a response of breast tumors is common with the use of chemotherapy drugs, a cure is not common. This is due in part to a lack of selectivity of the chemotherapy drugs, attacking cancer cells as well as normal cells, causing considerable negative side effects and limiting the tolerated doses. So far, chemotherapy has shown a better response on estrogen receptor (ER) negative breast cancers than on ER positive breast cancers.

Currently, the rapid expansion in the understanding of the molecular basis of breast cancer biology provides potential targets for novel therapies and allows to improve specificity by targeting drugs to cancer cells and key drivers of the disease. For example, hormone therapy, which aims at removing hormones or at blocking their action in order to stop hormone-responsive cancer cells from growing, is the first and most successful example of targeted therapy. Hormone or endocrine therapy is the first-line therapy for
patients with estrogen receptor (ER) positive or progesterone receptor (PR) positive metastatic breast cancer. The use of pure ER antagonists and novel selective ER modulators (SERMS) (tamoxifen and aromatase inhibitors) which target the estrogen receptor, are already available or currently in clinical trials and have shown more promise in ER positive breast cancer patients (Hussain S. et al., 2004).

Tamoxifen is the standard endocrine therapy for hormone receptor-positive breast cancer and is one of the most widely used drugs for the treatment of early stages and advanced breast cancer in both pre- and post-menopausal women. Tamoxifen competes for the binding of estrogen to the receptor, resulting in inhibition of hormone action. However, the partial estrogenic properties of tamoxifen limit its use and effectiveness because it increases the risk of endometrial cancer and stroke. In addition, the development of resistance to tamoxifen in many patients is another key limitation in the treatment of advanced breast cancer.

Fulvestrant, which is an ER antagonist that down-regulates the receptor without agonistic effect, is the new type of endocrine treatment (Howell A. et al., 2000). It was shown to be effective in inhibiting the growth of tumors resistant to tamoxifen and to increase treatment efficacy in postmenopausal women with advanced breast cancer (Morris C. and Wakeling A., 2002). Therefore, it may be integrated into the therapeutic sequences prior to, or subsequent to other hormonal therapy. This type of treatment is described as successive drug therapies.

The use of aromatase inhibitors (AIs) has also been investigated as an alternative to tamoxifen treatment. AIs block the synthesis of estrogen by blocking the aromatase enzyme that catalyses the conversion from androgen to estrogen, and have no estrogenic
activity. AIs (Formestane, anastrozole, and letrozole) have been shown to cause a decrease in the amount of circulating estrogen in postmenopausal women. The efficacy of AIs in the treatment of hormone receptor-positive breast cancer has already been demonstrated in various trials enrolling postmenopausal patients with metastatic breast cancer who had failed tamoxifen treatment (Smith I., 2004). These observations lead to an important change in the treatment of advanced breast cancer corresponding to a shift from adjuvant tamoxifen to adjuvant aromatase inhibitors in post-menopausal women. Unfortunately, in premenopausal women, the use of AIs does not translate to a reduction in circulating estrogen because the cycling ovary is negatively regulated by estrogen. Therefore, a decrease in circulating estrogen up-regulates hormone-dependent stimulation of estrogen synthesis (Hussain S. et al., 2004). Although, AIs have presented a major breakthrough for post-menopausal women with ER positive advanced breast cancer, the endocrine options for premenopausal women remain limited.

Another type of cancer treatment is the use of monoclonal antibodies as adjuvant therapy. Antibodies can be directed against accessible extracellular domain of molecules on cancer cells (receptor) or normal substances (ligand) that promote cancer cells grow. Blocking the action of these molecules by antibody binding can lead to cell growth inhibition, cell death or prevention of cancer cell invasion and metastasis. Monoclonal antibodies are given by infusion and may be used alone or to carry drugs, toxins or radioactive material directly to cancer cells.

A large number of monoclonal antibodies have been developed to target the EGF receptor for cancer therapy (Gill G. et al., 1984; Mendelson J., 2000; Ciardiello F. and Tortora G., 2001). Generally, the antibodies are directed to the extracellular domain of
the EGF receptor (EGFR) and bind with high affinity, thereby blocking the activation of the receptor intracellular tyrosine kinase by EGF or transforming growth factor alpha (TGF-α). For instance, the IMC-225, a chimeric human-mouse monoclonal IgG1 antibody, has been the first anti-EGFR targeted therapy to enter clinical evaluation in cancer patients in Phase II and III studies. Another example of monoclonal antibody is trastuzumab (Herceptin), which blocks specifically the effects of the growth factor receptor, HER2, overexpressed on the surface of breast cancer cells in some human breast cancers (Debrin J. et al., 1986; Hudziak R. et al., 1989; Harries M. and Smith I., 2002). The vascular endothelial growth factor (VEGF) has also emerged as a key target for the treatment of cancer. A humanized monoclonal antibody to VEGF, bevacizumab (Avastin), which has been used in Phase II clinical trials in patients with refractory metastatic breast cancer and has produced encouraging results leading to a Phase III trial (Rugo H., 2004).

The development of drug-resistant cells to endocrine therapy remains a major limitation for the treatment of advanced metastatic breast cancer. Until recently, the only option following the development of resistance to an endocrine agent was to change the endocrine drug (sequential manner), and upon exhaustion of endocrine sensitivity, to move to chemotherapy. Today, increased understanding of the molecular mechanisms underlying the development of resistance is now emerging and new strategies to overcome endocrine resistance are in development. In addition, secondary targets in critical pathways are identified to allow the elaboration of new drugs that can substitute for the primary treatment in order to deal with resistance. Evidence have shown that there are some cross-talks between the growth factor receptors (e.g., EGFR, HER2 and
insulin-like growth factor (IGF-1)) and ER signaling, which is responsible for endocrine therapy resistance phenomenon (Hussain S. et al., 2004; Osborne C. et al., 2005). When breast cancers become resistant to endocrine therapy, growth signals, which work through growth factor receptors and downstream kinases, become dominant pathways. This discovery provided the basis for the identification of new therapeutic targets and led to the development of novel drugs, the tyrosine kinase (TK) inhibitors (Levitzki A., 1992; Ciardiello F. and Tortora G., 2001). These inhibitors target the large family of kinases and are competitors with ATP for binding to the intracellular catalytic domain of the tyrosine kinase. To date, three Phase II clinical trials have examined the use of gefitinib, erlotinib or iressa, potent specific inhibitors of EGFR TK, in patients with advanced breast cancer (Hussain S. et al., 2004). Several other clinical trials are underway to investigate the role of combined endocrine therapy plus EGFR TK inhibition for patients with advanced breast cancer and also to examine gefitinib in combination with trastuzumab in patients with HER2-positive hormone-refractory breast cancer (Arteaga C. et al., 2002).

The farnesyltransferase inhibitors (FTIs) are a different type of drugs developed to target the RAS signaling pathway by blocking RAS activation through farnesylation. Some FTI are currently used in Phase II trial in combination with the anti-estrogen letrozole in patients with ER positive, tamoxifen-resistant breast cancer (Johnston S. and Kelland L., 2001).

Another approach to cancer treatment under development is the use of gene therapy (Hussain S. et al., 2004). This therapy is reliant upon an efficient vector system
to deliver the therapeutic effect specifically to tumor cells. One of its aspects corresponds to genetic ablation to correct the molecular lesion contributing to carcinogenesis. For example, antisense oligonucleotides can be used to target and sequester an oncogene at the transcriptional level (mRNA), blocking its activity. For breast cancer, several potential targets have been investigated, notably the HER2 receptor protein and the transforming growth factor (TGF) alpha (Kenney N. et al., 1993; Bertram J. et al, 1994; Roh H. et al., 2000; Tanabe K. et al. 2002). Other studies have used dominant-negative proteins to target HER2 and EGFR TKs at the post-transcriptional level. These dominant-negative proteins can heterodimerize with wild type receptor to reduce its affinity for ligand and thereby inhibiting signaling.

Immunotherapy for metastatic breast cancer treatment is under investigation. The aim of immunotherapy is to activate a systemic and tumor-specific immune response, which may be either cell mediated or antibody dependent. Immune responses to HER2 have been detected in some patients with advanced breast cancer, which suggest that HER2 is a potential target antigen for vaccine therapy. Preclinical studies have been performed using dendritic cells transduced with the \textit{HER2} gene as a vaccine. Subcutaneous injection of the vaccine into mice was able to induce protective immunity against subsequent challenge with HER2-positive breast cancer cells. These findings indicate a potential role for dendritic cell-based vaccination for advanced breast cancer and also for treatment in the adjuvant setting (Chen Y. et al., 2001; Sakai Y. et al., 2004).

These examples emphasize the advantage to develop combination therapies, which consist of using multiple drugs that target more than one trait of cancer cells and which interfere with signal transduction, gene function, angiogenesis or cytokine action.
at the same time. In some cases, a greater effect was achieved by giving a combination of two or more drugs at the same time.

1.5. **HER2/neu Oncogene**

1.5.1. **Receptor Tyrosine Kinase Signaling Pathways**

The tyrosine kinase receptor (RTK) family is constituted of four receptors: HER1/ErbB1 also known as EGFR, HER2/ErbB2, HER3/ErbB3 and HER4/ErbB4. These receptors are involved in regulation of a number of different cellular processes, including mitogenesis, tumorigenesis and differentiation. It has been recognized that ErbB receptors cooperate in *in vitro* cellular transformation and that alterations in their activation and expression have been implicated in numerous human malignancies, including breast cancer, lung cancer and glioblastoma.

The four receptors share an overall structure consisting of a ligand-binding extracellular domain, a single hydrophobic transmembrane α-helix and an intracellular domain composed of juxtamembrane region, a tyrosine kinase domain and a carboxyl tail harboring autophosphorylation sites (Lodish H. *et al.*, 2001; Bazley L. and Gullick W., 2005). ErbB3 is an exception because it doesn’t possess any tyrosine kinase domain. The receptors are activated by a variety of soluble or membrane-bound protein ligands, including epidermal growth factor (EGF), HB-EGF (heparin binding), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factor-α (TGF-α), nerve growth factor (NGF), beta-cellulin, amphiregulin, epiregulin, epigen, insulin and the neuregulins (NRGs) 1–4 (Olayioye M. *et al.* 2000). A significant proportion of these are initially expressed as membrane-anchored proteins that require proteolytic cleavage either to achieve activity in solution or bind to cell surface proteoglycans from
where they can act as a ligand-reservoir available for receptor binding. These ErbB-ligands are structurally divergent for the most part with the exception that they all encode an extracellular EGF-like domain, which confers ligand-binding capacity. ErbB1 has been shown to bind and become activated by different members of the EGF family of ligands, including EGF, TGF-α, epiregulin, amphiregulin and beta-cellulin. ErbB3 functions as a receptor for neuregulin 1 (also known as heregulin (HRG)) and neuregulin 2. ErbB4 serves as a receptor for HRG 1, 2, 3 and 4, as well as for beta-cellulin and epiregulin (Belsches-Jablonski A. et al., 2001). ErbB2 is the only one for which no known ligand has been identified. Such diversity of interaction between ligands and their receptors dictates the strength of the signaling kinetics and consequently the magnitude and specificity of the biological activity.

These receptors can homodimerize or heterodimerize with each other forming 10 different combinations: four homodimers and six heterodimers. ErbB2 activation is most likely occurring through homodimerization or heterodimerization with ligand-activated ErbB1, ErbB3 or ErbB4. The ErbB2/ErbB3 heterodimer is preferred and has been shown to be the most tumorigenic of the possible combinations. The binding of a ligand can stimulate receptor intrinsic protein kinase activity, which subsequently stimulates a signal-transduction cascade leading to changes in cellular physiology and/or patterns of gene expression, triggering a specific biological response (Figure 1). Upon dimerization, the protein kinase of each receptor monomer phosphorylates a distinct set of tyrosine residues in the intracellular domain of its dimer partner, a process termed autophosphorylation (Lodish H. et al., 2001). The receptor tyrosine kinase then phosphorylates other sites in the cytosolic domain. The resulting phosphotyrosines serve
as docking sites for other proteins that contain conserved Src homology (SH) 2 domain or phophotyrosine-binding domain (PTB). It has been reported that the pattern and the sequence context of phosphorylated tyrosines on the receptor will determine the type of SH2 or PTB domain-containing proteins that are recruited to the receptor and consequently, the downstream pathway activated (Olayioye M., 2001a). Furthermore, the phosphorylation pattern on a given receptor is modulated by the dimerization partner. These SH2 and PTB domain-containing proteins include the adaptor proteins such as GRB2, GRB7, SHC, GAB1, the lipid kinases such as Src and phosphatidylinositol 3-kinase (PI3K), phospholipase C and the protein phosphatases such as SHP1 and SHP2 (Lodish H. et al., 2001). They serve to transmit the signal received at the receptor to either the cytoplasm or the nucleus.
Figure 1. Schematic representation of the Receptor Tyrosine Kinase (RTK) signaling pathway. In response to stimuli, the RTK forms homo- or hetero-dimers with other family members. Dimerization results in the activation of the phosphotyrosine kinase followed by autophosphorylation of the receptors cytosolic domain. The resulting phosphotyrosine residues serve as docking site for various adaptor proteins (indicated as green circles) which can initiate distinct downstream signaling pathways. The Grb2 adaptor protein coupled to Sos guanine nucleotide exchange factor can bind directly to phosphotyrosine sites in the receptor. These protein interactions bring Sos in close proximity to RAS, allowing for RAS activation, which subsequently activates the RAF-MAPK and JNK signaling pathways. The activation of these pathways further activates various transcription factors (indicated as white circles) that control gene expression and contribute to cell proliferation and differentiation. The PI3K can also be recruited to and activated by RTKs, leading to consequent activation of AKT which sends survival signals and block pro-apoptotic effectors. Other adaptor proteins such as the phospholipase C (PLC), the Src tyrosine kinase and the Janus kinase (Jak2) can be recruited to and activated by RTKs to initiate various downstream cascades which activate gene transcription.
For example, the GRB2 adaptor protein has one SH2 and two SH3 domains which can bind to the phosphotyrosine residues on the activated receptor and then to the Sos cytosolic protein through the SH3 domains. SOS is activated by binding to GRB2 and functions as a guanine nucleotide-exchange factor (GEF) which can participate in the conversion of inactive GDP-bound RAS to the active GTP-bound form.

The type and amplitude of activated downstream signaling cascades depend on the type and the number of receptors expressed in a particular cell and on the type and amount of each ligand that stimulates the cells. Two major downstream signaling pathways of the ErbB family, commonly referred to, are the RAS-RAF-MAPKK-MAPK pathway and the PI3K-AKT pathway (Bazley L. and Gullick W., 2005). All of the HER receptors can activate the RAS-RAF-MAPKK-MAPK pathway which plays a critical role in the regulation of cell growth and differentiation. The MAP kinases function in a mitogen activated protein cascade and are activated by phosphorylation by upstream MAPK kinases (also called ERK kinases), themselves activated by RAF-like molecules. Aberrations of these enzymes or of the signaling cascades that regulate them have been implicated in a variety of human diseases. Activated MAP kinases can translocate to the nucleus and phosphorylate various nuclear transcription factors such as c-FOS, c-JUN, E2F, ETV1 and ELK-1 which then bind to DNA response elements resulting in transcriptional activation of downstream genes that contribute to proliferation. AKT, also referred to as PKB, plays a critical role in controlling the balance between survival and apoptosis. The activated phosphoinositide 3-kinase (PI3K) converts the membrane-bound PIP2 to PIP3 whose formation can regulate the AKT kinase. The PI3K-AKT pathway promotes cell survival by inhibiting apoptosis and by regulating cell cycle entry
through up-regulation of cyclin D1. In addition, the stress-activated protein kinase/Jun-
amino-terminal kinase (SAPK/JNK) can be activated by growth factors and G-protein
coupled receptor (GPCR) agonists, although it is potently and preferentially activated by
a variety of environmental stresses. Evidence suggests that the pathway can also be
activated following ErbB family activation (Karunagaran D. et al., 1996). When active
as a dimer, SAPK/JNK translocate to the nucleus where it regulates gene transcription
through its effects on c-JUN, ATF-2 and other transcription factors. There is also
evidence for a functional interaction between c-Src family of non-receptor tyrosine
kinases and ErbB family members in breast cancer cell lines leading to the activation of
the MAPK pathway (Belsches-Jablonski A. et al., 2001; Olayioye M., 2001a). Another
target of the ErbB receptors signaling is the JAK-STAT pathway (Bazley L. and Gullick
W., 2005; Yamauchi T. et al., 2000), which plays a major role in tumorigenesis.
Activated JAKs (Janus kinases) phosphorylate tyrosine residues on STAT proteins
(Signal Transducers and Activators of Transcription) and other downstream signaling
proteins such as GRB2 adaptor protein. Phosphorylated STAT proteins dimerize and
translocate to the nucleus where it binds to DNA response elements resulting in gene
transcription.

Although active homodimers can naturally form for both ErbB1 and ErbB4,
eligand-stimulated heterodimerization is a prerequisite for active signaling for both ErbB2
and ErbB3 receptors. ErbB2 binds no known ligand with high affinity and can only be
recruited as a co-receptor with another ErbB member. No known ligand can activate
ErbB2 homodimers. Conversely, ErbB3 binds a number of ligands with high affinity, but
has a defective tyrosine kinase and thus, requires co-recruitment with another ErbB
member to be transactivated. Various developmental genetic studies support the “heterodimerization model”, which implies that a receptor requires two other signaling components in order to be activated: a co-receptor and a high affinity ligand (Harari D. and Yarden Y., 2000). This model is important for understanding early stages of HER2-related cancers since it dictates that ErbB2 must be activated in concert with at least two other molecular components.

Overexpression of ErbB2 in cell culture leads to cell transformation (Hudziak R et al., 1987). The mechanism underlying ErbB2 overexpression at the cell surface and its tumorigenic action has been investigated. It has been shown that ErbB2 overexpression, in the absence of exogenously added ligand, correspond to a relatively high basal level of ErbB2 tyrosine kinase autophosphorylation (Lonardo et al., 1990). The overexpression of ErbB2 at the cell surface may induce spontaneous formation of homodimers and increases ErbB2 availability for forming heterodimers when a ligand binds for its direct receptor. In fact, the transforming ability of ErbB2 has been shown to be significantly increased when co-expressed with either ErbB1 or ErbB3 in the presence of a respective ligand (Wallasch et al., 1995). This implies that ErbB2 overexpression may promote tumorigenesis primarily in the context of a ligand-driven heterodimer.

Studies showed that ErbB2 can interact strongly with the MAPK and PI3K pathways supporting the fact that ErbB2 can activate cell proliferation and cell survival. ErbB2 signaling abilities appear unique and are believed to result from a decrease in the normal inactivation processes, which typically include dissociation of ligand-receptor complexes, dephosphorylation of the activated receptor, rapid internalization through clathrin-coated pits and degradation of active receptors (Harari D. and Yarden Y., 2000).
It has been documented that ErbB2 overexpression slows down the processes, with the exception of the dephosphorylation step. The stability of ErbB2 dimers and especially heterodimers with ErbB2 is higher than that of other receptor combinations. Thus, overexpression of ErbB2 decreases the ligand dissociation rate causing a prolonged intracellular signal. In addition, the internalization rate of ErbB2 receptors is reduced which impairs efficient down-regulation of ErbB2 homodimers and heterodimers at the plasma membrane, resulting in long-lived intracellular signal. The receptor degradation of ErbB2-containing heterodimer is also reduced due to impaired lysosomal targeting. The sequences contained within the carboxyl terminus of ErbB-2 confer a weak and ineffective coupling between the receptor and the c-Cbl (ubiquitin ligase) and prevent the association with the AP-2 plasma membrane-coated pit adaptor complex, impairing receptor internalization. On the contrary, the receptor recycling to the cell surface is increased. In summary, several distinct mechanisms allow prolonged retention of ErbB2 at the cell surface and thereby extend the duration of signaling by the heterodimeric partners.

The ability of ErbB receptors to form both homodimers and heterodimers with distinct signaling properties in response to a myriad of ErbB-specific ligands, generates a complex signaling network with an enormous potential for signal amplification and diversification. The mapping of intracellular pathways activated by this family of RTKs is further complicated by the observations that it exists some crosstalks between ErbB receptors and other classes of receptors such as integrin receptors (Yarwood S. and Woodgett J., 2001), G-protein coupled receptors, and prolactin (PRL) and growth hormone (GH) cytokine receptors (Yamauchi T. et al., 2000; Huang Y. et al., 2006).
1.5.2. HER2/neu Breast Cancer

The human epidermal growth factor receptor-2 (HER2) oncogene, also called erbB2, encodes a 185 kDa transmembrane glycoprotein with intrinsic tyrosine kinase activity (p185). The HER2 gene was originally identified as a viral oncoprotein of c-erbB2, which is involved in carcinogenic transformation. Therefore, the official name of this gene is “v-erb-b2” which stands for avian erythroblastic leukemia viral oncogene homolog 2.

Slamon D. and colleagues (1987) initially reported that the ErbB2 receptor was overexpressed in 20-30% of human breast cancers. In the vast majority of cases, overexpression of the receptor is due to amplification of the HER2 gene in multiple copies in the nuclei of affected cells. Amplification results in increased levels of mRNA and, consequently, protein. It was estimated that HER2 amplification results in a 50 to 100-fold increase in the number of surface ErbB2 receptors on cancer cells compared to the normal mammary epithelium (Wilson C. et al., 2005). HER2 gene amplification is a relatively early event in the clinical pathogenesis of human breast cancer based on its frequent occurrence in pre-invasive lesion, ductal carcinoma in situ (DCIS). However, its amplification in invasive DCIS has been reported to be as high as 77%. The observation of a positive correlation between HER2 overexpression and the DCIS subtype of breast cancer suggests that HER2 aberrant activity plays a functional role in breast cancer tumor initiation and progression. The overexpression of HER2 in breast cancer correlates with a number of histological prognostic features including tumor size, high grade, high percentage of S-phase cells, aneuploidy and lack of steroid receptors (Slamon D. et al., 1987). Importantly, HER2 overexpression has been associated with a poor prognosis, an aggressive tumor type and resistance to chemotherapy. Increased expression of HER2 is
also implicated in tumor progression of other human epithelial cancers including ovarian, gastric, lung and prostate cancers.

The “heterodimerization model” is important for understanding early stages of HER2-related cancers since it dictates that ErbB2 must be activated in concert with at least two other molecular components, leading to the assumption that another type of ErbB receptor is implicated in HER2-dependent cancers. Therefore, co-expression analysis of different ErbB receptors was investigated. It was found that ErbB1 overexpression or amplification was often observed in DCIS and that it correlated with poor prognosis and inversely correlated with estrogen receptor status. However, co-expression analyses did not find any positive or negative correlation between ErbB1 and ErbB2 overexpression. These results implied that ErbB1 played a similar, but not necessarily inter-dependent, role in HER2-related cancer, although their synergy in a subset of tumors with more aggressive phenotype did indirectly implicate co-receptor interaction (Harris A. et al., 1989; Torregrosa et al., 1997). There are conflicting reports as to the clinical significance of ErbB3 and ErbB4 overexpression in breast cancer (Gasparini et al., 1994; Travis A. et al., 1996; Knowlden J. et al., 1998; Vogt U. et al., 1998); however, a positive correlation with estrogen receptor status and tumor size was observed for both receptors and an increased survival rate for ErbB3 (Knowlden J. et al., 1998). It remains an open question whether or not ErbB3 and/or ErbB4 play a significant role in the co-activation of ErbB2 in breast cancers.

Aberrant signaling through these receptors is believed to play a direct role in malignant transformation and/or progression. Overexpression of ErbB2 receptors in tumor cell was shown to induce important phenotypic changes, including increased
growth in vitro, decreased anti-estrogen response, increased production of angiogenic factors, as well as increased tumorigenicity and metastatic potential in vivo (Hudziak R. et al., 1987; Pierce J. et al., 1991). These changes parallel the observed aggressive clinical behavior of human tumors that contain an amplified HER2 gene. Other experimental data, supporting the role of HER2 in breast cancer initiation, comes from transgenic experiments in which wild-type or activated HER2 expressed in mouse mammary epithelium leads to a high frequency of mammary carcinomas (Muller W. et al., 1988; Guy C. et al., 1992).

The HER2 gene amplification and oncogenic mutations (Val 664 to Glu) can constitutively activate the ErbB2 homodimeric tyrosine kinase in a ligand-independent manner (Lonardo F. et al., 1990). Elevated HER2 activity can reduce the growth factor dependence of ErbB2 amplified cells and prolonged stimulation of the Ras-Raf-MAPK pathway (Di Fiore P. et al., 1987; Ben-Levy R. et al., 1994). It is also increasingly clear that the high cell-surface ErbB2 density that accompanies gene amplification alters the normal equilibrium of ErbB dimers in favor of ErbB2 containing heterodimers, thus altering ligand dependent signaling mechanisms. The oncogenic potency of heterodimers such as ErbB1/ErbB2, is significantly enhanced compared to ErbB1 homodimers by several processes that prolong receptor signaling activity.

It has been documented that HER2 overexpression can promote the growth and malignancy of mammary epithelial cells in part by conferring resistance to the growth inhibitory effects of TGF-β signaling, which corresponds to the dominant system opposing the stimulatory effect of growth factors and early oncogene activation in many tissues including the mammary gland (Brandt R. and Ebert A., 1998; Wilson C. et al.,...
Resistance to the anti-proliferative effects of TGF-β appears at an early stage of tumor progression in a number of human malignancies.

In addition, a link between HER2 amplification and cyclin D1 overexpression in breast cancer has also been established. The cyclin D1 gene has been reported to be amplified in up to 20% of human breast cancer, while cyclin D1 protein is overexpressed in over 50% of human mammary carcinomas and particularly in DCIS (75%) (Weinstein-Saslow D. et al., 1995; Harari D. and Yarden Y., 2000; Yu Q. et al., 2001). Aberrant overexpression of D-type cyclins can reduce or overcome the dependency of mitogenic stimulation for a cell and, thus, play a role in the process of oncogenic transformation. It is believed that ErbB2 amplification results in hyper-activation of a signaling network, which deregulates the G1/S checkpoint of the cell cycle by up-regulation of cyclin D and of the cyclin-dependent kinase partners (CDK-4 and -6) (Harari D. and Yarden Y., 2000; Yang C. et al., 2004). Furthermore, ErbB2-overexpressing breast tumors can evade apoptosis and resist to chemotherapeutic cytotoxic agents such as Taxol. The CDK inhibitor, p21\textsuperscript{Waf1}, which plays a central role in ErbB2’s anti-apoptotic machinery, was found up-regulated in a number of ErbB2-overexpressing cell lines (Yu Q. et al., 2001).

Interestingly, a negative correlation between HER2 and ER expression has been observed in breast cancers. Patients with ErbB2 overexpressing tumors have demonstrated poor response to endocrine therapy, commonly used for ER-positive patients. This inverse relationship may reflect the existence of different molecular programs responsible for the development of distinct breast cancer subtypes. However, this inverse relationship is not absolute. Studies on ER-positive/HER2-positive breast
tumors suggested that ErbB2 signaling can override the tumor-inhibitory effect of anti-estrogen since the patients responded poorly to endocrine therapy.

The main therapeutic strategy for targeting HER2 positive breast cancer is the use of trastuzumab (Herceptin), a humanized monoclonal anti-HER2 antibody that targets cell surface ErbB2 receptors (Miles D., 2001; Harries M. and Smith I., 2002).

The first inhibitory antibody against the product of an oncogene (growth factor) was developed in the early 1980s. In 1986, Debrin J. et al. produced a monoclonal antibody against the mutated rat HER2 receptor and showed that it was inhibiting the growth and the tumorigenic potential of HER2/neu-transformed breast cancer cells implanted into nude mice. Later on, several groups raised a number of murine monoclonal antibodies to the extracellular domain of HER2 (Hudziak R. et al., 1989). These antibodies were able to inhibit the growth of cells overexpressing the receptor but had very little or no effect on cells without elevated levels of ErbB2. One of them, the muMab 4D5 demonstrated to be a potent inhibitor of growth of human breast cancer xenografts, and selected for further clinical development (Baselga J. and Mendelsohn J., 1994). To reduce the potential for generating a human anti-mouse immune response, the 4D5 murine monoclonal was humanized to form a chimeric antibody, called trastuzumab, which is 95% human and 5% murine, with retained a high affinity for the HER2 epitope (Carter P. et al., 1992; Sandhu J., 1994). The hypervariable region of the antibody was conserved but the kappa light chains and the IgG1 constant region were replaced by human version. Trastuzumab showed anti-proliferative effect in in vivo breast cancer xenografts as well as significant therapeutic effects in patients with strongly HER2-positive breast cancer.
Several putative mechanisms by which trastuzumab exerts its anti-tumor effects have been hypothesized and correspond to: 1) down-regulation of ErbB2 receptors by accelerating endocytic degradation; 2) reduction of homo- and heterodimers capable of signaling, 3) induction of G1 cell cycle arrest which blocks cell division and growth and 4) induction of a host tumor response via antibody-dependent cell cytotoxicity (ADCC) (De Santes K. et al., 1992; Petit A. et al., 1997; Harries M. and Smith I., 2002). Trastuzumab is the only FDA-approved therapeutic for HER2 overexpressing metastatic breast cancer. It is approved for first-line treatment in combination with the chemotherapeutic drug paclitaxel, and as a single treatment for heavily pre-treated patients with advanced breast cancer. Its use has been shown to be effective and is associated with higher response rates, longer time to progression and improved survival when compared to chemotherapy alone (Miles D., 2001). In a recently published joint efficacy, the benefits of combining trastuzumab to standard adjuvant chemotherapeutic agents have been calculated to correspond to a 52% reduction in events (i.e. recurrence, second primary cancer, or death before recurrence) (Slamon D. et al., 2006).

1.6. **RAS Oncogene**

1.6.1. **RAS Small GTPase Family**

The RAS gene codes for proteins involved in signal transduction. RAS proteins belong to a large superfamily of small GTPases which comprise more than 150 proteins identified to date. This family is structurally organized into at least five families including the RAS, RHO, RAB, RAN, and ARF families. These proteins are involved in different cellular process such as apoptosis, cell proliferation and differentiation, cytoskeleton reorganization and movement and membrane and vesicle trafficking. They
are small proteins (20-25 kDa) that act as molecular switches, cycling between an inactive guanosine diposphate (GDP)-bound state and an active guanosine triphosphate (GTP)-bound state. The exchange of GDP for GTP induces a conformational change in the proteins that allows them to interact with downstream effectors and carry various specific biological functions. The activation state is normally tightly regulated by the concerted action of guanine nucleotide exchange factors (GEFs) such as SOS and GTPases-activating proteins (GAPs) such as NF1. GEFs catalyze the release of GDP, allowing the binding of the more abundant GTP and the activation of the proteins, whereas GAPs catalyze their inactivation (Lodish H. et al., 2001).

The RAS family now includes at least 21 members, including the most common H-RAS, K-RAS (A and B isoforms), N-RAS, R-RAS, TC21/R-RAS2, R-RAS3/M-RAS, RAP1 (a and b), RAP2 (a, b and c), RIT, RIN, and RAL (A and B) (Rodriguez-Viciana P. et al., 2004). K-RAS, H-RAS and N-RAS (21 kDa) are commonly referred as the RAS proteins. In normal cells, they can be activated by a wide variety of extracellular stimuli including the receptor tyrosine kinases and G-protein coupled receptors to cause transient activation of RAS. In turn, RAS associates with and activates multiple effectors that stimulate cytoplasmic signaling pathways that regulate cell proliferation and differentiation, survival and apoptosis.

RAS proteins are synthesized as a precursor with a C-terminal CAAX motif (C= cysteine, A= aliphatic amino acids, X=variable) that undergoes a series of post-translational modifications. The first and most crucial modification is catalyzed by the farnesyltransferase (FTase enzyme) and corresponds to the covalent attachment of a farnesyl isoprenoid lipid (hydrophobic chain) to the cysteine residue at the carboxyl
termini of RAS proteins. This modification facilitates RAS attachment to the inner face of the plasma membrane which is required for its activity. In some cases the 15-carbon farnesyl chain is substituted by a 20-carbon geranylgeranyl chain. These post-translation modifications differ slightly between the different RAS isoforms (H-, N- and K-isoforms) due to sequence differences in the carboxyl terminus (Choy E. et al. 1999).

1.6.2. RAS Effector-mediated Signaling Pathways

RAS proteins can interact with a wide array of effectors. These effectors have been classified into two groups depending on their ability to regulate the anti-apoptotic or apoptotic actions of oncogenic RAS (Figure 2). The best characterized effectors are the RAF kinases, the p110 catalytic subunit of class I PI3Ks and a family of RAL guanine nucleotide exchange factors (RalGEFs). Other effectors have been described and include RIN1, Tiam1, phospholipase C (PLC), AF6, NORE1 and RASSF1 (Katz E. and McCormick F., 1997; Lodish H. et al., 2001; Rodriguez-Viciana P. et al., 2004). RAS effectors interact with RAS protein through a small region of approximately 100 amino acids called the RAS binding domain (RBD) to induce diverse cellular signals. Among the effectors, three distinct domains have been found which share structural topology of an ubiquitin superfold but little primary sequence identity: 1) the RAF-type of RBD, 2) the PI3K-type of RBD, and 3) the RA (RalGDS/AF6, Ras associating) domain (Rodriguez-Viciana P. et al., 2004; Herrman C, 2003). The RA domain is found in a wide variety of proteins including RalGEFs, Rin1, AF6, PLC, NORE1 and RASSF1. There is an incredibly large number of proteins with RBD domain and thus potential existence of other Ras effector not identified yet. In order to recognize a molecule as RAS effectors, a set of three conditions must be demonstrated: 1) interaction between
endogenous RAS with the full-length effector; 2) modulation of the effector’s function by interaction with Ras; and 3) dependence of RAS biological activity on effector’s function (Repasky G. et al., 2004).
Figure 2. Schematic representation of the RAS activation of multiple effector-mediated signaling pathways. Activated GTP-bound RAS can bind to and regulate diverse downstream effectors to mediate pro- or anti-apoptotic functions. RAS can bind to the RAF kinase and activate the RAF-MAPK cascade which ultimately leads to the activation of various transcription factors that promote gene expression. MAPK can also phosphorylate RSK leading to cell cycle arrest and differentiation. RAS interacts with and activates the PI3K which converts the membrane-bound PIP2 to PIP3, whose formation can regulate AKT kinase. AKT has an anti-apoptotic action which is achieved by phosphorylation of multiple targets. AKT blocks the pro-apoptotic effects of Bad, inactivates caspase-9 (casp9) and the forkhead transcription factor (FKH). AKT also activates IKK, which leads to NF-κB release and the expression of anti-apoptotic genes. Activated RAS can bind to Tiam effector and activate the anti-apoptotic effect of Rac that activates NF-κB. RalGEFs represent another group of RAS downstream effectors. RalGEFs are GDP-GTP-exchange factors and activators of Ral GTPases involved in proliferation/survival and in cell cycle division through Cdc42 activation. In contrast to the anti-apoptotic effectors, NORE1 and RASSF1 effectors up-regulate apoptosis. Activated RAS can bind to NORE1 and RASSF1. NORE1 and RASSF1 form homo- and hetero-dimers, which form a complex with and regulate the pro-apoptotic serine/threonine kinase MST1.
To study these biochemical properties, different experimental approaches have used Ras effector domain mutants, dominant negative mutants (S17N), pharmacological inhibitors and ectopic overexpression of activated mutant (G12V) form of RAS.

It is increasingly recognized that RAS proteins can mediate positive and negative functions in modulating cell growth and death (Cox A. and Der C., 2003). These opposing biological actions are achieved by interaction with and activation of distinct specific effectors. In normal conditions, RAS signaling results in a constant balance between the anti-apoptotic and the apoptotic effects. Whether oncogenic RAS promotes proliferation or cell death is also influenced by cell type, context, signals nature, signaling intensity, and tissue origin. Therefore, to understand the biological functions of the different RAS proteins and their individual contributions to human diseases, such as cancer, it is crucial to understand which effector pathway they can regulate.

Voice J. et al. (1999) demonstrated that the four Ras homologs (H-RASA, H-RASB, N-RAS and K-RAS) significantly differ in their abilities to activate RAF-1 effector, induce focus formation, anchorage-independent growth or cell migration in a distinct hierarchy manner. Results showed that K-RASB was the most effective for RAF-1 activation and cell migration, whereas H-RAS was most effective for focus formation but weak for RAF-1 activation and enable to induce anchorage-independent growth.

The differences in post-translational modification of the RAS isoforms seem to be responsible for distinct compartmentalization within the plasma membrane subdomains and may explain the different functions of the RAS isoforms in vivo (Jiang X. and Sorkin A., 2002). The complexity of RAS signaling is also reflected by the overlap in the
abilities of RAS proteins to activate different effectors pathways (Gille H. and Downward J., 1999). It is clear that RAS proteins interact with effectors in a very specific manner and even with some striking isoform-specific differences (Yan J. et al., 1998). This adds to the already complex system since different isoforms tend to show different pattern of expression. These selective interactions may have important biological consequences, depending on the specific properties and functions of different isoforms, the pattern of expression of the various GTPases and the effector isoforms in different cell types.

The use of fragments of effectors, typically the RBD domain only, to study in vitro interactions between RAS proteins and RAS effectors have revealed some conflicting results between laboratories. It has been shown that these minimal binding regions may not accurately reflect interaction with the full-length proteins (Linneman T. et al., 1999; Rodriges-Viciana P. et al., 2004). It is suggested that these differences in results highlight the possibility that another domain may interact with RAS proteins, aside from the higher-affinity RBD, as for instance, the lower-affinity cysteine-rich domain (CRD) present in RAF-1 effector which may play a role in modulating specificity (Hu D. et al., 1995; Chong H. et al., 2003a). Plus, most assays do not account for possible contributions from the post-translational modifications or subcellular localization of the RAS proteins (Pacold E. et al., 2000).

The three RAF serine/threonine kinases, A-RAF, B-RAF and c-RAF (RAF-1) are the main effectors recruited by GTP-bound H-RAS, N-RAS and K-RAS which activate the MAP kinase pathway, resulting in either apoptosis or survival. Activation of RAF-1 is the best understood and involves phosphorylation at multiple activating sites (Chong H. and Guan L., 2003b). Other RAS proteins such as TC21, R-RAS3 and Rit have been
found to activate the RAF-1 effector as well (Rodriguez-Viciana P. et al., 2004). However, not all RAS proteins can activate RAF-1 and more importantly, the magnitude of their stimulation varies greatly (Woods D. et al., 1997; Sewing A. et al., 1997; Pritchard C. et al., 1997). The lipid kinase PI3K is another RAS effector in many cell types and appears to provide an universal survival signal downstream of RAS by activating the AKT kinase which inhibits apoptosis by inhibiting the actions of Bad and Caspase 9. RAS protein can also activate RAC protein, independently of PI3K, through direct interaction with Tiam1, leading to the activation of the transcription factor NF-κB which produces potent pro-survival signals (Lambert M. et al., 2002). RalGEF is another group of well-studied RAS effector, with guanine-nucleotide exchange factor activity for RAL proteins (Linneman T. et al. 2002). H-RAS, N-RAS and K-RAS, TC21, R-RAS3 and Rit proteins can interact with the three members of the RalGEF family: RalGDS, RGL and RGL2/Rlf (Repasky G. et al., 2004). However, these studies indicate that the ability to associate with the protein in vivo does not correlate with the ability to stimulate the enzymatic activity of the downstream effector (Rodriguez-Viciana P. et al., 2004). Eckert L. et al. (2004) highlighted that elevated activity of RAS protein was not always associated with activation of RAF-MAPK and PI3K-AKT pathways, the two key RAS effector pathways in human breast cancer cell lines. Thus, this underlines the significant cell context variations and suggests that other effector pathways may be important for RAS-mediated oncogenesis.

The pathways mediating RAS regulation of apoptosis remain poorly understood. Recent studies have indicated that RAS effectors include protein products not only of oncogenes, but also of putative tumor suppressor genes. Thus, such effectors might
account for the growth inhibitory and apoptotic activity of RAS (Cox A. and Der C., 2003). This suggests that RAS proteins may directly regulate proteins with tumor suppressor properties. Indeed, a new family of genes, the Ras Association domain (RalGDS/AF-6) Family (RASSF 1-6) has been discovered and four of its members, RASSF1, RASSF2, RASSF4 (also known as AD037) and NORE1 (also called RASSF5) have been identified as RAS effectors and tumor suppressors (Hesson L. et al., 2003; Vos M. et al., 2003; Eckfeld L. et al. 2004; Agathanggelou A. et al., 2005). They are all located on different chromosome and RASSF1 has been the most studied one of all the members. The proteins are all characterized by a RA (RalGDS/AF-6, RA associating) domain at the C-terminus followed by a SARAH (Sav/RASSF/Hpo) domain. The RA domain mediates interactions with RAS proteins and other small RAS GTPases, whereas the SARAH domain is a novel interaction motif that can mediate heterotypic and homotypic interactions.

The role of the RASSF genes as tumor suppressors is supported by the loss of expression in human tumor cell lines and tumors due to hypermethylation of CpG islands in the promoter region (Agathanggelou A. et al., 2005). The identification of the RASSF proteins as RAS effectors is demonstrated by their interaction with specific RAS proteins in a GTP-dependent manner (Vos M. et al., 2000; Vos M. et al., 2003; Eckfeld K. et al, 2004). The evidence that RASSF proteins facilitate RAS-mediated growth inhibition and/or apoptosis are diverse and restricted to ectopic overexpression analyses. It was shown that ectopic overexpression of NORE1 or RASSF1 caused growth inhibition and apoptosis, effects which where enhanced by co-expression of activated H-RAS and antagonized by co-expression of dominant inhibitory H-RAS (Vos D. et al., 2000). A
number of studies have started to investigate the molecular mechanism leading to their growth-inhibition effects (Agathanggelou A. et al., 2003). Evidence show that RASSF1A can interact with different target molecules including microtubules to increase their stability, MST1 pro-apoptotic kinase to induce apoptosis and with cell cycle regulators to arrest cell cycle progression (Ortiz-Vega S. et al., 2002; Shivakumar L. et al., 2002; Vos D. et al., 2004; Agathanggelou A. et al., 2005). Finally, the physiological significance of the RASSF proteins as RAS effectors is unclear since it was shown that NORE1- and RASSF1A-mediated growth inhibition of human tumor cells did not require interaction with RAS proteins. Thus, whether the RASSF proteins are physiological important effectors that influence the role of mutated RAS in cancer development is still under debate (Repasky G. et al., 2004).

1.6.3. RAS in Cancer

RAS mutations are observed in approximately 40% of all human cancers and often translate to poor prognosis and treatment outcome. The highest incidence of RAS mutations are found in adenocarcinomas of the pancreas (90%), the colon (50%) and the lung (30%) as well as in thyroid tumors (50%) and in myeloid leukemia (30%) (Bos L., 1989). The high frequency of RAS mutations supports a critical role for aberrant RAS activation (GTP-bound form) in the progression and maintenance of these cancers. Constitutively activated RAS can induce multiple facets of malignant transformation by promoting cell cycle progression, uncontrolled cellular proliferation and by deregulating processes that control apoptosis. The most common oncogenic RAS mutation found in tumors corresponds to a change of glycine 12 to aspartate (G12D), which prevents RAS inactivation by the GAP proteins, possibly by increasing the overall rigidity of the protein
(Lodish H et al., 2001). Different alleles of RAS gene are mutated in cancers with K-RAS and N-RAS in most cancers and H-RAS in head/neck cancers (King R., 2000). K-RAS mutations have been found in colorectal cancer, non-small-cell lung cancers (NSCLC), melanomas and ovarian cancers (McLellan E. et al., 1993; Aviel-Ronen S. et al., 2006; Reifenberger J. et al., 2004; Cuatrecasas M. et al., 1997). Other RAS proteins such as TC21, R-RAS3 and RIT have been found to be activated by mutation in human cancers and thereby to induce transformation of a variety of cells (Rodriguez-Viciana P. et al., 2004).

Interestingly, RAS and RAF mutations have been reported in the same types of human cancers, suggesting that mutationally activated RAS and RAF are functionally equivalent. For example, approximately 70% of melanomas harbor mutated alleles of B-RAF and 25% harbor N-RAS mutations but in essentially non-overlapping occurrence. Similar observations of non-overlapping mutations of these two genes have been described for colorectal cancers, papillary thyroid carcinomas, ovarian carcinomas and other cancers (Repasky G. et al., 2004; Reifenberger J. et al., 2004). This suggests that B-RAF and RAS mutations share equivalent roles in oncogenesis and that oncogenic RAS involves RAF activation.

Some cancers have aberrant activation of RAS signaling even though they don’t have mutated forms of RAS. Aberrant activation have been found to result from alterations in other molecules such as in NF1, whose defect leads to a loss of its GTPase-activating potential, in childhood chronic leukemia (King R., 2000). In breast cancers, RAS mutations are rarely found (less than 5%); however, there is considerable evidence that aberrant RAS activation and signaling may promote breast cancer development.
Clark G. and Der C., 1995; Von Lintig F. et al., 2000). In fact, RAS can be activated by persistent upstream signaling from EGFR, ErbB2 and ER receptors which are often overexpressed in breast cancer and thereby promote tumor formation (Von Lintig F. et al., 2000).

The identification of mutated RAS genes in human tumors prompted great interest in the development of anti-RAS targeted strategies for cancer treatment. Among the anti-RAS strategies under evaluation in the clinic, a number of pharmacologic inhibitors where tested to prevent: 1) RAS association with the plasma membrane (FTases inhibitors (FTI)), 2) downstream signaling pathways (RAF and MEK protein kinase inhibitors), 3) autocrine growth factor signaling (EGFR inhibitors), or 4) gene expression of H-RAS and RAF-1 (Caponigro F., 2002; Dancey E., 2002; Cox A. and Der C., 2002; Wickstrom E., 2001). Although a number of these inhibitors have demonstrated potent anti-tumor activities in preclinical models, Phase I-III clinical trials have revealed unexpected complexities in RAS function and targeted therapies (Cox A. and Der C., 2002).

1.7. The Use of Transgenic Mouse Technology in Cancer Research

1.7.1. Transgenic Mouse Technology

Transgenic technologies have a broad range of applications, from studying the basic mechanism of gene regulation to the generation of the models for human diseases, and are used to create systems utilized in pathophysiological and therapeutic studies. The study of a transgene function in the context of an intact organism provides a much more complete and physiological relevant picture than any other methods could achieve. The use of transgenic techniques has evolved from the development and convergence of the
gene-transfer methods in murine cell culture systems, the methods to manipulate early mouse embryo and the embryonic stem cell technology (Hofker M. and Van Deursen J., 2002). The molecular genetic approaches towards the study of gene function in mice have traditionally relied on either overexpression or gene ablation using transgenic mouse or knockout mouse strategies respectively.

The first transgenic lines of mice containing foreign genes were produced in 1976 by infecting pre-implantation stages of embryos with a retro-virus (Jaenisch R., 1976). The retro-virus method showed numerous limitations and led to the development of a new dominating technique consisting in microinjection of DNA into the pronucleus of a developing zygote (Hofker M. and Van Deursen J., 2002). Gordon J. and colleagues were the first to report in 1980 the establishment of a transgenic mice line with stable integration of foreign DNA in the mouse genome by pronuclear microinjection (Gordon J. et al., 1980). The stable integration led to the expression of the foreign genes and was transmitted to the offspring. Therefore, by definition, a transgenic animal is an animal that has a foreign gene (transgene) stably incorporated into its genome through human intervention (Gordon J. et al., 1980; Auerbach A., 2004). The microinjected DNA integrates at random insertion sites and usually at more than one site in the genome. Usually integration occurs in only one chromosome resulting in the development of hemizygous founders for the transgene (Gordon J. and Ruddle F., 1981).

Although the technique of DNA pronuclear microinjection is widely used, a number of disadvantages have been reported (Auerbach A., 2004). For instance, it has a low efficiency of transgenic mouse production, with a low number of transgenic mice founders expressing the transgene appropriately, i.e. with suitable levels and correct
spatial/temporal expression. Many studies have also demonstrated dramatic differences in the expression levels of the same transgene in a given tissue between lines or even between individual founder siblings due to different integration loci (Auerbach A., 2004). Plus, the location of transgene insertion can play a great role in influencing the transgene expression and phenotype between the lines (Palmiter R. et al., 1983). Therefore, it is recommended to generate several independent founders from the same construct and to compare the observed phenotypes of independent transgenic lines.

Many of the transgenic models have used different regulatory regions called promoters to control the transgene expression and define the tissue and the cell type affected, plus the temporal onset of the phenotype. For example, there are the “ubiquitous” cytomegalovirus (CMV) (Furth P. et al., 1991), immediate early and metallothionein (MT) promoters (Hennighausen L., 2000), the lineage-specific Eμ-immunoglobulin heavy chain gene enhancer/promoter for the B-cell lineage (Adams J. et al., 1985), the tissue-specific promoters such as the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Stewart T. et al., 1984), the whey acid protein (WAP) gene and the bovine lactoglobulin promoter for the mammary gland (Hennighausen L., 2000; Largaespada D., 2003).

The knockout technique, which consists in disrupting a target gene in the germline by the insertion of a selectable marker, is a useful technique in defining the effects of repressor genes (Capecchi M., 1989; Kuhn R. and Schwenk F., 2003). Since the conventional knockout mice are usually homozygous for a null allele in the germline, they proved very useful to study inherited disease, leading to embryonic or early post-natal lethality (Kuhn R. and Schwenk F., 2003). The two first knockout models were the
Rb1 and the p53 tumor suppressor knockouts (Jacks T. et al., 1992; Jacks T. et al., 1994). The latter demonstrated that not all tumor suppressor knockouts are lethal in the homozygous state since homozygous p53\(^{-/-}\) mice are all fertile and viable. However, it appeared that the germline knockout mice did not constitute the best technical approach for analyzing other aspects of the repressor genes function in the adult mice. Therefore, a refined knockout strategy, termed conditional gene targeting, was developed that permits the inactivation of the target gene in somatic cells, only at a specific developmental stage and/or in a specific organ.

The latest technologies involve the ability to turn transgenes (e.g. oncogenes) on and off in vivo or to inactivate genes (e.g. tumor suppressor) in specific tissues (Kuhn R. and Schwenk F., 2003; Largaespada D., 2003). This was achieved by the development of conditional and inducible systems which allowed a spatial (tissue specific) and temporal control of the onset of transgene expression.

Common examples of these novel systems involve inducible promoters such as the tetracycline transactivator-responsive promoters and conditional system utilizing site-specific DNA recombinases such as the Cre-recombinase or Flp recombinase (Kilby N. et al., 1993; Utomo A. et al., 1999; Auerbach A, 2003). For example, in the case of tetracycline-regulated transgene, treatment with doxycycline, an inhibitor of Tet transactivator, can block the expression of a specific oncogenic transgene (Gossen M. and Bujard H., 1992). This system is reversible and allows to determine if continued oncogene expression is required for the maintenance of the transformed phenotype in vivo. The Cre-recombinase tool can also be used to keep a transgene from being expressed (Kilby N. et al., 1993; Largaespada D., 2003). For example, if the transgene is
separated from the promoter with a recombination-activated gene expression (RAGE) cassette which contains stop codons in all three reading frames and flanked by LoxP sites, the \textit{in vivo} induction of Cre-recombinase will cause the excision of the RAGE cassette at the LoxP sites and thereby, the expression of the transgene. In the opposite, the Cre-recombinase system can also be used to inactivate the expression of tumor suppressor genes by excision of a critical exon in the gene (Largaespada D., 2003). In addition, this can be achieved in a tissue-specific manner by controlling Cre-recombinase expression by tissue-specific promoter and in a controlled temporal manner by placing the Cre-recombinase under an inducible promoter (Uomo A. \textit{et al}., 1999). Controlled temporal systems are useful when the inactivation of the two alleles of a tumor suppressor gene is lethal at the embryonic stage (Kuhn R. and Schwenk F., 2003).

\textbf{1.7.2. Transgenic Mouse in Cancer Research}

Transgenic mouse models have offered tremendous opportunities for studies of the genetic basis of cancer. They proved useful in the characterization of the biological function of oncogene and tumor suppressor in specific tissues. Transgenic animals have been used for overexpression studies of genes encoding transcription factors (\textit{c-Myc}), cell surface receptors (HER2/neu) and structural proteins, as well as for loss-of function experiments by utilizing constructs encoding antisense RNA or dominant negative genes and also by DNA interference (Largaespada D., 2003). There has been a growing trend for using such models in cancer research over the last decade and more models are generated. Transgenic mice models carrying the \textit{c-Myc} oncogene were among the first one to be published (Stewart T. \textit{et al}., 1984). These models proved, for the first time, that putative cancer genes could actually initiate \textit{bona fide} cancer in a living organism.
However, one single model does not cover alone the full spectrum of cancer, but individual models address distinct aspects.

Considering that cancer development is a complex multi-step process involving more than one genetic alteration, researchers have developed mouse models that carry more than one transgene in order to study the cooperativity between genetic alterations in tumorigenesis. Leder and colleagues were the first one to develop bi-transgenic mouse models carrying the c-Myc and Ras oncogenes and to demonstrate a synergy between the two oncogenes (Sinn E. et al., 1987). These models confirmed that tumor progression is a multistep process involving different pathways and, most importantly, helped to identify parallel and interconnected pathways in tumor progression.

The mouse models have demonstrated many advantages over human genetic studies which include the controlled crosses, the ability to control environmental variables, the use of recombinant inbred and the ability to generate large numbers of genetically identical animals. However, some challenges come from the variability observed from different mouse strains which can translate into different tumor latency and even tumor type for one transgenic oncoprotein (Threadgill W. et al., 1995). Plus, the biology of tumors of genetically engineered mice has indicated some similarities but also some differences compared to human tumors (Hennighausen L., 2000).

Genetically engineered mouse models of mammary cancer have been precious in elucidating molecular pathways and signaling events associated with the initiation, promotion and progression of breast cancer. The different mouse models helped to identify some of the agents that can induce mammary tumors in transgenic mice (growth factors, growth factor receptors, lactogenic hormones and cell cycle regulators) and deep
insight into their function has been deduced from the different mouse models. A number of mouse models for studying genetic alterations, pathway activations and pharmacological agents in breast cancer have been developed. For example, the mouse models of neu overexpression, targeted to the mammary gland under the direction of the murine mammary tumor virus (MMTV) promoter, have been used to test the tumorigenic potential of the neu oncogene in the mammary epithelium (Guy C. et al., 1992). However, it is important to note that mouse models of breast cancer have some potentially important limitations relative to human cancer. For instance, there are endocrinological, hormonal and life style differences represented in the two biological systems (Hennighausen L., 2000). In addition, the histopathology descriptions of mouse mammary tumors do not mirror the most frequent forms of human breast tumors, e.g. invasive ductal carcinomas (Hennighausen L., 2000). Therefore, the extrapolation of the genetics of the mouse mammary tumorigenesis to human breast cancer must be done with caution.

1.8. The Use of Gene Expression Profiling in Cancer Research

Cancer is a highly variable disease with multiple heterogeneous genetic and epigenetic changes. Functional studies are essential to understanding the complexity and polymorphisms of cancer. The development of DNA microarray technology a decade ago has led to the establishment of functional genomics, one of the most active and successful scientific disciplines today (De Risi J. et al., 1996). Microarray technology is a new powerful method that provides researchers with the opportunity to analyze the expression patterns and the interactions of tens of thousands of genes in a short time and in one experiment that is impossible using conventional analysis.
Cancer causes the deregulation of normal cellular processes such as growth, proliferation, migration, differentiation and apoptosis. These different processes correspond to gene expression changes that cause and/or result from this deregulation. The changes in the expression of the large number of genes involved in the particular processes can be detected and quantified by cDNA microarray technologies in concert. This technique is used to identify particular expression patterns leading to genomic signatures of many systems. The correlation of the genomic signatures to specific features of phenotypic variation can provide the basis for an improved classification of cancers or of a particular type of cancer into subtypes. For example, Sorlie T. et al. (2001) reported that variations in gene expression patterns in 40 grossly dissected human breast carcinomas analyzed by cDNA microarrays and hierarchical clustering provided a distinctive "molecular signature" of each tumor and that the tumors could be classified into subtypes based solely on differences in these patterns. Furthermore, the tumor characteristics could be correlated to clinical outcome. In a similar manner, Dressman M. et al. (2003) were able to differentiate breast tumor types by gene expression profiling of breast biopsies in patients and demonstrated that this technology is valuable for adequate characterization and further prediction of prognostic and treatment choice.

The identification of genes regulated by a particular oncogene or tumor suppressor gene can reveal several novel targets for potential therapeutic interventions. A number of studies have used cDNA microarray technology to identify the genes that are associated with HER2 overexpression or amplification in breast cancer cells and tumors (Mackay A. et al., 2003; Kauraniemi P. et al., 2002). Alaoui-Jamali M. and colleagues (2003) assessed the gene transcriptional changes upon ErbB receptors and
looked at markers involving angiogenesis, signaling and cell-cell structure. They showed that the overall number of cancer related genes affected by ErbB receptors was higher in cells and xenograft tumors expressing paired combination of ErbB receptors, compared to single receptor. This correlated with the finding that heterodimers are more mitogenic. They were able to identify a unique gene expression profile for each specific heterodimer combination and reported that the affected genes corresponded to genes known to play a key role in signaling, angiogenesis, and cell-cell interaction. In addition, their results highlighted the existence of a broad diversity of ErbB-regulated cancer-associated genes.

Other researchers have used genetic profiling to define a metastasis signature, specific to bone marrow, by identifying distinct profiles between primary breast tumors with bone marrow metastasis and primary breast tumor without metastasis. They found that the bone marrow metastasis positive signature was mainly characterized by transcriptional repression, implying the role of protein as metastasis suppressors (Woelfle U. et al., 2003). Some scientists studied the dysfunction of transcription factors, key regulators of cell growth and differentiation, following different treatments (estrogen vs. tamoxifen) in order to obtain a comprehensive view of the differences in response of cancer cell lines to these two treatment agents (Jiang X. et al., 2006). This emphasized the use of this technique as a tool for the prediction of response to treatment and for the identification of novel therapeutic targets.

In conclusion, microarray technology is a promising tool to discover and study the underlying molecular mechanisms of tumorigenesis and in addition this technique has allowed the identification of an increasing number of molecular markers with prognostic and diagnostic potential in a broad range of human cancers. Thus, there are great
expectations from the use of this technology in clinical oncology. However, routine application of microarrays in clinical practice requires significant improvements to standardize the array manufacturing techniques, assay protocols and analytical methods used for the interpretation of the data. This new technology still faces major challenges, for example, the design of experimental controls that will permit comparison of quantitative expression profiles obtained from diverse biological contexts (Lee P. et al., 2001).
2. HYPOTHESIS AND OBJECTIVES

To study HER2/neu-related breast cancer, we chose the MMTV/neu transgenic mouse line from The Jackson Laboratories (N#202) as an animal model. It has been well documented that the overexpression of the neu oncogene leads to the development of mammary gland adenoma in approximately 50% of virgin female MMTV/neu transgenic mice at the age of 7 months (t_{50}=205 days) and nearly 80% at the age of 11 months or older. We turned our attention towards the fact that a small proportion of the virgin female MMTV/neu transgenic mice did not develop mammary tumors despite the presence of high levels of the neu oncogene.

The hypothesis of this study is that there may be one or more tumor suppressor genes that are somehow activated or overexpressed in the mammary gland of these tumor-resistant MMTV/neu transgenic mice.

The objectives of this study are first, to use comparative microarray technology to identify the alterations in gene expression in the mammary gland of tumor-resistant MMTV/neu female transgenic mice and possibly identify the candidate genes which may be responsible for the resistance to HER2/neu-initiated mammary tumor development, and secondly, to select one candidate gene for further characterization of its role in HER2/neu-initiated breast cancer through in vitro and in vivo studies.
3. MATERIAL AND METHODS

3.1. Animal Model

The virgin female MMTV/neu transgenic mice from The Jackson Laboratories (N#202) were chosen as an animal model to study HER2/neu-related breast cancer. In this study, the virgin female MMTV/neu transgenic mice were divided into 2 groups: the tumor-susceptible MMTV/neu transgenic mice (S) that develop mammary tumors and the tumor-resistant MMTV/neu transgenic mice (R) that do not develop tumors (Figure 3A). Concerning the tumor-susceptible MMTV/neu transgenic mice, a further distinction was made between the tumor tissue and the adjacent normal mammary gland. The latter is defined as tumor-resistant mammary gland (R-MG). A third group of mice, the FVB non-transgenic mice was used as control (C).

3.2. RNA Isolation

3.2.1. Total RNA Isolation from Tissue

Approximately 100-150 mg of tissue or 50-70 mg of tumor tissue was homogenized using a Polytron PT1200 motorized homogenizer (Polytron; Bad Wildbad, Germany) in 2 ml of cold TRlzol (Invitrogen, Carlsbad, CA). Four hundred µl of chloroform (Fisher Scientific, Pittsburgh, PA) was used to extract the RNA from the homogenate. Samples were centrifuged at 12,000 times gravity (g) for 15 min at 4°C.
The aqueous upper phase (~600 µl) was collected and RNA was precipitated with 900 µl of isopropyl alcohol (Sigma, St. Louis, MO). Samples were centrifuged again at 12,000 g for 15 min at 4°C. RNA precipitates were washed twice with 1 ml of cold 75% ethanol and allowed to dry thoroughly. The RNA precipitates were dissolved in 100 µl of DEPC water (ICN Biomedicals, Irvine, CA) and stored at -80°C. RNA was quantified by UV spectrophotometry.

3.2.2. Total RNA Isolation from Cells

Approximately 375 µl of cold TRIZol was added per well (35 mm) to lyse cells. Cell lysates were then collected and RNA was extracted with 100 µl of chloroform. Samples were centrifuged at 12,000 g for 15 min at 4°C. The aqueous upper phase (~200 µl) was collected and RNA was precipitated with 250 µl of isopropyl alcohol (Sigma). Samples were then handled in a similar manner than for the tissue RNA as described above.

3.3. Microarray Analysis

Microarray technology was used to compare the gene expression profiles of the mammary glands (R-MG, R and C) of the three groups of mice specified in 3.1. above. The experimental design is schematically represented in Figure 3B.

3.3.1. Sample Preparation

The mammary gland tissues were dissected from three mice (n=3) of 11 months old for each group of mice (R-MG, R and C). Total RNA was extracted from each mammary gland and equal amounts of the three total RNA samples were pooled into one sample per group. Pooling of samples was performed to help control for variations from one mouse to the other and for variations in the stages of mammary gland differentiation.
depending on the period of the estrous cycle. These RNA samples were further purified with Qiagen RNeasy cleanup procedure following manufacturer’s instructions (Qiagen, Valencia, CA). The purity and quantity of each sample was assessed on an Agilent Technologies Bioanalyzer and the RNA 6000 Nano Lab Chip, following manufacturer’s instructions (Agilent Technologies, Santa Clara, CA). Sample concentration was adjusted to approximately 1 µg per µl with DEPC water.
Figure 3. Nomenclature of the different groups of mice used in the study and flow chart of the experimental design. A) Three groups of virgin female mice were defined as follows: 1) Tumor-susceptible MMTV/neu transgenic mice that develop mammary tumors (S), 2) Tumor-resistant MMTV/neu transgenic mice that do not develop tumors (R) and 3) non-transgenic FVB mice used as control (C). Mammary gland isolated from the S group is defined as resistant mammary gland (R-MG). B) The experimental design consisted in the following steps: 1) dissecting the mammary glands from mice (R-MG, R and C) (n=3/group), 2) extracting the total RNA, 3) microarray analysis (Affymetrix Resource Group) and 4) comparison of the gene expression profiles.
3.3.2. **Affymetrix Microarray**

Samples were sent to the Keck Affymetrix Resource Group at Yale University (http://info.med.yale.edu/wmkeck/affymetrix) which is a full service cost recovery unit that carries out sample processing (cDNA and cRNA preparation), array hybridization and array data analysis. The GeneChip Mouse Expression MOE430A (Affymetrix) was employed for analysis. It contains approximately 22,000 probe sets representing transcripts and variants from over 14,000 well-characterized mouse genes. The Affymetrix Microarray 5.0 (MAS) software, which provides instrument control, data acquisition and data analysis for the entire GeneChip platform, was used to inspect hybridization artifacts and to detect changes in gene expression between samples by comparison analysis of fluorescence intensity values for each probe. The software formats the comparative analysis of two samples into one .txt file which contains the change fold, change call and the associated p-value which indicates statistical significance for detection and change calls. Microsoft Excel and GeneSpring GX (Agilent Technologies) software programs were then used for further data analysis to determine significant changes in gene expression profiles. The microarray analysis was repeated with new total RNA samples prepared from a second set of mice in order to obtain two sets of microarray data.

3.4. **Reverse Transcription-PCR (RT-PCR)**

Reverse Transcription (RT)-PCR technique was used to determine the expression levels of mRNA of various genes in a semi-quantitative manner. Specific pairs of forward and reverse primers were designed for the various RT-PCR analyses as listed in Table 1. One microgram of total RNA was added to the PCR mix included in the
Reverse Transcription-PCR kit (Promega, Madison, WI) with 1 µl of appropriate primers (10µM) and 0.5µl of reverse-transcriptase enzyme in a 25 µl total reaction. The RT-PCR analysis was carried in a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA). Total RNA was first reverse-transcribed for 45 min at 48°C. The cDNAs were initially denaturated at 95°C for 1 min and then amplified by 35 cycles of PCR using the following standard conditions: denaturation at 94°C for 1 min, annealing for 30 sec (optimal annealing temperatures varied depending on the melting temperature of the primers) (see Table 1), and extension at 72°C for 1 min. A final extension step at 72°C for 6 min was added. PCR conditions were modified for the amplification of the Rassf3 gene and consisted of: denaturation at 94°C for 1 min followed by a combined step of annealing and extension at 68°C for 1.5 min. The final extension step was carried at 68°C for 6 min. RT-PCR samples were stored at -80°C until further use. Ten µl of the RT-PCR product was resolved on a 1% agarose gel-electrophoresis and visualized by ethidium bromide staining and autoradiography. To control the total amount of RNA used, amplification of the G3PDH housekeeping gene was carried simultaneously.

Total RNA samples from pairs of human tumors and adjacent normal tissues were purchased from Ambion Inc. (Austin, TX). For these samples, RT-PCR analyses were conducted in duplicate with 0.5 µg of total RNA and with specific human RASSF3 primers. The number of PCR cycles was limited to 25 cycles. The photographs of gel electrophoreses were further analyzed with the Kodak 1D Image Analysis software (Eastman Kodak Company Molecular Imaging Systems; Rochester, NY) to compare the mean intensity of the bands. The mean intensity was set to 100% for the normal breast
tissue. The data were calculated as relative percentage of RASSF3 expression in the different samples.

Table 1. Pairs of primers used for RT-PCR and PCR analyses with mouse (m) or human (h) tissues and cell lines and corresponding PCR annealing temperature.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers Name</th>
<th>Sequences</th>
<th>Amplicon (bp)</th>
<th>T (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mMup1</td>
<td>mMup1 for mMup1 rev</td>
<td>5'-AAATGAAGATGCTGCTGCTG-3' 5'-ATTCTTTCAATTCTCGGGCCCTG-3'</td>
<td>549</td>
<td>54</td>
</tr>
<tr>
<td>SOCS2*</td>
<td>Socs2 for Socs2 rev</td>
<td>5'-TTGACTCATCTCTCCCATGACC-3' 5'-GCTGCATTCCGGAGATAGTCT-3'</td>
<td>688</td>
<td>54</td>
</tr>
<tr>
<td>mEtv1</td>
<td>mEtv1 for mEtv1 rev</td>
<td>5'-GGAGCAGAATGGATGATT-TT-3' 5'-GGGTTACTCATGTGATGACAC-3'</td>
<td>1400</td>
<td>54</td>
</tr>
<tr>
<td>hETV1</td>
<td>hEtv1 for hEtv1 rev</td>
<td>5'-GGAGCAGAATGGATGATT-TT-3' 5'-GTTAATAACCTACGTACCTCG-3'</td>
<td>1400</td>
<td>54</td>
</tr>
<tr>
<td>mRassf1</td>
<td>mRassf1 for mRassf1 rev</td>
<td>5'-ACCTTCCTTCCAATAGCCACATCCACAGCAG-3' 5'-GGACTTTTAAAGGACTGGGCAC-3'</td>
<td>300</td>
<td>68</td>
</tr>
<tr>
<td>mRassf2</td>
<td>mRassf2 for mRassf2 rev</td>
<td>5'-ACACGATGACGACCGGCGTACAGCAG-3' 5'-GAAGACTTTTAAGGACTGGGCAC-3'</td>
<td>400</td>
<td>68</td>
</tr>
<tr>
<td>mRassf3</td>
<td>mRassf3 for mRassf3 for</td>
<td>5'-GCTGACTGACGACCGGCTACGACAG-3' 5'-ACCCGTCCTCCGCTACTCCAC-3'</td>
<td>690</td>
<td>68</td>
</tr>
<tr>
<td>hRASSF3</td>
<td>hRassf3 for hRassf3 rev</td>
<td>5'-GCTGACTGACGACCGGCTACGACAG-3' 5'-GTCGACCTTAATGCAAGGCTTCCAC-3'</td>
<td>690</td>
<td>68</td>
</tr>
<tr>
<td>mRassf4</td>
<td>mRassf4 for mRassf4 rev</td>
<td>5'-CCTGCGCTGCGCTAATTGTAAATTC-3' 5'-ATGGACTTGGGAACTGCTTTGGG-3'</td>
<td>500</td>
<td>68</td>
</tr>
<tr>
<td>mRassf5</td>
<td>mRassf5 for mRassf5 rev</td>
<td>5'-CCTGCGCTGCGCTAATTGTAAATTC-3' 5'-ATGGACTTGGGAACTGCTTTGGG-3'</td>
<td>600</td>
<td>68</td>
</tr>
<tr>
<td>G3PDH *</td>
<td>G3PDH for G3PDH rev</td>
<td>5'-ACCACGATCCATGCACAC-3' 5'-TCCACACACCTGTGCTGTA-3'</td>
<td>453</td>
<td>Varied</td>
</tr>
</tbody>
</table>

*: used for both mouse tissues and human cell lines or tissues.
3.5. Cloning and Plasmid Construction

The 699 bp mouse Rassf3 cDNA was first PCR amplified (Clontech PCR Kit, Mountain View, CA) from 17 day-mouse embryo cDNA using a forward primer (5’-GCT AGC ATG AGC AGC GGC TAC AGC AG-3’) with a Nhe I restriction site and a reverse primer (5’-ACC GGT GCC GGG CTT CCA CAC CTC GC-3’) containing an Age I restriction site, following the conditions described above. The stop codon of Rassf3 was replaced with the Age I restriction site. The PCR product was ligated with pCR2.1 T/A cloning vector and transformed into E. coli TOP10 cells obtained from Invitrogen. Positive clones were identified by plating the cells on Luria-Bertani (LB) agar plates containing 100 µg/ml ampicillin and 80 µg/ml 5-bromo-4-chloro-3-indolyl-bD-galactoside (X-gal). The plasmids were isolated using the QIAprep® Spin Miniprep Kit (Qiagen). The Rassf3 DNA sequence was confirmed by sequencing analysis. The E. coli TOP10 cell cultures, producing the positive clones, were mixed with glycerol and frozen at -80°C for storage.

The Rassf3 DNA was then subcloned into the pcDNA3.1-His Tag expression vector (Invitrogen) and fused to the cytomegalovirus (CMV) promoter/enhancer. The Rassf3 DNA was isolated by restriction digestion with Kpn I and Age I restriction sites, separated by electrophoresis, purified from the agarose gel using the QIAquick® Gel Extraction Kit (Qiagen), and ligated with Kpn I and Age I cut pcDNA3.1-His Tag vector to create the pcDNA3.1/Rassf3-His Tag plasmid. Chemically competent E. coli TOP10 cells were transformed with pcDNA3.1/Rassf3-His Tag and propagated overnight at 37°C with agitation in LB broth containing 100 µg/ml ampicillin.
The positive clones were identified and handled in a similar manner as described above. The Rassf3-His Tag DNA sequence was also confirmed by sequencing analysis.

To subclone the 723 bp Rassf3-His Tag DNA into the pMSG expression vector (driven by the mouse mammary tumor virus long terminal repeat MMTV LTR) (Amersham Biosciences, Arlington Heights, IL), the DNA fragment from the pcDNA3.1/Rassf3-His Tag vector was amplified using the same forward primer as described above, but with a different reverse primer (5’-CCG GAG TTA CTA CTA CTA CTA C-3’) which was designed to hybridize to the 6x Histidine Tag and stop codon, containing an Xho I restriction site at the 3’ end. The PCR product was handled in a similar manner as described above. The Rassf3-His Tag fragment was isolated by restriction digestion with Nhe I and Xho I restriction sites, separated by gel electrophoresis, purified and ligated with Nhe I and Xho I cut pMSG vector to create the pMSG/Rassf3-His Tag plasmid. The sequence was confirmed by sequencing analysis as well. The pcDNA3.1/H-RAS G12V-Ha vector and pcDNA3.1/H-RAS S17N-Ha vector were purchased at UMR cDNA Resource Center (Rolla, MO). The maps of the different plasmids are presented in Figure 4.
Figure 4. Maps of the plasmids used in the study. The mouse Rassf3 gene was PCR amplified from mouse embryo tissue and inserted into the pCR2.1 T/A cloning vector (pcR2.1/Rassf3). After sequence verification, the Rassf3 cDNA was subcloned into a mammalian expression vector (pcDNA3.1/Rassf3-His). The Rassf3 cDNA was further subcloned into the bacterial expression pET22b(+) vector (pET22b(+)/Rassf3-His) for production of the RASSF3 protein. The Rassf3 cDNA was also subcloned in the pMSG expression vector (pMSG/Rassf3) for transgenic mice production. The pcDNA3.1/H-RAS(G12V)-HA and pcDNA3.1/H-RAS(S17N)-HA were purchased from UMR cDNA Resource Center and used for transient transfection assays.
3.6. **RASSF3 Protein Production**

The *Rassf3* cDNA was amplified by PCR from the pcDNA3.1/His Tag vector with specific primers. The forward primer sequence (5′-GAC ATA TGA GCA GCG GCT ACA GCA G-3′) was designed to contain a *Nde I* restriction site and the reverse primer (5′-CCG GAG TTA CTA CTA CTA CTA CTA C-3′) was designed to hybridize to the 6x Histidine Tag and stop codon, and contained an *Xho I* restriction site at the 3′ end. The *NdeI-Rassf3-His* Tag cDNA fragment was amplified by 30 cycles using the following conditions: denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1.5 min, followed by a final extension step at 72°C for 6 min. The PCR product was ligated with pCR2.1 T/A cloning vector, then excised using external *Nde I* and *Xho I* sites and ligated into pET22b(+) vector (Novagen, Madison, WI). Fifty microliters of chemically competent *E. coli* BL21 (DE3) Rosetta cells (Invitrogen) were transformed with pET22b/*Rassf3-His Tag* plasmid and propagated overnight at 37°C with agitation in LB broth containing 100 µg/ml ampicillin. The next morning, 20 ml of starter culture was inoculated in 500 ml of LB broth for additional growth until the O.D.₆₀₀ reaches 1.0 and then induced for protein production with 1mM isopropyl β–thiogalactoside (IPTG) (Alexis Biochemicals, San Diego, CA). After three hours of induction the cells were harvested by centrifugation at 5000 g for 10 min and resuspended in Solution 1 (0.2M NaPO₄; 10 mM EDTA, pH 8.0; 0.5% Triton X-100; pH 8.0) to which was added 0.1 mg/ml lysozyme (Fisher Scientific). The cells were incubated for 1 hour at room temperature to weaken the cell membranes and then sheared on ice with five 1-min ultra-sonic dismemberment using a 550 Sonic Dismembrator (Fisher Scientific). The insoluble fraction containing the inclusion bodies was recovered
by centrifugation at 12,000 g for 30 min at 4°C and resuspended in Solution 2 (0.2M NaPO₄; 10 mM EDTA, pH 8.0; 0.5% Triton X-100; 1 M urea; pH 7.0). The inclusion bodies were then collected by centrifugation at 12,000 g for 30 min at 4°C and frozen at -80°C. Approximately 0.01 g of pellet was solubilized and denatured in 10 ml of Solution 3 (0.2M NaPO₄; 8 M urea; 1% v/v β-mercaptoethanol; pH 8.0) and the protein concentration was determined by the Bradford method (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA). Protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Pierce, Rockford, IL) and stained with SYPRO® Orange staining (Molecular Probes, Eugene, OR) for purity identification. The RASSF3 protein was used as a positive control for each immunoblotting with customized anti-RASSF3 antibody.

3.7. Custom Antibody Design and Production

Custom polyclonal antibodies to mouse RASSF3 protein were produced by Proteintech Group Inc. (Chicago, IL) (http://www.PTGLab.com). General rules were used to select two antigen peptides: the RASSF3-Nt (Li621) peptide composed of the following sequence N'-MSSGYSSLEEDTFFART-C and the RASSF3-AA54 (Li622) consisting of the following sequence N'-KEKVHKYNSAVTDKLKMT-C. Approximately 40 mg of the two antigen peptides were synthesized by Proteintech Group Inc. at approximately 90% purity for 20 mer-peptides with Mass Spectrum data to ensure the finest sample for immunization. Ten mg of peptide was conjugated with 5 mg of Keyhole-limpet hemocyanin (KLH) carrier protein. The conjugated peptides were used to immunize rabbits (#S1228-1, S1228-2, S1229-1, and S1229-2) (two rabbits per peptide). The standard long protocol of 3.5 months was preferred. This protocol was
composed of the following steps: Day 0: pre-immune bleed; Day 1: primary injection; Day 28: boost 1; Day 42: boost 2; Day 56: test bleed and Elisa for Titer; Day 60: boost 3; Day 74: production bleed; Day 78: boost 4; Day 88: production bleed; Day 102: Final bleed. Rabbits were given 4 booster injections to increase the success rate of the immunization protocol.

Titer levels of the test bleeds were tested by ELISA as indicated in Figure 5A. The titer levels are 1:1,000,000 for rabbit S-1228-1, 1:1,000,000 for rabbit S-1228-2, 1:1,000,000 for rabbit S-1229-1 and 1:100,000 for rabbit S-1229-2. Each rabbit yielded approximately 13 ml of serum in each production bleed and 60 ml of serum in the final bleed, corresponding to a total of about 86 ml of antiserum per rabbit per antigen peptide. The crude antiserums were stored at -20°C until use. Once thawed, sodium azide was added to a final concentration of 0.02% and the antiserums were stored at 4 °C for up to a month.

The RASSF3-Nt serum from rabbit S-1228-1 turned out to be more reactive against RASSF3 protein than RASSF3-Nt serum from rabbit S-1228-2 and from RASSF3-AA54 serums, determined by immunoblot analysis (data not shown). The RASSF3-Nt (S-1228-1) from the first production bleed (day 74) was tested at a dilution of 1:500 for blotting of a nitrocellulose membrane containing various amounts of RASSF3 protein as indicated in Figure 5B. This latter antiserum was used as RASSF3 antibody throughout the experiments.
Figure 5. Elisa testing of the anti-mouse RASSF3 polyclonal antibodies and Western blot analysis of RASSF3-Nt (S-1228-1) antibody. A) The titer levels of test bleeds from rabbits immunized with either RASSF3-Nt (Li621) or RASSF3-AA54 (Li622) peptides were determined by ELISA. B) The specificity of RASSF3-Nt (S-1228-1) antibody to RASSF3 protein was tested by Western blot analysis. The RASSF3-Nt (S-1228-1) serum from the first bleed was tested at a dilution of 1:500 for blotting of a nitrocellulose membrane containing various amount of RASSF3 protein as indicated.
3.8. **Cell Culture and Reagents**

The following Human Breast Cancer cell lines, SKBR3, BT-474, MDA-MB-453, BT-483, MDA-MB-134, T-47D, MCF-7, MDA-MB-468, MDA-MB-436 and MDA-MB-231 were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained in medium containing 10 µg/ml gentamicin at 37°C with humidity and 5% CO₂ unless otherwise mentioned. HC11 Murine Mammary Cell line was kindly provided by Dr. Ameae Walker (University of California, Riverside, CA) and the MCNeuA and N202Fb3 Murine Mammary Cells by Dr. Mike Campbell (University of California, San Francisco, CA).

All media and supplements were purchased from Invitrogen unless otherwise mentioned. BT-474, SKBR3, T-47D, MCF-7 and MDA-MB-436 cells were maintained in RPMI Medium 1640 supplemented with 10% fetal bovine serum (FBS) (Invitrogen). BT-483 cells were maintained in RPMI Medium 1640 supplemented with 20% fetal bovine serum (FBS), 2.5 mg/ml glucose (Sigma), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM sodium pyruvate, and 10 µg/ml insulin (Sigma). MDA-MB-134 cells were maintained in Leibovitz's L-15 Medium supplemented with 20% FBS without CO₂. MDA-MB-453 and MDA-MB-231 cells were maintained in Leibovitz's L-15 Medium supplemented with 10% FBS without CO₂. MDA-MB-468 cells were maintained in Leibovitz's L-15 Medium supplemented with 10% FBS and 2 mM L-glutamine without CO₂. MCNeuA and N202Fb3 cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1 mM sodium pyruvate. HC11 cells were maintained in RPMI Medium 1640.
supplemented with 10% FBS, 1 mM sodium pyruvate, 10 µg/ml insulin and 10ng/ml EGF.

EGF solution was purchased from Cambrex (East Rutherford, NJ) and prepared to a final concentration of 200 ng/ml, aliquoted, and stored at -20°C. Camptothecin was purchased from Sigma and dissolved in dimethyl sulfoxide (DMSO) (20mM) and stored at 4°C. Working solutions of EGF (60 ng/ml) and camptothecin (50 mM) were prepared in culture medium immediately before use.

3.9. **Protein Isolation and Western Blot Analysis**

3.9.1. **Protein Isolation from Tissues**

Fresh mammary gland, brain or mammary tumor tissue was excised, frozen immediately on dry ice, and stored at -80°C until use. Approximately 100-150 mg of frozen tissue was used for protein extraction. Tissue was homogenized in 1ml of Lysis Buffer (50 mM Tris·HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA, pH 8.0) containing protease inhibitors (1 µg/ml aprotinin; 1 µg/ml leupeptin; 1 µg/ml pepstatin; 170 µg/ml phenylmethylsulphonyl fluoride (PMSF); 180 µg/ml sodium orthovanadate; 50mM sodium fluoride), using a Polytron PT1200 motorized homogenizer. The lysates were transferred to 1.5 ml tubes, passed six times through 21-gauge needles, incubated on ice for 20 min and then clarified by centrifugation for 15 min at 14,000 rpm at 4°C. The lysates were then transferred into a new 1.5 ml tubes and stored at -20°C. The protein content was determined against bovine serum albumin (BSA) standards using the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL).
3.9.2. Protein Isolation from Cells

The cells were washed with ice cold phosphate buffer saline (PBS) and lysed in 200 µl of Lysis Buffer (50 mM Tris·HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA, pH 8.0) containing protease inhibitors (1 µg/ml aprotinin; 1 µg/ml leupeptin; 170 µg/ml PMSF; 180 µg/ml sodium orthovanadate). The lysates were agitated for 10 min on an orbital shaker, transferred to 1.5 ml tubes and processed as described above.

3.9.3. Western Blot Analysis

Samples containing the same amount of proteins (50-100 µg) were mixed with 6X SDS-PAGE sample buffer and heated at 100°C for 5 min. The proteins were separated using 4-15% (for Cytochrome C and Bax proteins) or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Laboratories, Hercules, CA) at 100 volts for 80 min. Proteins were transferred to a Hybond Nitrocellulose membrane (Amersham Biosciences) at 25 volts overnight. A full range molecular weight marker (Amersham Biosciences) was used to confirm the protein size as well as the efficacy of transfer. Following transfer, the membranes were blocked in tris-buffer saline (TBS) containing 5% non-fat powdered milk and 0.05% Tween-20 for 1 to 2 hours (TBS-T). Membranes were incubated with the primary antibody at different dilution in TBS-T/5% milk, overnight at 4°C with gentle agitation. The membranes were washed once with TBS-T for 5 min and then incubated with the secondary antibodies for 2 hours at room temperature. The membranes were washed additionally three times with TBS-T before incubation with the ECL™ Western Blotting Detection Reagents (Amersham Biosciences), as per the manufacturer’s instructions. To visualize the bands, the
membranes were exposed to Kodak Biomax MR film (Fisher Scientific) and developed with Konica SRX-101A processor (Konica Minolta Medical Imagining, Wayne, NJ).

For additional probing, membranes were stripped by incubation in 100mM β-mercaptoethanol /2%SDS/62.5 mM Tris-HCl pH 6.7 at 50°C for 30 min and re-probed with another primary antibody. To confirm that equivalent amounts of total protein were added to each well, the membranes were stripped and re-probed with anti-β-actin monoclonal antibody for analysis of cell lysates or anti-β-tubulin monoclonal antibody for analysis of tissue homogenates.

3.9.4. Antibodies

The following primary antibodies were used at the following dilutions: 1:1000 anti-phospho-HER2/neu (Lab Vision; Fremont, CA) for cell lysate and 1:400 anti-phospho-HER2/neu (Santa Cruz Biotechnology, Santa Cruz, CA) for tissue homogenate respectively; 1:1000 anti-HER2/neu (EMD Biosciences; Darmstadt, Germany); 1:500 anti-Cyclin D1 (Invitrogen) for cell lysates; 1:1000 anti-phospho-JNK (Cell Signaling Technology, Danvers, MA); 1:1000 anti-RAS (Cell Signaling Technology); 1:1000 anti-Cytochrome C (Santa Cruz Biotechnology); 1:10,000 anti-β-Actin (Millipore, Billerica, MA); 1:10,000 anti-β-Tubulin (Sigma). Anti-phospho-Akt 1/2/3; anti-phospho-Erk1/2 and anti-BAX were obtained from Santa Cruz Biotechnology and used at 1:1000. The secondary antibodies, goat anti-mouse IgG- and goat anti-rabbit IgG-horseradish peroxidase-conjugates were obtained from Bio-Rad Laboratories (Hercules, CA) and used at 1:2000.
3.10. Transient Transfection

Cells were seeded in six-well plates (35mm) at a concentration ranging from 3.2 to 3.8x10^5 cells per well, depending on the cell type, in order to reach 60-80% confluency, 24 hours after seeding. Cells were starved for 2 hours with Opti-MEM® Reduced Serum Medium (Invitrogen) before transfection. Transient transfections of 1, 2, 3 or 4 µg of DNA plasmids (pcR3.1 vector, pcDNA3.1/Rassf3, pcDNA3.1/H-RAS (G12V) or pcDNA3.1/H-RAS (S17N)) alone or in different combinations (co-transfections) were performed using LipofectAMINE 2000 Reagent (Invitrogen) according to the manufacturer’s recommendations. The total amount of transfected DNA was maintained constant between compared samples. The DNA-lipofectamine transfection mixtures were applied on cells for 6 to 8 hours and then replaced by fresh growth media. Transient transfected cells were harvested after 24 hours for protein lysates and after 48 hours for apoptosis and proliferation assays.

3.11. Cell Proliferation MTS/PMS Assay

The transient transfected cell lines were harvested 48 hours after transfection, counted and seeded in 96-well culture plates (6 to 8 wells per cell line) at a density of 10,000 cells per well in 200 µl of appropriate culture medium and grown as recommended. After 24, 48, 72, 96 or 120 hours, the culture medium was removed and 100 µl of MTS/PMS solution (Promega Corp., Madison, WI) in PBS buffer (1:6 dilution) was added to each well. The mixture was incubated for 3.5 hours at 37°C in a humidified 5% CO₂ atmosphere. The viability of the cells was determined by colorimetric measurement of the reduction of MTS by the living cells using a Benchmark microplate reader (Bio-Rad) measuring absorbance at 490 nm. All transfection assays were
performed in triplicate for BT-474, MCNeuA and HC11 cell lines and repeated 8 times for SKBR3 cell line. The cell viability was calculated as a percentage of control, corresponding to pcR3.1 vector transfected cells. The data are represented as the mean ± standard error of the mean (SEM) for all experiments.

3.12. Apoptosis Assay

SKBR3 cells were seeded in six-well plates at a concentration of 3.6x10^5 cells per well. After 24 hours, cells were transiently transfected with 2 µg of pcR3.1 vector, 4 µg of pcR3.1 vector, 2 µg of pcDNA3.1/Rassf3 and 4 µg of pcDNA3.1/Rassf3 as described above. Untransfected SKBR3 cells and camptothecin-treated SKBR3 cells (50µM; 24 hours) were used as negative control and positive control for induction of apoptosis, respectively. Each sample was prepared in triplicate. Six hours later, the transfection mixture was removed and 1.5 ml of fresh media was added per well. After 48 hours the nonadherent apoptotic cells in the medium and the viable adherent cells from each individual well were collected and transferred into 1.5 ml tubes and centrifuged at 310 g for 5 min at 4°C. The pellets were re-suspended in approximately 150 µl of growth media and kept on ice. The re-suspended cells were diluted 1:10 to 1:20 in Guava® ViaCount® Reagent (Guava Technologies, Hayward, CA) which contains two DNA-binding dyes, one of which selectively penetrates apoptotic and dead cells characterized by compromised membrane integrity and one which penetrates all nucleated cells. Following a five minute incubation, the stained cells were analyzed using a Guava Personal Cytometer. Each sample was analyzed using Guava CytoAnalysis software. The amount of live/healthy cells, apoptotic cells and dead cells were expressed as a
percentage of the total cellular population for each sample. Data are expressed as mean ± SEM and are representative of two experiments.

3.13. **Generation and Study of Transgenic Mice**

3.13.1. **Generation of MMTV/Rassf3 Transgenic Mice**

The 723 bp mouse *Rassf3-His Tag* cDNA was subcloned into the pMSG expression vector (pMSG/Rassf3-His Tag) and fused to the MMTV-LTR promoter. The 3,180 bp MMTV-Rassf3-His Tag cDNA fragment was digested with *Tth111 I* and *BamH I* enzymes, separated by electrophoresis, purified from the agarose gel, eluted in microinjection TE buffer and microinjected into fertilized mouse single-cell embryos of FVB mice (The Jackson Laboratory, Bar Harbor, ME) following standard protocol (Wagner T. *et al.*, 1981). The tyrosinase gene, responsible for the agouti fur color was co-injected with the transgene of interest in order to have a convenient color marker. Founder animals identified as transgene-positive mice were selected for further analysis and for breeding. Three founder lines were generated but only the MMTV/Rassf3 transgenic mice line #13 was expanded by mating MMTV/Rassf3 male mice with FVB female mice. All animals were maintained in barrier facilities and cared for in accordance with the Institutional Animal Care and Use Committees (IACUC) guidelines and policies.

3.13.2. **Generation of MMTV/Rassf3-neu Bi-Transgenic Mice**

The MMTV/neu transgenic mice line (N#202) was purchased from the The Jackson Laboratory (Bar Harbor, ME) and expanded in our animal facility. MMTV/Rassf3 heterozygotic male mice were bred with MMTV/neu homozygous females to produce a new MMTV/Rassf3-neu bi-transgenic mice line. All offspring were
heterozygous for MMTV/neu\(^{+/−}\) transgene and 50% were positive for the MMTV/Rassf3 transgene and had the agouti fur color.

### 3.13.3. Identification of Transgenic Mice

The incorporation of the transgene was confirmed by PCR analysis using genomic DNA isolated from ~0.5 cm mouse tail and digested with proteinase K in Direct PCR (tail) Lysis Reagent (Viagen Inc., Los Angeles, CA). Specific primers for the transgene were used. The forward primer (5′-TGT TTG TGT CTG TTC GCC AT-3′) was designed to anneal to a region on the MMTV promoter sequence whereas the reverse primer (5′-TTT GCA GAG TTC CAT CTG CAC-3′) was designed to anneal to a region located in the middle of the Rassf3 sequence. PCR was performed on a thermocycler using 35 cycles of the following program: 94°C for 30 sec, 58°C for 1 min, 72°C for 1 min, followed by a final extension step at 72°C for 6 min. PCR products were analyzed by electrophoresis on a 1% agarose gel. Transgene incorporation was checked for every colored mouse from F1, F2 and F3 generations as well as for some white littermate mice as negative control. Since every colored mouse showed positive transgene insertion and the white littermates were always transgene-negative, the fur color was then the only marker used to further identify the positive transgenic mice.

### 3.13.4. Tumor Incidence and Recurrence

Virgin female MMTV/Rassf3-neu bi-transgenic mice and virgin female MMTV/neu\(^{+/−}\) littermates were checked weekly for the development of primary mammary tumors. The data are plotted as the percentage of tumor-free animals as a function of age in days. Primary tumors were surgically removed from anesthetized mice once the tumor dimension reached between 7 to 11 mm and the wounds were closed with...
surgical staples. At the time of surgery, the tumor dimensions were recorded and calculated on the assumption of an ellipsoidal volume: \( V = \frac{4}{3} \pi \left( \frac{A}{2} \right)^2 \left( \frac{B}{2} \right) \) with “A” as the short dimension and “B” as the long dimension. The mice were then inspected for the recurrence of the primary tumor at the site of surgery or for the development of a secondary tumor at a different site, in one of the other mammary glands. The index of the inverse of tumor aggressiveness (S) was calculated based on the following formula: \( S = T_1 + T_2 \times \frac{1}{V} \). The formula was developed based on the fact that the tumor aggressiveness (S) is a function of different factors including the mouse’s age at the time of appearance of the primary tumor (T₁), the mouse’s age at the time of primary tumor recurrence or secondary tumor development (T₂) and the tumor volume (V) at the time of surgery. The formula indicates that the higher the index is, the less aggressive is the tumor. S is expressed in arbitrary units. The statistical differences between the groups were determined using the non-parametric Kruskal-Wallis and Dunn’s multiple comparison tests.


The posterior (fourth or fifth inguinal) mammary gland was dissected from female mice in the diestrus stage of the estrous cycle and placed on a glass slide. The fresh tissue was fixed overnight in freshly prepared Carnoy’s fixative at room temperature. The following day, the glands were systematically re-hydrated in ethanol/water solutions of decreasing ethanol concentration over a period of 2 hours. The glands were then stained overnight in Carmine Alum Stain. The next day, the glands were dehydrated with ethanol/water solutions containing systematically increasing ethanol concentration over a period of 90 min. The fat pad was cleared with xylene (Sigma) for approximately 1 hour.
A cover-slip was then secured over the glands with SecureMout (Fisher Scientific). The glands were documented via digital photography and photos were compared for mammary gland morphology.

3.15. **Statistical Analyses**

Statistical analyses were performed using GraphPad Prism software. Survival curves were computed using the method of Kaplan-Meier and the statistical differences were calculated using the Log Rank test. Statistical differences between the groups were determined using one-way ANOVA and Tukey’s multiple comparison tests unless otherwise mentioned.
4. RESULTS

4.1. Comparison of the Mammary Gland Morphology of the MMTV/neu Transgenic Mice

The morphology of the mammary gland of 6 month old virgin female MMTV/neu mice, which overexpress the *neu* oncogene in the mammary gland, was compared to the mammary gland morphology of age-matched non-transgenic virgin female FVB mice as shown in Figure 6. Similar degrees of ductal elongation and density were observed; however, the MMTV/neu mice displayed a significant increase in terminal end bud formation and lobulo-alveolar structures as compared to the non-transgenic mice. These changes reflected a higher degree of proliferation in the mammary gland of the MMTV/neu mice.
Figure 6. Images of the mammary gland whole mounts of MMTV/neu transgenic mice (R) and non-transgenic FVB mice (C). The fourth inguinal mammary glands were dissected from MMTV/neu transgenic mice (R) and non-transgenic FVB mice (C) at 6 months of age. The mammary glands were mounted and stained in carmine alum stain. The whole mounts were digitally photographed to compare the branching density, the bud formation and lobulo-alveolar structures in the mammary glands. The images are representative of three mice from each group. Inserts correspond to an enlargement of the smaller regions indicated by the boxes.
4.2. Identification of Differentially Expressed Genes by cDNA Microarray Analysis

The total RNA samples prepared from mammary glands of tumor-susceptible MMTV/neu transgenic mice (R-MG), tumor-resistant MMTV/neu transgenic mice (R) and FVB non-transgenic (C) mice were processed for hybridization to an oligonucleotide microarray to compare the gene expression profiles via the MAS 5.0 software from the Keck Affymetrix Resource Group. The three comparative analyses (R-MG vs. R, R-MG vs. C and R vs. C) are represented by scatter plots as shown in Figure 7. On each scatter plot, the log of the signal intensity of the probe sets are reported for the two compared samples. Attention was accorded to the genes demonstrating at least a 2 fold change in expression between the two compared samples, thus, corresponding to the probe sets located above the upper diagonals or below the lower diagonals indicated on the scatter plots. Interestingly, the majority of the genes that are differentially expressed (2 fold) in R group when compared to C or R-MG are mostly up-regulated (76% and 65% respectively), whereas the genes that are differentially expressed (2 fold) in R-MG group when compared to C (69%) or R are mostly down-regulated.

Two approaches were designed to analyse the microarray data in order to identify the candidate genes which may be responsible for the resistance to HER2/neu-initiated mammary tumor development in tumor-resistant MMTV/neu mice.
Figure 7. Scatter-plot representation of the comparative analysis of samples analyzed by microarray technology. The plots represent the 3 comparative analyses (R vs. R-MG, R vs. C and R-MG vs. C) obtained from microarray analyses. Each dot corresponds to one probe set on the microarray chip. The graphs show the log of the signal intensity of probe sets in the compared samples. The probe sets located above the upper diagonal or below the lower diagonal show a 2 fold difference in signal intensity between the two compared samples. The probe sets colored in red correspond to the genes that show a 2 fold increase in signal intensity in the mammary gland of tumor-resistant MMTV/neu transgenic mice (R) compared to tumor-susceptible MMTV/neu transgenic littermates (R-MG).
4.2.1. Approach 1: Search for Differentially Expressed Genes in Tumor-resistant Mice

The first approach aimed at searching for genes that exhibited an opposing pattern of expression between R and R-MG mammary glands. Particular attention was accorded to the genes that are up-regulated in the mammary glands of tumor-resistant mice (R) but down-regulated in the mammary glands of tumor-susceptible mice (R-MG). The result of this comparison is presented in Table 2. This list includes genes that are involved in different cellular functions such as signal transduction, cell/tissue structure, transport and metabolism. We particularly turned our attention towards genes involved in signal transduction and towards genes which could fit and interact with members of the HER2/neu receptor tyrosine kinase pathway. Three candidate genes were selected for further analysis. Two of these genes encode for intracellular proteins involved in signal transduction and correspond to the Socs2 gene, which is a well-characterized suppressor of cytokine signaling and the novel Rassf3 gene which belongs to the Ras Association domain Family (RalGDS/AF-6) gene. The third gene is the Etv1 gene which belongs to the ETS family of transcription factors.
Table 2. List of genes that are overexpressed in the mammary glands of tumor-resistant MMTV/neu female transgenic mice compared to tumor-susceptible MMTV/neu littermates, obtained by cDNA microarray analysis.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>GenBank</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Signal Transduction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Intracellular signaling molecules</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soc2</td>
<td>NM_007706</td>
<td>2.8</td>
</tr>
<tr>
<td>Rassf3</td>
<td>BC011511</td>
<td>2.4</td>
</tr>
<tr>
<td><strong>Transcription factors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cited4</td>
<td>BC025116</td>
<td>2.2</td>
</tr>
<tr>
<td>Etv1</td>
<td>NM_007960</td>
<td>2.2</td>
</tr>
<tr>
<td>Tcfap2c</td>
<td>BC003778</td>
<td>2.1</td>
</tr>
<tr>
<td>Etv5</td>
<td>BG966751</td>
<td>2.1</td>
</tr>
<tr>
<td>Hey1</td>
<td>BG966751</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>Growth factors and growth factor receptors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mia</td>
<td>NM_019394</td>
<td>3.2</td>
</tr>
<tr>
<td>Tm4sf3</td>
<td>BC025461</td>
<td>2.1</td>
</tr>
<tr>
<td><strong>Cell tissue structure</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cytoskeleton</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mlpc</td>
<td>NM_010861</td>
<td>5.8</td>
</tr>
<tr>
<td>Tncc</td>
<td>NM_009393</td>
<td>3.6</td>
</tr>
<tr>
<td>Tpm3</td>
<td>NM_022314</td>
<td>3.2</td>
</tr>
<tr>
<td>Homer2</td>
<td>AB017136</td>
<td>2.9</td>
</tr>
<tr>
<td>Tnnt1</td>
<td>NM_011618</td>
<td>2.9</td>
</tr>
<tr>
<td>Mlc</td>
<td>X67685</td>
<td>2.9</td>
</tr>
<tr>
<td>Myh7</td>
<td>NM_080728</td>
<td>2.5</td>
</tr>
<tr>
<td>Myh2</td>
<td>BC008538</td>
<td>2.2</td>
</tr>
<tr>
<td>Tnni1</td>
<td>NM_021467</td>
<td>2.1</td>
</tr>
<tr>
<td><strong>Extracellular matrix protein, cell adhesion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Col9a1</td>
<td>NM_007740</td>
<td>3.7</td>
</tr>
<tr>
<td>Col11a1</td>
<td>NM_007729</td>
<td>3.3</td>
</tr>
<tr>
<td>Ceacam10</td>
<td>NM_007675</td>
<td>3.0</td>
</tr>
<tr>
<td>Npnt</td>
<td>AW553512</td>
<td>3.0</td>
</tr>
<tr>
<td>Comp</td>
<td>NM_016685</td>
<td>2.2</td>
</tr>
<tr>
<td>Ceacam1</td>
<td>NM_011926</td>
<td>2.1</td>
</tr>
<tr>
<td><strong>Protein modification, proteases and proteases inhibitor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papln</td>
<td>BC005747</td>
<td>2.4</td>
</tr>
<tr>
<td>Spag5</td>
<td>BM208112</td>
<td>2.4</td>
</tr>
<tr>
<td>Timp4</td>
<td>BI788452</td>
<td>2.0</td>
</tr>
</tbody>
</table>
Table 2. List of genes that are overexpressed in the mammary glands of tumor-resistant MMTV/neu female transgenic mice compared to tumor-susceptible MMTV/neu littermates, obtained by cDNA microarray analysis (Continued).

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>GenBank</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transporter</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Electron transporter</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2410043F08Rik</td>
<td>NM_133754</td>
<td>2.3</td>
</tr>
<tr>
<td><strong>Ion transporter</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kcnn4</td>
<td>NM_008433</td>
<td>2.9</td>
</tr>
<tr>
<td>Kcnk1</td>
<td>NM_008430</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>Other transporter</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aqp5</td>
<td>NM_009701</td>
<td>4.6</td>
</tr>
<tr>
<td>Slc29a1</td>
<td>AF305501</td>
<td>3.0</td>
</tr>
<tr>
<td>Lman1</td>
<td>AK011495</td>
<td>2.0</td>
</tr>
<tr>
<td>Slc21a2</td>
<td>NM_033314</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>Metabolism</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fatty acid biosynthesis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scd2</td>
<td>BG060909</td>
<td>2.0</td>
</tr>
<tr>
<td>B4galt6</td>
<td>BG066773</td>
<td>2.0</td>
</tr>
<tr>
<td>9430020A05Rik</td>
<td>BI153391</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>Glycolysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aldo3</td>
<td>BM941201</td>
<td>3.6</td>
</tr>
<tr>
<td>Hk1</td>
<td>NM_010438</td>
<td>3.0</td>
</tr>
<tr>
<td><strong>Amino acid metabolism</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Got1</td>
<td>AA792094</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2310047E01Rik</td>
<td>AK009873</td>
<td>3.0</td>
</tr>
<tr>
<td>Egln3</td>
<td>NM_028133</td>
<td>2.8</td>
</tr>
<tr>
<td>4930555L11Rik</td>
<td>BG066916</td>
<td>2.4</td>
</tr>
<tr>
<td>Cp</td>
<td>BB531328</td>
<td>2.3</td>
</tr>
<tr>
<td>Rdh12</td>
<td>BC016204</td>
<td>2.1</td>
</tr>
<tr>
<td>Ckmt2</td>
<td>AK009042</td>
<td>2.1</td>
</tr>
<tr>
<td><strong>Other genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xlr3a</td>
<td>NM_011726</td>
<td>3.6</td>
</tr>
<tr>
<td>Scrg1</td>
<td>NM_009136</td>
<td>3.4</td>
</tr>
<tr>
<td>---</td>
<td>BB798279</td>
<td>2.4</td>
</tr>
<tr>
<td>4931430I01Rik</td>
<td>BC019531</td>
<td>2.2</td>
</tr>
<tr>
<td>---</td>
<td>BM239368</td>
<td>2.1</td>
</tr>
<tr>
<td>Emu1-pending</td>
<td>NM_080595</td>
<td>2.1</td>
</tr>
</tbody>
</table>
4.2.2. Approach 2: Search for Differentially Expressed Genes in Tumor-resistant Mammary Tissue

The second analytical approach was designed to identify the candidate genes that were overexpressed in the mammary glands of both tumor-resistant mice (R) and tumor-susceptible mice (R-MG) compared to the control mice (C). The genes up-regulated in the two following comparisons R-MG vs. C (94 genes) and R vs. C (20 genes) were first identified, and then the genes present in both comparisons were selected. Thirteen genes were identified through this second approach out of which only ten have been characterized and reported in the literature (Table 3). This list includes genes involved in cell/tissue structure, cell metabolism and transport. Among those genes, we turned our attention towards a novel gene whose function has not been reported yet. The transporter gene *Mup1*, or major urinary protein 1 which encodes for a secreted protein, was selected as a candidate gene.
Table 3. List of genes that are overexpressed in the mammary glands of both female tumor-resistant MMTV/neu transgenic mice and tumor-susceptible MMTV/neu transgenic mice, compared to the mammary gland of non-transgenic mice.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gen Bank</th>
<th>Fold Change</th>
</tr>
</thead>
</table>
| **Cell tissue structure**  
*Extracellular matrix protein, cell adhesion*  
Col9a1 | Procollagen, type IX, alpha 1 | AK004383 | 11.7 |
| Npnt | Nephronectin | AA223007 | 10.6 |
| **Transporter**  
Aqp5 | Aquaporin 5 | NM_009701 | 9.5 |
| Mup1 | Major urinary protein 1 | NM_031188 | 7.2 |
| **Metabolism**  
Fatty acid biosynthesis  
Facl4 | Acyl-CoA synthetase long-chain family member 4 | AB033886 | 4.0 |
| **Other**  
Lalba | Lactalbumin, alpha | NM_010679 | 9.1 |
| Car6 | Carbonic anhydrase 6 | NM_009802 | 3.7 |
| **Other**  
Secreted protein  
C2a-pending | Androgen binding protein beta | BC024677 | 7.0 |
| Csnk | Casein kappa | NM_007786 | 2.7 |
| Spp1 | Secreted phosphoprotein 1 | NM_009263 | 2.0 |

4.3. Analysis of the Differentially Expressed Candidate Genes

4.3.1. Confirmation of the Differentially Expressed Candidate Genes by RT-PCR Analysis

The expression patterns of the candidate genes selected from the two analytical approaches were confirmed by semi-quantitative RT-PCR analyses on the total RNA samples isolated from the mammary glands of the three groups of mice. As demonstrated in Figure 8, Rassf3, Socs2 and Etv1 genes are overexpressed in the mammary glands of tumor-resistant MMTV/neu mice (R) compared to tumor-susceptible MMTV/neu mice (R-MG) and control mice (C), whereas, the Mup1 gene is overexpressed in the
mammary gland of both tumor-resistant mice (R) and tumor-susceptible mice (R-MG) compared to control mice (C). These observations confirm that Rassf3, Socs2, Etv1 and Mup1 genes are differentially expressed between the R, R-MG and C mammary glands as indicated in the microarray analysis.

![Image of microarray data](image.png)

**Figure 8. Confirmation of the microarray data for the four selected candidate genes by RT-PCR analysis.** Total RNA was isolated from the mammary glands of 11 months old tumor-resistant (R) and tumor-susceptible (R-MG) MMTV/neu transgenic mice and of age-matched non-transgenic mice (C). RT-PCR analyses were conducted using appropriate primers for Rassf3, Socs2, Etv1 and Mup1 genes and the RT-PCR products were resolved on 1% agarose electrophoresis-gels. G3PDH was used as a loading control.
4.3.2. Comparison of Gene Expression of Rassf3, Soc52, Etv1 and Mup1 in Mammary Tumors and Adjacent Normal Tissues

The expression patterns of Rassf3, Soc52, Etv1 and Mup1 genes in the mammary tumors and the adjacent normal mammary tissues were compared. Total RNA was isolated from four pairs of mammary tumors (T) and adjacent normal tissues (R-MG) dissected from MMTV/neu mice that developed tumors at the age of 6, 7, 9 or 11 months and used for RT-PCR analyses (Figure 9). Surprisingly, the results showed that the expression levels of the Rassf3 gene are consistently higher in the mammary tumors (T) than in the adjacent normal mammary tissues (R-MG), independently of the age at which the tumor appeared. Sequencing analyses of the Rassf3 gene cloned from 6 mammary tumors revealed no consensus mutation in the gene (data not shown). The expression levels of the Etv1 gene and, to a lesser extent, the Soc52 gene presented a similar pattern than Rassf3. In contrast, the expression of the Mup1 gene was high in the adjacent normal mammary tissues (R-MG), but totally absent in the tumor tissues (T).
Figure 9. Comparison of gene expression in mammary tumors and adjacent normal tissues by RT-PCR analysis. Total RNA was isolated from pairs of mammary tumors (T) and adjacent normal tissues (R-MG) of MMTV/neu transgenic mice that developed tumors at the age of 6, 7, 9 and 11 months (mo). RT-PCR analyses were conducted using appropriate primers for Rassf3, Socs2, Etv1 and Mup1 genes and the RT-PCR products were resolved on 1% agarose electrophoresis-gels. G3PDH was used as a loading control.

To further study the expression pattern of Rassf3, Socs2, Etv1 and Mup1 genes in tumor tissues, the expression levels of these genes were compared in epithelial cells (MCNeuA) vs. fibroblasts (N202Fb3), both derived from a Her2/neu mammary tumor (Campbell M. et al., 2002). Interestingly, even though Rassf3 expression is high in Her2/neu primary tumor, Rassf3 expression appeared to be absent in both established cell lines (Figure 10A). On the other hand, the Mup1 gene was not detectable in MCNeuA and N202Fb3 cells. This observation was expected since Mup1 expression was not detectable in Her2/neu tumor. The expression of Socs2 and Etv1 genes was significantly
higher in MCNeuA cells compared to N202Fb3 cells, indicating that Socs2 and Etv1 genes are preferentially expressed in epithelial cells (Figure 10A).

The obvious discrepancy of Rassf3 expression levels in established tumor cell lines and the primary tumor tissue, reflects the fact that cells are exposed to different environmental pressure in culture dishes and in whole tissue which can result in a change in gene expression between tumor tissue and isolated cultured tumor cells.

The expression pattern of Rassf3 in the primary cultured cells from a Her2/neu mammary tumor was also examined at different passages (24 and 72 hours) by RT-PCR analysis. The results showed that Rassf3 gene is expressed in primary cells at early passage (24 hours), but that its expression is diminished after further passages (72 hours) (Figure 10B). At the same time, the morphology of the primary culture, recorded under a microscope at 24 hours and 72 hours passages, revealed a switch of the cell population with passages, corresponding to an increase in the fibroblast (F) population and a progressive loss of the epithelial cells (E) population (Figure 10C). This last observation is probably due to the difference in doubling time of these two cell types. This finding suggests that Rassf3 is preferentially expressed in the epithelial compartment compared to the fibroblast compartment.
Figure 10. Comparison of the gene expression patterns in the epithelial vs. fibroblast cells derived from a HER2/neu mammary tumor by RT-PCR analysis. A) Total RNA was isolated from established epithelial cells (MCNeuA) and fibroblasts (N202F3) derived from the mammary tumor of MMTV/neu transgenic mice and analyzed by RT-PCR experiments for Rassf3, Mup1, Socs2 and Etv1 gene expression. The RT-PCR products were resolved on 1% agarose electrophoresis-gels. G3PDH was used as a loading control. B) Total RNA was isolated from a primary HER2/neu mammary tumor of a MMTV/neu mice and from the primary cultured cells of the tumor at 24 hours and 72 hours passages and analyzed by RT-PCR experiment for Rassf3 gene expression. The RT-PCR products were resolved on a 1% agarose electrophoresis-gel. C) The cell morphology of the primary culture at 24 hours and 72 hours passages was recorded under a microscope to demonstrate the different cell populations (F, fibroblast, E, epithelium).
4.3.3. Comparison of the Gene Expression Pattern of the Ras Association domain Family genes (Rassf-1, -2, -3, -4, -5) in Various Mouse Mammary Tissues

The expression patterns of the members of the Rassf family in mammary tumors (T) and adjacent normal mammary tissues (R-MG) of MMTV/neu mice and in mammary glands of age-matched non-transgenic mice (C) were compared by RT-PCR analysis. The results indicated that the expression levels of Rassf3 are higher in tumors than in normal tissue and that it was the lowest in the mammary gland of non-transgenic mice (Figure 11). This expression pattern was consistent, whether the mice developed tumor at 6, 9 or 11 months. On the contrary, the expression levels of Rassf2, Rassf4 and Rassf5 were not significantly different between T, R-MG and C tissues, whereas, Rassf1 gene showed a different pattern of expression between the three tissues at the three different time points. Therefore, these observations strengthen the belief that the Rassf3 gene plays a particular role in HER2/neu-induced breast tumor.
Figure 11. Comparison of the gene expression patterns of the Ras Association domain Family genes (*Rassf*-1, -2, -3, -4, -5) in mouse mammary tissues by RT-PCR analysis. Total RNA was isolated from pairs of mammary tumors (T) and adjacent normal tissues (R-MG) of MMTV/neu transgenic mice that developed tumors at the age of 6, 9 and 11 months old (mo), as well as from mammary glands of age-matched non-transgenic mice (C). RT-PCR analyses were conducted using specific primers for *Rassf1*, *Rassf2*, *Rassf3*, *Rassf4* and *Rassf5* (also known as *Nore1*) genes and the RT-PCR products were resolved on 1% agarose electrophoresis-gels. *G3PDH* was used as a loading control.
4.3.4. Relationship between RASSF3, SOCS2 and ETV1 Gene Expression and HER2/p-HER2 Levels in Multiple Human Breast Cancer Cell Lines

A panel of 10 human breast cancer cell lines was analyzed by Western blot analysis for the levels of HER2 and p-HER2 proteins and by RT-PCR analysis for the expression level of RASSF3 gene. The results indicated that RASSF3 gene expression is inversely correlated to HER2 expression levels among the 10 cell lines (Figure 12A). RASSF3 expression is minimal or non-detectable in SKBR-3 and BT-474 cell lines, which express high levels of HER2 and p-HER2. On the contrary, the levels of RASSF3 expression are high in MB-231 and MB-436 cell lines, which did not show expression of HER2 protein. T47D cells show moderate levels of both endogenous RASSF3 and HER2.

The RT-PCR analysis was further conducted on a panel of 5 human breast cancer cell lines including SKBR-3, BT-474, T47D, MCF-7 and MB-231 to examine the expression levels of SOCS2 and ETV1 genes (Figure 12B). The levels of SOCS2 expression appeared to be inversely correlated to HER2 levels among the 5 cell lines. However, the inverse correlation between SOCS2 expression and HER2 levels was not as obvious as the trend observed for the RASSF3 gene. The ETV1 gene was expressed in MB-231 cells but non-detectable in the 4 other cell lines. The expression of Mup1 gene was not investigated in human breast cancer cell lines since it has no known human homolog.
Figure 12. Correlation between HER2/p-HER2 protein and RASSF3, SOCS2 and ETV1 mRNA levels in multiple human breast cancer cell lines by Western blot and RT-PCR analyses. Protein lysates and total RNA were collected from human breast cancer cells and used for Western blot analyses for HER2 and p-HER2 proteins and RT-PCR analyses for RASSF3, SOCS2 and ETV1 genes. A) HER2 and p-HER2 levels were compared to the levels of RASSF3 mRNA in 10 cell lines. B) HER2 and p-HER2 levels were compared to the levels of SOCS2 and ETV1 mRNA in 5 cell lines. β-ACTIN and G3PDH were used as loading controls.
4.3.5. RASSF3 Expression in Various Human Tumors versus Adjacent Normal Tissues

To assess the status of the RASSF3 gene expression in human tumors compared to adjacent normal tissues, total RNA samples from five pairs of human tumors and adjacent normal tissues from breast, lung, uterus, colon and cervix were used for RT-PCR analysis (Figure 13). The level of RASSF3 expression was higher in the tumor compared to the adjacent tissue of the breast and the cervix samples (p<0.05). On the contrary, the level of RASSF3 expression was lower in the tumor compared to the adjacent tissue of the lung, uterus and colon organs. These observations indicate that the expression pattern of RASSF3 is tumor-type specific.
Figure 13. RT-PCR analysis of *RASSF3* expression in tumors vs. adjacent normal tissues of various human organs. Total RNA from pairs of tumor and adjacent normal tissue from human breast, lung, uterus, colon and cervix were analyzed by RT-PCR experiment using specific human *RASSF3* primers to test the expression levels of *RASSF3* in various human tumor vs. adjacent normal tissue. The RT-PCR products were resolved on 1% agarose electrophoresis-gels. *G3PDH* was used as a loading control. Gel electrophoresis photography was analyzed with the Kodak 1D Image Analysis software to compare the mean intensity of the bands. The mean intensity was set to 100% for the normal breast tissue. The data are given in percentage and correspond to *RASSF3* relative expression in the different tissues. The data represent the mean ± SEM of two independent experiments. (*) p < 0.05.

4.4.1. Amino Acid Sequence Alignments of the Mouse RASSF3, Human RASSF3 and Human RASSF1

The mouse (m) Rassf3 and human (h) RASSF3 genes encode a 232 amino acid protein (M.W. 26.7 kDa) and a 247 amino acid protein (M.W. 28.6 kDa) respectively. The proteins contain a highly conserved Ras association (RalGDS/AF-6) (RA) domain at the C-terminus followed by a SARAH (Sav/RASSF/Hpo) domain like the other members of the RASSF genes (Figure 14A). The hRASSF1A and hRASSF1C isoforms encode for a 340 amino acid protein and 270 amino acid protein, respectively. The hRASSF1A isoform encodes for a larger protein with a cysteine-rich domain (CRD) at the N-terminus which is a putative diacylglycerol (DAG)-binding domain (Figure 14A).

Sequence alignment with ClustalW algorithm showed that the mRASSF3 and hRASSF3 sequences share 94% identity at the amino acid level, whereas the hRASSF1A and hRASSF1C isoforms share 81% identity at the amino acid level and that they differ mainly at the N-terminus (Figure 14B). In addition, it was found that mRASSF3 and hRASSF3 both share 40% and 44% identity with hRASSF1A and hRASSF1C proteins, respectively.
Figure 14. Schematic comparison of the protein domains and amino acid sequences of human RASSF1 (isoforms A and C), human RASSF3 and mouse RASSF3 proteins. A) The Ras Association (RA) (RalGDS/AF-6) domains, the putative diacylglycerol (DAG)-binding domain and the SARAH domains are represented by boxes and their amino acid positions are indicated below. B) The alignment of hRASSF1A (GenBank NM_007182), hRASSF1C (GenBank NM_170713), hRASSF3 (GenBank BC100951) and mRASSF3 (GenBank NM_138956) were generated using the ClustalW algorithm, using the MegAlign program from Lasergene (DNASTAR). Identical amino acids are highlighted in gray. The RA domains are defined by the boxes.
**4.4.2. RASSF3 Expression Reduces Cell Proliferation and Induces Apoptosis**

The SKBR3 human breast cancer epithelial cell line, which expresses very high levels of HER2 protein, was selected to examine the effects of Rassf3 expression on cell growth. As we were unable to generate stable cell lines expressing Rassf3, we used the transient transfection approach to study the effects of Rassf3 in vitro.

Different amounts of pcDNA3.1/Rassf3 plasmid or vector were transfected into SKBR3 cells. The results from the cell proliferation assay (at 24 hours) indicated that Rassf3 expression reduces cell proliferation in a dose-dependent manner (Figure 15A). With 1µg of pcDNA3.1/Rassf3 plasmid, a minimum inhibitory effect was observed. The inhibitory effect reached a maximum with 2 µg and 4µg of pcDNA3.1/Rassf3 plasmid. Rassf3 gene expression was confirmed by RT-PCR analysis and demonstrated a progressive increase of gene expression level with the increase of transfected DNA (Figure 15B).

The results from the time-course study indicated that the inhibitory effect of Rassf3 (2µg) transient transfection reached a maximum after 24 hours of proliferation (Figure 15C). RT-PCR analysis showed that Rassf3 transient expression was still detected after 72 hours (Figure 15D).
Figure 15. The effects of the Rassf3 gene expression on SKBR3 human breast cancer cell viability. A) SKBR3 cells were transiently transfected with 1, 2 and 4 µg of vector or pcDNA3.1/Rassf3 plasmid. The cells were plated 48 hours after transfection into 96-well plates to monitor their proliferation rate after 24 hours. Data are presented as mean ± SEM of a percentage of pcDNA3.1/Rassf3 inhibition compared to vector. B) Rassf3 gene expression was confirmed by RT-PCR analysis and gel-electrophoresis. G3PDH was used as a loading control. (Data not shown for 1µg and 4µg of vector) C) SKBR3 cells were transfected with 2 µg of vector or pcDNA3.1/Rassf3 plasmid. The cells were plated 48 hours after transfection into 96-well plate to monitor their proliferation rate with time. Data are expressed as mean ± SEM of a percentage of pcDNA3.1/Rassf3 inhibition compared to vector. D) Rassf3 gene expression was confirmed at 72 hours of proliferation by RT-PCR analysis and gel-electrophoresis. G3PDH was used as a loading control.
The inhibitory effect of \textit{Rassf3} on cell proliferation was further confirmed with multiple epithelial cell lines (SKBR3, BT-474, MCNeuA and HC11) which express high levels of HER2 protein. The cell lines were transfected with 2µg of pcDNA3.1/\textit{Rassf3} plasmid or vector and cellular proliferation was measured after 24 hours. The results showed that \textit{Rassf3} transient expression reduced cell proliferation by 20% in SKBR3, 8% in BT-474, 15% in MCNeuA and 10% in HC11 cells (Figure 16A). The expression of the RASSF3 protein in the four cell lines was confirmed after transient transfection with a specific anti-RASSF3 polyclonal antibody (Figure 16B).

To determine the potential mechanism behind the growth-inhibitory properties of \textit{Rassf3}, apoptosis assays were performed on transfected SKBR3 human breast cancer cells (Figure 17). SKBR3 cells were transfected with 2 or 4 µg of vector and pcDNA3.1/\textit{Rassf3} and then collected 48 hours later to run the Guava ViaCount™ Assay. The results indicated that \textit{Rassf3} transfection caused approximately 15%-20% reduction in the number of live/healthy cells and 15%-20% increase in the number of apoptotic and dead cells compare to non-specific vector transfection. These changes in cells numbers were statistically very significant (p<0.01) in both cases with 2µg or 4µg of pcDNA3.1/\textit{Rassf3} plasmid.
Figure 16. The effects of the *Rassf3* gene expression on cell viability of various cell lines. A) Two human breast cancer cell lines (SKBR3 and BT-474), one mouse mammary cancer cell line (MCNeuA) and one mouse mammary cell line (HC11) were transiently transfected with 2 µg of vector or pcDNA3.1/*Rassf3* plasmid. Forty-eight hours after transfection, the cells were plated into 96-well plate. The proliferation rates were monitored after 24 hours. Data are presented as mean ± SEM of a percentage of pcDNA3.1/*Rassf3* inhibition compared to vector. Means are obtained from 8 independent experiments for SKBR3 cells and 3 independent experiments for BT-474, MCNeuA and HC11 cells. B) RASSF3 protein expression was confirmed in the four cell lines at 24 hours of proliferation by Western blot analysis. β-ACTIN was used as a loading control and is representative of all the gels.
Figure 17. The effects of the *Rassf3* gene expression on apoptosis in SKBR3 human breast cancer cell line. SKBR3 cells were transiently transfected with 2 or 4 µg of vector and pcDNA3.1/Rassf3 and then collected 48 hours later to run the Guava ViaCount™ Assay, which allows total cell counts, viability assessments and apoptotic cell counts. Untransfected cells and camptothecin treated cells were used as negative and positive controls respectively. The amount of live/healthy cells, apoptotic cells and dead cells were expressed as a percentage of the total cellular population for each transfection or treatment. Data are presented as mean ± SEM of triplicates and represent one of two separate experiments. (**) p<0.01.
4.4.3. Effects of RASSF3 Expression on Cellular Signaling Pathways

To determine if RASSF3 expression in SKBR3 human breast cancer cells alters intracellular signaling pathways that are associated with overexpression of HER2 protein, the status of several common intracellular biomarkers involved in survival (p-AKT, AKT), proliferation and differentiation (p-ERK, ERK, JNK, RAS), cell cycle (Cyclin D1), and apoptosis (Cytochrome C, BAX) were analyzed after Rassf3 transfection (Figure 18). SKBR3 cells were transiently transfected with 2µg of pcDNA3.1/Rassf3 plasmid or vector and stimulated with EGF and the expression levels of the selected markers were assessed by Western blot analysis after 24 and 72 hours. Changes in protein levels of the intracellular markers due to RASSF3 protein expression were not obvious at both time points; however, it appears that RASSF3 expression may decrease AKT phosphorylation. The experiment was repeated with cell lysates collected 72 hours after transfection and similar results were observed.
Figure 18. Western blot analysis of the effect of Rassf3 gene expression on intracellular signal transduction pathways in SKBR3 human breast cancer cell line. SKBR3 cells were transiently transfected with 2 µg of vector and pcDNA3.1/Rassf3 plasmid. 24 hours after transfection, cells were treated with EGF (60 ng/ml) for 20 minutes and then proteins were extracted from cell lysates. The protein levels of RASSF3, p-AKT, AKT, p-ERK, ERK, p-JNK, RAS, Cytochrome C, BAX and Cyclin D1 were analyzed by Western blot analysis. β-ACTIN was used as a loading control and is representative of all the gels.
4.4.4. Possible Interaction between RASSF3 and H-RAS Proteins

SKBR3 cells were transiently co-transfected with various amounts of vector, pcDNA3.1/Rassf3 and pcDNA3.1/H-RAS (G12V) plasmids, such that the total amount of transfected DNA remained constant; cells were collected after 24 hours for Western blot analysis of RASSF3 and H-RAS protein levels. As shown in Figure 19, H-RAS protein level progressively increased as the amount of transfected pcDNA3.1/H-RAS (G12V) increased. Interestingly, the H-RAS protein level was significantly reduced when pcDNA3.1/Rassf3 plasmid was co-transfected (Figure 19). This suggests that RASSF3 transient expression can influence activated H-RAS protein level, possibly by interacting with it at the protein level.

To study the effects of this possible interaction between RASSF3 and H-RAS on cell proliferation, the Rassf3 gene was co-transfected with activated H-RAS (pcDNA3.1/H-RAS (G12V)) or dominant-negative H-RAS (pcDNA3.1/H-RAS (S17N)) in SKBR3 cells. The results showed that Rassf3 inhibited cell proliferation in a very significant manner compared to activated H-RAS or dominant-negative H-RAS alone (p<0.01) whose effects on cell proliferation were minimal (Figure 20A). However, the results obtained from the co-transfections indicated that Rassf3 growth-inhibition effect was enhanced in the presence of H-RAS (Figure 20B).
Figure 19. Western blot analysis of the interaction of RASSF3 protein with constitutively activated H-RAS in SKBR3 human breast cancer cell line. SKBR3 cells were transiently co-transfected with various combinations of vector, pcDNA3.1/Rassf3 or pcDNA3.1/H-RAS (G12V) plasmids. Proteins lysates were collected 24 hours after transfection and protein levels of RASSF3 and H-RAS were analyzed by Western blot analysis using anti-RASSF3 and anti-RAS antibodies. β-ACTIN was used as a loading control.
A. Single Transfections

- **Rassf3**
- **H-RAS (G12V)**
- **H-RAS (S17N)**

B. Co-Transfections

- **Vector + Rassf3**
- **Rassf3 + H-RAS (G12V)**
- **Rassf3 + H-RAS (S17N)**

**Figure 20.** The effects of co-transfection of Rassf3 with activated H-RAS or dominant negative H-RAS on cell viability in SKBR3 human breast cancer cell line. A) SKBR3 cells were transfected with 2 µg of vector, pcDNA3.1/Rassf3, pcDNA3.1/H-RAS (G12V) or pcDNA3.1/H-RAS (S17N). The cells were plated 48 hours after transfection into 96-well plates to monitor their proliferation rate after 24 hours. The data are presented as a percentage of Rassf3 cell proliferation inhibition compared to vector, H-RAS (G12V) effect compared to vector and H-RAS (S17N) effect compared to vector. The data correspond to the mean ± SEM of percentages obtained from 4 independent experiments. (**) p<0.01. B) SKBR3 cells were co-transfected with 2 µg of each plasmid in different combinations as indicated. Transfection with 4 µg of vector was used as a reference to calculate the percentages. The cells were plated 48 hours after transfection into 96-well plates to monitor their proliferation rate after 24 hours. The data correspond to the mean ± SEM of percentages obtained from 3 independent experiments.
4.5. Generation and Analysis of Novel Transgenic Mouse Lines: The MMTV/Rassf3 Transgenic Mice and MMTV/Rassf3-neu Bi-Transgenic Mice

In order to study the function of the Rassf3 gene in an in vivo model, two novel transgenic mouse lines were generated: the MMTV/Rassf3 transgenic mice and the MMTV/Rassf3-neu bi-transgenic mice.

4.5.1. Confirmation of the Transgenic Mouse Lines

The incorporation of the Rassf3 transgene was confirmed by PCR analysis on genomic DNA isolated from mouse tail biopsies (Figure 21). Various tissues from female MMTV/Rassf3 mice and their littermates were analyzed by RT-PCR for the expression of the Rassf3 gene. The results indicated that the Rassf3 gene is expressed in the mammary gland as well as in the brain, the small intestine and the muscle tissues of MMTV/Rassf3 transgenic mice (Figure 22).
Figure 21. Representative PCR analysis of the genomic DNA from MMTV/Rassf3 transgenic mice, MMTV/Rassf3-neu bi-transgenic mice and their littermates. The presence of the transgene was confirmed by PCR analysis of genomic DNA from tail biopsies of the MMTV/Rassf3 transgenic mice (1, 1’), FVB non-transgenic littermates (2, 2’), MMTV/Rassf3-neu bi-transgenic mice (3, 3’) and MMTV/neu+/- littermates (4, 4’). A pair of specific primers that span the MMTV promoter and the Rassf3 cDNA sequence was used for PCR analysis. The PCR products were resolved on 1% agarose electrophoresis-gels and G3PDH was used as a loading control.

<table>
<thead>
<tr>
<th></th>
<th>MMTV/Rassf3 mice</th>
<th>Littermates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1’</td>
<td>2</td>
</tr>
<tr>
<td><strong>Rassf3</strong></td>
<td><strong>G3PDH</strong></td>
<td></td>
</tr>
</tbody>
</table>

- +        - +        - +        - +

<table>
<thead>
<tr>
<th></th>
<th>MMTV/Rassf3-neu mice</th>
<th>MMTV/neu+/- littermates</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3’</td>
<td>4</td>
</tr>
<tr>
<td><strong>Rassf3</strong></td>
<td><strong>G3PDH</strong></td>
<td></td>
</tr>
</tbody>
</table>

Figure 22. RT-PCR analysis of the total RNA from various tissues of MMTV/Rassf3 transgenic mice and their littermates. Total RNA was isolated from the mammary gland, brain, small intestine and muscle tissues of MMTV/Rassf3 female transgenic mice (+) and of their littermates (-). Rassf3 gene expression was investigated by RT-PCR analysis and RT-PCR products were resolved on a 1% agarose electrophoresis-gel. G3PDH was used as a loading control.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Brain</th>
<th>Mammary Gland</th>
<th>S. Intestine</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Rassf3</strong></td>
<td><strong>G3PDH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
To further study the expression of *Rassf3* gene, Western blot analysis was conducted on mammary glands and brain tissues using a specific anti-RASSF3 polyclonal antibody. For the mammary gland tissues, the RASSF3 protein was not detected on the immunoblots for the MMTV/*Rassf3* mice and MMTV/*Rassf3-neu* bi-transgenic mice, although the *Rassf3* mRNA was present and amplified by RT-PCR analysis of mammary gland homogenates (Figure 23A). However, the RASSF3 protein was detected in the brain tissue of all the MMTV/*Rassf3* transgenic mice and of the MMTV/*Rassf3-neu* bi-transgenic mice whereas it was not detected in all the littermates (Figure 23B).

There was no apparent overt phenotype in both male and female MMTV/*Rassf3* transgenic mice up to currently 21 months of age. The MMTV/*Rassf3* female mice were fertile with normal litter size and showed no difference in maternal behavior compared to the FVB non-transgenic littermates.
A. Mammary Gland

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>RASSF3 (Western Blot)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rassf3 (RT-PCR)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3PDH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. Brain

<table>
<thead>
<tr>
<th></th>
<th>4 mo</th>
<th>5 mo</th>
<th>7 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>RASSF3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-TUBULIN</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>6 mo</th>
<th>3 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>RASSF3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-TUBULIN</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 23. Western blot and RT-PCR analyses of RASSF3 expression in mammary gland and brain tissues of the transgenic mice. A) Tissue lysates and total RNA were isolated from mammary gland tissues of transgenic mice and their littermates. Protein lysates were used for Western blot analysis with an anti-RASSF3 antibody and total RNA samples were used for RT-PCR analysis. RT-PCR products were resolved on 1% agarose electrophoresis-gel. B) Tissue lysates were prepared from brain of transgenic mice and their littermates at different ages (mo: months) for Western blot analysis with an anti-RASSF3 antibody. 1: MMTV/Rassf3 mice; 2: non-transgenic FVB mice; 3: MMTV/Rassf3-neu bi-transgenic mice; 4: MMTV/neu+/- littermates; and C: Purified RASSF3 protein as positive control. β-TUBULIN and G3PDH were used as loading controls.
4.5.2. Comparison of the Mammary Gland Morphology of the Transgenic Mice

To determine the effects of Rassf3 on mammary gland development, the mammary gland whole mounts obtained from the MMTV/Rassf3 transgenic mice and their non-transgenic FVB littermates (Figure 24A) at the age of 3 and 7 months were compared. The observed morphological differences are minimal. The overall ductal density and end bud formation are similar; however, it appears that the amount of side branching is slightly reduced in the MMTV/Rassf3 transgenic mice compared to their littermates at the two time points. No morphological differences were detected between the mammary gland whole mounts from the MMTV/Rassf3-neu bi-transgenic mice and from the MMTV/neu$^{+/-}$ littermates at the age of 3 and 6 months (Figure 24B).
Figure 24. Images of the mammary gland whole mounts of MMTV/Rassf3 transgenic mice, FVB non-transgenic mice, MMTV/Rassf3-neu bi-transgenic mice and MMTV/neu+/- littermates. The fourth inguinal mammary glands were dissected from 3, 6 and 7 months old (mo) mice, mounted and stained in carmine alum stain. The mammary gland whole mounts were digitally photographed to compare the morphology. Pictures are representative of mammary gland images of MMTV/Rassf3 transgenic mice and littermates (n=2/group; panel A) and of MMTV/Rassf3-neu bi-transgenic mice and MMTV/neu+/- littermates (n=3/group; panel B). Inserts correspond to an enlargement of the smaller regions indicated by the boxes.
4.5.3. Comparison of the Mammary Tumor Incidence in the MMTV/Rassf3-neu Bi-Transgenic Mice and in their Littermates

To assess the effect of Rassf3 overexpression on HER2/neu mammary tumorigenesis, the MMTV/Rassf3-neu transgenic mice and the MMTV/neu<sup>+-</sup> littermates were monitored for the development of palpable mammary tumors. The comparison of the mammary tumor incidence revealed that there was a delay in tumor formation (p=0.0552) in the bi-transgenic mice (Figure 25). The $t_{50}$ of MMTV/Rassf3-neu bi-transgenic mice ($n=37$; $t_{50}=262$) was increased by 37 days compared to the $t_{50}$ of MMTV/neu<sup>+-</sup> littermates ($n=32$; $t_{50}=225$).
Figure 25. Comparison of the mammary tumor incidence in the MMTV/Rassf3-neu bi-transgenic mice and the MMTV/neu+/− littermates. The data are plotted as the percentage of tumor-free female mice (y-axis) as the function of age in days (x-axis). The MMTV/Rassf3-neu bi-transgenic mice (n=37) are represented by closed circles (●), and the MMTV/neu+/− littermates (n=32) are represented by closed squares (■). The t50 of MMTV/Rassf3-neu bi-transgenic mice and their littermates correspond to 262 and 225 days respectively. Log rank test, P = 0.0552.
Western blot analysis of mammary tumors indicated that RASSF3 protein is expressed in the tumors of MMTV/Rassf3-neu bi-transgenic mice and of MMTV/neu+/− littermates (Figure 26). The expression levels of RASSF3 and p-HER2 proteins appeared to be both higher in the tumors from the bi-transgenic mice than in the tumors from the littermates.

![Western blot analysis of RASSF3 expression in mammary tumors of the MMTV/Rassf3-neu bi-transgenic mice and their littermates.](image)

**Figure 26.** Western blot analysis of RASSF3 expression in mammary tumors of the MMTV/Rassf3-neu bi-transgenic mice and their littermates. Protein lysates were isolated from mammary tumors of MMTV/Rassf3-neu bi-transgenic mice (6 to 8 months old) and of MMTV/neu+/− littermates (7 to 9 months old) for Western blot analysis with anti-RASSF3 and anti-phospho-HER2 antibodies. Purified RASSF3 protein was used as a positive control (C). β-TUBULIN was used as a loading control.
Primary mammary tumors were surgically removed from several mice to investigate the incidence of recurrent mammary tumors and of secondary mammary tumors. The results suggested that Rassf3 overexpression did not influence the recurrence of primary tumors nor the development of secondary tumors in MMTV/Rassf3-neu bi-transgenic mice as indicated in Table 4. On the other hand, the development of secondary tumors appeared to be more frequent than the recurrence of primary tumors in MMTV/neu+/- littermates. However, the chi-square test indicated that the distribution of recurrence and secondary tumor between the two groups of mice was not significant (p> 0.05).

Table 4. Mammary tumor recurrence and secondary mammary tumor development in female MMTV/Rassf3-neu bi-transgenic mice compared to their MMTV/neu+/- littermates.

<table>
<thead>
<tr>
<th></th>
<th>MMTV/Rassf3-neu bi-transgenic mice</th>
<th>MMTV/neu+/- littermates</th>
<th>All mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secondary tumor</td>
<td>8 (47.1%)</td>
<td>7 (63.6%)</td>
<td>15 (51.7%)</td>
</tr>
<tr>
<td>Recurrence</td>
<td>9 (52.9%)</td>
<td>4 (36.4%)</td>
<td>13 (48.3%)</td>
</tr>
<tr>
<td>Total of Mice</td>
<td>17 (60.7%)</td>
<td>11 (39.3%)</td>
<td>28 (100%)</td>
</tr>
</tbody>
</table>

The index of tumor aggressiveness (S) was calculated for several recurrent primary tumors and secondary mammary tumors. In the case of the MMTV/Rassf3-neu bi-transgenic mice, there was no significant difference in the index of tumor aggressiveness between the two types of tumors, recurrent primary tumors (Mean of
S=252 units) and secondary tumors (Mean of S=209 units) (Figure 27). On the contrary, the indexes of tumor aggressiveness were significantly different between the two types of tumors for the MMTV/neu+/- littermates, with the recurrent primary tumors (Mean of S=173 units) corresponding to a more aggressive type than the secondary tumors (Mean of S=253 units).

Figure 27. Analysis of the recurrence of mammary tumors in MMTV/Rassf3-neu bi-transgenic mice and their littermates after surgical removal of the primary tumor. After surgical removal of the primary tumors, the mice were monitored for primary tumor recurrence (Recurrence) or for the development of a secondary tumor from other site (Secondary). The data represent the index of the inverse of tumor aggressiveness (S) calculated by the formula: $S = T1 + T2 \times \frac{1}{V}$ which includes the mouse’s age at the time of appearance of the primary tumor (T1), the mouse’s age at the time of primary tumor recurrence or secondary tumor development (T2) and the tumor volume (V) at the time of surgery. Tumors from MMTV/Rassf3-neu bi-transgenic mice are represented by circles (● & ○), and tumors from the MMTV/neu+/- littermates are represented by squares (■ & □). (*) p < 0.05.
5. DISCUSSION

5.1. HER2/neu Human Breast Cancer and its Mouse Models

Amplification and/or overexpression of HER2/neu have been found in one-third of human breast cancers (Slamon D. et al., 1987; Slamon D. et al., 1989). The HER2/neu overexpression is associated with a poor clinical outcome in that women with a HER2/neu-positive tumor experience earlier disease relapse and shorter survival time. Evidence support that HER2/neu overexpression plays a direct causal role in pathogenesis of the malignancy of breast cancer (Di Fiore P. et al., 1987; Hudziak R. et al., 1987). The transforming potential of the HER2/neu protein is closely related to its intrinsic tyrosine kinase activity (Guy C. et al., 1992). Overexpression of HER2/neu oncogene in human breast cancer is also associated with enhanced metastatic potential upon breast tumor cells (Paterson et al., 1991).

The molecular mechanism(s), by which HER2/neu overexpression stimulates neoplastic cell growth and renders cancer cells chemoresistant, have not been completely defined. It has been documented that HER2 overexpression can promote the growth and malignancy of mammary epithelial cells in part by conferring resistance to the growth inhibitory effects of TGF-β signaling which corresponds to the dominant system opposing the stimulatory effect of growth factors and early oncogene activation in many tissues, including the mammary gland (Brandt R. and Ebert A., 1998). This resistance to
the anti-proliferative effects of TGF-β appears at an early stage of the tumor progression in a number of human malignancies (Wilson C. et al., 2005). In addition, it has been established that a link exists in breast cancer between HER2 amplification and cyclin D1 overexpression. The cyclin D1 gene has been reported to be amplified in up to 20% of human breast cancers, while cyclin D1 protein is overexpressed in over 50% of human mammary carcinomas and particularly in DCIS (75%) (Weinstat-Saslow D. et al., 1995; Harari D. and Yarden Y., 2000; Yu Q. et al., 2001). Aberrant overexpression of cyclins D can reduce and overcome the dependency of mitogenic stimulation for a cell, and thus plays a role in the process of oncogenic transformation. It is believed that HER2 overexpression results in hyper-activation of a signaling network that deregulates the G1/S checkpoint of the cell cycle by up-regulation of cyclins D and of the cyclin-dependent kinase partners (CDK-4 and -6) (Harari D. and Yarden Y., 2000; Yang C. et al., 2004). Hudziak R. and colleagues (1988) showed also that HER2/neu oncogene expression allowed the tumor cells to resist to the cytotoxic effects of tumor necrosis factor alpha (TNF-alpha) or macrophages and thereby to evade host immune defenses. Furthermore, it was demonstrated that HER2-positive breast tumors can evade apoptosis and resist to chemotherapeutic cytotoxic agents by up-regulating the CDK inhibitor p21\textsuperscript{Waf1}, which plays a central role in HER2 anti-apoptotic machinery (Yu Q. et al., 2001).

To directly test the tumorigenic potential of the HER2/neu oncogene in the mammary epithelium, transgenic mouse models carrying the rat neu oncogene under the transcriptional control of the mouse mammary tumor virus promoter/enhancer (MMTV) have been established (Muller W. et al, 1988; Guy C. et al. 1992). Two different models
have been developed with either the normal form of \textit{neu} or an activated form which corresponds to a mutated form of \textit{neu} with a change at position 664 in the transmembrane domain from valine to glutamic acid (Bargmann C. and Weinberg R., 1988).

In several strains of MMTV/activated \textit{neu} mice, early onset of the transgene expression in the mammary gland resulted in the development of multifocal mammary adenocarcinomas in both sexes by 78 to 95 days, with occasional progression to pulmonary metastasis (Muller W. \textit{et al}., 1988; Bouchard L. \textit{et al}., 1989). These results suggested that activated \textit{neu} through mutation was sufficient (single step) for mammary tumorigenesis (Muller W. \textit{et al}., 1988).

On the other hand, expression of the normal form of \textit{neu} in MMTV/\textit{neu} transgenic mice resulted in the development of mammary tumors with a longer latency (120-337 days) than when activated \textit{neu} is expressed (Muller W. \textit{et al}., 1988). It is believed that overexpression of the unactivated NEU protein is the primary mechanism contributing to human breast cancer, since primary human breast cancers fail to reveal any comparable mutations (Lemoine N. \textit{et al}., 1990). Guy C. and colleagues (1992) reported also that overexpression of the unactivated form of \textit{neu} in the mammary epithelium resulted in the appearance of focal mammary adenocarcinomas that metastasized with high frequency to the lung in transgenic mice, suggesting that overexpression of \textit{neu} can confer an enhanced metastatic potential to the mammary tumor cells. For one of their best characterized line (N#202), the study of the kinetic of tumor occurrence revealed that approximately 50\% of female virgin mice developed mammary tumors at the age of 7 months ($t_{50}$=205 days) and about 80\% of the female virgin mice at the age of 11 months or older (Guy C. \textit{et al}., 1992). In addition, they reported that the
neu transgene was expressed in both tumors and adjacent mammary epithelium, with higher levels in tumors, whereas the NEU-associated tyrosine kinase showed high activity in tumors but not in the adjacent mammary tissues. The delay in tumor onset in the MMTV/neu transgenic mice implies that additional genetic events beyond neu overexpression are required for mammary tissue transformation leading to tumor formation (Guy C. et al., 1992). This reflects the multistep model of tumorigenesis where more than one genetic event are involved. Therefore, elucidation of the molecular alterations that occur during HER2/neu breast cancer initiation and progression is crucial in the identification and development of therapeutic approaches to targeting HER2/neu-positive tumors in breast cancer patients.

The HER2/neu animal model has been used in many studies since its establishment (Muller W. et al, 1988). However, the focus of nearly all of those studies was to investigate the mechanism by which the overexpression of HER2/neu oncogene in epithelial cells initiates tumorigenesis. In the present study, we used the previously developed MMTV/neu female transgenic mouse model but we focused on the small proportion (approximately 20 %) of MMTV/neu transgenic mice which did not develop mammary tumors at 11 months or older despite the overexpression of the neu oncogene (Guy C. et al., 1992) and which seem to have naturally developed resistance to HER2/neu-tumorigenesis. We hypothesized that there may be one or more tumor suppressor genes that are up-regulated in the mammary glands of these tumor-resistant mice which might suppress the development of mammary tumors.
5.2. Identification of the Genes Responsible for the Tumor Resistant Phenotype in MMTV/neu Transgenic Mice by cDNA Microarray Analysis

To test our hypothesis, cDNA microarray analysis was used to compare the differences in gene expression profiles of mammary glands of R, R-MG and C mice, in order to identify genes that may be responsible for the resistance to HER2/neu mammary tumor development in the tumor-resistant mice. Two approaches were used in this study to analyze the microarray data.

The first approach consisted in searching for differentially expressed genes in tumor-resistant MMTV/neu mice (R) compared to tumor-susceptible MMTV/neu mice (R-MG). The rationale of this first approach supported the notion that the genes that are up-regulated in tumor-free mice but down-regulated in tumor-susceptible mice might be responsible for the resistance to tumor formation (Table 2). Among the genes identified, two genes encoding for intracellular signaling proteins associated with a suppressive character, have attracted our attention: the Rassf3 and the Socs2 genes.

The Rassf3 gene is a novel gene that has been previously reported but remains relatively uncharacterized. Indeed, Tommasi S. and colleagues (2002) identified two homologues of RASSF1 gene by BLAST searches using the predicted amino-acid sequence of RASSF1 RA domain and named them RASSF3 and Rassf3 for the human and mouse genes respectively. They found that RASSF3 was expressed ubiquitously in normal human tissues and tumor cell lines in a similar manner than the isoform C of RASSF1. Based on their sequence homology with the tumor suppressor RASSF1 and on the presence of a conserved RAS association domain (RA) at the C-terminus followed by a SARAH (Sav/RASSF/Hpo) domain, the RASSF3 gene has been classified as a member of the Ras Association domain (RalGDS/AF-6) Family gene (RASSF), which contains
characterized RAS effectors and tumor suppressor genes (Hesson L. *et al*., 2003; Vos M. *et al*., 2003; Eckfeld L. *et al*., 2004; Agathanggelou A. *et al*., 2005). The belonging to the RASSF family suggests that RASSF3 may function in signal transduction pathways involving RAS GTPases proteins. However, no functional studies of human RASSF3 and mouse Rassf3 genes have been reported. RASSF1 and NORE1 genes have been the most extensively studied members of the RASSF Family. They encode both two major isoforms, RASSF1A (340 aa), RASSF1C (270 aa) and NORE1A (418 aa), NORE1B (265 aa) respectively. Only RASSF1A and NORE1A isoforms contain an additional functional domain at the N-terminus, the cysteine-rich domain (CRD) which is a putative diacylglycerol (DAG)-binding domain. (Vos M. *et al*., 2003; Agathanggelou A. *et al*., 2005).

The role of the RASSF genes as tumor suppressors is supported by the loss of expression in human tumor cell lines and tumors due to hypermethylation of CpG islands in the promoter region (Agathanggelou A. *et al*., 2005). In fact, the RASSF1A isoform (but not the RASSF1C isoform) has been reported to be down-regulated at the expression level by promoter hypermethylation in the majority of cancer cell lines from lung, glioma, breast (MCF-7, MDA-MB-157, MDA-MB-231, T47D and ZR75-1) and ovaries, and to be inactivated in a wide range of human sporadic cancers such as in 80% of lung (NSCLC) cancers, 60% of breast cancers (Dammann R. *et al*., 2001; Burbee D. *et al*., 2001), 70% of prostate cancers, 90% of hepatocellular cancers, as well as in ovarian, kidney, prostate, neuroblastoma, gliomas, thyroid and gastric cancers (Tommasi S. *et al*., 2005; Agathanggelou A. *et al*., 2005). NORE1A promoter region was also found to be hypermethylated in breast, lung, colorectal, kidney and glioma tumor cell lines and in
24% of NSCLC primary tumors (Vos D. et al., 2003b). In a similar way, RASSF2 expression is reported to be frequently down-regulated or silenced by promoter hypermethylation in human lung and gastric cancer cell lines and primary gastric cancers (Endoh M. et al., 2005). RASSF4 is broadly expressed in normal tissue but its expression is also down-regulated by promoter hypermethylation in breast, lung, colorectal and kidney cancer cell lines as well as in primary breast and lung tumors (Agathanggelou A. et al., 2005). Some differences have been observed in promoter methylation between the two isoforms of RASSF1 (A and C) and of NORE1 (A and B) which reflects tissue-dependent differences in biological activity of the isoforms. In addition, re-expression of RASSF1A in lung cancer cell lines by treatment with an unmethylating agent was shown to reduce colony formation, suppress anchorage-independent growth and to inhibit tumor formation in nude mice (Burbee D. et al., 2001). These characteristics all together reinforce a potential role for RASSF1A as a tumor suppressor gene. The Rassf3 gene has been found unmethylated in human cancers, which may suggest the existence of a different mechanism of regulation for Rassf3 expression (Agathanggelou A. et al., 2005).

In addition, it has been documented that the RASSF proteins can interact with specific RAS proteins in a GTP-dependent manner, therefore supporting a role as RAS effectors. It has been shown that RASSF1 and NORE1 interact specifically with H-RAS, whereas RASSF2 and RASSF4 both interact specifically with K-RAS (Vos M. et al., 2000; Vos M. et al., 2003; Eckfeld K. et al, 2004).

The Socs2 gene encodes for the intracellular suppressor of cytokine signaling-2 protein which belongs to a family of proteins involved in the regulation of cytokine
responses from the growth hormone receptor, insulin-like growth factor receptor and the prolactin receptor. These SOCS proteins act in a classical feedback loop (Hilton D., 1999; Johnston J., 2004). Their gene expression is induced by exposure to cytokines, hormones, and growth factors. Once produced, they bind to various components of the signaling apparatus and inhibit further signal transduction. The SOCS proteins can block the cytokine signaling either by direct inhibition of the adaptor protein JACK2 or by binding to the tyrosine phosphorylated receptor to prevent binding with other SH2 and PTB-domain containing adaptor proteins (Raccurt M. et al., 2003). In fact, SOCS2 has been found to bind to JAK2 and suppress its phosphorylation, thereby inhibiting the JAK-STAT intracellular signaling pathway (Larsen L. and Ropke C., 2002). The inhibitory actions of SOCS proteins are also combined with a mechanism of targeting associated signaling molecules for poly-ubiquitination and proteasome mediated degradation (Johnston J., 2004).

Various effects of SOCS proteins have been demonstrated in the immune system and immune pathology (Alexander W. and Hilton D., 2004; Elliot J. and Johnston J., 2004); however, the role of SOCS proteins in other diseases is less well known. Several studies have shown that the loss or inactivation of SOCS proteins (1, 2 and 3) correlates with a growth promoting effect (Schultheis B. et al., 2002; Johnston J., 2004; Miller M. et al., 2004). Others have shown that SOCS proteins can regulate cell proliferation and differentiation and are involved in cross-talk between signaling pathways (Leung K., 2003; Johnston J., 2004). It seems that SOCS2 behavior shows some differences compared to its cognate receptors SOCS1 and SOCS3. Favre H. et al. (1999) showed that SOCS2 had two opposite effects, i.e. it inhibited growth hormone-induced STAT5-
dependent gene transcription at low concentrations, but restored growth hormone signaling when present at higher concentrations. These findings suggested that SOCS2, which is often induced later than SOCS1 and SOCS3 in response to cytokines, may restore the sensitivity to cytokines by suppressing the initial inhibitory effect of SOCS1 and SOCS3. In breast cancer, conflicting data have been reported on SOCS proteins (Raccurt M. et al., 2003; Miller M. et al., 2004) and therefore further studies are necessary. Based on the facts reported in the literature, it seems that the Socs2 gene may interfere with HER2/neu signaling pathway by interacting with the JAK2 adaptor protein.

In addition, through the first analytical approach, a number of transcription factors were found up-regulated in tumor-resistant mice. These transcription factors such as the ETV1 are correlated with HER2/neu expression. The ETV1 transcription factor (also known as ER81) belongs to the ETS family of transcription factors, characterized primarily by a highly conserved DNA binding domain known as the ETS domain. The ETS transcription factors play an important role in mammalian development and hematopoietic development. Loss of function of various ETS transcription factors has been indeed correlated with embryonic lethality (Gilliland G., 2001). They have also been implicated in the pathogenesis of a spectrum of human cancers and seem to function by influencing the promoter activity of TGF-β type II receptor, which behaves as a tumor suppressor (Im Y., 2000; Gilliland G., 2001; Dowdy C. et al., 2003; Kopp J. et al., 2004). ETV1 belongs to the PEA3 transcription factor subfamily and its activity has been found to be regulated and enhanced by the HER2/NEU tyrosine kinase (Goel A. and Janknecht R., 2004) through RAS-RAF-MAPK and/or JNK downstream signaling pathways. Phosphorylation of ETV1 transcription factor leads to its association with the Cbp/p300
transcriptional co-activator in the nucleus and further stimulation of gene transcription such as the human telomerase reverse transcriptase hTERT involved in the control of replicative capacity and senescence (O’ Hagan R. and Hassell J., 1998; Goueli B. and Janknecht R., 2004).

In respect to the second analytical approach, the rationale to analyzing the microarray data comes from evidence demonstrating that a primary tumor sends circulating signals to its surrounding environment to suppress the development of metastases. Folkman and colleagues were the first ones to show that the inhibition of metastases by a primary mouse tumor is mediated, at least in part, by an angiogenesis inhibitor angiostatin (O’Reilly M. et al., 1994; Folkman J., 2002; Naumov G. et al., 2006). In consequence, we realized that in our model of study, the normal adjacent mammary gland, dissected from tumor-susceptible MMTV/neu mice that had developed tumors, may express considerable suppressive signals. Thus, the adjacent normal mammary glands could be considered as tumor-resistant mammary glands (R-MG). Therefore, we hypothesized that the mammary glands of tumor-resistant mice (R) and of tumor-susceptible mice (R-MG) may present similar patterns of gene expression.

Among the genes identified through this second approach, the Mup1 gene, characterized by a high fold change, was further studied. Mup1 or major urinary protein 1 gene belongs to a large family consisting of 35 to 40 highly homologous genes and pseudogenes. It is expressed in a number of different secretory tissues of the mouse, including the liver, the submaxillary gland and the lachrymal, sublingual, parotid and mammary glands (Shaw et al., 1983). The expression of Mup mRNA is under the control of different developmental and hormonal stimuli in different tissues (Shaw et al., 1983).
The secreted MUP proteins belong to the lipocalin superfamily and share with other members of this family the capacity to bind hydrophobic molecules such as pheromones (Shahan et al., 1987). No known function has yet been identified for Mup1 gene. However, Mup genes have been defined as negative tumor markers of mouse hepatocarcinomas (Dragani T. et al., 1989), because the expression of Mup genes was found to be decreased in the early development of mouse liver tumors compared to normal.

5.3. Analysis of the Candidate Genes

The overexpression of the Rassf3 gene in mouse mammary tumors compared to adjacent normal mammary tissues may be the result of a cellular defensive response to the high levels of HER2/NEU proteins in the mammary tumors of the MMTV/neu transgenic mice (Figure 9 and 11). This particular expression pattern has been previously documented for genes with tumor suppressor activity, notably for TGF-β, p53 and SOCS tumor suppressors (Oft M. et al., 1998; Gonzalez-Palacios F. et al., 1997; Raccurt M. et al., 2003; Rowland B. and Peeper D., 2006). Contrary to the notion that tumor suppressor proteins are down-regulated in cancers, it has been found that there is a marked increase in their expression in specific cancers.

Evidence report that TGF-β demonstrates a biphasic action during multistage carcinogenesis, acting as a tumor suppressor by inhibiting cell-cycle progression and tumor growth during early tumor development but enhancing the malignant phenotype (invasiveness and metastasis) in late stage human carcinogenesis of the pancreas, colon, stomach, lung, endometrium, prostate, breast, brain and bone (Gold I., 1999). Late-stage tumors show an increased expression of TGF-β accompanied by a loss of the growth-
inhibitory response to TGF-β (Oft M. et al., 1998). The mechanism for up-regulation of TGF-β is still unknown; however, the loss of TGF-β growth-inhibitory effect appears to result from the decrease in the levels of cyclin-dependent kinase inhibitors p21\textsuperscript{Waf} and p27\textsuperscript{Kip1} in tumors (Gold I., 1999; Rowland B. and Peeper D., 2006). Because of this dual role, members of the TGF-β signaling pathway are being considered as predictive biomarkers for progressive tumorigenesis, as well as molecular targets for prevention of cancer and metastasis (Bachman E. and Park B., 2005).

The overexpression of the tumor suppressor p53 has been observed in various forms of cancer and was not always indicating the presence of a gene mutation (Soong R. et al., 1966). In fact, overexpression of p53 has been observed in invasive breast carcinomas, lung adenocarcinomas and colorectal carcinomas. (Gonzalez-Palacios F. et al., 1997). Accumulation of the p53 protein has been found to occur during the transition from early to advanced stages of the cancer (Kawasaki M. et al., 1996). In invasive colorectal carcinomas, p53 overexpression was found to be associated with increased metastatic potential (Sory A. et al., 1997). These observations suggested that overexpression of the tumor suppressor p53 could be used as a useful clinical indicator of the degree of tumor malignancy and as a strong prognostic marker.

Overexpression of the SOCS(-1,-2,-3) genes and increased SOCS protein levels have been found in breast ductal carcinomas compared to normal breast tissue (Raccurt M. et al., 2003). Farabegoli F. et al. (2005) reported that SOCS2 expression was associated with high differentiation and low proliferation rate, but not with an overall survival and that it was inversely correlated with the cyclin A, the retinoblastoma protein (pRb) and the epidermal growth factor receptor (EGFR). This supports a role for SOCS2
in the regulation of cell proliferation and tumor growth in breast carcinoma which may vary depending on its level of expression (Farabegoli F. et al., 2005). Others postulated that the increase of SOCS expression in breast cancers may be a response to the induction of an intense production of inflammatory cytokines, mammotrophic, PRL and GH hormones, all involved during tumorigenesis and invasion (Raccurt M. et al., 2003).

In comparison, we speculate that Rassf3 may function in a classical feedback loop. Overexpression of the Rassf3 gene may be induced by HER2/neu proteins in mammary tissues. In our experiments, we also found that Rassf3 expression was not detectable in a spontaneous mammary tumor (neu negative) derived from a non-transgenic FVB mouse (data not shown). The analysis of human tumors and adjacent normal tissues also revealed that RASSF3 overexpression pattern varies between tissue/cancer types (Figure 13). These observations suggest that Rassf3 overexpression in tumors is dependent on the tumor type and the context (HER2/neu oncogene-specific). Therefore, Rassf3 overexpression may become an interesting biomarker in breast cancer.

As also observed in our results, the Etv1 transcription factor has been reported to be overexpressed in mammary tumors of MMTV/neu transgenic mice and is believed to play a role in neu-mediated mammary oncogenesis (Shepherd T. et al., 2001). The expression of the Mup1 gene in normal mammary tissue, but its absence in mammary tumors, suggest that Mup1 may be a negative tumor marker of mouse mammary tumor in a similar way that it is for hepatocarcinomas (Dragani T. et al., 1989)

The comparison of HER2 protein and RASSF3 mRNA levels among multiple human breast cancer cell lines, revealed an inverse correlation of expression between these two genes (Figure 12). After literature searching, the inverse correlation of
expression between an oncogene and a tumor suppressor gene has been reported in
different cancers and often suggests a cooperative role between the two genes in
carcinogenesis.

Xing M. et al. (2004) have reported that RASSF1A epigenetic inactivation by
methylation was an early step in thyroid tumorigenesis and that RASSF1A methylation
status and B-RAF mutations were inversely correlated in thyroid tumor cell lines with a
mutually exclusive relationship. This indicates that thyroid tumor cell lines characterized
by RASSF1A epigenetic inactivation did not simultaneously contain activating mutations
in B-RAF. An identical inverse correlation with mutual exclusivity was documented in
non-small cell lung cancers (NSCLC) between K-RAS mutation and NORE1A
methylation (Irimia M. et al., 2004). The same inverse correlation between K-RAS
mutation and RASSF1A methylation was observed in colorectal cancers (Van Engeland
M. et al., 2003) and pancreatic adenocarcinomas (Damman R. et al., 2003). Similarly,
Hesson L. and colleagues (2005) reported that K-RAS mutation and RASSF2 promoter
hypermethylation are mutually exclusive. These observations suggest that inactivation of
RASSF gene by methylation or activating RAS mutation can provide alternative
pathways for affecting RAS signaling pathway (Xing M. et al., 2004). The mutual
exclusivity of the two alterations in genes involved in the same pathway suggests that
these genes play a critical and cooperative role in human tumorigenesis (Agathanggelou
A. et al., 2005). However, in some cancer patients, epigenetic inactivation of RASSF1A
was found with K-RAS mutations in NSCLC or with B-RAF mutations in melanomas
and was associated with a poorer outcome which suggested a synergistic mechanism of
action between the alterations (Reifenberger J. et al., 2004).
Other types of correlation/cooperativity have been reported in the literature involving different genes including the telomerase enzyme activity and the expression of the p16 cell cycle negative regulator in NSCLC (Gonzalez-Quevedo R. et al., 2001), where telomerase activity is often correlated with loss of expression of the tumor suppressor p16 and with poorer prognosis.

Therefore, in comparison, the inverse correlation between HER2 protein levels and RASSF3 expression in human breast cancer cell lines may suggest that HER2 and RASSF3 genes have a cooperative role in breast carcinogenesis and that they are involved in the same pathway.

5.4. Functional Studies of the Rassf3 Gene in HER2/neu-positive Cell Lines

The complexity of genetic alterations in cancer is increased by the recently emerging evidence that some genes seem to have dual functions, i.e. the same gene can have tumor suppressor-like activities in one type of cancer, but function as an oncogene in a different cancer setting (Rowland B. and Peeper D., 2006). Remarkably, although RAS oncoproteins are usually associated with loss of growth control and tumorigenic transformation (Lowy D. et al., 1993), increasing evidences have demonstrated that RAS proteins have the ability to activate a variety of growth-inhibiting pathways including apoptosis and cell cycle arrest (Chen C. and Faller D., 1995; Chen, C. et al., 1998; Shao J. et al., 2000; Khokhlatchev A. et al., 2002). These contrasting activities suggest that the activation of powerful oncogenes such as RAS can promote conflicting biological processes in a potential tumor cell. This capacity to induce transformation or death has also been reported for other oncoproteins, as for example c-MYC, NOTCH1 and CDKN1A (Hueber A. and Evans G., 1998; Rowland B. and Peeper D., 2006).
The recent discovery of the RASSF family of RAS effectors helps to understand partly the growth inhibitory action of the RAS proteins. It has been demonstrated that these effectors serve as tumor suppressors directly activated by RAS. Indeed, it was shown that ectopic overexpression of NORE1 or RASSF1 genes induced growth inhibition and apoptosis, effects which were enhanced by co-expression of activated H-RAS and antagonized by co-expression of dominant inhibitory H-RAS (Vos D. et al., 2000). The same observations were obtained for RASSF2 and RASSF4 ectopic expressions with activated K-RAS (Vos D. et al., 2003a; Ecfeld K. et al., 2004). The similarity between RASSF2 and RASSF4 behavior was reinforced by the fact that they shared 60% identity at the amino acid level (Ecfeld K. et al., 2004). Additional evidence suggested that cyclin D1 regulation is responsive to native RASSF1A activity and that RASSF1A can induce cell cycle arrest in NSCL (A549) cell lines (Shivakumar L. et al., 2002).

In our study, we have identified through microarray analyses another member of the RASSF family, the mouse homologue of the RASSF3 gene noted Rassf3. Due to the close homology of Rassf3 gene with the other member of the RASSF family, we hypothesized that Rassf3 may serve as a negative regulator of RAS proteins which have been shown to be aberrantly activated (Clark G. and Der C., 1995; Von Lintig F. et al., 2000) due to persistent upstream signaling from the HER2 receptors, often overexpressed in breast cancer (Von Lintig F. et al., 2000). Therefore, since the novel Rassf3 gene has remained uncharacterized, we devoted most of our efforts to determine its role in HER2/neu tumorigenesis.
We demonstrated that Rassf3 ectopic expression inhibits cell proliferation of various cell lines including the SKBR3 and BT474 human breast cancer cell lines, the MCNeuA mouse cancer cell line and the HC11 mouse cell line, all characterized by the expression of high levels of HER2 protein (Figure 16). The investigation of the mechanism by which Rassf3 promotes growth inhibition in SKBR3 cells suggested that Rassf3 promotes cell apoptosis (Figure 17). These findings support a role for Rassf3 as a tumor suppressor gene. In addition, our results suggested that activated H-RAS protein interacts indirectly with RASSF3 protein (Figure 19) and enhances its growth-inhibitory properties (Figure 20), insinuating that RASSF3 is a RAS effector. The limited augmentation of Rassf3 cell growth-inhibition effect by activated H-RAS co-transfection can be explained by the fact that SKBR3 cells exhibit elevated levels of RAS-GTP proteins and have therefore persistent RAS activation (Eckert L. et al., 2004). The observation that a dominant-negative form of H-RAS did not abrogate Rassf3 growth-inhibition may suggest that RASSF3 biological activity is not entirely dependent on H-RAS activity in SKBR3 cells or that RASSF3 does not function downstream of H-RAS for promoting its growth-inhibition effect (Figure 20). In addition, RASSF3 effector activity may be influenced by a RAS isoform other than H-RAS. Indeed, differences in effector binding and signaling between the different RAS isoforms have been reported for the RASSF proteins (Yan J. et al., 1998; Rodriguez-Viciana P. et al., 2004). It is important to note that effector utilization and activation by RAS proteins are complex and have shown significant cell context variations (Kivinen L. et al., 1999; Coleman M. et al., 2003).
To further understand the mechanisms contributing to the biological activity of the RASSF proteins, a number of studies have started identifying RASSF-interacting proteins and potential target genes that are differentially regulated by RASSF proteins by transfecting these proteins into various RASSF-negative cell lines (Agathanggelou A. et al., 2003). Evidence show that RASSF1A can interact with different target molecules including microtubules to increase their stability, with MST1 pro-apoptotic kinase to induce apoptosis and with cell cycle regulators to arrest cell cycle progression (Khokhlatchev A. et al., 2002; Shivakumar L. et al., 2002; Agathanggelou A. et al., 2005). Liu L. et al. (2003) presented evidence that RASSF1A and RASSF1C may play a role in genomic stability by binding to and regulating microtubules dynamics at the mitotic apparatus (Song M. et al., 2004; Vos M. et al., 2004). Other scientifics showed that exogenous RASSF1A can also block the JNK pathway in lung cancer cells (Whang Y. et al., 2005), whereas NORE1A can inhibit MAPK activation with no apparent effect on AKT.

The mechanism by which RASSF1 and NORE1 can promote apoptosis has been further investigated. Researchers revealed that activated RAS can bind to RASSF1 and NORE1 which can efficiently homodimerize and heterodimerize with each other and further form a complex with the MST1 (Mammalian sterile 20-like kinase 1) protein kinase through the SARAH domain to mediate a novel RAS-regulated apoptotic pathway (Ortiz-Vega S. et al., 2002; Scheel H. and Hofman K., 2003; Praskova M. et al., 2004; Oh J. et al., 2006). The MST1 protein is a serine/threonine kinase that contains caspase cleavage sites and that can become activated by autophosphorylation to initiate apoptosis (Khokhlatchev A. et al., 2002). The SARAH domain is a novel interaction motif which
has been shown to mediate heterotypic and homotypic interactions between tumor suppressors and MST kinases (Scheel H. and Hofman K., 2003). Since RASSF3 possess also the SARAH domain it is not excluded that it may participate into a similar pathway (Figure 14). We speculate that activated RAS may bind to RASSF3 and induce homodimerization or heterodimerization with other members of the RASSF family and then further complex with the MST kinase to promote apoptosis.

An attempt was made to identify the molecular signaling pathways altered by transient transfection of Rassf3 in SKBR3 cells (Figure 18). No obvious changes in protein levels of intracellular markers involved in RAF-MAPK or JNK pathways due to ectopic expression of RASSF3 protein were detected, which suggested that RASSF3 growth-inhibition must occur by different mechanisms. The slight decrease in p-AKT levels may suggest that RASSF3 growth-inhibition results in a decreased activity of the survival PI3K-AKT pathway. These observations may vary in other cell lines since the genetic background of individual cell lines can influence the expression profile of candidate RASSF3 target genes. Thus, the effect of Rassf3 transient expression on intracellular signaling pathways needs further investigation.

On the other hand, the observation that RASSF3 protein reduces the level of H-RAS protein following co-transfection suggest a role for RASSF3 as a negative regulator of H-RAS proteins (Figure 19).

5.5. **Functional Study of the Rassf3 Gene in Transgenic Mice**

The use of transgenic mouse technology is a powerful tool to study specific questions in developmental and cancer biology. Transgenic mice that carry one particular transgene can provide deep insight into the function of a particular transgene in
a specific tissue. So far, only one animal model used for studying a member of the RASSF family, the RASSF1A, has been described by Tommasi S. et al. (2005). They created a mouse knockout for Rassf1a, where the Rassf1a was specifically inactivated, to closely mimic the situation in human tumors. They showed that Rassf1a+/− and Rassf1a−/− were prone to spontaneous tumorigenesis in advanced age (18 to 20 months) and more susceptible to chemical carcinogen-induced tumor formation. The tumors included lung adenomas, lymphomas and breast carcinomas. These data reinforced the role of RASSF1 gene as a tumor suppressor.

Because tumor progression is a complex multi-step process involving different signaling pathways, oncogenes or tumor suppressor genes (Huand D. et al., 1997), the development of transgenic mice carrying more than one transgene have been shown to be more adequate to address simultaneously multiple aspects of tumorigenesis and to investigate the synergy between transgenes. The aim of the second part of our study was to develop a bi-transgenic mouse line to investigate the synergy between the neu oncogene and the Rassf3 gene in mammary glands, in order to further elucidate the role of Rassf3 in HER2/neu-initiated breast cancer. To reach this goal, we generated two novel transgenic mouse lines. First, the MMTV/Rassf3 transgenic mouse line was generated by standard cDNA microinjection technique (Wagner T. et al., 1981). Second, the MMTV/Rassf3-neu bi-transgenic mouse line was produced by cross-breeding MMTV/Rassf3 transgenic mice with MMTV/neu transgenic mice.

A number of bi-transgenic mouse models carrying simultaneously the neu oncogene and other transgene have been reported in the literature. These models have been very useful in studying the synergy between the neu oncogene and other oncogenes,
tumor suppressors or signaling mediators and in identifying parallel and interconnected pathways involved in HER2/neu tumorigenesis. For example, bi-transgenic mice generated by crossing the MMTV/neu transgenic mice with MMTV/p53 mutant mice or null mice have demonstrated a direct cooperativity between HER2/neu and p53 pathways in human breast cancer (Li B. et al., 1997; Blackbum A. and Jerry J., 2002). It was reported that the introduction of the mutant p53 transgene in bi-transgenic mice reduced tumor latency to 154 days (Blackbum A. and Jerry J., 2002). These observations demonstrated that p53 mutation is an important event in HER2/neu-mediated tumorigenesis. Several bi-transgenic models studied the interaction between neu and TGF-β, a naturally occurring potent inhibitor of cell growth. These models highlighted that TGF-β overexpression in mammary gland is not sufficient to block neu tumorigenic potential (Muraoka R. et al., 2003; Siegel P. et al., 2003; Gorska A. et al., 2003) and furthermore, that TGF-β may contribute to tumor invasion and metastasis once the carcinomas have developed. These observations reinforce the notion of a biphasic role for TGF-β which has an anti-mitogenic effect and a prometastatic effect simultaneously (Gold I., 1999; Rowland B. and Peeper D., 2006). Yu Q. et al. (2001) reported the cross between cyclin D1 -/ knockout mice with MMTV/neu mice. Remarkably, they showed that the bi-transgenic mice bearing the neu oncogene but lacking cyclin D1 were completely resistant to neu-induced mammary carcinogenesis as the mice remained tumor-free. These findings indicated that intact cyclin D1 functions are essential for transformation by HER2/neu. Others showed that overexpression of the p16 tumor suppressor, which specifically blocks cyclin-dependent kinase (CDK) -4 and -6 activity, was also able to block neu-induced tumorigenesis (Yang C. et al., 2004).
consequence, it was discovered that the deregulation of the cyclin D1-CDK4/6 interactions, involved in the cell-cycle machinery, is an essential target for HER2/neu function in breast cancer. This last discovery raised the exciting possibility that inhibitors of cyclin D1 could specifically target human breast cancers that overexpress the HER2/neu oncogene (Chodosh L., 2002).

The production of the novel MMTV/Rassf3 transgenic mouse line which overexpresses the Rassf3 gene in the mammary glands under the control of the MMTV promoter suggested that overexpression of Rassf3 does not impair mammary gland development since the mice were viable and fertile with normal litter size and parental behavior during weaning. The observation that the transgene transcript is also detected in lower amounts in other tissues (e.g. brain, muscle and small intestine) beside the mammary gland has been documented in the literature (Figure 22) (Ross S. et al., 1990; Wagner K. et al., 1997; Guy C. et al., 1992); however, the high amount of transgene expression in the brain tissue was a surprise.

The bi-transgenic mouse model overexpressing both the Rassf3 gene and the neu oncogene in the mammary glands under the transcriptional control of the MMTV LTR promoter permitted to determine the influence of Rassf3 overexpression on HER2/neu mammary tumor formation in an in vivo model. The results revealed that Rassf3 overexpression delays the tumor onset in the bi-transgenic mouse line (Figure 25). The appearance of tumors in the bi-transgenic mice indicates that Rassf3 overexpression alone is insufficient to fully suppress tumor initiation. Therefore, the resistance to HER2/neu tumor development in the naturally tumor-resistant MMTV/neu mice may be due to the activation of more than one suppressor or suppressor pathway. This reflects the multi-
step model of HER2/neu tumorigenesis where more than one genetic event are involved (Guy C. et al., 1992). Thus, tumor heterogeneity and multiple step-tumorigenesis emphasize the difficulty to pin point one single factor that is responsible for altering the process of tumorigenesis and reinforce the advantages of using combined therapies which are aimed at attacking different pathways involved in cancer development at the same time or in a sequential manner.

The detection through Western blot analysis of the RASSF3 protein in the brain tissue but not in the mammary gland suggested that the expression level of the RASSF3 protein is too low in the mammary glands to react with the polyclonal anti-RASSF3 antibody rather than concluding to the total absence of RASSF3 protein (Figure 23). In addition, the presence of RASSF3 protein in the brain of the MMTV/Rassf3 and the MMTV/Rassf3-neu transgenic mice and its absence in the littermates confirmed the generation of Rassf3-positive transgenic mice. Therefore, the increase in mammary tumor latency in the MMTV/Rassf3-neu bi-transgenic mice compared to their littermates can be attributed to the expression of the Rassf3 gene in mammary tissues. We believe that if RASSF3 expression levels and activity was boosted in the mammary gland of the bi-transgenic mouse line, the delay in tumor onset would have been further increased for the bi-transgenic line compared to their littermates.

RASSF3 and p-HER2 protein levels in mammary tumors of the MMTV/Rassf3-neu bi-transgenic mice and the MMTV/neu<sup>+/−</sup> littermates are positively correlated, suggesting that RASSF3 protein does not interfere directly with HER2 activity (Figure 26). We speculate that the high level of RASSF3 found in tumors is not the causal factor but actually reflects the results of a cellular/host defensive response in this case. It
appears that RASSF3 expression is induced in response to HER2 activity in HER2/neu-positive tumors and that RASSF3 inhibits tumor growth by acting as a negative regulator of constitutively activated RAS.

Finally, the findings that Rassf3 is overexpressed in the mammary gland of tumor-resistant MMTV/neu female transgenic mice and in addition that Rassf3 delays tumor onset in the bi-transgenic mouse model are strong indicators that a particular RAS-effector pathway, involving the RASSF3 effector, plays a role in HER2/neu-tumorigenesis.
The overexpression of the HER2/neu oncogene in human breast cancer is associated with increased disease recurrence and worse prognosis. A better understanding of the fundamental biology and pathogenetic effects of HER2/neu overexpression in human breast cancer remains a major focus. In this study, we used the well-characterized MMTV/neu female transgenic mouse model to study HER2/neu breast cancer and in particular the factors involved in the inhibition of HER2/neu-initiated tumorigenesis.

By focusing on the MMTV/neu mice that naturally become tumor-resistant and by using comparative genetic profiling, we identified a novel gene, the Ras Association domain (RalGDS/AF-6) Family 3 (Rassf3), as one of the candidate genes that may influence the mammary tumor incidence in female MMTV/neu transgenic mice.

We provided evidence suggesting an important role for the Rassf3 gene in the process of HER2/neu-initiated mammary tumorigenesis. Our data demonstrated: 1) that the Rassf3 gene is overexpressed in the mammary gland of tumor-resistant MMTV/neu mice compared to the tumor-susceptible MMTV/neu transgenic littermates or age-matched non-transgenic FVB mice, and 2) that the Rassf3 gene is significantly up-regulated in neu-specific mouse mammary tumors compared to adjacent normal
tissues. The expression pattern of the \textit{Rassf3} gene in mammary tumors and adjacent mammary glands varies depending on the tissue/cancer type and the context (\textit{HER2/neu} oncogene).

Furthermore, we reported the first functional study for the \textit{Rassf3} gene using \textit{in vitro} and \textit{in vivo} models. Our results showed that \textit{Rassf3} overexpression inhibits cell proliferation in \textit{HER2/neu}-positive human and mouse breast cancer cell lines, possibly through induction of apoptosis. In addition, our results suggest a possible interaction between RASSF3 and H-RAS proteins. Through the establishment of a novel MMTV/\textit{Rassf3-neu} bi-transgenic mouse line which overexpresses both \textit{Rassf3} and \textit{neu} genes in the mammary glands, we demonstrated that \textit{Rassf3} overexpression delays the onset of mammary tumor formation.

These findings suggest that the \textit{Rassf3} gene exhibits the properties of a RAS effector and tumor suppressor gene similar to other members of the RASSF family already characterized by other researchers. Overexpression of the \textit{Rassf3} gene may be induced by the high levels of \textit{HER2/neu} proteins in the mammary gland. \textit{Rassf3} may then inhibit cell proliferation and tumor growth by acting as a negative regulator of activated RAS. We speculate that the high levels of RASSF3 found in tumors are the results of a cellular defensive response in this case. However, the molecular mechanism of growth-inhibition of the \textit{Rassf3} gene and its particular role in \textit{HER2/neu} tumor initiation and progression needs further investigation.
Finally, this study presented an innovative approach for studying the already well-documented MMTV/neu transgenic mouse model. The focus on the naturally tumor-resistant MMTV/neu female transgenic mice allowed the identification of the novel Rassf3 gene as a candidate tumor suppressor involved in the resistance to HER2/neu-initiated mammary tumorigenesis.
BIBLIOGRAPHY


Im Y.H., Kim H.T., Lee C., Poulin D., Welford S., Sorensen P.H., Denny C.T., Kim S.J.,
EWS-FLI1, EWS-ERG, and EWS-ETV1 oncoproteins of Ewing tumor family all
suppress transcription of transforming growth factor beta type II receptor gene.

Irimia M., Fraga M.F., Sanchez-Cespedes M., Esteller M. CpG island promoter
hypermethylation of the Ras-effector gene NORE1A occurs in the context of a wild-


Jacks T., Remington L., Williams B.O., Schmitt E.M., Halachmi S., Bronson R.T.,

Jaenisch R. Germ line integration and Mendelian transmission of the exogenous Moloney

Jiang X., Sorkin A. Coordinated traffic of Grb2 and Ras during epidermal growth factor
receptor endocytosis visualized in living cells. Mol Biol Cell. 2002 May;13(5):1522-
35.

Jiang X., Roth L., Lai C., Li X. Profiling activities of transcription factors in breast


Johnston J.A. Are SOCS suppressors, regulators, and degraders? J Leukoc Biol. 2004

Johnston S.R., Kelland L.R. Farnesyl transferase inhibitors-a novel therapy for breast

Karunagaran D., Tzahar E., Beerli R.R., Chen X., Graus-Porta D., Ratzkin B.J., Seger R.,
Hynes N.E., Yarden Y. ErbB-2 is a common auxiliary subunit of NDF and EGF

Katz M.E., McCormick F. Signal transduction from multiple Ras effectors. Curr Opin

Kauraniemi P., Barlund M., Monni O., Kallioniemi A. New amplified and highly
expressed genes discovered in the ERBB2 amplicon in breast cancer by cDNA


161


Woods D., Parry D., Cherwinski H., Bosch E., Lees E., McMahon M. Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by p21Cip1. Mol Cell Biol. 1997 Sep;17(9):5598-611.


