

## **ABSTRACT**

**HOULE, CHRISTOPHER DAVID.** Cooperative Effects of Tumor Suppressor Genes and Oncogenes on the Dynamic Process of Tumorigenesis. (Under the direction of Dr. John Cullen)

Tumorigenesis is a multistep process involving a sequence of genetic alterations and subsequent functional changes over time. Two major classifications of genes, oncogenes and tumor suppressor genes, are known to play a significant role in the process of tumorigenesis. The general theme of this dissertation was to evaluate cooperative interactions between certain tumor suppressor genes and oncogenes and assess how this promotes tumorigenesis. Various phases of the tumorigenic process were evaluated including early changes involving alteration in growth control as well as late stages involving metastasis. The overall hypothesis was that certain genetic mutations or altered proteins would have a greater tumorigenic effect if acting cooperatively rather than independently. Three studies were conducted in support of this hypothesis and each involved female reproductive tissues, including the mammary gland and ovary.

The first study evaluated cooperative interactions between the tumor suppressor genes Brca2 and p53. The effects of mutation of these genes on cell proliferation and apoptosis were evaluated in the developing mouse mammary gland with and without exposure to irradiation. The hypothesis for this study was that combined mutation of both genes would produce a more deleterious response than mutation of either gene alone. Results demonstrated that individual mutation of Brca2 or p53 could either increase or decrease apoptosis and cell

proliferation but had little effect on the growth index (apoptosis:proliferation ratio).

Combined mutation of both genes, however, did alter the growth index, but only in response to irradiation. Nonetheless, this finding did support the hypothesis that Brca2 and p53 can interact cooperatively in processes that would promote tumorigenesis.

The second study evaluated spontaneous, ethylene oxide-, and benzene-induced mouse mammary tumors for p53 and H-ras mutations. The hypothesis for this study was that both p53 and H-ras mutations would commonly occur together in the chemically induced tumors but not in spontaneous tumors. The results supported the assumption that p53 and H-ras mutations would be common events in benzene- and ethylene oxide-induced mammary tumors, however, a comparable number of mutations were also found in spontaneous tumors. The mutational spectra between chemically induced and spontaneous tumors, however, was different suggesting that benzene and ethylene oxide exposure could induce mammary specific genetic alterations predisposing to mammary tumorigenesis. Overall, the results revealed that p53 and H-ras mutation were common events in these tumors, regardless of cause, and suggested that cooperative interaction between these genes is important in the genesis of mouse mammary tumors in general.

The third study evaluated the protein expression of four different cell adhesion molecules known to be involved in the metastatic process (KAI1, CD9, E-cadherin, and N-cadherin). Expression was compared between various grades of primary ovarian epithelial tumors and metastases. The hypothesis was that more than one but not all of these adhesion molecules would be downregulated as tumors progressed towards a metastatic phenotype. The results

supported this hypothesis by showing a remarkable variation in expression between these adhesion molecules. KAI1 and CD9 expression decreased as tumors progressed, while N-cadherin expression increased. In contrast, E-cadherin expression remained elevated throughout all tumor grades and metastases. Overall, these results revealed the complexity of adhesion molecule modulation during tumorigenesis and metastasis and suggested several potential mechanisms of cooperative interaction that could promote the metastatic process.

In conclusion, this dissertation evaluated cooperative interactions between several different classes of cancer-related genes and proteins and provided support for the hypothesis that particular cancer-related genes/proteins can work cooperatively and promote tumorigenesis more effectively in combination rather than individually.

**Cooperative Effects of Tumor Suppressor Genes and Oncogenes  
on the Dynamic Process of Tumorigenesis**

by

**CHRISTOPHER DAVID HOULE**

**A dissertation submitted to the Graduate  
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**COMPARATIVE BIOMEDICAL SCIENCES**

**RALEIGH**

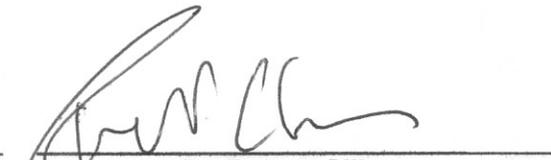
**2005**

**APPROVED BY:**

  
\_\_\_\_\_  
**Dr. Philip Sannes**

  
\_\_\_\_\_  
**Dr. David Malarkey**

  
\_\_\_\_\_  
**Dr. John M. Cullen**  
(Chair of Advisory Committee)

  
\_\_\_\_\_  
**Dr. Robert Sills**  
(Co-chair of Advisory Committee)

## **DEDICATION**

I dedicate this dissertation to my wife Janis, my son Jared, and my mom and dad, David and Gloria Houle.

## **BIOGRAPHY**

**Christopher (Chris) David Houle** was born April 17, 1970 in Newport, New Hampshire to David and Gloria Houle. Chris was raised predominantly in New Hampshire where he graduated from Concord High School in 1988 and then went on to obtain a Bachelor of Science degree in 1992 from the University of New Hampshire. Chris attended veterinary school at North Carolina State University where he received his Doctor of Veterinary Medicine degree in 1997. Following graduation from veterinary school, he completed a 2-year residency in anatomic pathology at the University of Tennessee. In 1999 he received an NIH Intramural Research Training Award for a research position at the National Institutes of Environmental Health Sciences (NIEHS). While at NIEHS, Chris entered graduate school in the Comparative Biomedical Sciences program at North Carolina State University. While working on his dissertation research at NIEHS, Chris also achieved certification by the American College of Veterinary Pathologists in 2000. In the Fall of 2001, Chris joined Experimental Pathology Laboratories as a staff pathologist while he continued to pursue his Ph.D. degree on a part-time basis.

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## LIST OF ABBREVIATIONS

$^{137}\text{Cs}$	cesium 137
8OHdG	8-hydroxydeoxyguanosine
A, C, G, T	adenine, cytosine, guanine, thymine
AB	automation buffer
$\text{Brca2}^{-/-}$	brca2-mutant
BrdU	5-bromo-2-deoxyuridine
cDNA	complementary deoxyribonucleic acid
DAB	3, 3'-diaminobenzidine
dKO	double-mutant ( $\text{Brca2}^{-/-}\text{p53}^{-/-}$ )
DMBA	7, 12-dimethylbenz[a]anthracene
DNA	deoxyribonucleic acid
DSB	double strand breaks
E	embryonic day
ES cells	embryonic stem cells
EtO	ethylene oxide
FA	Fanconi's Anemia
GAPs	GTPase activating proteins
GEFs	guanine nucleotide exchange factors
GDP	guanosine diphosphate
GOG	Gynecologic Oncology Group
Growth Index	proliferation:apoptosis ratio
GSTT1	glutathione S-transferase theta 1
GTP	guanosine triphosphate
Gy	gray
HIER	heat induced epitope retrieval
HR	homologous recombination
IARC	International Agency for Research on Cancer
IHC	immunohistochemistry

IR	irradiation
LI	labeling index
LOH	loss of heterozygosity
LT	large T
MAPK	mitogen-activated protein kinase
MCA	3-methylcholanthrene
MCL	mononuclear cell leukemia
MEF	mouse embryo fibroblasts
MMTV	mouse mammary tumor virus
MNU	N-methyl-N-nitrosourea
mRNA	messenger ribonucleic acid
MT	middle T
NHEJ	nonhomologous end-joining
NLS	nuclear localization signal
NMU	nitrosomethylurea
NQO1	NADPH-quinone oxidoreductase 1
NTP	National Toxicology Program
OSE	ovarian surface epithelium
P53 <sup>-/-</sup>	p53-mutant
PCR	polymerase chain reaction
PhIP	2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine
Pidd	p53-induced protein with a death domain
PIGs	p53-induced genes
ROC	Report on Carcinogens
ROS	reactive oxygen species
RPA	replication protein A
RTK	receptor tyrosine kinases
SCE	sister chromatid exchange
SSA	single-stranded annealing

ssDNA	single stranded deoxyribonucleic acid
TEB	terminal end bud(s)
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling
WAP	whey acidic protein
WT	wildtype (Brca2 <sup>+/+</sup> p53 <sup>+/+</sup> )

## GENERAL INTRODUCTION

It is well known that the initiation of cancer requires several different alterations and thus is probably better described as a process rather than a single event. In each stage of this process, the nascent cancer cell experiences a different set of challenges that must be overcome. Fortunately, cells have a number of safeguards that help prevent initiation or progression of the tumorigenic process. To overcome these safeguards, a cancer cell must accumulate genetic and/or epigenetic alterations that work cooperatively in the tumorigenic effort. Alterations can occur in any one of the many cancer-related genes or proteins, however, it cannot be assumed that all combinations will work cooperatively.

In contrast to the large number of cancer-related genes/proteins, there are only a relatively few cancer-related pathways. This speaks to the high level of redundancy among the various genes/proteins involved in regulating cellular function as well as to the high level of cross-talk between pathways. It is rare to find two or more alterations affecting a single linear pathway in the same tumor, particularly when one alteration would be sufficient. For example, human breast cancers are known to exhibit a high level of signaling activity in the ras pathway, yet ras mutations are relatively rare (<5%)(127). In the human tumors, ras activity is primarily driven by overexpression of upstream growth factor receptors such as HER2/neu (erbB2). In contrast, animal studies, including that described in **manuscript two**, have demonstrated frequent ras mutations in mammary tumors. Nevertheless, the end result in both the human and animal tumors is the same, increased ras activity, only the mechanism

and the region of the pathway affected are different. This example illustrates how two apparently different mechanisms can potentially lead to the same outcome and also shows why it would be unnecessary to have two or more of these common mechanisms in the same tumor. Acquisition of both alterations in the same tumor cell would provide little benefit and therefore such a combination would not be considered a cooperative interaction. This makes sense from a clonal evolutionary point of view in that once one pathway has been altered in a manner that promotes the tumorigenic process there would be little selective advantage for a second alteration in that same pathway.

In this dissertation, I have explored gene/protein interactions that I hypothesized would be selected for during the tumorigenic process. In this context, these gene/protein interactions can be described as being cooperative with the common goal of promoting some aspect of the tumorigenic process. Not all mutations are created equal and it is certainly reasonable to assume that the significance of any particular mutation would depend on the cellular context. Although two individual mutations may work towards the common goal of promoting tumorigenesis that does not necessarily imply that the two mutant proteins are working cooperatively. Cooperation, as defined in this dissertation, indicates that the combination of two particular mutant genes/proteins would have a greater effect towards promoting tumorigenesis than would be predicted based on mutation of each individual gene. This does not necessarily imply that the two mutant gene/proteins are directly interacting only that alteration of one or both genes creates a cellular environment more conducive to the tumorigenic effects of the mutant combination. Furthermore, cooperation in this context does not imply that the effects of these genes/proteins necessarily produce an additive or

synergistic response, only that the alteration of a single gene on its own would not be able to induce the same overall effect.

# **CHAPTER ONE**

## **COOPERATION IN CARCINOGENESIS**

For the past 50 years, Armitage and Doll's (1954) multistage theory of cancer has provided the conceptual foundation for the age-specific incidence of cancer. The multistage theory suggests that normal tissues are transformed into cancerous tissues by means of a series of discrete stages. The stages may be somatic mutations, genomic rearrangements, or changes in tissue interactions and environment. Alterations such as these accumulate in cells changing their behavior from normal growth to unrestrained growth and eventually lead to invasion into surrounding tissue and metastasis (14, 149, 221). The precise nature of each stage does not affect the predictions of the multistage theory. What matters is that individuals, as they age, move stochastically through the various stages of transformation. Eventually there is a regular probability distribution of individuals who have progressed to precancerous stages or all the way to the malignant stage. What happens to an individual is highly random and cannot be predicted, however, a distinct and predictable pattern emerges at the population level. It was Knudson's (1971) insight to compare incidences in inherited and non-inherited forms of retinoblastoma to test the predictions of multistage theory (149, 260). In experimental models, such as mouse skin tumorigenesis, the process has been broken down into at least three distinct steps: initiation, promotion, and progression. From the perspective of the organism, the multistep nature of tumorigenesis is easily rationalized; each step in the process represents a physiological barrier that must be overcome in order for a cell to progress towards a malignant phenotype. Such barriers ensure that successful completion of the tumorigenic process is rarely achieved. For this reason, the initiating mutagenic event is

almost always insufficient to cause carcinogenesis by itself and thus requires one or more secondary/cooperative events to convert a mutated cell into a tumor cell (221, 566).

The concept of gene cooperation is not limited to cancer as there are several examples of similar events occurring in normal cellular activities. One example of this is the competence and progression model of cell cycle regulation (454). It has been shown that fibroblast cell lines, such as Balb/c 3T3 cells, in the quiescent state do not respond to single growth factors, but rather to a combination of at least two factors (416). Growth factors in one group (e.g., PDGF and FGF) can convert cells into a competent state, then competent cells can respond to a second set of growth factors (e.g., EGF and IGF-1), which stimulate the cells to progress in the cell cycle. An exposure to each type of growth factor is required to elicit a response. The decision point at which the progression factors act has been given several names, and may correspond to the restriction point, which is as far a cell can progress in G1 without additional protein synthesis (402). An additional example of this dual signaling system occurs in the immune system, in which two signals are required to initiate growth of T- and B-lymphocytes. As an example, mitogenesis of T-lymphocytes requires signals from both the IL-2 receptor and the antigen receptor (91, 221). Dual signal requirements such as these act as a fail-safe mechanism to prevent accidental triggering of quiescent cells into the cell cycle by transient exposure to mitogenic growth factors. These fail-safe mechanisms are also an underlying factor in the necessity for gene cooperation in tumorigenesis.

There are a number of experimental systems in which cooperation between oncogenes can be observed. An early indication that oncogenes could cooperate was the finding that certain

combinations of oncogenes could transform primary rodent fibroblasts or kidney cells in culture, whereas individually the same oncogenes could not (566). The first such evidence for oncogene cooperation came from experiments carried out with combinations of polyomavirus oncogenes (426), and this was shortly followed by similar studies with cell-derived oncogenes (221, 274). Interestingly, only certain combinations of cooperating oncogenes gave rise to cellular transformation in these assays, further supporting the hypothesis that several distinct signal transduction pathways have to be disrupted for malignant progression to occur. Similar results have also been reported in tissue culture models of human cells (369).

The necessity of several mutations cooperating during human carcinogenesis is also supported by epidemiological studies. Predictions on the number of genetic alterations needed to produce a malignant phenotype often vary depending on the tumor type but generally fall within the range of 2 to 7 (140, 199, 411, 431).

Although the evidence presented above strongly supports the role of oncogene cooperation in both the initiation and progression of tumorigenesis, it is also evident that not all combinations of cancer-related genes have the same effect. For example, no acceleration of tumorigenesis was observed in p53 null/bcl-2 transgenic mice suggesting that these particular genes do not cooperate and that perhaps the reason is that they function in the same tumorigenesis pathway (325). In addition, it is possible that certain genetic alterations will have to occur in a specific sequence in order to achieve the maximal effect. Furthermore, certain combinations of altered cancer genes that can transform cells in one setting may be

harmless in another. It is likely that non-genetic or epigenetic features of different cell types modulate their susceptibility to transformation. For example, different cells might express different components of the cell-cycle machinery, different transcription factors, or signaling molecules (e.g. H-ras or K-ras) (123). Likewise, it's important to keep in mind that, although some tumor types may have certain patterns of genetic alteration it is unlikely that the same patterns will occur in every individual (36).

### ***1.1 BIGENIC MICE – COOPERATION***

Compelling evidence for oncogene cooperation has come from studies with transgenic mice. Transgenic animal models allow one to study the functional effects of a single altered gene in an animal and then, by crossing these animals, allows for the assessment of altered gene combinations. If the bi-transgenic (bigenic) offspring of the cross develop tumors at a faster rate than their parents, then cooperativity has occurred. Experiments such as these have also helped support the notion that a single altered gene is not sufficient to induce tumorigenesis.

In terms of mammary tumorigenesis, there have been a number of models exploring the potential cooperativity between various cancer-related genes. For example, some of the first bigenic mice to be generated were the result of matings between MMTV/c-myc and MMTV/v-Ha-ras monogenic mice. While 50% of MMTV/c-myc and MMTV/v-Ha-ras females developed mammary tumors at 325 and 168 days, respectively, half of the females carrying both genes possessed mammary tumors by 46 days of age (61, 479, 486). In addition, it has been shown that mammary tumorigenesis in the MMTV-v-Ha-ras transgenic mice can be greatly accelerated by the additional loss of p53 function (113, 219), a finding of

significance to the study described in **manuscript two**. Cooperative interactions for mammary tumorigenesis have also been demonstrated in transgenic mice co-expressing MMTV/c-myc and MMTV/TGF-alpha transgenes (479). The mean onset of tumor formation in these animals was 66 days compared to 298 days for MMTV/c-myc monogenic mice. No tumors developed in MMTV/TGF-alpha mice after 1 year of observation (479).

Transgenic mice that overexpress both TGF-alpha and the neu proto-oncogene (erbB2/HER2) in the mammary epithelium develop mammary tumors at a greatly accelerated rate as compared to transgenics expressing either TGF-alpha or neu alone. Similar results were observed in bigenic animals expressing the neu proto-oncogene and a mutant form of p53 (p53-172H). The median age of tumor onset in the p53-172H/neu bigenic animals was 154 days compared to 254 days for the neu overexpressing monogenic strain. In contrast, mice with only the mutant p53-172H gene did not develop mammary tumors after more than 300 days. Interestingly, the mammary tumors that arose in these bigenic animals were highly anaplastic and displayed both aneuploidy and tetraploidy (76, 479).

Breeding of transgenics with mice bearing a disrupted targeted gene (knockout mice) have been used to examine the effects combining various overexpressed transgenes in a p53-deficient background. The first of these studies involved crossing transgenic mice expressing a Wnt-1 transgene with mice deficient for p53 (113). Both females and males expressing Wnt-1 in a p53-deficient background developed mammary tumors at an accelerated rate compared to mice expressing Wnt-1 on a wildtype p53 background. Additionally, the lack of p53 resulted in mammary tumors that were predisposed to aneuploidy. More recently a

modest increase in the number of mammary tumors was demonstrated in  $Brca1^{+/-} p53^{-/-}$  mice compared to  $Brca1^{+/-} p53^{+/-}$  mice (310). These observations are supported by data from conditionally targeted inactivation of *Brca1* in the mammary gland. Although mice lacking *Brca1* in the mammary gland developed mammary tumors, the additional loss of *p53* function greatly accelerates the onset of mammary tumor formation. Similar studies have confirmed cooperativity between *p53* and *Brca2* in mammary tumorigenesis (479).

## ***1.2 VIRALLY INDUCED CANCER – COOPERATION***

The study of the transforming proteins of DNA tumor viruses have been very revealing, and strongly support the idea that cooperation is an essential feature of oncogenesis. Some of the original evidence for the ability of oncogenes to cooperate came from the demonstration that polyomavirus large T (LT) and middle T (MT) antigens were both required for the transformation of primary rat embryo fibroblasts (426). Polyomavirus LT antigen binds to and inactivates the Rb family of proteins. The polyomavirus MT antigen binds to a completely different set of cellular proteins, including pp60c-src, phosphatidylinositol 3-kinase, and PP2A (61, 221). A related virus known as SV40 was first thought to have the ability transform cells by inactivating a single cancer-related gene, however, it was later learned that its version of the large T antigen was actually multifunctional and had the ability to bind and inactivate both *p53* and Rb (284). In the case of adenovirus, both of its transforming proteins (E1A and E1B) are required to achieve oncogenesis. E1A binds Rb and the E1B protein binds *p53* (573). There is a similar story for human papillomavirus, where E7 binds Rb, and E6 binds to *p53* (453).

Likewise, there are a number of retroviral systems where a pair of cooperating oncogenes has been acquired in the genome of a single virus. For example, the avian carcinoma virus (MH2) has v-mil/raf and v-myc; the avian erythroblastosis virus (AEV ES4) has v-erbA and v-erbB; and the E26 leukemia virus has v-myb-ets (fused c-myb and c-ets-1) (400). In each case, mutational inactivation of one of the two genes leads either to complete loss of transforming activity or to reduced transformation potential, depending on which gene is mutated (221). This implies a true cooperation between the resident oncogenes.

The avian erythroblastosis virus, mentioned above, also provides a nice example of oncogene cooperation in the induction of cancer. One of its genes, v-erbB, codes for a constitutively active EGF-like receptor that provides a continuous growth signal, whereas its other gene, v-erbA, blocks expression of genes needed for erythroid differentiation (602). Together they induce erythroid leukemia. (221). Particular isolates of the avian erythroblastosis virus that only contain one of the two oncogenes (v-erbB) are much less potent as tumor inducers (61).

### ***1.2.1 Mouse Mammary Tumor Virus - Cooperation***

The mouse mammary tumor virus (MMTV) is a type B retrovirus that infects mammary epithelial cells and randomly inserts its proviral DNA into somatic cell DNA during its replicative cycle (265, 433). Bittner's "milk agent", as it was originally called, was discovered by Bittner in 1936 (43). This was the first virus universally accepted in the US as a cancer-causing virus. In addition to this exogenous form of MMTV, some mouse strains also carry an endogenous form of MMTV integrated into their genome (201). In these mice,

the virus has been inserted into the DNA of the egg or sperm. Both the endogenous and exogenous forms of virus result in infected mice, but strains of mice in which the virus has become endogenous usually exhibit higher rates of cancer (394).

Rather than carry its own oncogenes, the MMTV induces mammary tumors by acting as an insertional mutagen (64). Proviral insertion causes alteration of structure and expression of tightly regulated cellular genes. This can lead to the overproduction of a natural protein product or could lead to overexpression of truncated proteins (76). Molecular analysis of proviral integration sites in tumors has led to the discovery of a number of cellular proto-oncogenes. An interesting common theme of these genes is that most are involved in short-range signaling between cells. This includes several members of the FGF growth factor family, and two Wnt genes: Wnt-1 (formerly known as int-1) and Wnt-3. Another gene identified this way, int-3, is not a secreted molecule, but rather has homology to the *Drosophila* Notch gene, a receptor molecule (542). A majority of the MMTV-induced mammary tumors have multiple viral insertions, and several examples have been found where more than one gene is activated in a single tumor. One investigator found 50% of MMTV-induced tumors to have both Wnt-1 and *fgf3* rearranged in the same sample (542). Other examples of cooperative events include tumors in which *fgf3* and *fgf4* are activated by a single proviral insertion or tumors in which Wnt-3 is activated along with amplified copies of the Wnt-2 gene (542).

Several mouse models described elsewhere in this dissertation have taken advantage of the MMTV's specificity for the mammary gland by using its long terminal repeat promoter

sequence as a means to target the mammary gland. Although not completely specific for mammary gland, this has been an effective means for getting relatively specific gene expression in the mammary gland. In addition to the mammary gland, this promoter is active in the salivary gland and basal cells of the epidermis. Males also exhibit expression in the seminal vesicle and Leydig cells (198).

## CHAPTER TWO

### LITERATURE REVIEW FOR MANUSCRIPT ONE

#### **2.1      *MOUSE MAMMARY GLAND DEVELOPMENT***

Mammary gland development depends on branching morphogenesis, a developmental process shared by several tissues including the lung, kidney, and salivary gland. In each case, an epithelial tube penetrates mesenchyme or connective tissue and, through repeated bifurcations, forms tissues with elaborate, tree-like architectures (481). In the mammary gland, ductal morphogenesis results in an arborizing ductal network consisting of a large primary duct and numerous branches of smaller secondary and tertiary ducts extending in and ultimately filling the mammary fat pad by 3 months of age (432). Unlike the development of amorphous glandular tissues, ductal morphogenesis requires a dynamic structure in which the continuous addition of new cells coincides with canalization and forward movement. In the mammary gland, terminal end buds serve this purpose (575). When ducts approach the edge of the fat pad or become surrounded by other ducts, growth is inhibited and end buds are transformed into blunt-tipped structures with few or no dividing cells (481).

#### **2.1.1      *Fetal Development – Mammary Gland Development***

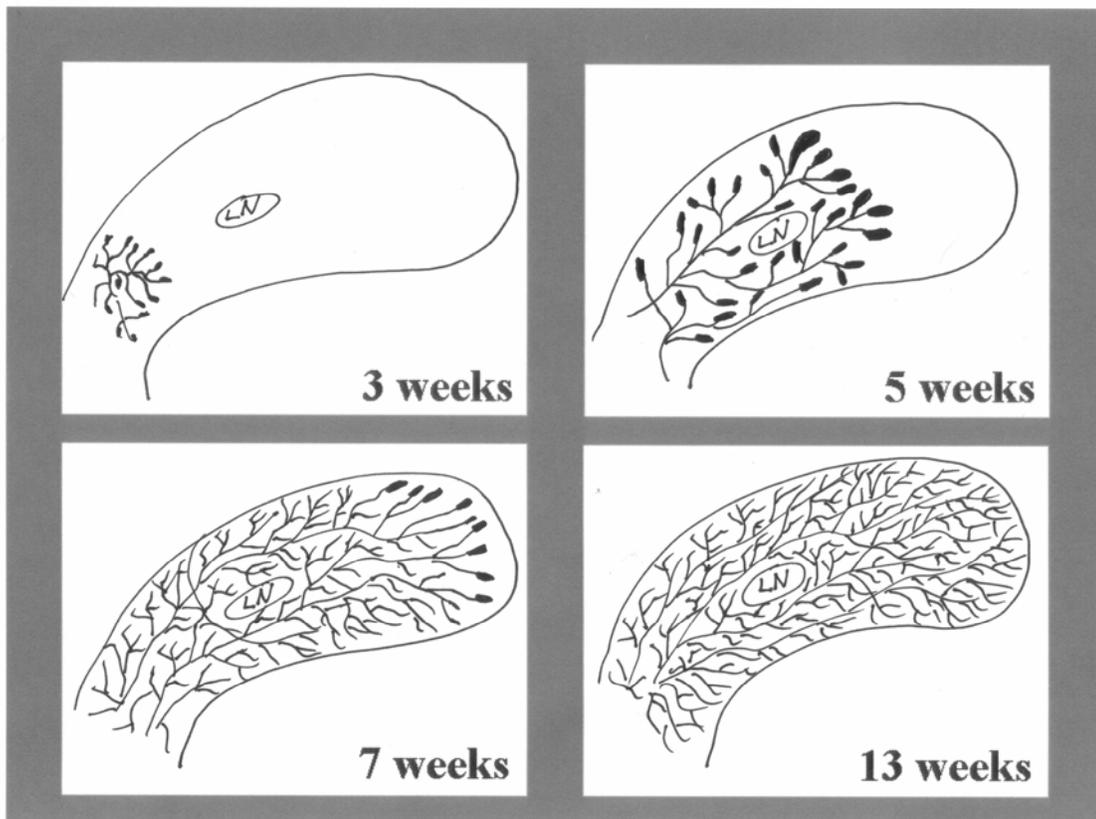
Mammary gland development begins during embryogenesis but is not finished until completion of puberty. Although mammary epithelium is derived from the epidermis, its initiation is dependent on the presence of a specialized mesenchyme called the mammary fat

pad (95). Signals from the mammary fat pad underlying the epidermis direct epidermal cells towards a mammary differentiation pathway. In the mouse fetus, the mammary gland is visible as early as day 10-11 of gestation and consists of rudimentary epithelial ducts ending in club-like structures known as end buds (163). Gonadal differentiation occurs late on embryonic day 13 (E13) resulting in sexual differentiation of the mammary gland by day 14. Males then begin to produce androgens resulting in inhibition of mammary development and ultimately degeneration (122). Mammary development in females remains quiescent until E16 at which time there is another round of cellular proliferation and differentiation in response to maternal hormones (163).

### ***2.1.2 Postnatal Development – Mammary Gland Development***

Unlike most organ systems, the mammary gland is unique in that the majority of its development occurs postnatally (24). Postnatal growth of the rodent mammary gland is chronologically divided into five periods: neonatal, juvenile, prepubertal, pubertal, and mature virgin. In the mouse mammary gland, these growth periods correspond to 1-3 weeks of age, 3-4 weeks of age, 4-5 weeks of age, 5-7 weeks of age, and 7-9 weeks of age and beyond, respectively (529). At birth, the mammary gland consists of a rudimentary branching network of about 15-20 ducts (122), however, with the onset of the prepubertal growth period, the mouse mammary gland becomes increasingly sensitized and responsive to elevations in local and systemic reproductive hormones (529, 575). It is during this prepubertal growth period that the gland grows allometrically with respect to the animal and the highly mitotic terminal end bud (TEB) structures emerge to drive ductal morphogenesis.

Figure 1 shows four different stages of mouse mammary gland development. The bulbous structures along the leading edge of the developing ducts are TEB and are particularly prominent at five weeks then gradually decrease in size. During the latter stages of the pubertal growth period, as the expanding mammary ductal mass reaches the limits of its fat pad, the TEB structures are permanently replaced by mitotically quiescent terminal end ducts and alveolar buds (ductules) (529, 575). However, all regions of the mammary epithelium retain the capacity to regenerate the entire mammary structure if necessary (490).



**FIGURE 1.** Mouse mammary gland development.

### ***2.1.2.1 Terminal End Bud Structure – Mammary Gland Development***

TEB exhibit high rates of cell proliferation, as they elongate and branch throughout the mammary fat pad, and high rates of apoptosis, as subtending ducts are formed (122, 217). Both processes are critical for normal mammary ductal morphogenesis (217, 218, 506). Systemic hormones (e.g., estrogen, growth hormone) and locally acting growth factors (e.g., TGF-alpha, TGF-beta, EGF, and IGF-1) regulate the proliferative state of the TEB (24). The TEB contains two histologically distinct cell types: the body cells and the cap cells. The cap cells are the outermost layer of the TEB and interact through a thin basal lamina with the surrounding stroma. These morphologically distinct cap cells are considered pluripotential stem cells of the developing gland and are known to give rise to the intermediate, luminal, and myoepithelial cells of the advancing duct (24). The interior of the teardrop-shaped TEB is filled with several layers of body cells. Body cells are characterized by the expression of specific cytokeratins and cadherins (218). Apoptosis, which plays a critical role in mammary ductal morphogenesis, is most prevalent in the body cells adjacent to the developing lumen (217). Proximal to the TEB is the neck region, which contains several layers of body cells and acts as the transition zone between the TEB and the subtending duct. Adjacent to the neck region is a simple duct with one to three layers of ductal epithelial cells surrounded by a single layer of attenuated myoepithelial cells (217).

### ***2.1.2.2 Terminal End Bud Kinetics – Mammary Gland Development***

In every tissue, normal or abnormal, cell composition consists of a balance of three different cell populations: cycling cells, resting cells (cells in G0), and dying cells (cell loss). The

growth of normal tissue involves the net increase in cell number resulting from more cells being born than dying. In the differentiated or in the adult tissue, in which growth has ceased, the number of cells produced per unit of time is equal to the number of cells that die (445). Normal development of the mammary gland is dependent upon both periodic proliferation and removal of populations of mammary epithelial cells by apoptosis (506). Humphries *et al.* showed that apoptosis occurred predominately in defined zones of the TEB; 14.5% of the cells within three cell layers of the lumen (body cells) were undergoing apoptosis compared to 7.9% outside this boundary (218). DNA synthesis in the TEB demonstrated a reciprocal pattern; 21.1% in the outside three cell layers and 13.8% within (218). Apoptosis is thought to be required for the “hollowing out” of the solid TEB creating the ductular structures, whereas the proliferating cells on the periphery maintain continued growth and extension into the fat pad (218). Appropriate balance between these two processes is critical for preventing malformation of the mammary gland as well as neoplastic transformation.

### ***2.1.3 Mammary Gland Carcinogenesis – Mammary Gland Development***

The window of opportunity for embryonic growth in some organs is very narrow. It is critical to have the capability to maximize proliferative capacity during the available timeframe and fulfill the requirements of pattern formation and growth required for normal organogenesis. Therefore, the capacity for simultaneous proliferation and apoptosis is a distinct advantage for many developing systems. Consequently, tight or joint regulation of these two processes is required in order to avoid overpopulation or deletion of needed cells (217). However, this time of development also represents a period of exquisite sensitivity to environmental

exposures (it is because of this sensitivity that the mice described in **manuscript one** were irradiated at 5-weeks of age). Imbalance of the developmental process can have significant consequences in terms of future carcinogenesis. Proliferation that is not balanced by apoptosis can contribute to neoplastic development. Alterations in the number of cells undergoing apoptosis or failing to complete execution of apoptosis can result in accumulation of epithelial cells or premalignant hyperplasia that contributes to development of mammary neoplasia (506).

TEB are the primary structures in the rodent mammary gland that give rise to carcinogen-induced mammary tumors (447). The higher susceptibility of the TEB to neoplastic transformation is attributed to the fact that this structure is composed of an actively proliferating epithelium with a high growth fraction (445). It is the presence of the mitotically active cap cells that is thought to represent a time of particular sensitivity to carcinogens and subsequent susceptibility to tumorigenesis (575). Russo *et al.* found that exposure of the rat mammary gland to carcinogens at an early age resulted in significant mutations associated with malignant transformation in the undifferentiated cell populations of the TEB (447). In rodents, maximal incidence of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary cancer occurs when the carcinogen is administered to young virgin cycling rats, but the same carcinogen fails to induce tumors when given to rats after a full-term pregnancy (444). Epithelial cells in the TEB also metabolize carcinogens differently than mature mammary epithelium. TEB cells metabolize DMBA to more polar and less phenolic metabolites than mature mammary epithelium (445). In addition, the highly proliferative TEB epithelium metabolizes DMBA faster producing more epoxides than

mature epithelium, ultimately resulting in a greater number of DNA adducts. TEB cells have also been shown to be less efficient in removing DNA adducts as compared to mature epithelial cells (445).

### ***2.1.3.1 P53 and Brca2 – Mammary Gland Development***

Studies have shown that p53 null mice have only a slight reduction in TEB apoptotic levels as compared to wildtype mice and ductal development appears to occur normally in these mice suggesting that p53 is not significantly involved in mammary ductal morphogenesis (217). Studies have shown that the Bcl family of proteins play a greater role in the regulation of apoptosis in the developing mammary gland (218). There is data, however, to show that p53 is critical upon carcinogen exposure during mammary gland development (218).

Radiation induced apoptosis appears to be greatest during times of peak proliferative stages of mammary gland development (218). In addition, Minter *et al.* (360) showed that p53-mediated responses in the mammary gland vary depending on the developmental stage suggesting that the highly carcinogen sensitive periods of mammary gland development may be related to p53 status.

Brca2 mutant mice have shown a reduction in mammary ductal development and branching complexity, however, as with the p53 mutant mice, they ultimately develop fully functional mammary glands (34). The mechanisms for this developmental delay are unclear; however, it is possible that alterations in apoptosis and/or proliferation may be involved. This possibility was explored further in **manuscript one** of this dissertation.

## **2.2      *GENETICALLY MODIFIED MOUSE MODELS OF MAMMARY CANCER***

The use of genetically modified mice has provided important insights into the molecular basis of mammary tumorigenesis and has helped evaluate the effect of gene-environment interactions. Advances in gene targeting techniques have made it possible to generate mice with a wide variety of defined genetic alterations (55). Genes either can be removed or added using knockout or transgenic techniques, respectively. One important consideration when using genetically modified animals is the influence of the parent background strain. It has been known for many years that different mouse strains vary in their susceptibility to tumorigenesis.

### **2.2.1      *Knockout Mice – Genetically Modified Mice***

Knockout mice have provided important insights for many genes during normal growth and differentiation, as well as during neoplastic development (55, 114). Using knockout mouse models to study tumor suppressor genes such as *Brca2* is especially attractive since loss of function seems to be required for neoplastic progression. Embryonic stem cells (ES cells) and homologous recombination are utilized to inactivate an endogenous gene from a host's genome. ES cells are derived from 3-day old embryos and are undifferentiated but remain totipotent. These cells are transfected in cell culture with a vector that contains a homologous portion of the gene of interest. The vector consists of two regions of homology at either end, the disrupted gene of interest, and two additional genes to allow for selection of properly

transfected cells. The regions of homology are located at either end of the vector and are complementary to specific sites on the genome surrounding the gene of interest. The vector binds to the genome at these two points of homology. The cells own nuclear machinery recognizes the identical stretches of sequence and swaps out the existing gene with the artificial piece of DNA. This is a process known as homologous recombination, which is a regular genetic event that occurs in all cell types at low frequency. Through positive and negative selection, homologous recombinants can be identified and then characterized by the polymerase chain reaction (PCR) analysis to confirm the presence of the defective gene. These ES cells of interest are injected into a blastocyst and then transferred into a pseudopregnant female. The mice born from these procedures are referred to as chimeras. Chimeric animals, which are capable of transmitting the mutant genetic locus to their offspring, are typically bred to wildtype mice, producing F1 animals. F1 animals are crossed to generate F2 animals homozygous for the target locus (488, 489, 599, 608). Unfortunately, inactivation of some tumor suppressor genes results in mice particularly susceptible to development of specific tumor types making certain mouse models unsuitable for the study of particular cancer types (105, 177).

#### ***2.2.1.1 P53 Deficient Mice – Genetically Modified Mice***

P53 deficient mice are generally considered developmentally normal, however, some studies have shown that p53 null mice exhibit reduced apoptotic levels in comparison to wildtype mice (114, 154). P53 null mice are highly susceptible to neoplastic development with a 74% tumor incidence before 6 months of age (114). Malignant lymphomas are the predominant

tumor type, although a variety of other sarcomas also develop (189). The rapid development of these tumors has limited the ability to study mammary carcinogenesis in these mice. Mice heterozygous for germline p53 mutations may or may not develop mammary tumors depending on their genetic background. P53 heterozygotes on a Balb/c background have shown a 55% incidence of mammary tumors, however, other strains such as C57BL/6 and 129 appear to be relatively mammary tumor resistant (271). Recent studies using mammary specific (conditional) knockout of both p53 alleles have been very successful revealing a complete penetrance of mammary tumors (307). Others have had success by transplanting p53 null mammary epithelial cells into the cleared mammary glands of wildtype p53 mice (105). In these studies, 66% of the mice develop mammary tumors by 400 days post-transplantation.

#### ***2.2.1.2 Brca2 Deficient Mice – Genetically Modified Mice***

Early functional studies in Brca2 deficient mice examined the effects of Brca2 truncations in the 5' region of exon 11, omitting all of the BRC repeats (these are Rad51-binding domains described in detail later on). These studies were hindered by the embryonic lethal phenotype that developed around embryonic days 6.5 to 8.5 (253). Interestingly, it was found that the addition of a p53 or p21 null background into these embryos extended survival by 1 or 2 days (310). It was eventually found that truncation of Brca2 must allow retention of three or more BRC repeats in order for the embryo to survive. Disruption of Brca2 after the third BRC repeat results in the live birth of approximately 50% of the homozygous mutants. Disruption after the seventh BRC repeat leads to enhanced embryonic survival although these mice are

growth retarded, infertile, and develop thymic lymphomas by 5 months of age (458). These tumors also frequently had mutations in checkpoint genes (e.g., p53, Bub1, Mad3L) resulting in defective spindle assembly. The length of embryonic survival also depends to some degree on the genetic background of the mouse being utilized. Transfer of embryos from a 129/SvEv background to a Balb/cJ background resulted in prolonged embryonic survival suggesting the presence of modifier genes capable of influencing the phenotype (33). Mouse embryo fibroblasts (MEF) derived from Brca2 null embryos exhibit elevated p53 levels, impaired cell proliferation, and hypersensitivity to irradiation. In addition, these cells spontaneously develop a variety of different chromosomal aberrations after only a few cell divisions including gross chromosomal rearrangements such as translocations, inversions, large deletions, and partial or complete loss of chromosomes (101, 368, 460, 470). Double strand breaks (DSB) as well as tri-radial and quadri-radial chromosomes are also seen suggesting a role for Brca2 in mitotic recombination (377, 378, 404).

### ***2.2.2 Conditional Knockouts – Genetically Modified Mice***

There are several different ways to make conditional knockout models, however the most widely used method is the Cre-loxP recombinase system. Cre recombinase is an enzyme that works like scissors to cut out a gene that is in between two target sequences called loxP (438). Because this enzyme is expressed only in certain cell types, the targeted gene will be knocked out of only those cells. Placing Cre under the control of a mammary specific promoter allows for expression of the recombinase followed by gene deletion only in cells of the mammary gland. For such experiments, two types of genetically engineered mouse strains are generated: one contains the "floxed" (flanked by loxP sites) gene of interest, and

the other has the Cre-recombinase transgene. After intercrosses between these two strains, both genetic alterations appear in all cells of the progeny. As a result, Cre-recombinase that is selectively expressed in mammary epithelial cells catalyzes excision of the "floxed" tumor suppressor gene in a tissue-specific manner so that only mammary cells become predisposed to cancer development. This method was successfully applied for conditional inactivation of the Brca1, Brca2, and p53 tumor suppressor genes in mammary epithelial cells (241, 584). In agreement with the known role of these genes in human breast cancer, mice with targeted deletion of these genes also developed mammary carcinomas. Mammary specific Brca2 knockout mice show a high incidence (77%) of mammary adenocarcinomas after a long latency period (311). These tumors often have a variety of different chromosomal aberrations and the majority are also positive for p53 and/or p21 expression. Jonkers *et al.* developed conditional mutants with Brca2 and/or p53 inactivated in various epithelial tissues, including mammary gland epithelium (241). Although no tumors arose in mice carrying conditional Brca2 alleles, mammary and skin tumors developed frequently in females carrying conditional Brca2 and p53 alleles. The presence of one wildtype Brca2 allele resulted in a markedly delayed tumor formation (241). Based on these studies, it was concluded that inactivation of both Brca2 and p53 enhances mammary tumorigenesis and disruption of the p53 pathway is pivotal in Brca2-associated mammary cancer.

### **2.2.3 Transgenic Mice – Genetically Modified Mice**

Transgenic mouse models have proven useful for studying the function of oncogenes (35). A transgenic organism is one that has exogenous DNA in its genome. In order to achieve

inheritance of exogenous DNA (transgene), integration must occur in cells that can give rise to germ cells. This is accomplished by introducing the transgene into a fertilized egg cell or into embryonic stem cells (488). Within a few hours after mating, zygotes can be removed from a female mouse before the female and male pronuclei fuse. The pronucleus from the male is injected (the male pronucleus is larger) with a construct containing the gene of interest. After injection of the DNA into the male pronucleus, the zygotes are placed in the oviducts of a 0.5-day pseudopregnant female. About 15% of the offspring will contain the transgene and are designated transgenic hemizygous animals. Transgenic mice can be identified by Southern analysis of a DNA sample purified from a tail clipping. Transgene positive mice are referred to as founders and can be mated with wildtype mice to produce offspring that also contain the transgene confirming germline transmission. Although this technique is most commonly used to overexpress a protein, antisense, as well as dominant negative approaches can also be used to reduce protein expression (486, 488).

The constructs used for generating these mice can be targeted to specific tissues in the adult animal by the use of specific promoters. For example, an albumin promoter will target the expression to the liver, certain keratin promoters will target the expression to the epidermis, and an actin promoter (housekeeping gene) will result in expression of the transgene in all cells. For targeting transgene expression to the mammary gland, MMTV and WAP promoters are frequently used (66). The MMTV promoter is derived from the mouse mammary tumor virus described earlier in this dissertation. The WAP promoter is a part of the whey acidic protein gene, which is synthesized in normal mammary epithelial cells during the last half of

pregnancy and during lactation. As a result, expression of a transgene under the control of this promoter depends on the physiological status of the animal.

Transgenic technology has allowed direct testing of numerous human oncogenes for their transforming potential in mouse mammary cells. Many oncogenes have shown the ability to induce mammary tumors in the mouse. Such genes include mammary epithelial cell growth factors such as transforming growth factors (TGF) alpha and beta, heregulin, hepatocyte growth factor (HGF), and fibroblast growth factors (FGF) as well as receptors for these growth factors (e.g. TGF-beta DNIIR, erbB2/neu, etc.). Transgenics have also been used to study second messengers in signal transduction pathways (e.g. ras, Pten), and regulators of the cell cycle (e.g. myc, cyclin D, and cyclin E) and differentiation (e.g. Wnt and Notch4) (25, 197, 221, 479).

### ***2.2.3.1 Ras Transgenics – Genetically Modified Mice***

Expression of ras oncogenes induces mammary carcinoma development in mice and rats (184). MMTV/H-ras transgenic mice are highly susceptible to the development of mammary gland tumors and p53 deficiency has been shown to accelerate the formation of these tumors (113, 220). Mice carrying the MMTV/v-Ha-ras transgene manifest two distinct disturbances of cell growth. The first is a benign hyperplasia of the Harderian gland, and the second involves focal development of malignancies of mammary, salivary, and lymphoid tissue (485). Yu *et al.* found that expression of the ras and neu/erbB-2 oncogenes leads to exclusive induction of cyclin D1 in mouse mammary epithelial cells, whereas expression of c-myc and Wnt-1 results in induction of both cyclins D1 and D2 (595). Furthermore, rats carrying the

human c-Ha-ras protooncogene demonstrated an increased susceptibility to chemical carcinogens targeting the mammary gland. All of the rats developed pre-neoplastic mammary lesions within 20 days after injection of MNU and mammary carcinomas within 8 weeks of treatment with a variety of chemical carcinogens (184).

## **2.3      *BREAST CANCER***

Breast cancer is the most common malignant disease in women worldwide (574). Each year breast cancer is diagnosed in over 900,000 women worldwide with greater than 370,000 women dying from the disease. Most of the cases occur in industrialized countries. In the United States, it is estimated that about 211,000 women will be diagnosed with breast cancer in 2005, which represents 32 percent of all incident cancers in the United States. An estimated 40,000 US women will die from the disease in 2005 (4). In contrast, male breast cancer is a rare disease with an incidence rate about 1 percent of that for female breast cancer.

### **2.3.1      *Etiology – Breast Cancer***

A variety of different risk factors has been associated with the development of breast cancer in women. These include parity, age, hormonal factors, environmental exposures, and a family history of breast cancer (90).

#### **2.3.1.1      *Parity – Breast Cancer***

Parity and an early first full-term pregnancy both decrease the long-term breast cancer risk (90). Before pregnancy, mammary gland cells are in a vulnerable undifferentiated state and only reach full differentiation during pregnancy. Because of this, it is thought that pregnancy decreases the pool of vulnerable mammary epithelial cells.

### **2.3.1.2 Age – Breast Cancer**

Breast cancer is very rare before 30 years of age, after which incidence rises steeply up to about 50 years of age (90). Thereafter, incidence still increases with age, but more slowly. The strong dependence on age is similar to that seen for many other adult cancers, although the apparent change in slope of the age-incidence curve at 50 years of age is unique for breast cancer. It is presumed that this occurs because of hormonal changes associated with menopause, a time when circulating estrogen levels are reduced.

### **2.3.1.3 Hormones – Breast Cancer**

Estrogens and other hormones are thought to affect the neoplastic progression of initiated mammary gland cells (90). This has been clearly shown in rodent studies, where chemical- or radiation-associated mammary tumors are most abundant after additional hormonal stimulation. Moreover, it has been shown that women who have a late menarche and/or early menopause have a reduced breast cancer risk. This is thought to occur due to a smaller number of menstrual cycles and therefore shorter exposure to ovarian hormones during the reproductive years. In addition, exogenous hormones such as oral contraceptives and post-menopausal hormone replacement therapy have been linked to increased breast cancer risk.

In addition to the promoting effects of estrogen, it has been shown that estrogen metabolites may be a source of DNA damaging agents. When injected into the rat mammary gland, catechol estrogen-3, 4-quinones have been shown to cause unstable DNA adducts; a form of

DNA damage resulting in depurination. Whether such events occur *in vivo* from natural concentrations of these metabolites is not known (574).

#### **2.3.1.4 Environmental Exposures – Breast Cancer**

Using a database of almost 45,000 twins, Lichtenstein *et al.* estimated that 73% of all breast cancers are related to environmental factors (290). These include dietary or environmental xenobiotics, heterocyclic amines, aromatic amines, polycyclic aromatic hydrocarbons, nitropolycyclic aromatic hydrocarbons, epoxides such as ethylene oxide, and organochlorines including chlorinated pesticides, polychlorinated biphenyls, and polychlorinated dibenzo compounds (501, 574).

Many classes of carcinogens are known to form chemically stable DNA adducts (574). The presence of aromatic and/or hydrophobic DNA adducts in human mammary tissue has been detected in a number of studies. In some studies, the adducts have been shown to be partly characteristic of those formed by polycyclic aromatic hydrocarbons, whereas in another study, a contribution from aromatic amine-like species was implicated. Oxidative damage has also been implicated in human breast cancer development and the formation of adducts. DNA adducts formed by malondialdehyde, a product of lipid peroxidation, were found at significantly higher levels in tissue from breast cancer patients as compared to tissue from disease-free individuals. Furthermore, levels of another marker of oxidative DNA damage, 8-hydroxydeoxyguanosine (8OHdG), have been found to be substantially lower in normal breast tissue as compared to breast tumor tissue (574).

The high lipid content of the mammary gland allows an accumulation of lipid soluble compounds, including possible carcinogens (574). Mutagenic activity has been detected in nipple aspirates and breast cyst fluid. Furthermore, extracts of human breast milk have been shown to be genotoxic, supporting the notion that functional elements of the mammary gland may be exposed to potential tumor initiators (574). In addition, human milk is known to contain a large number of compounds of exogenous origin, but whether they are present at biologically significant levels is uncertain. Monocyclic aromatic amines have been detected in human milk at parts-per-billion levels, including o-toluidine, which is a mammary carcinogen in rats (574).

Although many studies have shown chemical exposures to be associated with breast cancer, few if any have provided a definitive link. The only exogenous agent for which there is a significant amount of strong evidence for breast cancer causation is ionizing radiation, both among atomic bomb survivors and in cohorts of women exposed to therapeutic doses of radiation (574). The role of radiation in breast cancer will be described later on in this dissertation.

### **2.3.1.5 Polymorphisms – Breast Cancer**

Polymorphisms in several metabolic and detoxifying enzymes (e.g., GSTM1, CYP1A1, CYP17, NAT2, SULT1A1, COMT, SOD2) have been shown to influence breast cancer

susceptibility suggesting that metabolism (or lack thereof) of environmental carcinogens may indeed be a significant contributing factor to human breast cancer development (417).

#### **2.3.1.6 Family History – Breast Cancer**

Although numerous risk factors for the development of breast cancer have been identified, family history is one of the strongest known risk factors. Breast cancer risk increases with the number of affected first-degree relatives. Risk also seems to be greater for women with a first-degree relative diagnosed with breast cancer at a young age (usually defined as less than 50 years). It is estimated that 15 to 20 percent of women with breast cancer have a family history of the disease, with approximately 5 percent of all breast cancers attributable to dominant susceptibility alleles. Two major breast cancer susceptibility genes (Brca1 and Brca2) have been identified, whereas others are actively being sought (90).

#### **2.3.2 Hereditary – BRCA associated – Breast Cancer**

In 1990, evidence was found for linkage of early onset breast cancer pedigrees to a single locus on chromosome 17q21 (356). In 1994, the Brca1 gene was identified (356). That same year a second breast cancer associated locus was identified on 13q and Brca2 was subsequently identified in 1995 (579). Germline mutations in these two genes appear to be responsible for a high proportion of cancers in women with familial cancer histories.

Brca1 is a highly penetrant breast cancer susceptibility gene that is thought to account for 20 to 30% of inherited breast cancers. Families with germ-line mutations in Brca1 have an

autosomal dominant inheritance pattern of breast cancer as well as an increased incidence of ovarian cancer. The mutation spectrum of Brca1 is well defined, and functional links to transcription regulation, cellular response to DNA damage, and development are becoming evident. Women who inherit a mutant Brca1 allele have a 90% lifetime risk of developing breast cancer and between 40 and 66% for ovarian cancer (90).

Brca2 is a highly penetrant breast cancer susceptibility gene that is thought to account for 10 to 20% of inherited breast cancers. Families with germ-line mutations in Brca2 also have an autosomal dominant inheritance pattern of breast cancer. Female Brca2 carriers have a lifetime risk of breast cancer of 85% and between 10 to 20% for ovarian cancer. In addition, male Brca2 carriers have a lifetime risk for breast cancer of 7% (508), which represents a 100-fold increase over the expected male population risk (378). Brca2 carriers are also predisposed to a number of other tumor types such as cancer of the stomach, prostate, ovary, thyroid, gallbladder, bile duct, colon, pancreas, larynx, and the eye (ocular melanoma) (90, 124, 125, 248, 257, 569, 579).

Several hundred different germline mutations in Brca1 and Brca2 have been reported and most are predicted to result in protein truncation caused by frame-shift, nonsense, or splice-site alterations (19). Many of these mutations appear to be unique to particular families (19). Several founder mutations in Brca1 and Brca2 have been reported. Three founder mutations have been described in the Ashkenazi Jewish population (Brca1 185delAG, 5382insC, and Brca2 6174de1T) with a combined frequency of over 2% in the population. Approximately 20% of Ashkenazi women diagnosed with breast cancer under the age of 40 carry the

185delAG mutation, and nearly 12% of Ashkenazi Jewish breast cancer cases of all ages may be attributable to founder mutations in the Brca1 or Brca2 genes (560). Different founder mutations have been reported in other populations from Iceland, Finland, Sweden, and Canada (French Canadians) (19).

BRCA-associated breast tumors display unique pathological features compared to nonfamilial breast tumors (19). A higher frequency of medullary carcinomas is associated with Brca1 mutations and lobular carcinomas are associated with Brca2 mutations. Brca1 mutation carriers generally present with high grade, highly proliferative, aneuploid cancers. These features have also been reported for Brca2-related tumors although they are generally not as dramatic as that seen with Brca1 tumors. Brca1-related tumors are typically estrogen and progesterone receptor negative, whereas Brca2-related tumors are usually positive for both receptors (19). Both Brca1 and Brca2-related cancers exhibit a higher frequency of p53 mutations as compared to sporadic cancers (93). Despite the fact that germline mutations in Brca1 and Brca2 occur at high frequency in hereditary forms of breast cancer, few mutations in these genes have been reported in nonhereditary cancers (157, 507). Decreased expression of Brca1 mRNA and protein have been reported in association with breast cancer, however, suggesting that Brca1 is involved in the progression of nonfamilial forms of breast cancer (605). The identification of aberrant methylation of the Brca1 promoter suggests a possible mechanism for this finding. Such methylation has not been detected for Brca2, however, a recently described mechanism has provided evidence that Brca2 may also be involved in sporadic breast cancers (216). A protein known as EMSY was shown to suppress Brca2

activity by binding to exon 3 of the Brca2 gene. Furthermore, EMSY maps to chromosome 11q13.4-5, a region frequently amplified in sporadic breast cancers (216).

### **2.3.3 *Hereditary – Non-BRCA associated – Breast Cancer***

Although germline mutations in the Brca1 and Brca2 genes account for many cases of familial breast cancer, a large proportion of cases are not caused by mutations in either gene (termed BrcaX families). There is evidence that some hereditary breast cancers may be associated with a locus on the short arm of chromosome 8 (394), however, to date, no "Brca3" gene has been found (272).

In addition to the Brca genes, other familial cancer-related syndromes can also be associated with breast cancer development. These include Li-Fraumeni syndrome (p53 or chk2), Ataxia telangiectasia (Atm), Cowden disease (Pten), Peutz-Jeghers syndrome (STK11), Familial adenomatous polyposis (APC), and Hereditary nonpolyposis colon cancer (MLH1, MSH2, MSH6, and others) (380).

### **2.3.4 *Sporadic - Breast Cancer***

In addition to the genes involved in familial forms of breast cancer, there are a number of other genes associated with the development and/or progression of sporadic breast cancers. These include genes/proteins such as cyclin D1, cyclin E, epidermal growth factor receptor

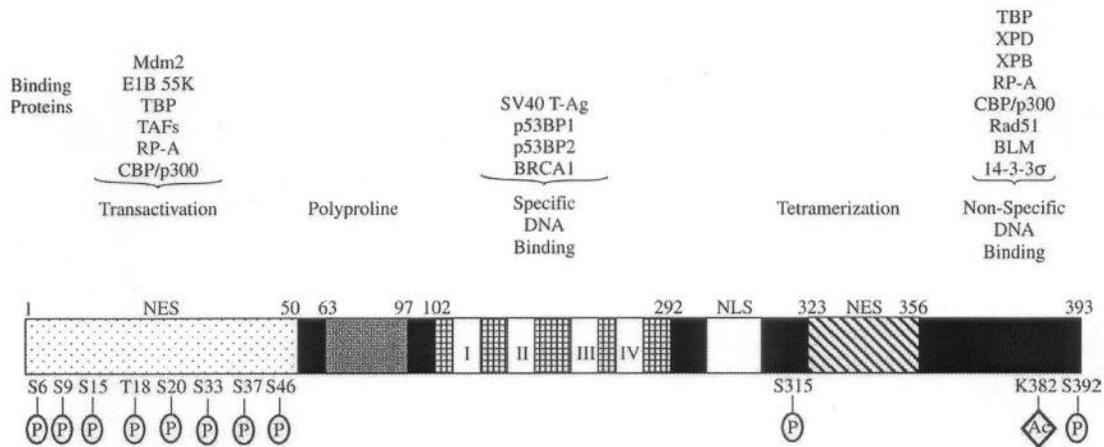
(EGFR/HER1), HER2/neu/erbB2, Bcl-2, insulin-like growth factor receptor 2 (IGF-II), myc, beta-catenin, cathepsin D, Rb, p53, p21, p27, and E-cadherin (13, 234, 251, 394, 434).

## **2.4 P53 REVIEW**

P53 has been called the "guardian of the genome" because it plays an essential role as a tumor suppressor protein. P53 is involved in a number of critical cellular processes including cell cycle regulation, senescence, apoptosis, DNA repair, and maintenance of genetic stability (187, 371). The p53 pathway is composed of a network of genes and their products that are targeted to respond to a variety of environmental and intracellular stresses including hyper-proliferation, hypoxia, or DNA damage due to agents such as gamma or ultraviolet irradiation, alkylation of bases, depurination of DNA, or reaction with oxidative free radicals (174, 187, 353, 549).

### **2.4.1 Gene/Protein Structure – P53**

P53 is a phosphoprotein with a molecular weight of 53,000 encoded by a 20 kb gene containing 11 exons. Exons 2-11 code for an mRNA of 2.2-2.5 kb (94, 497). The human and mouse p53 genes are conserved between species, sharing 78% identity, and are located on chromosomes 17 and 11, respectively (94, 497). Two promoters have been demonstrated at the 5' end of the human p53 gene (94). The first is located upstream of the first exon and is responsible for transcription of the major p53 mRNA species. The second (p53p2) is a stronger promoter and is located within the 10,738 base pair first intron, approximately 1,000 base pairs downstream of exon 1. The human and mouse p53 proteins are very similar with 393 amino acids in the human protein and 387 in the mouse. It is composed of three discrete structural and functional domains (Figure 2):



**FIGURE 2.** P53 protein structure (94).

The transactivation domain is found in the amino-terminus and binds to several components of the RNA polymerase machinery (75, 94, 308, 467). Within this domain, protein-protein interactions facilitate or inhibit the transactivation capacity of p53. Just downstream of the transactivation domain is a polyproline region containing multiple copies of the PXXP sequence (X = any amino acid). This region is thought to mediate transcription-independent growth suppression and apoptotic functions of p53 (94, 449, 547). The amino-terminus also contains a nuclear export signal (spanning residues 11-27) whose activity can be modified by post-translational phosphorylation at serines 15 and 20 following irradiation.

The middle core domain of p53 (amino acids 100-300) contains the sequence-specific DNA binding activity of p53. Within this central core are four evolutionarily conserved regions

that play a role in facilitating DNA-protein interactions (82, 94). It is within this region that approximately 90% of the mutations identified in human cancers have been found (this is also the region examined in the study described in **manuscript one** of this dissertation). A large percentage of these mutations affect just five “hotspot” amino acid residues (codons 175, 248, 249, 273, and 282) that play a key role in maintaining the structural integrity of the DNA binding motifs (94, 333). The middle core domain can also facilitate protein-protein interactions with the SV40 large T antigen protein and the p53BP1 proteins. Finally, the DNA repair proteins, Brca1, BLM (Bloom's Syndrome protein), and Rad51 interact with the middle and carboxy-terminal domains. This interaction with Rad51 suggests a role for p53 in Rad51/Brca2-mediated homologous recombination during the repair of DNA double strand breaks (94, 118, 581).

The carboxy-terminus contains an oligomerization domain that facilitates the ability of p53 to form tetramers, the optimal configuration for p53 function (94, 267, 414). Biogenesis of p53 involves co-translational dimerization of monomers followed by post-translational dimerization of the resulting dimers (94, 381). Once in its activated tetrameric form, p53 protein binds with high affinity to DNA and activates transcription of promoters with the consensus binding site 5' -PuPuPuC(A/T)(T/A)GpyPyPy-3' (94, 526). Mutation or loss of just one p53 allele can reduce oligomerization and thus result in a similar reduction of p53 tetramers. The carboxy-terminal region also contains nuclear localization signal motifs (NLS) that regulate nuclear translocation following DNA damage. Furthermore, the carboxy-terminus regulates the ability of p53 to bind nonspecifically to double and single stranded DNA or to damaged DNA (23, 94, 238, 429).

## **2.4.2     *Function - P53***

Although p53 is involved in a variety of different cellular functions, its tumor suppressive functions are generally attributed to its involvement in cell cycle regulation, DNA repair, and cell survival (85, 396). In many cell types, p53 acts to induce cell cycle arrest in the G1 and G2 phases of the cell cycle (142). In highly radiosensitive cell types (e.g., lymphocytes, thymocytes, and germ cells), p53 facilitates radiation-induced apoptosis, thus removing potentially oncogenic cells from rapidly proliferating tissues or the germline. Although p53 dependent cell cycle arrest and apoptosis are not necessarily mutually exclusive functions, there are control points where one function is favored over the other. This process is not completely understood but there are a number of factors that likely contribute. For example, many studies have suggested that the nature and intensity of the stress signal, the level of p53 expressed, and the presence of other interacting proteins arbitrate the two potential choices (16, 85). Cell type, extracellular stimuli and the location and type of protein modifications on the p53 protein likely also influence the choice (85, 187, 422). Specifically, for irradiation, the intensity of DNA damage appears to be an important determinant. Lower doses of irradiation initiate reversible cell growth arrest, whereas higher doses of irradiation trigger apoptosis (246). Studies of p53 signaling upon irradiation of primary mouse lymphocytes demonstrated that G0 lymphocytes rapidly undergo p53-dependent apoptosis, whereas stimulated lymphocytes go into p53-dependent, p21-mediated growth arrest. The switch in p53 response upon stimulation was not the result of a switch in transcriptional activation of

p53 target genes but was instead mediated by proliferative signals from the cells themselves (85, 194).

#### ***2.4.2.1 Cell Cycle Regulation and Checkpoints – P53***

One well-characterized cellular response to DNA damage is to slow progression through the cell cycle, a process mainly mediated through p53 (142). This provides time for repairing damage and thus prevents damaged DNA from being propagated. When the damage is irreparable, the checkpoints enforce a cell's death by inducing apoptosis (142). Loss of these checkpoints results in decreased DNA repair, increased mutation rates, genomic instability, and ultimately neoplastic transformation.

##### *G1 Arrest*

A body of evidence indicates that p53 is required for proper G1 checkpoint function (112, 246, 247, 261, 269, 422). Activated p53 protein stimulates transactivation of p21<sup>WAF1</sup>, a protein whose amino-terminal domain interacts with and inhibits Cdk-cyclin dependent kinases (79, 167, 261). This in turn, maintains the Rb-E2F complex and thus inhibits E2F proteins from promoting the transcription of genes necessary for progression to the S-phase (167, 261). The result is cell cycle arrest.

##### *G2 Arrest*

P53 is also involved in cell cycle regulation at the G2/M restriction point although its role here is much less clear (187). An intact p53 carboxy-terminal domain is necessary to induce G2 arrest (187, 374). The primary mechanism involves inhibition of Cdk1 (also called cdc2).

In normal cells, Cdk1, in complex with cyclin B, becomes activated through phosphorylation by CAK (Cdk-activating kinase), allowing the cell to progress into mitosis (187, 415). In damaged cells, p53 inhibits Cdk1 through a number of mechanisms. P53 transcriptionally activates three inhibitors of Cdk1 including Gadd45, p21<sup>WAF1</sup>, and 14-3-3-sigma (187, 523). Gadd45 directly inhibits the activity of the Cdk1/cyclin B complex by physical interaction with Cdk1 (187, 603). p21<sup>WAF1</sup> is a universal Cdk/cyclin inhibitor essential for controlling both the G1, as noted previously, and G2 checkpoints (57, 187). 14-3-3 sigma acts by sequestering cdc25c in the cytoplasm. cdc25c is a protein necessary for activation of the Cdk1/cyclin B complex, thus its failure to enter the nucleus will prevent cells from entering the mitotic phase of the cell cycle (187). P53 can also regulate the G2 checkpoint directly by binding to the catalytic subunit of the Cdk1/cyclin B kinase (1). In addition, p53 has been implicated in the G2/M checkpoint through its ability to inhibit DNA topoisomerase II alpha gene expression (557). Inhibition of this enzyme results in failure of chromatin condensation and decatenation, thus preventing cells from entering mitosis (121). Finally, some studies have suggested a relationship between p53 and the G2/M checkpoint in relation to its role in ubiquitin-mediated degradation of mitotic cyclins (359).

#### **2.4.2.2 Apoptosis – P53**

P53 plays a critical role in apoptosis and its function is required for apoptosis to proceed after failed DNA damage repair (344). The mechanisms by which p53 modulates activation of apoptosis are not completely understood but many of these functions seem to be localized to the carboxy-terminal domain between residues 364-393 and the proline-rich domain between

residues 64-91 (187). P53-mediated apoptosis can generally be classified into two categories: transcription-independent and transcription-dependent.

#### *Transcription-independent apoptosis*

Evidence of transcription-independent p53-mediated apoptosis is not abundant but is accumulating (62, 187). Mihara *et al.* reported that p53 could directly induce permeabilization of the outer mitochondrial membrane by forming complexes with the protective Bcl-XL and Bcl-2 proteins, resulting in cytochrome c release (355). Additional evidence for the transcription-independent proapoptotic activities of p53 include the p53-mediated translocation of the Fas/CD95 death receptor to the plasma membrane and the p53-mediated translocation of Bax from the cytoplasm to the mitochondria, resulting in cytochrome c release (187). Transactivation-independent p53-mediated apoptosis may also occur through p53's ability to repress gene expression. For example, p53 can inhibit the transcription of anti-apoptotic genes such as Bcl-2 (428).

#### *Transcription-dependent apoptosis*

P53's transcription-dependent role in apoptosis is related to the large number of pro-apoptotic target genes it regulates. These generally fall into three groups based on their subcellular location (142). The first group of genes encodes proteins that localize to the cell membrane (e.g., CD95, Killer/DRS, Perp) (18, 142); the second group is localized to the cytoplasm, including Pidd and PIGs (142); and the third group of genes encodes proteins that localize to

the mitochondria (e.g., Bax, Noxa, Puma, p53AIP1, Bid) (142, 363, 391, 392). Many of these p53-regulated genes enhance the secretion of cytochrome c from the mitochondria (e.g., Bax, Noxa, Puma) (346). During apoptosis, loss of mitochondrial membrane integrity is followed by release of cytochrome c into the cytosol. Cytochrome c interacts with Apaf-1 (another p53-regulated gene) to initiate the caspase cascade, leading to activation of caspase-9 and then caspase-3 followed by apoptosis. This is the intrinsic apoptotic pathway and is initiated by a number of stress signals that activate the p53 pathway including the proapoptotic gene Bax. Bax has been shown to contain p53-binding sites in its promoter site and is upregulated in response to DNA damage and increased p53 expression (346, 429). Reactive oxygen species (ROS) are also powerful activators of mitochondrial damage and apoptosis. A number of genes that increase production of ROS have been found to be induced by p53; these are known as p53-induced genes or PIGs (303, 346). In addition to the intrinsic pathway, p53 stimulates expression of genes involved in the extrinsic apoptotic pathway including Killer/DRS, Fas, and p53-induced genes with a death domain (Pidd) (346, 551). These cause increased caspase-8 and caspase-3 activities and subsequently apoptosis. P53 can also directly transactivate the transcription of caspase-1 and caspase-6 (176, 314, 346).

#### **2.4.2.3 DNA Repair – P53**

P53's role in DNA repair is remarkably complex, as it seems to influence numerous different DNA repair mechanisms either through its role as a transcription factor or through direct interaction with other DNA repair proteins or DNA itself. P53 has been implicated in a number of well known DNA repair processes such as nucleotide excision repair (NER), base

excision repair (BER), and mismatch repair (MMR) (314, 465, 491, 610), however, its role in DNA double-strand break repair is perhaps best understood. Further details of p53's role in this process are described later on in this dissertation (chapter 2.6).

DNA repair processes are frequently initiated by activation of a variety of different enzyme activities that subsequently modify p53 amino acid residues allowing for p53 protein stabilization and activation. The p53 protein undergoes extensive post-translational modifications of at least 18 sites along its length often involving phosphorylations and acetylations (10). The nature of the stress signal is transmitted to the protein by a code inherent to the post-translational modification (86). For example, gamma-radiation activates the Atm and Chk2 kinases, both of which phosphorylate p53 at specific sites, while ultraviolet radiation activates Atr, Chk1, and casein kinase-2 (CKII), resulting in phosphorylation of a different set of amino acid residues (11). Thus, it is the nature of these post-translational modifications that direct p53 towards one of the many potential p53-mediated DNA repair mechanisms.

#### **2.4.2.4 Cellular Senescence – P53**

P53 is known to be involved in cellular senescence, although its role in this process is not completely understood. Senescence has typically been studied in normal cells taken from an *in vivo* setting and cultured *in vitro*. As these cells undergo multiple divisions, chromosomal ends (telomeres), in the absence of telomerase, shorten to a point at which they eventually trigger activation of p53. P53 then activates the program for cellular senescence. Cellular

senescence can also be activated by overexpression of a number of different oncogenes (E2F-1, beta-catenin, myc, ras, and adenovirus E1A), all of which can induce p53 activation (102, 187, 399, 609).

#### **2.4.2.5 Protein Secretion – P53**

In addition to genes regulating DNA repair, cell cycle arrest, senescence, and apoptosis, the p53 protein regulates a set of genes that produce secreted proteins including plasminogen activator inhibitor, maspin, and thrombospondin. These secreted proteins may be used to communicate signals to surrounding cells informing them of a stress response (187) or help to regulate angiogenic signals (387). In addition, p53-activated cells undergoing apoptosis might sensitize adjacent cells for enhanced apoptotic cell death or altered gene expression. Several experiments have indicated that p53 activated cells can provide a “bystander effect” to surrounding cells in a tissue (187, 367).

#### **2.4.2.6 Chromosomal Maintenance – P53**

Loss of p53 function may result in accumulation rather than deletion of genetically unstable cells (17, 271, 418, 539). Several studies have indicated that p53 plays an important role in preventing gene amplification (188, 548). Embryonic fibroblasts from p53-null mice are permissive to gene amplification as are primary human cells from Li-Fraumeni patients (297). Absence of p53 can also lead to centrosome amplification and chromosomal mis-segregation (535). Centrosomes are involved in nucleating mitotic spindles and forming

poles of division during mitosis. An abnormal number of centrosomes can result in chromosome mis-segregation.

### **2.4.3 Regulation – P53**

P53 activation is a complex mechanism affected by a range of proteins that either enhance or suppress p53 activity. The abundance and activity of p53 are regulated by many different post-translational modifications including phosphorylation, methylation, acetylation, ribosylation, O-glycosylation, ubiquitination, and SUMOylation (187, 348). In addition, the activated p53 protein appears to interact with a number of proteins that are important for its transcriptional activity such as PML bodies (promyelocytic leukemia bodies), and the Werner helicase (44). Once the p53 protein is activated, it initiates a transcriptional program that reflects the nature of the stress signal, the protein modifications, and proteins associated with the p53 protein (187). Activation of p53 occurs in three stages 1) an increase in p53 protein concentration either by enhanced translation or increased half-life; 2) transformation of the p53 protein from latent to active form; and 3) translocation of p53 protein from the cytoplasm to the nucleus (187). A number of studies over the years have identified ten positive or negative feedback loops in the p53 pathway. Each of these loops is composed of proteins whose activities or rates of synthesis are influenced by the activation of p53. Of these, seven are negative feedback loops (Mdm2, Cop-1, Pirh-2, p73 delta N, cyclin G, Wip-1, and Siah-1) and three are positive feedback loops (Pten-Akt, p14/19<sup>ARF</sup>, and Rb) (187). Each of these networks are autoregulatory in that they are either induced by p53 activity at

the transcriptional level, transcriptionally repressed by p53 (p14/p19<sup>ARF</sup>), or regulated by p53-induced proteins (187).

#### **2.4.3.1 Negative Regulation- P53**

One of the best-characterized negative feedback loops involves ubiquitin ligases. Mdm2 (murine double minute; the human protein is called HDM2) is the best known and the first discovered although there are two others (Cop-1 and Pirh-1) that also form an autoregulatory loop resulting in lower p53 activity (85, 120, 281). All three of these genes are transcriptionally activated by p53. In the absence of DNA damage, p53 basal expression is low. Low level of expression is maintained through high turnover rates of p53, which has a protein half-life of 10-20 minutes (15). In most cases, the high turnover is maintained through binding of Mdm2 to the amino-terminal domain of p53, targeting it for proteolysis in both the cytoplasm and the nucleus (15). Low levels of Mdm2 activity induce mono-ubiquitination and nuclear export of p53, whereas high levels of Mdm2 promote p53 poly-ubiquitination and nuclear degradation (288). In addition, Mdm2 can inhibit the ability of p53 to activate transcription of its downstream target genes by binding directly to the transactivation domain of p53 (293). Furthermore, Mdm2 can inhibit p300-mediated p53 acetylation by forming a ternary complex with p53 and p300 (262). This significantly represses p53 transcriptional activity without affecting p53 protein levels (240). Moreover, Mdm2 can mediate translocation of p53 to the cytoplasm thereby removing it from its site of action, and can recruit the histone deacetylase HDAC1 to deacetylate lysine residues on the carboxy-terminus of p53, thus making them available for ubiquitination. After activation, p53

itself transactivates Mdm2 transcription thus keeping p53 levels low and completing the auto-regulatory feedback loop (12).

P53 protein activation may also involve phosphorylation of serines located at residues 33 and 46 by the p38 MAP kinase. The p38 MAP kinase is itself activated by phosphorylation (regulated by the ras-raf-mek-erk pathway) that can be reversed or inactivated by the Wip-1 phosphatase. Wip-1 is a p53-responsive gene forming a negative autoregulatory loop and connecting the p53 and ras pathways (519).

In addition, activated p53 protein positively regulates the transcription of the ubiquitin ligase Siah-1 (145). Siah-1 negatively regulates p53 by degrading the positive regulator, beta-catenin (230). Beta-catenin levels can regulate the p14/19<sup>ARF</sup> gene (described below), which in turn negatively regulates Mdm2 and results in higher p53 levels. Siah-1 thus connects the Wnt-beta-catenin-APC pathway to the p53 pathway.

Another negative feedback loop involves a member of the p53 family of transcription factors, which includes p53, p63, and p73. After a stress response, the p53 gene is activated, which in turn stimulates the transcription of a particular spliced mRNA from the p73 gene called p73 delta N. This translates to a p73 protein without its amino-terminal transactivation domain. As with p73 itself, the p73 delta N protein can still bind many p53-regulated genes, however, absence of its transactivation domain makes it act as a repressor or competitor of p53 transcriptional activation. In this way, a negative feedback loop is set up and p53 activity declines (172, 245).

Additional cellular proteins capable of inhibiting p53 activity include MdmX (307, 478), phosphatidylinositol 3-OH-kinase (Akt) (585), and Brca2 (328), as will be described later on in this dissertation.

#### **2.4.3.2 Positive Regulation- P53**

Although multiple stress signals are known to activate p53, the best understood pathways involve DNA damage or uncontrolled cell growth induced by oncogenes. The key players that pass along the appropriate signals to p53 during stress are the kinases Atm, Chk2, and CKII, which phosphorylate p53, and the tumor suppressor protein p19<sup>ARF</sup> (p14<sup>ARF</sup> in human) (307).

The principal p53 activation pathways often involve Mdm2 inhibition leading to reduced p53 degradation. For example, p53 activation under cell stress depends to a large extent on the presence of c-Abl (Abelson murine leukemia cellular oncogene), which protects p53 from the inhibitory effect of Mdm2 (307). Likewise, p14<sup>ARF</sup>/p19<sup>ARF</sup> are strong activators of p53 and work by binding Mdm2 and promoting its rapid degradation (307). P19<sup>ARF</sup> is unusual in that it is encoded as an alternative reading frame of the Ink4a locus. This locus contains two independent promoters that give rise to exons 1-alpha and 1-beta which, when spliced to common exons 2 and 3, result in expression of p16<sup>INK4A</sup> and p19<sup>ARF</sup>, respectively. Since exons 1-alpha and 1-beta are in different reading frames, they encode totally unrelated proteins. It is well established that p16<sup>INK4A</sup> plays a role in cell cycle regulation (307). P19<sup>ARF</sup>

activates p53 by sequestering Mdm2 to the nucleolus and blocking its E3-ubiquitin ligase activity. Transcription of the p14/p19<sup>ARF</sup> gene is positively regulated by E2F1 (607) and beta-catenin (97) and negatively regulated by p53 itself. In addition, the levels of p14/p19<sup>ARF</sup> protein can be increased by ras and myc activities.

In some cell types, the p53 protein induces the transcription of the Pten gene. Pten physically interacts with p53 and prevents its degradation by excluding a portion of the p53 protein from the p53-mdm2 complex. The Pten-p53 complex also enhances p53 DNA binding and transcriptional activity. Additionally, Pten inhibits Akt, a key kinase responsible for phosphorylation of Mdm2 and its subsequent nuclear translocation (74, 187, 384).

Another protein that inhibits p53-mdm2 binding is Chk2 (307). Chk2 is a serine/threonine kinase that influences chromatin remodeling, cell cycle progression, DNA repair, and apoptosis by phosphorylating multiple targets, including p53. Specifically, Chk2 phosphorylates p53 at serine 23 (S20 in humans), which lies at the edge of the Mdm2 binding site. Phosphorylation of serine 23 inhibits Mdm2 from binding p53, thereby stabilizing p53 protein, and promoting its activation (307).

Atm is activated in response to DNA damage and as a result, it phosphorylates a number of proteins in an effort to enforce cell-cycle checkpoints. Specifically, Atm phosphorylates p53 at serine 18 (S15 in humans), and this is believed to set off a cascade of post-translational modifications ultimately leading to p53 stabilization, DNA binding, and transcriptional activation. Atm also phosphorylates Chk1 and Chk2, which in turn phosphorylate p53 at

serine 23 (S20 in humans) (77, 307). In addition, p53 and Mdm2 are phosphorylated by Atm directly at serine 15 and serine 395, respectively (307). Phosphorylations at these sites inhibit p53-mdm2 binding and therefore protect p53 from ubiquitination and degradation (307).

There also exist a number of other p53 activation pathways unrelated to Mdm2 such as casein kinase II, which phosphorylates p53 at serine 389 thereby promoting p53-mediated DNA binding (307). Acetylation of the p53 carboxy-terminal domain by PCAF (p300/CREB-binding protein associated factor) and CBP/p300 also promotes p53 activation by stimulating its sequence-specific DNA-binding activity (85, 173). Two additional proteins that promote p53 activation include Brca1 (397, 459) and poly(ADP-ribose) polymerase-1 (Parp) (570).

#### **2.4.4 Mutation - P53**

P53 mutation is the most common genetic alteration found in human cancers and breast cancer is no exception. Forty to sixty percent of sporadic breast cancers have at least one mutant p53 allele (168). Breast cancers with mutant p53 are, in general, highly aggressive, associated with negative steroid receptor status, and exhibit a high histological grade (131). The frequency of p53 mutations and/or protein overexpression can differ between histological subtypes (110, 357). Positive p53 immunostaining is frequently found in medullary and ductal carcinomas of the breast, but not in lobular tumors (380). In addition, as noted previously, individuals that inherit germ-line mutations of p53 (Li Fraumeni Syndrome) are also at increased risk for developing breast cancer with an incidence that

approaches 100% in individuals that survive to adulthood (105). Li-Fraumeni patients are also particularly susceptible to radiation induced breast cancer (316).

Close to 90% of reported p53 mutations are localized within exons 5-8 (codons 126-306), a highly conserved and functionally significant domain (168). Most of the remaining p53 mutations are in exons 4 and 10. Missense mutations are the most common type (79%) in the conserved midregion, although this is in contrast to the findings in other tumor-suppressor genes, such as the APC gene, in which only about 9% of mutations are of the missense type (292). Missense mutations are uncommon in the amino- and carboxy-termini of p53, where non-missense mutations (nonsense, frameshift, splicing, and silent mutations) predominate (168). The rarity of missense mutations in the amino- and carboxy-termini suggests that in these regions the integrity of a single amino acid residue is not essential to p53 function or conformation (168). Non-conserved residues within exons 5-8 also show a low proportion of missense mutations, supporting the hypothesis that substitutions of non-conserved amino acids do not provide cells with a positive selection advantage (168). The most commonly mutated amino acids in the DNA-binding region are close to the protein-DNA interface and these often result in a protein that fails to transcribe p53-responsive genes (187).

The wildtype p53 protein usually resides in the cell nucleus, but has a very short half-life (10-20 minutes) and is present in such small quantities that it cannot be detected by routine immunohistochemistry (IHC) (168). In contrast, mutant p53 proteins may have a prolonged half-life of up to 1-2 hours resulting in a 3-10-fold increase in p53 concentration (482). As a result, p53 protein can often be identified using IHC methods allowing investigators to use

IHC as a screening test prior to DNA sequencing. Greenblatt *et al.* looked at 84 studies in which both IHC and sequencing were used in the same tumor sets (168). Positive staining was found in 44% of tumors by IHC, while 36% contained mutations. The sensitivity of IHC in these studies was determined to be just 75% (range, 36-100%), and the positive predictive value was only 63% (range, 8-100%), with considerable variation among tumor types. Thus, the status of the p53 protein by IHC cannot always be directly equated with a wildtype or mutant genotype. In contrast, other studies have shown a good correlation between positive immunohistochemical staining and p53 mutations. Hsia *et al.* demonstrated that most p53 mutations could be identified by immunohistochemical staining in human hepatocellular carcinomas with 94% sensitivity and 73% specificity (211). These inter-study differences indicate that tumor type may play an important role in the correlation between p53 protein expression and p53 mutation. Discrepancies between IHC and sequencing may be due to false negative and positive results from technical aspects of sample preparation and analysis or the result of unrecognized biological phenomena. Mutations that result in deletion or truncation of the protein (nonsense and frameshift) do not cause protein accumulation, thus making IHC unsuitable for screening tumors with this mutation type. In addition, some tumors, including melanoma and testicular carcinoma, are frequently immunopositive for p53 even in the absence of p53 mutation (168). Other potential mechanisms for positive IHC staining in the absence of mutant p53 include stabilization of p53 protein by binding to viral or cellular proteins or increased expression because of DNA damage from chemical or physical genotoxic agents. In addition, mutations occurring in a gene downstream of p53, such as p21<sup>WAF1</sup>, could potentially interrupt feedback loops necessary for regulating p53 protein expression (168).

The presence of mutated p53 protein in tumors suggests that cells acquire a selective growth advantage from p53 mutation and/or loss. Analysis of all p53 mutations in human cancers reveals a nonrandom distribution within the p53 molecule, implying that specific mutant proteins possess different growth stimulatory properties (94). In addition to loss of normal p53 function, mutant p53 may acquire added oncogenic properties resulting in enhanced tumorigenic potential, enhanced drug resistance, and even allele-specific phenotypes (509). Moreover, p53 missense mutant proteins can exhibit altered transcriptional activities (187, 252, 471) and some p53 mutants have been shown to bind and inhibit p73, thus reducing p73-dependent apoptosis (326). It should also be mentioned that p53 does not fully comply with the Knudson model in that only a reduction of p53 expression may be sufficient for tumor predisposition, thus suggesting a gene-dosage effect for p53 function.

## **2.5 BRCA2 REVIEW**

Germline mutations of the Brca2 gene confer susceptibility to early onset, hereditary breast cancer in women but also increase susceptibility to a variety of other cancers (as noted earlier in this dissertation). In addition, germline inheritance of certain bi-allelic Brca2 mutations can result in development of Fanconi's anemia (476), a condition also associated with increased cancer susceptibility. The Brca2 gene encodes a large protein proposed to function in DNA repair, homologous recombination, chromatin remodeling, and regulation of transcription (589). Brca2 plays a pivotal role in maintaining genomic stability and behaves as a classic tumor suppressor in that inactivation of both alleles is required for tumor development (546).

### **2.5.1 Gene/Protein Structure – Brca2**

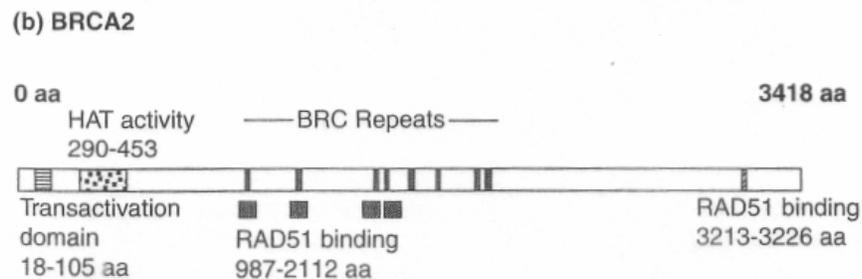
**Alternative names:** breast cancer 2, early onset, FACD, FAD, FAD1, FANCB, FANCD, FANCD1

The Brca2 gene contains no homology to any known genes (457). Both the human and mouse gene have 27 exons distributed over roughly 70 kb of genomic DNA. This includes a very large exon 11 containing eight highly conserved copies of a 30-110 amino acid motif, the so-called BRC repeat (311, 395). Brca2 genes are also not unique to mammals, as homologues have been found in both plants and fungi (587). The number, sequence, and spacing between these motifs are conserved during vertebrate evolution, suggesting functional relevance (476). The human gene is located on chromosome 13q12.3 while the

mouse gene is on the distal end of chromosome 5 (469). It is expressed as an 11.5-12 kb (human) or 11-11.5 (mouse) mRNA transcript. The Brca2 protein is very large consisting of 3418 amino acids in the human and 3329 amino acids in the mouse (101, 451) (Figure 3). It does not exhibit any significant similarity to any other known proteins (545). Overall the amino acid sequence homology between the human and mouse proteins is 59% with 72% similarity, however, multiple functional domains have been evolutionarily conserved including 6 of the 8 BRC repeats and portions of the amino and carboxy domains (335, 451). Despite some of these differences, many of the protein-protein binding domains are maintained between the human and rodent Brca2 proteins. The carboxy domain of Brca2 reveals 77% identity and 86% similarity and one particular stretch of the carboxy domain (residues 2501-2616) has 92% identity (89, 335). A comparison of Brca2 protein between dog, mouse, rat, and chicken orthologs (561) revealed an average identity of 68% for the carboxy domain as opposed to 42% for the entire protein (587).

There are several known Rad51 binding domains in the central portion of the protein and one in the carboxy-terminus. In addition, the carboxy-terminus of Brca2 contains a nuclear localization signal (NLS), and a DNA binding domain (587). Details on the Rad51 and DNA binding capabilities of Brca2 will be provided in the section on radiation-induced DSB repair. The NLS in the carboxy-terminus of human Brca2 appears critical for nuclear localization of the protein since loss of this region results in cytoplasmic localization of Brca2 (498). In fact, some cases of human cancer have been reported with loss of only the last 224 residues of the Brca2 carboxy-terminus (498). In contrast, the mouse Brca2 protein does not appear to require the carboxy-terminus in order to maintain nuclear localization,

indicating the presence of additional NLS elsewhere in the mouse Brca2 protein (451). Despite this, mouse cells lacking only the last exon (27) exhibit many of the same properties seen in cells with larger Brca2 truncations, including hypersensitivity to gamma-irradiation and deficiency in error-free homology-directed DNA repair (33, 451). This suggests that the carboxy-terminus is essential for normal sensitivity to DNA damaging agents in both the rodent and human.



**FIGURE 3.** Brca2 protein structure (380).

### 2.5.2 *Expression - Brca2*

The tissue expression patterns of the Brca2 protein are remarkably similar between man and mouse (83). Brca2 expression is regulated in a cell cycle dependent manner with mRNA levels increasing at the G1/S boundary and peaking during S-phase, while the gene products remain abundant throughout the S and G2 phases (460, 558). Brca2 has proven to be indispensable for embryonic development where it is widely expressed in the mid-gestational embryo around the time of gastrulation (E 7.5-13.5) and subsequently downregulated around E18.5 (516). Brca2 is also expressed in several adult tissues including most rapidly dividing cells or cells involved in high levels of recombination such as the thymus, testis, and ovary

(228, 404, 535, 604). Rajan *et al.* demonstrated that Brca2 mRNA expression is tightly regulated during mammary epithelial proliferation and differentiation, where it is coordinately regulated with Brca1 expression (424).

Brca2 is a hormonally responsive gene. During pregnancy, Brca2 is highly expressed in the mammary gland early on in gestation and then declines to the lowest levels during lactation and involution (311). Interestingly, it has also been shown that Brca2 expression in the mouse mammary gland is consistently higher in parous mice as compared to virgin mice. Similarly, Jernstrom *et al.* found that human carriers of Brca2 mutations who have children are significantly more likely to develop breast cancer by age 40 than carriers who are nulliparous (239). Each pregnancy is associated with an increased cancer risk. This is unlike that described for sporadic breast tumors in which pregnancy has a protective effect.

### **2.5.3 Mutation – Brca2**

In humans, over 100 independent germline Brca2 mutations have been identified most (~90%) of which are frame-shift or nonsense mutations resulting in premature termination of translation and subsequent truncation of the protein (153). These mutations have proven to be highly penetrant carrying a high lifetime risk for cancer development, however, variation in risk may be influenced by genetic background (modifier genes) as well as environmental factors (378). The carboxy terminal region contains 27% of the tumor-derived missense mutations (517) indicating that this region has an important role in the tumor suppressing function of Brca2.

## **2.5.4     *Function - Brca2***

### **2.5.4.1     *DNA Repair - Brca2***

Brca2's primary known function is in DNA repair. Along with Rad51, Brca2 plays a critical role in the DNA repair process known as homologous recombination. Details of its role in this process are described in the section on radiation induced DNA double strand break repair. In addition, Brca2 may be involved in DNA damage repair following oxidative stress. Cells deficient in Brca2 have shown an impaired ability to repair 8-hydroxydeoxyguanosine (8OHdG), which carries a high mutagenic potential (276).

### **2.5.4.2     *Chromosome Maintenance - Brca2***

Brca2-deficient cells exhibit marked instability in chromosome structure, which is exacerbated after DNA damage leading to the accumulation of gross chromosomal rearrangements such as translocations, large deletions, and end-to-end fusions (404, 535, 596). Chromosomal aberrations such as these lead to cell cycle arrest and/or apoptosis. Mouse embryonic fibroblasts derived from Brca2 null embryos (deleted exon 11) develop micronuclei, also indicating the presence of genetic instability, and in addition display amplification of their centrosomes (535), much like that described in p53 deficient cells. This suggests that the combined loss of Brca2 and p53 would result in accelerated genomic instability and tumorigenesis. Finally, Brca2 deficiency was shown to impair the completion of cell division by cytokinesis (98). Brca2 inactivation in mouse embryo fibroblasts (MEF)

and HeLa cells by targeted gene disruption or RNA interference either delayed or prevented cell cleavage (98).

#### **2.5.4.3 DNA Replication - Brca2**

Lomonosov *et al.* presented evidence that Brca2 has a role in the cellular response to blocked DNA replication (301). The Y-shaped DNA junctions normally found at stalled replication forks disappeared during replication arrest in Brca2-deficient MEFs and this was accompanied by double-strand DNA breakage. Activation of the replication checkpoint kinase Chk2 was unaffected, suggesting that Brca2 stabilized the DNA structures at stalled forks. It was hypothesized that the breakdown of replication forks in Brca2 deficiency triggers spontaneous DNA breakage, leading to mutability and cancer predisposition (301).

#### **2.5.4.4 Cell Proliferation - Brca2**

Brca2 null cells are very slow growing and have a severely impaired ability to undergo cell proliferation. This phenotype is in part associated with elevated expression of p53 and p21 (516). This finding suggests a potential role for Brca2 in regulating the cell cycle indirectly through its ability to inhibit the transcriptional activity of p53 (328). Additional evidence suggests that Brca2's interaction with Smad3 might be a mechanism for modulating the cell cycle (419). Preobrazhenska *et al.* showed that loss of the Brca2-Smad3 interaction could negatively effect TGF-beta signaling thus promoting the release of cells from its growth inhibitory action and possibly leading to increased cell proliferation (419).

#### **2.5.4.5    *Transcription - Brca2***

Milner *et al.* showed that the amino-terminal portion of human Brca2 encoded by its third exon (a highly conserved region) shares homology with the activation domain of c-jun, a known transcription factor (358). This is suggestive of a role for Brca2 in transcriptional regulation although its exact role in this process is still uncertain.

#### **2.5.4.6    *Checkpoint Regulation - Brca2***

Brca2 has been shown to interact with and become phosphorylated by hBubR1 (a mitotic checkpoint protein) when spindle fibers become disrupted suggesting that Brca2 is involved in the mitotic checkpoint (156). The mitotic checkpoint is necessary to assess kinetochore activity and determine if chromosomes have aligned correctly at the spindle.

#### **2.5.4.7    *Fanconi's Anemia - Brca2***

Bi-allelic hypomorphic mutations in the carboxy-terminal region of Brca2, comparable to the carboxy-terminal deletion of the Brca2 deficient mice described in **manuscript one**, have been associated with another genetic disorder known as Fanconi's Anemia (FA) (209, 503). This was surprising because it was previously thought that humans with mutations in both Brca2 alleles would be non-viable. Patients with FA share similar phenotypes to several Brca2 mouse knockout models including the tendency to develop squamous cell carcinomas (334). Furthermore, knockout mice deficient with another FA protein (FANCD2) also reveal a phenotype quite similar to that described previously in the Brca2 deficient mice from **manuscript one (appendix two)** (208, 334). In addition, mutations in the Brca2 and FA

genes as well as Rad51 all lead to a similar cellular phenotypes including increased chromosomal instability and corresponding cancer susceptibility (601). Moreover, this association of Brca2 with FA may support a role for Brca2 in apoptotic function since deregulation of apoptosis appears to be a primary characteristic of this rare genetic disorder (46).

## **2.6 IONIZING RADIATION AND DNA REPAIR**

Ionizing radiation is one of the most widely acknowledged and studied human carcinogens. Radiation from natural sources is ubiquitous in the environment; it includes cosmic rays, terrestrial radiation, and internally deposited radionuclides, such as radon (446). Exposure to radiation, either accidentally or for therapeutic reasons, has long been associated with a greater incidence of neoplasms, primarily hematopoietic, gonadal, and breast cancers. The mammary gland is one of the most highly radiosensitive tissues with a high susceptibility to radiation-induced carcinogenesis. Breast cancer development in irradiated women shows a strong association with young age at the time of exposure (446). Epidemiological studies of survivors of the atomic bombs in Hiroshima and Nagasaki show that only women younger than 29 at the time of the bombing developed breast cancer, whereas older women developed benign breast diseases (338). Excess risk decreases with increasing age at exposure. The highest relative risks are observed for women exposed between the ages of 10 and 20, while there is little risk for those greater than 40. Parity, in addition to age, modifies the risk of developing radiation-induced breast cancer, since the risk is greater in nulliparous women. Young women successfully treated for Hodgkin's disease are at high risk for breast cancer development (96, 158, 186, 235). Studies have consistently demonstrated that women treated for Hodgkin's disease at a median age of 21 to 24 years, subsequently develop breast cancers after a median interval of 15 years. Women irradiated between the time of puberty and the age of 30 are at the highest risk of developing cancer (446).

Numerous studies have outlined the cellular response to ionizing irradiation (IR) and many of the molecular mechanisms involved in this process have been characterized (287). IR induces a large variety of DNA lesions, including single- and double-strand breaks (DSB) as well as base and sugar damage (222, 559). Among these, DSB are considered the most dangerous. The generation of DSB dramatically increases the demand for proteins involved in DNA repair, cell cycle regulation, and apoptosis (287). Radiation-induced damage also leads to genomic instability, an event frequently associated with initiation of the carcinogenic process (166, 418, 539, 540). According to one model, radiation induced instability places the structural integrity of virtually all genes at risk for mutation and thus cancer (418, 464).

Radiation can reduce the latency for tumor development in mice (250) and in particular, it has been shown to enhance tumorigenesis in preneoplastic mammary outgrowth lines (344). However, very few studies have specifically addressed the possible additional sensitivity or susceptibility to radiation for women or animal models carrying defective Brca2 or p53 alleles. The study outlined in **manuscript one** utilized ionizing irradiation as a carcinogen because of its ability to induce apoptosis in the mouse mammary gland and create DSB, a lesion that involves p53 and Brca2 for repair (250, 343). In addition, Balb/c mice (the mice used in **manuscript one** were 50% Balb/c) are inefficient at repairing DSB and are known to be susceptible to radiation-induced mammary carcinogenesis (393, 505, 540). The rationale for irradiating mice at five weeks of age was based on the studies showing young age is being a particularly sensitive time to the effects of radiation (527).

### **2.6.1 Cell Cycle Checkpoints – Radiation**

Mammalian cells respond to DNA damaging agents by activating cell cycle checkpoints. Transit through the cell cycle is arrested at two checkpoints when normal cells are exposed to irradiation (372). The G1/S checkpoint, dependant on p53 (56, 104, 309) prevents the replication of damaged templates, whereas the G2/M checkpoint prevents segregation of damaged chromosomes (132, 372, 405). When irradiated cells undergo p53-dependent G1 arrest they do not subsequently arrest in G2. Cells irradiated past the G1 checkpoint do arrest in G2 but do not delay in the subsequent G1 phase (407). G1 arrest is thought to give the cell time to repair damaged DNA prior to DNA replication thereby preventing the propagation of genetic lesions to progeny cells. Following DNA repair, the cell can resume the cell cycle or if it is damaged beyond repair, it can undergo apoptosis (287).

### **2.6.2 Double Strand Break Repair – Radiation**

DNA breaks are a chronic problem for cells, arising from endogenous sources such as free radicals and the replication of damaged DNA, as well as from exogenous sources such as chemical mutagens and ionizing radiation (179). Low levels of DSB also occur during normal DNA replication with an estimated 10 such lesions occurring every time a cell divides (179). DSB are considered the most detrimental form of DNA damage because they tend to be either lethal or mutagenic leading to chromosomal breakage and rearrangement events that can result in apoptosis or tumorigenesis (100, 179, 583). There are four separate pathways for repairing DSB in eukaryotes: 1) homologous recombination (HR); 2) nonhomologous end-joining (NHEJ); 3) single-stranded annealing (SSA); and 4) break-

induced replication. HR and NHEJ are the most commonly utilized repair methods (244, 254). In NHEJ, two DNA ends are aligned but it does not require extensive homology between the two recombining ends and as a result, this can be an error-prone repair process. The components involved in NHEJ include DNA-PK, Ku70, Ku80, ligase IV, XRCC4, and others. NHEJ is perhaps best known through its role in creating antibody diversity through V(D)J recombination. In contrast, HR is considered an error free repair process critical for maintaining genomic integrity by repairing DSB before cell division (525, 583). HR involves proteins such as Atm, c-Abl, Rad51/51C/51D/52/54, XRCC2/3, BLM, Brca1, Brca2, and others. In addition, the complex of Rad50, MER11, and NBS1 is involved in both HR and NHEJ (254). P53 also plays a role in both HR and NHEJ, whereas Brca2 is a critical component of HR but not NHEJ. Interestingly, wildtype p53 can bind to and modulate the activity of Rad51 thus acting as a negative regulator of HR. Loss of p53 function results in elevated HR frequencies, increased loss of heterozygosity (LOH), and decreased chromosomal stability (583). In contrast, Brca2 is capable of binding to and inhibiting the transcriptional activity of p53. The contrasting roles of these two proteins on the HR process suggests some type of autoregulatory loop, however, the exact details of this process are not presently clear. It is possible that early on in the DNA damage response, p53 is activated to promote cell cycle arrest, however, at the same time it inhibits the HR process until everything is in place and ready for repair. Once appropriate, Brca2 can reduce p53 transcription eliminating its inhibitory effects on the HR process and thus allow DSB repair to proceed.

### **2.6.2.1 *Brca2 and Double Strand Break Repair – Radiation***

Since Brca2 is only involved in the HR repair pathway, its deficiency does not completely prevent DSB repair, but it does force cells to utilize error-prone routes of repair such as NHEJ or SSA (476). Xia *et al.* (2001) provided the first direct functional evidence that the human Brca2 gene promotes DSB repair via homologous recombination, however, one of the earliest indications of Brca2's involvement in DSB repair was revealed when it was found associated with Rad51 (583).

Rad51 is a highly conserved recombinase protein with homologues identified in bacteria (RecA) and yeast (317). Mutation of Rad51 in mammalian cells results in chromosomal loss (317) and Rad51 knockout mice exhibit an embryonic lethal phenotype very similar to that seen in Brca2 null mice (587). Rad51 plays a distinct role in normal meiotic and mitotic recombination where it searches for homologous regions between two double-stranded DNA molecules and promotes strand exchange (described below). It is also involved in recombinational repair of DSB and chromosome segregation.

Brca2 interacts with Rad51 following irradiation forming distinct nuclear foci approximately 0.5 hours after exposure (460); however, these foci fail to form in Brca2-defective cells (266). Rad51 does not contain a nuclear localization signal and without Brca2, it is inefficiently transported into the nucleus (101). Several domains of the Brca2 protein interact directly with Rad51, the most prominent of these being the BRC repeats in exon 11. Interestingly, the BRC repeats of Brca2 mimic a motif in Rad51 that serves as an interface for oligomerization between individual Rad51 monomers. The Rad51 oligomerization motif

is highly conserved among RecA-like recombinases, highlighting a common evolutionary origin for the mechanism of nucleoprotein filament formation (described below). Pellegrini *et al.* showed that cancer-associated mutations that affect the BRC repeats disrupt its predicted interaction with Rad51, yielding structural insight into its potential role in cancer susceptibility (408). Another highly conserved Rad51-binding domain is located in exon 27 of the Brca2 carboxy-terminus (368). The carboxy-terminus of Brca2 also contains a DNA-binding domain capable of binding directly to single stranded DNA (ssDNA). This domain has proven critical for the processing of DNA breaks by HR.

Current views of eukaryotic HR suggest that DSB are processed to yield 3' ssDNA overhangs, which are then protected by replication protein A (RPA) (513). Rad52 then helps displace RPA allowing for Rad51 (in complex with Brca2) to bind the ssDNA (512, 515). Once bound, Brca2 promotes the oligomerization of 100's of Rad51 monomers onto the ssDNA forming a nucleoprotein filament essential for strand-pairing reactions during DNA recombination (587). The nucleoprotein filament enables the ssDNA to invade and pair with homologous bases in duplex DNA initiating strand exchange and eventually recombination between the paired DNA molecules. Thus, the direct interaction between Brca2 and Rad51 through the evolutionarily conserved BRC motifs suggests that a primary function of Brca2 is linked to DNA recombination reactions mediated by Rad51 (476).

### **2.6.2.2 P53 and Double Strand Break Repair – Radiation**

The dual role of p53 in cell cycle arrest and apoptosis suggests that the p53 status of the cell is a fundamental component of radiosensitivity (304, 305, 563). DSB induced by radiation activate the DNA damage checkpoint followed by numerous phosphorylation events, which ultimately result in cell cycle arrest coupled with DNA damage repair or apoptosis. P53 is thought to be able to “sense” DNA damage at several stages throughout the cell cycle and then determine whether cell cycle arrest at the subsequent checkpoint is necessary (407). Cells unable to repair DSB are typically eliminated via p53-mediated apoptosis. Cells lacking functional p53 may allow DNA replication to occur despite the presence of damaged DNA causing fixation of mutations into the genome, progressive loss of genomic stability, and potentially neoplasia (407). P53 protein has been co-localized with a number of DNA DSB sensing and repair proteins including gamma-H2AX, BLM, Brca1/2, Rad50, Rad51, DNA-PK, and Ku70 (185, 289, 410, 577). In addition to these indirect roles, p53 can bind nonspecifically to single- and double-stranded DNA within regions of damage and can directly promote annealing of single-stranded nucleic acids (261, 283). The p53 carboxy-terminus also has 3'-5' exonuclease activity and may be directly involved in DSB repair following gamma irradiation (318, 520). This activity appears to be intrinsic to wildtype p53, being associated with the central core DNA-binding domain of the p53 protein (236). As mentioned earlier, an additional function for p53 is to restrain excessive DNA exchange between imperfectly homologous sequences (8). Up to a 20-fold increase in HR rates can be seen in p53 deficient cells (583). *In vitro* studies have shown that p53 mutations are associated with increased radioresistance (54, 339, 594). *In vivo*, the p53 status has proven to be an essential factor in determining the radiosensitivity of a variety of different tissues (53,

59, 200, 264, 305, 313, 350). P53-deficient mice are extremely susceptible to radiation-induced tumorigenesis (250) and radiation-induced apoptosis is inhibited in mammary preneoplasias with mutant p53 (352).

## CHAPTER THREE

### EXPERIMENTAL HYPOTHESES

My overall hypothesis for this dissertation was that **certain combinations of cancer-related genes/proteins would work cooperatively promoting tumorigenesis more effectively in combination rather than independently**. This hypothesis was tested with three separate studies each involving a different stage of the tumorigenic process.

The first study to support this hypothesis involved a comparison of the individual and cooperative effects of two tumor suppressor genes, Brca2 and p53. I looked at the effects of mutation of these genes on the processes of cell proliferation and apoptosis in the developing mouse mammary gland with and without exposure to ionizing irradiation. **My hypothesis was that combined mutation of both genes would produce a more deleterious response than mutation of either gene alone. I also proposed that the harmful effects of Brca2 mutation would not be fully realized without concurrent p53 mutation.** This study involved the use of genetically altered mice carrying germline mutations in Brca2 and/or p53. Although previous studies have outlined the effects of mutant p53 in response to radiation, few have specifically looked at the mouse mammary gland and even fewer have evaluated the cooperative effects between p53 and Brca2. Although this project did not directly involve the analysis of tumors, it did evaluate two processes commonly altered in the tumorigenic process (cell proliferation and apoptosis). In addition, the developing mammary gland represents a time of particular sensitivity to genotoxic insults such as radiation, thus providing an ideal model for identifying key points in the initial stages of tumorigenesis.

Subsequent studies using these mice were done to characterize the overall tumorigenesis in these mice as they aged with a primary focus of evaluating cooperative effects between p53 and Brca2 (**appendix three**). In this study, it was predicted that mice deficient in both genes would exhibit an increased tumor incidence and decreased tumor latency as compared to their wildtype counterparts.

The second study in support of my overall hypothesis continued analysis of p53 by characterizing its involvement in ethylene oxide- and benzene-induced mammary tumors and explored how this was related to concurrent mutation of the oncogene H-ras. Previous studies have shown that ras can induce the expression of p53 leading to cell cycle arrest. In addition, cells that lack p53 are known to be highly susceptible to ras induced transformation suggesting that mutations in both genes would have a cooperative effect in promoting tumorigenesis. Although there are many potential factors involved in ethylene oxide- and benzene-induced tumorigenesis the exact mechanisms remain unknown. In addition to evaluating ethylene oxide- and benzene-induced mouse mammary tumors, a number of spontaneously induced mammary tumors were also evaluated as controls. P53 mutations were looked for in the DNA binding region including exons 5, 6, 7, and 8. In addition, these tumors were evaluated for abnormal p53 protein expression (IHC) with the intent of correlating this with the p53 mutational data. Ras mutations were evaluated in codon 61, a potential hotspot identified in other rodent and human tumor types. **My hypothesis for this study was that both p53 and H-ras mutations would commonly occur together in ethylene oxide- and benzene-induced mammary tumors but not in spontaneously induced mammary tumors.**

The third phase of this dissertation evaluated a later stage in the tumorigenic process involving the acquisition of invasive and metastatic properties. As in the early stages, this process also requires the multistep accumulation of genetic modifications and most certainly involves cooperative interactions between genes or proteins. Modulation of cell adhesion is a biological function critical to the development of invasive and metastatic properties. In fact, many cell adhesion molecules are also classified as tumor suppressor genes for this reason. Development of invasive and metastatic properties involves both the loss of cell adhesion, as tumor cells look to dissociate from surrounding cells and tissues, as well as a gain in (or retention of) cell adhesion, as tumor cells attempt to gain access to distant tissues. The development of both of these antagonistic properties in the same cell suggests that more than one adhesion molecule is involved in the process and it is therefore likely that cell adhesion molecules function cooperatively in the effort to gain an invasive and/or metastatic phenotype. For this phase of the project, I looked at the protein expression of four different cell adhesion molecules (KAI1, CD9, E-cadherin, and N-cadherin) known to be involved in invasive and metastatic processes. I compared the expression of these adhesion molecules between various grades of primary ovarian epithelial tumors and observed how the expression changed as the primary tumors progressed towards a metastatic phenotype. **I hypothesized that more than one but not all of these adhesion molecules would be downregulated as tumors progressed towards a metastatic phenotype.** In theory, it would be predicted that even the metastatic tumor cells would need to maintain some cell adhesive properties in order to become established at distant sites. It is also likely that some metastatic tumors regain expression of previously downregulated cell adhesion molecules.

## CHAPTER FOUR

### MANUSCRIPT ONE

(Accepted for publication in *Experimental and Toxicologic Pathology*)

## MUTANT BRCA2/P53 MICE EXHIBIT ALTERED RADIATION RESPONSES IN THE DEVELOPING MAMMARY GLAND

### 4.1 ABSTRACT

Appropriate balance between proliferation and apoptosis is critical for mammary gland development and is often altered during tumorigenesis. Carcinogens like radiation induce DNA damage and activate protective responses such as cell cycle arrest and apoptosis. Here mice carrying *Brca2*<sup>-/-</sup> and/or *p53*<sup>-/-</sup> mutations were used to evaluate the individual and combined effects of these genes on cell proliferation and apoptosis in the developing mammary gland. Mice were exposed to 5 Gy of radiation or chamber exposure (controls) followed by injection with BrdU. Mammary glands were collected 6 hours post-radiation exposure and evaluated for proliferation (BrdU) and apoptosis (TUNEL) in terminal end buds (TEB) and ducts. Under control conditions, the *Brca2* mutation reduced proliferation and apoptosis in TEB but not ducts, whereas the *p53* mutation reduced apoptosis in TEB and ducts but did not influence proliferation. Despite these alterations in proliferation and/or apoptosis, neither mutation, either individually or combined, significantly altered the overall balance between the two as measured by the proliferation to apoptosis ratio (growth index). Following irradiation, the *Brca2* mutation had no significant effect on proliferation or apoptosis, whereas the *p53* mutation resulted in reduced apoptosis in TEB and ducts but did not significantly influence proliferation. Neither mutation by itself altered the growth index

in the TEB after irradiation although combined Brca2/p53 mutation caused significantly increased proliferation, reduced apoptosis, and an elevated growth index in TEB and ducts. These results reveal both independent and collaborative growth regulatory roles for Brca2 and p53 under normal and adverse environmental conditions. Additionally, these results demonstrate the importance of gene-environment interactions by showing that Brca2- and p53-deficient mice can compensate for their genetic deficiencies under control conditions but not after exposure to radiation. These results also demonstrate distinct spatial differences in the cellular functions of Brca2 and p53 and show that combined mutation of both genes is more detrimental than loss of either gene alone.

## **4.2 INTRODUCTION**

Germline mutations in Brca2 confer an increased risk of breast cancer in both women and men (126). Several lines of evidence suggest that loss of p53 function may be an important event in the genesis of Brca2-associated cancers, although the mechanism of how this occurs is not known (93, 169, 171, 241, 345, 425, 528). Brca2 and p53 physically and functionally interact (328) and recent studies suggest their relationship is antagonistic. P53 has an inhibitory effect on the Brca2 promoter and is capable of inhibiting homologous recombination, a process mediated by Brca2 (347, 511, 581). Brca2 can negatively regulate p53-mediated transcriptional activity (328), whereas deficiency of Brca2 can result in elevated p53 expression (88, 404). Loss of either gene results in genomic instability and an impaired ability to respond to radiation-induced DNA damage (39, 88, 147, 470, 535, 536).

Ionizing radiation activates a network of signaling pathways involved in cell cycle arrest, apoptosis, and DNA repair (287). P53 has a role in all three of these processes (305, 313, 422), although the function of Brca2, beyond its role in DNA repair, is much less certain and sometimes conflicting. For example, one study revealed decreased apoptosis in thymocytes from Brca2-mutant mice exposed to the DNA damaging agent etoposide (146), whereas another study reported increased rates of spontaneous apoptosis in Brca2-null T-lymphocytes (81). In contrast, Patel *et al.* (1998) determined that apoptotic mechanisms were largely unaffected in Brca2-deficient lymphoid cells and similar observations have been made with Brca2-deficient embryos (516). Studies utilizing the hamster cell line V-C8, which carries mutant Brca2, revealed increased levels of apoptosis in response to the DNA damaging agent mitomycin C (401). Similarly, Yan *et al.* showed that a mouse derived Brca2-heterozygous cell line and Capan-1 cells, which carry a mutant Brca2 gene, are more sensitive to apoptosis when exposed to 7, 12-dimethylbenz[*a*]anthracene (DMBA) (586).

A potential role for Brca2 in cell cycle regulation has been suggested, although it is often difficult to differentiate direct Brca2-mediated effects from secondary effects related to its role in DNA repair. Studies showing increased Brca2 expression in rapidly proliferating cells and those that describe Brca2's cell cycle specific expression provide support for involvement in cell cycle regulation (424, 544). Early studies with Brca2-deficient embryos showed a marked proliferation defect that was less severe on a p53-null background (153, 310). Brca2-deficient mouse embryonic fibroblasts revealed a similar proliferation defect characterized by cell cycle arrest in G1 and G2/M and increased expression of p53 and p21 (88, 404). Some reports suggest potential roles for Brca2 in cell cycle regulation through its

association with other proteins such as Brca2-associated factor 35 (327), PIK1 (280), BCCIP-alpha (296), DSS1 (330), and Smad3 (419). There have been several studies linking Brca2 with checkpoint regulation (156, 279, 340), although the results here can be conflicting as well. Brca2 is known to interact with mitotic checkpoint regulating proteins such as hBubR1 (156, 279), although others have demonstrated that Brca2-deficient cells maintain the G1/S and G2/M cell cycle checkpoints (366).

This wide variation in results and the number of conflicting reports illustrates how much uncertainty there is regarding Brca2's role in regulating cell growth. Much of this uncertainty could be related to cell type, tissue-specific, or developmental differences in function or perhaps differences in relation to the particular mutation being studied. To date, most studies looking for Brca2's role in cell growth have been conducted *in vitro* with cells not derived from mammary epithelium. The Brca2-mutant mice used in this study lack just the terminal exon 27 of Brca2 but still exhibit a high incidence of neoplasia, particularly carcinomas (334). These Brca2-mutant mice (Brca2<sup>-/-</sup>) along with p53-mutant mice (p53<sup>-/-</sup>) and double-mutant mice (dKO) carrying both mutations were used to study the role these genes have in regulating cell proliferation and apoptosis in the developing mammary gland. Differences in cell proliferation, as measured by BrdU incorporation, and apoptosis, as measured by the TUNEL assay, were used to assess the overall influence of genotype on growth of the 5-week old mouse mammary gland under control conditions (unirradiated) and in response to a single dose (5 Gy) of whole-body gamma-irradiation (irradiated). Furthermore, measurement of these parameters in histologic section allowed us to discriminate between distinct

epithelial structures (terminal end buds and ducts) in the developing mammary gland thus providing a unique look at spatial differences in the function of these genes.

### **4.3 MATERIALS AND METHODS**

#### **4.3.1 Animals**

$Brca2^{-/-}$  mice were created as previously described (334). Additional details are provided in Figure 5. These mice were on a C57BL/6 background and had a germline mutation eliminating exon 27.  $p53^{-/-}$  mice were purchased from a commercial source (The Jackson Laboratory, Bar Harbor, ME). These mice were on a Balb/cJ background and had a germline mutation removing approximately 40% of the coding region, which disrupts p53 protein expression (231). Mice hemizygous for the  $Brca2$  mutation were crossed with mice hemizygous for the  $p53$  mutation to create offspring hemizygous for both mutations. These double-hemizygous mutant mice were intercrossed to generate the F2 offspring used in this study (WT,  $Brca2^{-/-}$ ,  $p53^{-/-}$ , and dKO).

These mice exhibit a low penetrance of perinatal lethality, however, the majority reach adulthood. This type of  $Brca2$  truncation retains all of the Rad51 binding BRC repeats in exon 11, however, the carboxy-terminal Rad51 binding domain between amino acids 3196-3232 is deleted. These mice also lack several highly conserved nuclear localization signals (NLS) located in exon 27 in both the human and mouse genes (105, 404, 588). Additional studies with these mice have demonstrated that they are tumor prone with a tendency to develop a variety of different carcinomas as well as lymphomas (**appendix two**). These

findings strongly support the importance of this portion of the gene in mediating Brca2's tumor suppressor functions. As mentioned previously, these mice also exhibit stunted mammary ductal development although they do eventually develop fully functional mammary glands (34).

### **4.3.2 Genotyping**

Genomic DNA was recovered from tail biopsies and each mouse pup was genotyped using PCR-based techniques. Brca2 genotyping was done as previously described (146, 334). The mutant p53 allele was detected using one set of PCR primers, p53MF and p53WR, 5'-CTATCAGGACATAGCGTTGG-3' and 5'-TATACTCAGAGCCGGCCT-3'.

### **4.3.3 Experimental Design**

Thirty-two female mice were divided into unirradiated (control) and irradiated groups each consisting of subgroups representing four genotypes. Overall there were nine WT, eight Brca2<sup>-/-</sup>, eight p53<sup>-/-</sup>, and seven dKO mice in the study. At 5-weeks of age (+/- 2 days), mice were irradiated (<sup>137</sup>Cs source) with a single 5 Gy dose of whole-body irradiation or chamber-exposed without irradiation (controls). Five hours after exposure, each mouse was given an intraperitoneal injection of 5-bromo-2-deoxyuridine (BrdU) at a dose of 50 mg/kg and then sacrificed 1 hour later (6 hours post-irradiation). The optimal time for post-irradiation sacrifice was determined through results of a pilot study that showed maximal changes in apoptotic and cell proliferative parameters at the 6-hour time point (**appendix one**). Partial

necropsies were performed on all mice and the left fourth mammary gland, ovaries, uterus, and vagina were collected. Tissues were fixed in 10% neutral buffered formalin for 24 hours then transferred to 70% ethanol until further processing. All tissues were routinely processed and embedded in paraffin blocks. Hematoxylin and eosin stained slides of the reproductive tract and ovaries were used for staging of the estrous cycle. Two 5-micrometer sections were taken from each mammary gland block and used for the assessment of apoptosis and cell proliferation. In addition, the right fourth mammary gland was collected, fixed in 10% neutral buffered formalin for 24 hours, and stained with Carmine dye as previously described for whole-mount preparation (26). All procedures utilizing animals in this study complied with NIH guidelines for the humane care and use of animals.

#### ***4.3.4 Cell Proliferation and Apoptosis Analyses***

BrdU incorporation with immunohistochemical detection were used to assess cell proliferation as previously described (129). Briefly, immunohistochemistry was performed with an avidin-biotin peroxidase method on a Ventana automated stainer (Ventana, Tucson, AZ,) using a mouse anti-BrdU primary antibody (Neomarkers, Fremont, CA) for 30 minutes at 24°C. The Vector MOM kit (Burlingame, CA) was used to develop the antibody staining. The antibody complex was visualized using 3, 3'-diaminobenzidine (Ventana, Tucson, AZ). The ApopTag In Situ Apoptosis Detection Kit (Intergen, Purchase, NY) was used to detect apoptotic cells according to the manufacturer's instructions.

#### **4.3.5 *Image Analysis***

Final analysis of BrdU and TUNEL stained slides required capturing multiple digital images from each mammary gland section. Digital images were captured using a Spot Insight digital camera (Diagnostic Instruments, Sterling Heights, MI) attached to an Olympus BX41 microscope (Olympus America, Melville, NY). Images were taken of all epithelial structures (terminal end buds and ducts) using the 40X objective. For analysis, data were captured using Image-Pro Plus image analysis software, version 5.0 (Media Cybernetics, Silver Spring, MD) running on a PC-based computer. In each field, mammary epithelial cell nuclei were identified and the total nuclei present per field were measured using color thresholding techniques. All TUNEL and BrdU positive nuclei were manually counted using the manual tag feature of Image-Pro Plus. Data included the measured indices of total nuclei count and total positive nuclei count as well as the calculated labeling index (the number of BrdU or TUNEL positive nuclei / the total number of nuclei x 100). Terminal end bud and duct epithelium were analyzed independently. For each genotype, an average of at least 22000 epithelial cells was counted including 13000 or greater from TEB and 9000 or greater from ducts. Stromal cells were not included in the analysis.

#### **4.3.6 *Data Analysis***

A square root transformation was performed on the raw data so that it was approximately homoscedastic between genotypes. Standard analysis of variance procedures were used to assess whether radiation and genotype had an effect on apoptosis and cell proliferation in the

mouse mammary gland. Analyses were done using PROC GLM, with LSMEANS options for multiple comparisons, available in the statistical software package SAS (Release 8.2, 2001). Studentized residuals were used to identify outliers in the data. These included: apoptosis data from one irradiated WT mouse (TEB); cell proliferation data from two control p53<sup>-/-</sup> mice (TEB); and proliferation:apoptosis ratio data from one irradiated p53<sup>-/-</sup> mouse (ducts) and one irradiated dKO mouse (TEB). The weight by genotype interaction was evaluated independently by using Dunnett's test.

#### **4.4 RESULTS**

All mutant genotypes exhibited lower mean body weights as compared to WT mice; however, only Brca2<sup>-/-</sup> (p=0.0368) and dKO (p=0.0016) mice were statistically significant (Figure 1). Despite these differences, estrous cycle staging indicated that all mice were cycling at the time of exam and whole-mount analysis showed comparable levels of ductal extension and terminal end bud numbers between genotypes. Genotype specific differences in cell proliferation and apoptosis, under control conditions and in response to radiation, are summarized in Figure 2 (TEB) and Figure 3 (ducts). Representative images of TEB proliferation and apoptosis are shown in Figure 4A and Figure 4B, respectively. Significant differences between genotypes in terminal end bud and duct epithelium are summarized in Table 1 and Table 2, respectively, and in the text below.

#### **Cell proliferation and apoptosis in the developing mammary gland of Brca2 and p53 mutant mice.**

In unirradiated controls, there was significantly reduced cell proliferation in the TEB of  $Brca2^{-/-}$  ( $p=0.0210$ ) and dKO mice ( $p=0.0228$ ) when compared to that of WT mice (Figure 2A and Figure 4A). The degree of reduction in proliferation rates was similar between  $Brca2^{-/-}$  and dKO mice.  $p53^{-/-}$  mice revealed TEB proliferation rates that were reduced but not statistically different from WT mice. dKO mice revealed ductal proliferation rates that were close to being significantly reduced as compared to WT mice ( $p=0.0675$ ), although  $Brca2^{-/-}$  and  $p53^{-/-}$  mice revealed proliferation rates very similar to WT mice (Figure 3A).

There was a significantly reduced apoptotic rate in the TEB of  $Brca2^{-/-}$  ( $p=0.0061$ ),  $p53^{-/-}$  ( $p=0.0132$ ), and dKO mice ( $p=0.0034$ ) when compared to that of WT mice (Figure 2B and Figure 4B). The degree of reduction was similar for all three mutant genotypes and no significant differences were identified between them. In ducts, dKO mice ( $p=0.0336$ ) exhibited significantly reduced apoptosis as compared to WT mice (Figure 3B).  $p53^{-/-}$  ( $p=0.0112$ ) and dKO mice ( $p=0.0027$ ) also exhibited significantly less ductal apoptosis than  $Brca2^{-/-}$  mice (Figure 3B). No significant differences in duct apoptotic rates were identified between  $p53^{-/-}$  and dKO mice.  $Brca2^{-/-}$  mice displayed duct apoptotic rates slightly higher but comparable to those seen in WT mice (Figure 3B).

The ratio of cell proliferation to apoptosis was calculated to provide an estimate of the overall amount of mammary epithelial cell turnover (growth index). In unirradiated control mice, there were no statistically significant differences in growth index between any of the genotypes in either TEB or ducts (Figure 2C and Figure 3C).

## **Cell proliferation and apoptosis in the developing mammary gland of irradiated Brca2 and p53 mutant mice.**

Following irradiation, dKO mice exhibited significantly higher levels of cell proliferation in TEB as compared to Brca2<sup>-/-</sup> (p<0.0001), p53<sup>-/-</sup> (p=0.0002), and WT mice (p<0.0001), whereas Brca2<sup>-/-</sup> and p53<sup>-/-</sup> mice responded to radiation with proliferation rates much like WT mice (Figure 2A and Figure 4A). The number of ductal epithelial cells incorporating BrdU was not significantly different between any of the mutant genotypes and WT mice (Figure 3A).

dKO mice exhibited significantly decreased levels of apoptosis in TEB as compared to Brca2<sup>-/-</sup> (p<0.0001), p53<sup>-/-</sup> (p=0.0312), and WT mice (p<0.0001) (Figure 2B and Figure 4B). p53<sup>-/-</sup> mice had significantly reduced levels of apoptosis in TEB as compared to Brca2<sup>-/-</sup> (p<0.0001) and WT mice (p<0.0001) (Figure 2B and Figure 4B). There were no differences in TEB apoptotic rates between Brca2<sup>-/-</sup> and WT mice. In ducts, dKO mice exhibited reduced levels of apoptosis as compared to Brca2<sup>-/-</sup> (p<0.0001) and WT mice (p<0.0001) (Figure 3B). p53<sup>-/-</sup> mice had significantly reduced duct apoptosis as compared to Brca2<sup>-/-</sup> (p=0.0013) and WT mice (p<0.0001) (Figure 3B). There were no significant differences in ductal apoptotic rates between dKO and p53<sup>-/-</sup> mice or between Brca2<sup>-/-</sup> and WT mice.

In TEB, dKO mice exhibited a significantly higher growth index as compared to Brca2<sup>-/-</sup> (p<0.0001), p53<sup>-/-</sup> (p=0.0010), and WT mice (p<0.0001) (Figure 2C). There were no significant differences in TEB growth index between Brca2<sup>-/-</sup> and WT mice. p53<sup>-/-</sup> mice revealed growth indices increased over WT mice although the difference here did not show

statistical significance. In ducts, dKO mice exhibited a significantly higher growth index as compared to  $Brca2^{-/-}$  ( $p=0.0427$ ) and WT mice ( $p=0.0014$ ) (Figure 3C).  $p53^{-/-}$  mice exhibited a significantly higher ductal growth index as compared to WT mice ( $p=0.0439$ ) (Figure 3C). There were no significant differences in ductal growth index between dKO and  $p53^{-/-}$  mice.  $Brca2^{-/-}$  mice exhibited an elevated growth index as compared to WT mice although this finding lacked statistical significance.

#### **4.5 DISCUSSION**

Although a role for  $Brca2$  is well recognized in the pathogenesis of breast cancer, much remains unknown about its function, particularly regarding how it interacts with  $p53$ . These results demonstrate clear individual and collaborative functions for  $Brca2$  and  $p53$  during mammary gland development and in the acute response to radiation, a known carcinogen. Furthermore, these results show distinct spatial differences in the cellular functions of  $Brca2$  and  $p53$  and highlight the importance of gene-environment interactions by showing the ability of  $Brca2$ - and/or  $p53$ -mutant mice to compensate for their genetic deficiencies under control conditions but not after radiation exposure (as measured by changes in the growth index).

In the present study,  $Brca2$  mutation resulted in reduced cell proliferation in the mammary gland. Unirradiated  $Brca2^{-/-}$  mice revealed significantly lower levels of BrdU incorporation in TEB epithelium as compared to WT mice. Although this has been described previously in  $Brca2$ -mutant cells (88, 119, 366, 404, 516, 534), this is thought to be one of the first reports

of such an effect in mammary epithelial cells *in vivo*. This finding further reveals a role for Brca2 at specific times during mammary gland development. Interestingly, the Brca2<sup>-/-</sup> mice continued to display reduced TEB proliferation even when placed on a p53-mutant background. dKO mice exhibited lower baseline levels of cell proliferation, similar to those seen in Brca2<sup>-/-</sup> mice, suggesting that this phenotype is not associated with p53 activation and that other cell cycle regulators are likely involved. Alternatively, these observed reductions in cell proliferation could be related to the recently described association between Brca2 and delayed cytokinesis (98).

Apoptosis is a process critical for normal mammary gland development and these data demonstrate that both Brca2 and p53 are involved in its regulation in the TEB. Ductal apoptosis, on the other hand, appears to be predominantly mediated by p53. Unirradiated Brca2- and p53-mutant mice both exhibited reduced levels of TEB apoptosis, as did dKO mice. Considering that the apoptotic rates were similarly reduced in Brca2<sup>-/-</sup>, p53<sup>-/-</sup>, and dKO mice, there did not appear to be an additive effect with combined mutation of both genes. This suggests that Brca2 and p53 might be involved in the same or closely related apoptotic pathways, or the effect seen in Brca2 mutants could be a compensatory response related to the concurrent reduction in the cell proliferation rate.

Support for a compensatory response is provided by analysis of the growth index that shows similar growth indices between all unirradiated mutant and WT mice. Although mutation of Brca2 and/or p53 may alter cell proliferation and/or apoptosis, the developing mammary gland appears capable of adjusting to these changes with the appropriate counter-response

and thus maintains overall balanced growth characteristics. This may explain why Brca2- and p53-mutant mice can show alterations in mammary gland development but ultimately develop functional mammary glands (34, 218, 241).

The finding of comparable cell proliferation rates in the TEB and ducts of irradiated p53<sup>-/-</sup> and WT mice was somewhat unexpected considering the known role for p53 in radiation-induced cell cycle arrest. However, it should be noted that proliferation rates in the p53<sup>-/-</sup> mice were reduced as compared to WT mice just not to the level of statistical significance. In addition, it is possible that this finding represents a cell-type specific or developmentally regulated function of p53 not typical of its known function elsewhere (141, 313, 351, 354, 360). In contrast, there was a dramatic cell proliferative response to irradiation in the TEB of dKO mice characterized by a marked increase in cell proliferation as compared to all other genotypes. This strongly suggests a radiation-induced interaction between Brca2 and p53 in the TEB in that combined but not individual mutation results in an adverse response.

When interpreting these findings with regard to the function of Brca2 it should be emphasize that this mutation only involved exon 27 and thus a large portion of the gene remained intact. Despite this, these mice have been shown to exhibit an increased susceptibility to spontaneous tumorigenesis suggesting that this mutation is significant (334). This region of the gene is one of the most highly conserved regions of Brca2 and contains several important functional domains involved with nuclear localization and binding to Rad51 and single stranded DNA (334, 587, 588, 597). Cells lacking the carboxy-terminus are also known to be hypersensitive to gamma-radiation (366) and deficient in error-free, homology-directed

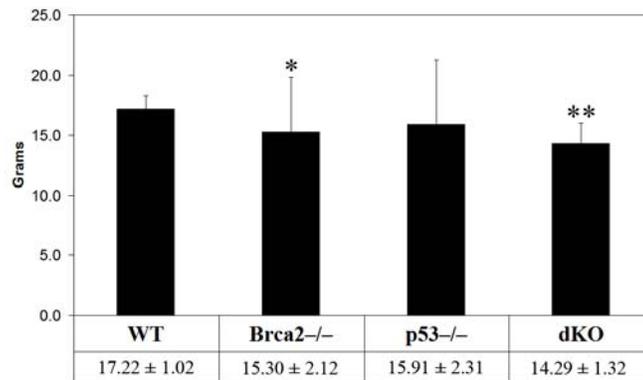
DNA repair (119). The results here suggest this region of the gene is also important for growth regulation of the developing mammary gland and, in cooperation with p53, the appropriate cellular response to radiation. In addition, it should also be noted that, since the genetic backgrounds of the Brca2- (C57BL/6) and p53- (Balb/cJ) mutant mice were different, the overall genetic makeup of each cross was likely not identical. This could have a potential influence on these results considering that the two respective mouse strains vary in their response to irradiation and susceptibility to mammary tumorigenesis. Balb/cJ mice appear to be more sensitive to radiation and more commonly develop mammary tumors as compared to C57BL/6 mice (31).

Overall, these results show several important independent and collaborative interactions between Brca2 and p53 and provide insight into their roles in mammary gland development and in the cellular response to radiation. These data reveal a clear distinction between TEB and duct epithelium in both mammary ductal morphogenesis and radiation response.

Considering the fact that these mutant mice do eventually develop mature mammary glands it would seem that alternative pathways are ultimately capable of compensating for these developmental deficiencies. However, it should be noted that the growth regulatory imbalances revealed in these mutant mice are likely to create an environment more susceptible to genotoxic agents. A change such as this could ultimately lead to tumorigenesis, particularly upon exposure to a genotoxic agent such as radiation. Indeed, these results do show that Brca2 and p53 have important roles in regulating the acute mammary epithelial response to radiation. P53 plays the dominant role in duct epithelium although, in TEB, Brca2 and p53 appear to function both independently and collaboratively. The most adverse

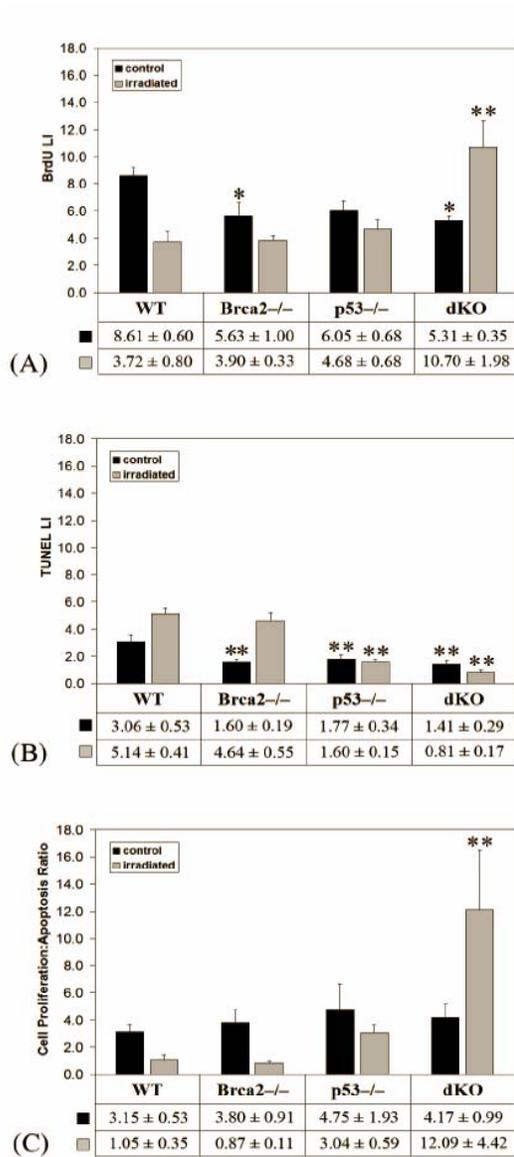
responses to radiation, however, consistently occurred in dKO mice suggesting that both genes are necessary to maintain an appropriate cellular response to radiation. This was particularly true in TEB where dKO mice responded to irradiation with decreased apoptosis, increased cell proliferation, and a significantly elevated growth index. Furthermore, this analysis of changes in growth index between unirradiated and irradiated mice highlights the significance of gene-environment interactions and shows how a particular genotype can significantly influence the response to an adverse environmental factor such as radiation.

## FIGURE 1



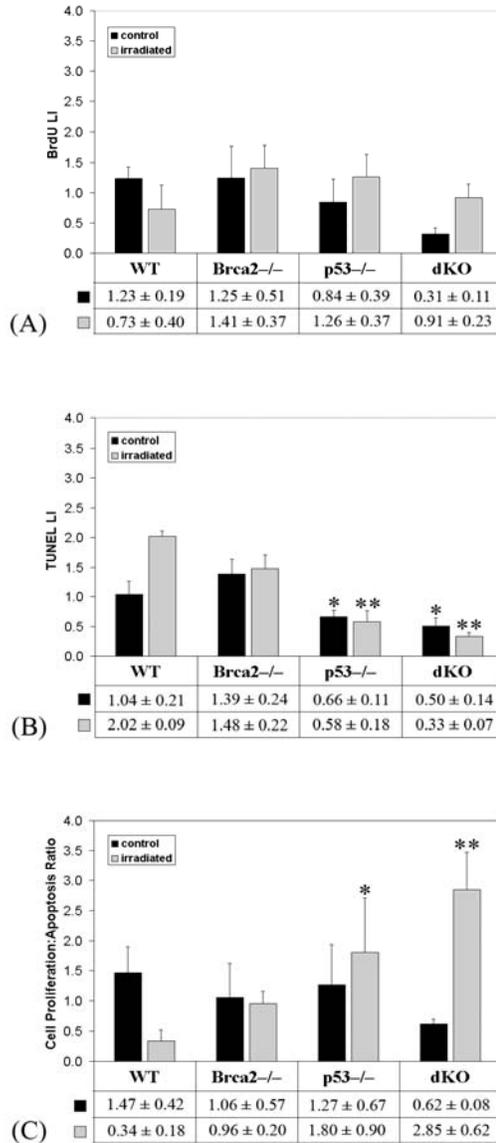
**Fig. 1.** Differences in group mean body weight  $\pm$  SE. Asterisks indicate values statistically different from corresponding WT mice (\* =  $p < 0.05$  and \*\* =  $p < 0.01$ ).

## FIGURE 2



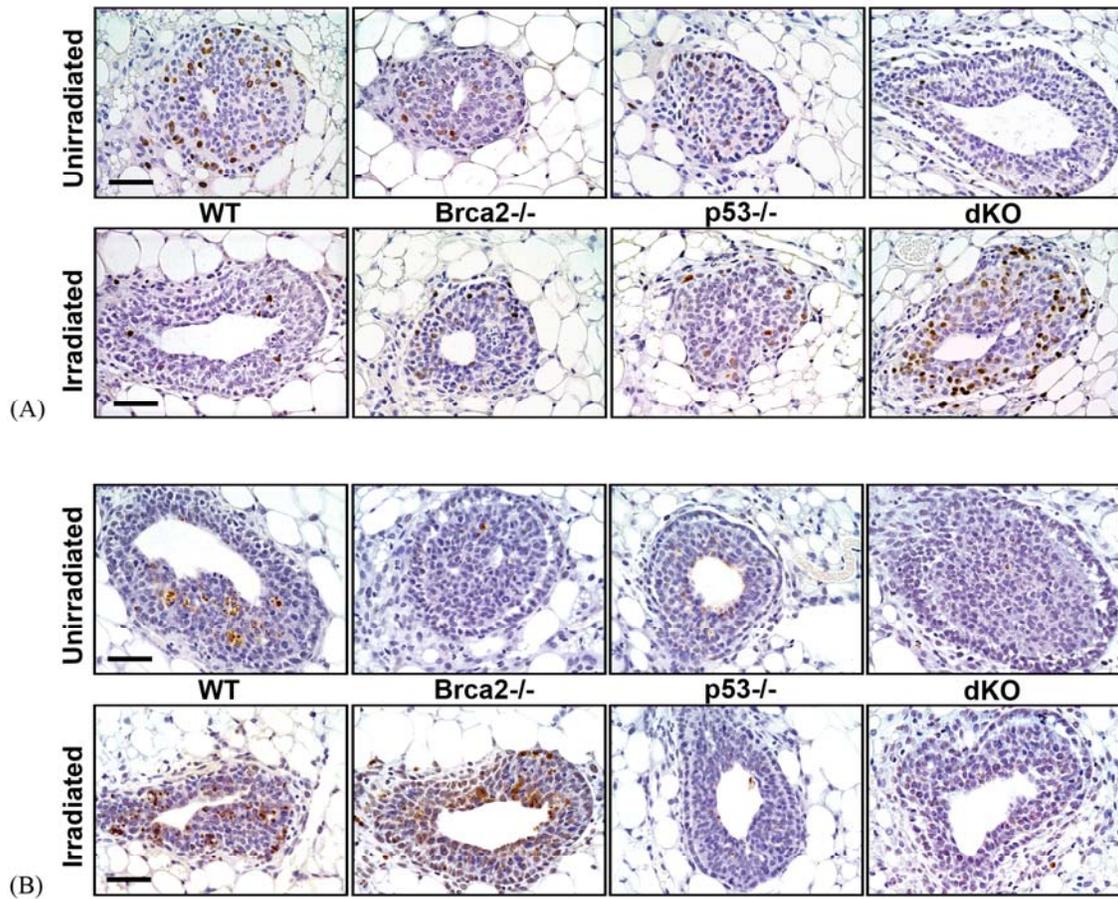
**Fig. 2.** Cell proliferation and apoptosis in terminal end bud epithelium of 5-week old *Brca2/p53* mutant mouse mammary gland. (A) Terminal end bud proliferation as measured by BrdU incorporation. The x-axis values and overlying bars represent the group mean labeling indices  $\pm$  SE from unirradiated (■) and irradiated (□) mice. (B) Terminal end bud apoptosis as measured by the TUNEL assay. The x-axis values and overlying bars represent the group mean labeling indices  $\pm$  SE from unirradiated (■) and irradiated (□) mice. (C) The ratio of cell proliferation to apoptosis (growth index) in terminal end bud epithelium from unirradiated (■) and irradiated (□) *Brca2/p53* mutant mice. Asterisks indicate values statistically different from corresponding WT mice (\* =  $p < 0.05$  and \*\* =  $p < 0.01$ ).

### FIGURE 3



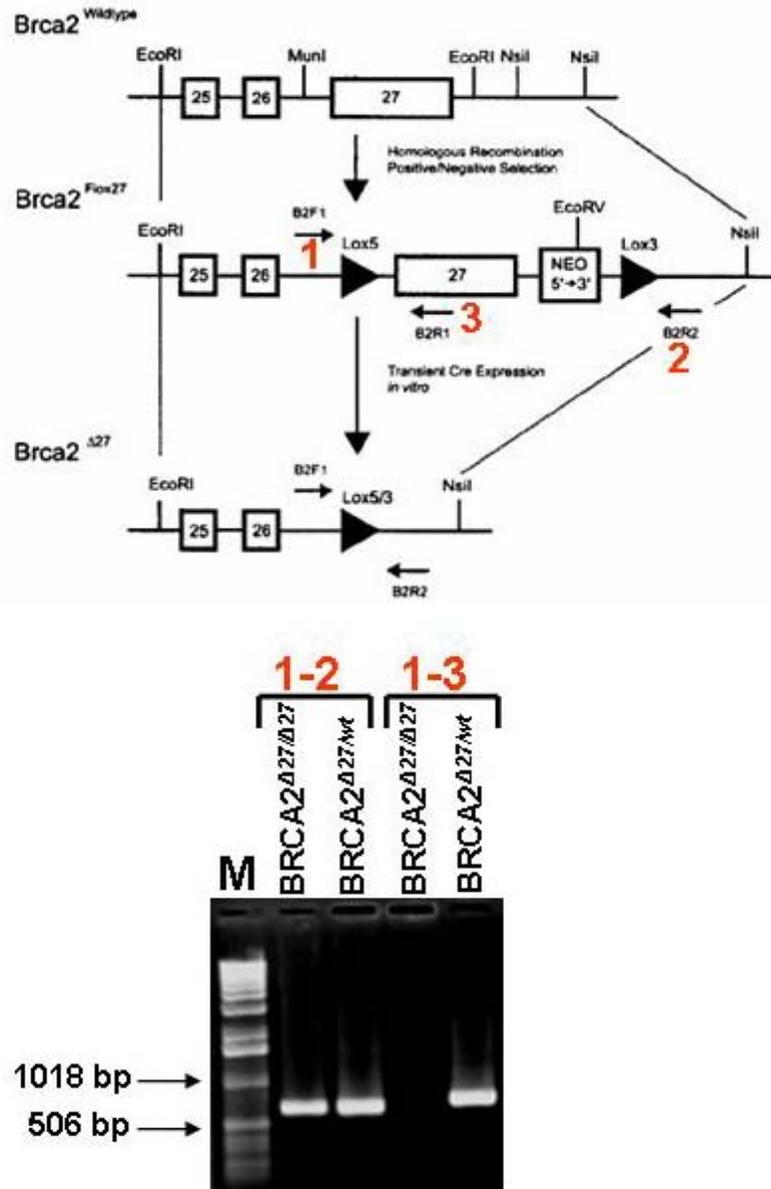
**Fig. 3.** Cell proliferation and apoptosis in the duct epithelium of 5-week old *Brca2/p53* mutant mouse mammary gland. (A) Duct proliferation as measured by BrdU incorporation. The x-axis values and overlying bars represent the group mean labeling indices ± SE from unirradiated (■) and irradiated (□) mice. (B) Duct apoptosis as measured by the TUNEL assay. The x-axis values and overlying bars represent the group mean labeling indices ± SE from unirradiated (■) and irradiated (□) mice. (C) The ratio of cell proliferation to apoptosis (growth index) ± SE in duct epithelium from unirradiated (■) and irradiated (□) *Brca2/p53* mutant mice. Asterisks indicate values statistically different from corresponding WT mice (\* = p<0.05 and \*\* = p<0.01).

**FIGURE 4**



**Fig. 4.** Cell proliferation and apoptosis in terminal end buds from 5-week old *Brca2/p53* mutant mouse mammary gland before (unirradiated) or 6 hours after (irradiated) treatment with 5 Gy of whole body  $\gamma$ -radiation. (A) Representative images of terminal end bud cell proliferation as measured by BrdU incorporation in immunohistochemically stained sections of mammary gland. Bar 50  $\mu\text{m}$  (200X). (B) Representative images of apoptosis in mammary gland terminal end buds as measured by the TUNEL assay. Bar 50  $\mu\text{m}$  (200X).

**FIGURE 5**



**Figure 5.** Targeting construct for Brca2 exon 27 knockout mice (334).

## TABLES 1 AND 2

**Table 1. Summary of statistical differences in terminal end bud epithelium**

	Cell Proliferation		Apoptosis		Proliferation:Apoptosis Ratio	
	Unirradiated	Irradiated	Unirradiated	Irradiated	Unirradiated	Irradiated
<i>Brca2</i> <sup>-/-</sup>	less than: WT (p=0.0210)	ND	less than: WT (p=0.0061)	ND	ND	ND
<i>p53</i> <sup>-/-</sup>	ND	ND	less than: WT (p=0.0132)	less than: <i>Brca2</i> <sup>-/-</sup> (p<0.0001) WT (p<0.0001)	ND	ND
dKO	less than: WT (p=0.0228)	greater than: <i>Brca2</i> <sup>-/-</sup> (p<0.0001) <i>p53</i> <sup>-/-</sup> (p=0.0002) WT (p<0.0001)	less than: WT (p=0.0034)	less than: <i>Brca2</i> <sup>-/-</sup> (p<0.0001) <i>p53</i> <sup>-/-</sup> (p=0.0312) WT (p<0.0001)	ND	greater than: <i>Brca2</i> <sup>-/-</sup> (p<0.0001) <i>p53</i> <sup>-/-</sup> (p=0.0010) WT (p<0.0001)

ND = no statistical differences with any other genotypes  
 WT = wild-type; *Brca2*<sup>-/-</sup> = *Brca2*-mutant; *p53*<sup>-/-</sup> = *p53*-mutant; dKO = double-mutant

**Table 2. Summary of statistical differences in duct epithelium**

	Cell Proliferation		Apoptosis		Proliferation:Apoptosis Ratio	
	Unirradiated	Irradiated	Unirradiated	Irradiated	Unirradiated	Irradiated
<i>Brca2</i> <sup>-/-</sup>	ND	ND	ND	ND	ND	ND
<i>p53</i> <sup>-/-</sup>	ND	ND	less than: <i>Brca2</i> <sup>-/-</sup> (p=0.0112)	less than: <i>Brca2</i> <sup>-/-</sup> (p=0.0013) WT (p<0.0001)	ND	greater than: WT (p=0.0439)
dKO	ND	ND	less than: <i>Brca2</i> <sup>-/-</sup> (p=0.0027) WT (p=0.0336)	less than: <i>Brca2</i> <sup>-/-</sup> (p<0.0001) WT (p<0.0001)	ND	greater than: <i>Brca2</i> <sup>-/-</sup> (p=0.0427) WT (p=0.0014)

ND = no statistical differences with any other genotypes  
 WT = wild-type; *Brca2*<sup>-/-</sup> = *Brca2*-mutant; *p53*<sup>-/-</sup> = *p53*-mutant; dKO = double-mutant

## CHAPTER FIVE

### LITERATURE REVIEW FOR MANUSCRIPT TWO

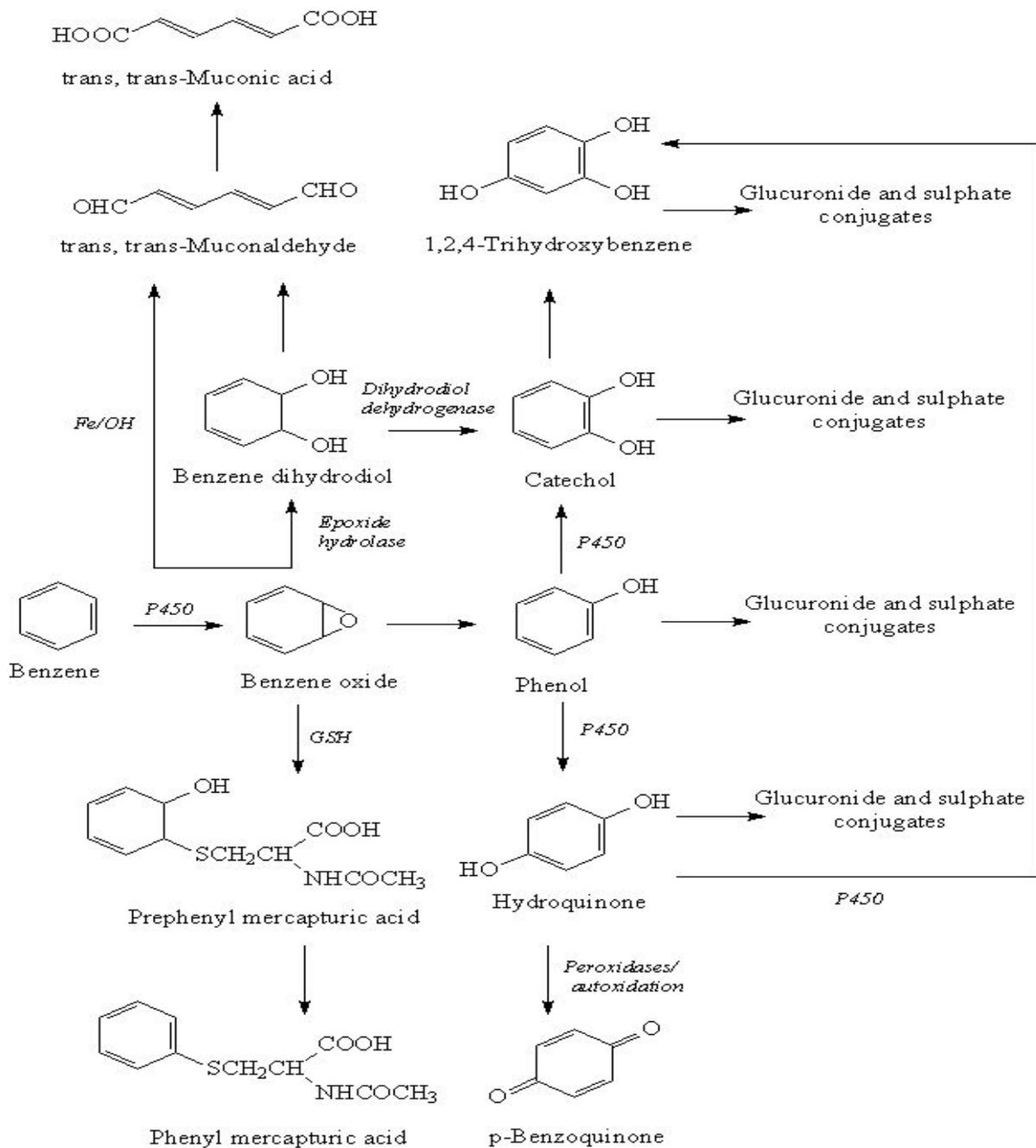
#### 5.1 *BENZENE*

Benzene is a ubiquitous environmental pollutant and a recognized human leukemogen and rodent carcinogen. It is used as a solvent in the chemical and pharmaceutical industries, a starting material and intermediate in the synthesis of numerous chemicals, and is found in gasoline, where it acts as an octane enhancer and an anti-knock agent (268). As a raw material, it is used in the synthesis of ethylbenzene (used to produce styrene), cumene (used to produce phenol and acetone), cyclohexane, nitrobenzene (used to produce aniline and other chemicals), detergent alkylate (linear alkylbenzene sulfonates), maleic anhydride, chlorobenzenes, and other products (435). The primary route of human exposure to benzene is inhalation of ambient air. Benzene is present in the atmosphere both from natural sources, which include forest fires and oil seeps, and from industrial sources, such as industrial emissions and fuel evaporation from gasoline (435). The major route of exposure for the general population, however, is breathing air contaminated from man-made sources such as cigarette smoke and vehicular emissions (268). Benzene is a lipid-soluble, volatile organic compound that is absorbed rapidly and efficiently in humans and experimental animals following inhalation. Since it is lipophilic, benzene tends to accumulate in adipose tissue but also accumulates in bone marrow and central nervous system tissue (268).

### **5.1.1 Metabolism – Benzene**

There is considerable evidence supporting a role for metabolism in benzene toxicity (437). Co-administration of toluene, a competitive inhibitor of benzene metabolism, reduced toxicity in mice. Partial hepatectomy also decreases benzene metabolism and protects against toxicity. Furthermore, benzene exposure of CYP2E1 knockout mice results in a 90% reduction in urinary benzene metabolites and a complete lack of benzene-induced myelotoxicity and cytotoxicity. Moreover, occupationally exposed workers with a phenotype corresponding to rapid CYP2E1 metabolism were found to be more susceptible to benzene toxicity (437).

Benzene is initially metabolized in the liver by CYP2E1 and to a lesser extent by CYP2B1 (437). It initially undergoes epoxidation resulting in benzene oxide, which then gives rise to all subsequent metabolites, including phenol as well as other polyphenolic metabolites such as 1,2,4-benzenetriol, catechol, and hydroquinone and their quinone oxidation products, 1,2 (o)- and 1,4 (p)-benzoquinone. Benzene oxide can also undergo a ring-opening process resulting in the metabolite *trans, trans*-muconaldehyde (268, 493) (Figure 1).



**FIGURE 1.** Benzene Metabolism (436, 496, 590).

The phenolic metabolites can be conjugated with either sulfate or glucuronic acid (268).

Schrenk *et al.* showed that incubation of benzene with isolated rat hepatocytes led to the

accumulation of up to eleven metabolites, including sulfate, glucuronide, and glutathione conjugation products of phenolic metabolites (456). After induction of CYP2E1, overall benzene metabolism was increased and eight of these metabolites accounted for 90% of the metabolism. Similar studies using mouse hepatocytes found that mice produced more polyhydroxylated metabolites, and high levels of hydroquinone sulfate, and 1,2,4-benzenetriol sulfate (437). In addition, the array of benzene metabolites in the urine of benzene-treated mice is more complex than found in the urine of benzene-treated rats (495). This supports the widely held conclusion that mice are more susceptible to benzene toxicity than rats (450).

Although each of these primary metabolites has toxic properties in their own right, a number of secondary metabolites undoubtedly contribute to the toxic properties of benzene. Since the CYP2E1 system is concentrated in the centrilobular region of the liver, benzene bypasses much of the hepatic conjugating system and is hydroxylated close to the point of exit from the liver. As a result, unconjugated metabolites are frequently found in the exiting perfusate, and therefore, could potentially contribute to toxicity in other tissues (495). Various tissues may contribute to secondary metabolism of benzene metabolites, however, it depends on the particular enzyme content of that tissue. For example, in the bone marrow benzene metabolites can be oxidized by peroxidases, such as myeloperoxidase or prostaglandin H synthase, forming reactive quinones including 1,4-benzoquinone and 1,2-benzoquinone (134, 161, 268). As oxidants, these quinones redox cycle with their semiquinones, producing an elevated level of reactive oxygen species (ROS) resulting in oxidative stress (69). These ROS are postulated to mediate key steps in the mechanism of benzene toxicity. Quinones can be

detoxified by NADPH-quinone oxidoreductase 1 (NQO1), which is a quinone reductase that maintains quinones in their reduced forms making them more readily conjugated and excreted (437). However, high levels of peroxidases and/or a lack of NQO1 can allow for increased formation of toxic semiquinones and quinones without the possibility of their being reduced (69). Interestingly, bone marrow contains high levels of myeloperoxidase as do other solid tumor target organs identified in rodents including the Zymbal's, Harderian, preputial, and mammary glands and the nasal and oral cavity (437).

An additional property of benzene metabolites is that they tend to become more toxic in combination (437, 495, 496). It has been shown that toxicity can be exacerbated when certain metabolites interact. Combinations of phenol and hydroquinone lead to increased loss of bone marrow cellularity in mice, increased peroxidative activation of hydroquinone, and increased DNA damage *in vitro* and *in vivo*. Catechol has also been found to stimulate the peroxidase-mediated activation of hydroquinone and produced a synergistic genotoxic effect in lymphocytes. Combinations of muconaldehyde and hydroquinone were shown to result in the greatest potency of all benzene metabolite combinations tested in a mouse model of erythropoietic suppression (437, 495, 496).

### **5.1.2 Mechanisms of Genotoxicity - Benzene**

Based on studies of the biochemical effects of benzene and its metabolites, four specific genotoxic mechanisms have been most frequently cited in the literature (268). These mechanisms include: 1) DNA-reactive benzene metabolites forming adducts or cross-links;

2) oxidative DNA damage; 3) damage to components of the mitotic apparatus; and 4) topoisomerase II inhibition (572). Benzene metabolites can also interfere with cellular functions in other ways such as inhibiting DNA polymerase, interfering the activation of cytokines, and inhibition of tubulin function (268, 495). There is also evidence that certain metabolites (hydroquinone and the benzoquinones) can inhibit caspase-3 activation resulting in suppression of apoptosis (224).

#### **5.1.2.1 DNA Adducts - Benzene**

Administration of benzene *in vivo* has resulted in studies showing diverse results. Some reports have shown large numbers of DNA adducts, whereas others have shown few to none (495). Several studies have been successful in identifying DNA adducts derived from most of the major benzene metabolites including benzene oxide, catechol, 1,2,4-benzenetriol, hydroquinone, 1,4-benzoquinone, 1,2-benzoquinone, and muconaldehyde (69, 134, 161, 164, 437, 495). Muconaldehyde has been shown to form cyclic adducts with the DNA bases adenine and guanine (164). 1,4-benzoquinone reacts with DNA to form benzetheno adducts with deoxycytidine, deoxyadenosine, and deoxyguanosine (80). Hydroquinone forms benzetheno adducts primarily with deoxyguanosine (80). Gaskell *et al.* demonstrated the formation of four major DNA adducts *in vitro* following the reaction of DNA or individual nucleotides with the benzene metabolite 1,4-benzoquinone (160). These adducts were identified as (3''-hydroxy)-3,N(4)-benzetheno-2'-deoxycytidine 3'-monophosphate, (3''-hydroxy)1,N(6)-benzetheno-2'-deoxyadenosine 3'-monophosphate, (3''-hydroxy)-1,N(2)-benzetheno-2'-deoxyguanosine 3'-monophosphate, and (3'',4''-dihydroxy)-1,N(2)-

benzetheno-2'-deoxyguanosine 3'-monophosphate. The 2'-deoxycytidine 3'-monophosphate adduct was the main adduct formed from the direct reaction of DNA with 1,4-benzoquinone, whereas the two 2'-deoxyguanosine 3'-monophosphate adducts were minor products of the reaction. Reaction of DNA with the benzene metabolite hydroquinone induced only one major DNA adduct, identified as (3''-hydroxy)-1,N(2)-benzetheno-2'-deoxyguanosine 3'-monophosphate (160, 161).

Quinones obtained by enzymatic oxidation of catechol, such as 1,2-benzoquinone, have been shown to form predominantly depurinating adducts at the N7 of guanine and the N3 of adenine. These adducts are considered analogous to those formed with DNA by enzymatically oxidized 4-catechol estrogens (69).

#### ***5.1.2.2 Reactive Oxygen Species - Benzene***

Benzene metabolites have been shown to induce oxidative DNA damage, lipid peroxidation, and increases in the levels of antioxidant enzymes, all findings that support a role for ROS in benzene toxicity (224, 572). Several benzene metabolites, including 1,4-benzoquinone, 1,2-benzoquinone, phenol, hydroquinone, and 1,2,4-benzenetriol, have been shown to generate ROS (134, 437). In fact, phenol and hydroquinone can act synergistically to potentiate the formation of 1,4-benzoquinone and subsequently ROS (134). Some benzene metabolites can undergo redox cycling through oxidation of the reduced form of a metabolite thus creating ROS such as hydroxyl radicals. One of the major products formed by attack of hydroxyl

radicals on DNA is 8-hydroxydeoxyguanosine (8OHdG) and this has been detected in increased amounts in animals exposed to benzene (164).

#### **5.1.2.3 Mitotic Apparatus Dysfunction – Benzene**

Another mechanism that has been suggested as a means for benzene-induced genotoxicity is disruption of the mitotic apparatus. Quinones, semiquinones, and ROS can all promote inhibition of microtubule assembly, which could conceivably lead to inhibition of DNA replication (164, 268, 437, 572). Benzene metabolites can also inhibit topoisomerase II, an enzyme involved in DNA replication (78). Many of these effects could lead to DNA strand breaks and chromosomal damage.

#### **5.1.2.4 Chromosomal Damage – Benzene**

A combination of events including damage to tubulin, histone proteins, and other DNA associated proteins may be responsible for causing DNA strand breaks, mitotic recombination, chromosome translocations, and aneuploidy (164). Benzene has been found to induce chromosomal aberrations in a number of species *in vivo*, as well as sister chromatid exchanges, and micronuclei in polychromatic erythrocytes (268, 572). Hydroquinone induces chromosomal damage in lymphocytes *in vitro* similar to that observed in benzene-exposed workers (437). 1,4-benzoquinone can also induce sister chromatid exchanges and micronuclei in human lymphocytes, and has the ability to induce DNA single strand breaks (161).

#### **5.1.2.5 P53 Dysfunction - Benzene**

Previous studies have suggested that p53 dysfunction is involved in benzene-induced toxicity (48, 203, 592). Yoon *et al.* showed that the cell cycle is still functional in benzene exposed p53 knockout mice, whereas wildtype mice undergo cell cycle arrest due to expression of checkpoint genes such as p53 (592). This suggests that inactivation of p53 during benzene exposure may lead to an increased mutation frequency and therefore could be a potential factor leading to benzene-induced carcinogenesis. Further support for this comes from studies with p53 heterozygous mice showing that inhaled benzene induces a high frequency of thymic lymphomas (111). An additional study demonstrated that nearly 90% of the benzene-induced thymic lymphomas in C57BL/6-p53 haploinsufficient mice exhibited loss of the functional p53 locus (47).

### **5.1.3 Carcinogenicity – Benzene**

#### **5.1.3.1 Human - Benzene**

The leukemogenic activity of benzene was suggested over 100 years ago (437). Since then numerous epidemiological studies of benzene exposure have been published. Some studies found that the risk of leukemia increased with increasing benzene exposure and that the risk of death from leukemia was very high in the groups with the highest exposure. A review of 18 community-based and 16 industry-based studies of benzene exposure suggested that benzene exposure was associated with an increased incidence of leukemia in general, rather than specifically with acute myelogenous leukemia, as is often cited (435). One study showed that 82% of the leukemia cases were acute non-lymphocytic leukemia, which includes acute

myelogenous leukemia (62% of the total). Chronic lymphocytic leukemia was also observed but at a much lower incidence (2%). Most studies find that benzene exposure increases the risk of total lymphatic and hematopoietic cancers including leukemias, however, little evidence is found for an association between benzene exposure and multiple myeloma or non-Hodgkin's lymphoma (435). A number of non-neoplastic effects are also attributed to chronic benzene exposure including leukopenia, thrombocytopenia, pancytopenia, and anemia. In addition, myelodysplasia is associated with chronic benzene exposure and is a precursor to leukemia (161).

#### **5.1.3.2 Rodent - Benzene**

The evidence in humans is supported by studies in experimental animals demonstrating that benzene is a multi-site carcinogen in rodents. Benzene was tested for carcinogenicity in mice and rats exposed by several routes, including oral administration, inhalation, injection, and dermal application. When administered orally, benzene caused Zymbal's-gland carcinomas and oral-cavity tumors in rats of both sexes; skin carcinomas in male rats; Zymbal's gland carcinomas, malignant lymphomas, and lung tumors in mice of both sexes; Harderian gland adenomas and preputial gland carcinomas in male mice; and ovarian tumors and mammary gland carcinomas in female mice (389). When administered by inhalation, benzene causes tumors at many sites in rats and a tendency towards lymphoid tumor induction in mice (435, 572). Male mice exposed to benzene by inhalation were significantly more susceptible to benzene-induced toxicities than females (135). Benzene administered by intraperitoneal injection caused benign lung tumors in male mice. No tumors were observed in mice

administered benzene by subcutaneous injection or dermal application. Benzene administered orally to heterozygous p53-deficient mice caused head and neck, thoracic cavity, and subcutaneous sarcomas (435, 572).

Several benzene metabolites, with the notable exception of phenol, are able to mimic the bone marrow depressant effects of benzene in animals, however, there are few animal models for benzene-induced leukemia (495). French and Saulnier *et al.* demonstrated that application of benzene to the skin of Tg.AC mice (v-Ha-ras transgenic) over a 6 month period resulted in both papillomas of the skin and granulocytic leukemia, but this was not reproduced when administered orally (152). Blood levels of benzene metabolites were identified in major organs including bone marrow. Mice treated dermally displayed higher levels of benzene metabolites in bone marrow than mice treated orally (495).

## **5.2      *ETHYLENE OXIDE***

Ethylene oxide was first listed in the Fourth Annual Report on Carcinogens in 1985 as *reasonably anticipated to be a human carcinogen* based on evidence of carcinogenicity in humans, including a combination of epidemiological and mechanistic investigations, and sufficient evidence in experimental animals. The listing was revised to *known to be a human carcinogen* in the Ninth Report on Carcinogens in 2000 (435). The International Agency for Research on Cancer (IARC) has determined that EtO is a definite (Group 1) human carcinogen, based on evidence from epidemiologic studies showing increased hematopoietic cancer in EtO exposed people. This was further supported by positive human cytogenetic evidence and sufficient evidence from animal carcinogenesis studies (435, 501).

### **5.2.1    *Use – Ethylene Oxide***

The major use of ethylene oxide is as an intermediate in the production of industrial chemicals (223). The remainder is used in the gaseous form as a sterilizing agent, disinfectant, fumigant, or insecticide. About 60% of the ethylene oxide is used to produce ethylene glycol (antifreeze). Other chemicals that are produced from ethylene oxide include non-ionic surfactants (used in industrial applications, detergents, and dishwashing formulations), glycol ethers, ethanolamines (used in soaps, detergents, and textile chemicals), diethylene glycol, triethylene glycol, polyethylene glycol, polyester fibers, and urethane polyols. In hospitals, ethylene oxide is used as a gaseous sterilant for heat-sensitive medical items and surgical instruments (103, 286, 435).

### ***5.2.2 Exposure and Metabolism – Ethylene Oxide***

The primary routes of human exposure to EtO are inhalation and ingestion, which may occur through occupational, consumer, or environmental exposures (223). The general population may be exposed to EtO through use of products that have been sterilized with the compound or people who live near industrial facilities may be exposed from uncontrolled industrial emissions. EtO is also found in tobacco smoke and automobile exhaust (435, 553). In addition, EtO is produced metabolically in the body from ethylene. Endogenous sources of ethylene include metabolic processes in intestinal microorganisms, lipid peroxidation, and oxidation of methionine (263).

Inhaled EtO is quickly absorbed in the lungs and distributed rapidly throughout all tissues; it forms dose-related hemoglobin adducts in people and rodents, and dose-related DNA adducts in rodents (103, 501). Although EtO is a direct acting mutagen, and therefore does not require metabolic activation to exert its toxic effects, there are two pathways described in its biotransformation. These include hydrolysis to ethylene glycol and conjugation with glutathione. EtO's metabolism to ethylene glycol is subsequently followed by metabolism to glycolic acid, a process involving the action of epoxide hydrolases and alcohol dehydrogenases (336). Glycolaldehyde is a putative intermediate in this reaction, although little is known about its toxicity (331). *In vivo*, glycolaldehyde is rapidly metabolized to glycolic acid, which is the primary cause for metabolic acidosis in ethylene glycol poisoning (196). Conjugation with glutathione is a major pathway of metabolism, resulting in the renal

excretion of a number of metabolites (190). The *in vivo* elimination half-life of EtO varies among different species (190). In humans, the half-life is estimated to be approximately 40-55 minutes, whereas in the mouse the half-life is estimated to be 6-8 minutes (190).

EtO is a substrate of the polymorphic enzyme glutathione S-transferase theta 1 (GSTT1). Support for the role of metabolism in EtO toxicity comes from epidemiological studies that group individuals into one of two categories; 1) a "highly sensitive group"; and 2) a "less sensitive group" (524). These differences are related to polymorphism in the glutathione transferase GSTT1 gene. Hallier *et al.* demonstrated that peripheral lymphocytes of people lacking the glutathione transferase GSTT1 gene (homozygote hGSTT\*1 negative or "non-conjugators") were much more susceptible to the sister chromatid exchange-inducing effect of EtO than were lymphocytes of hGSTT\*1 positive individuals ("conjugators") (182). Conjugators can be further subdivided into two groups, heterozygous "slow conjugators" and homozygous "high conjugators" (524).

### **5.2.3     *Mechanisms of Genotoxicity – Ethylene Oxide***

EtO is mutagenic and genotoxic in a number of biological systems, producing point mutations, sister chromatid exchanges, micronuclei, and chromosomal aberrations in both experimental animals and man (259, 553). In fact, EtO has been shown to be a powerful mutagen and clastogen at all phylogenetic levels. EtO can react directly with nucleophilic centers of cellular macromolecules, such as DNA, RNA, or proteins (259, 553). Significant dose-related increases in the frequency of chromosomal aberrations and sister chromatid

exchanges in lymphocytes, micronuclei in erythrocytes, DNA single-strand breaks in peripheral mononuclear blood cells, and *hprt* mutations in peripheral lymphocytes have been observed in workers occupationally exposed to ethylene oxide (223). Similar genotoxic effects have been observed in rodents exposed to ethylene oxide (223, 263). Furthermore, EtO causes gene mutations and heritable translocations in germ cells of exposed rodents.

#### **5.2.3.1 DNA Adducts – Ethylene Oxide**

Ethylene oxide is a monofunctional, direct acting, S<sub>N</sub>2 alkylating agent (103). The reactivity of this agent is attributable to the strain of the oxirane ring and the residual positive charge on the carbon atoms. The charge on the carbon atoms gives EtO an electrophilic character. EtO does not alkylate at random but alkylates preferentially on more nucleophilic sites (103). The ability of EtO to hydroxyethylate DNA has been shown in studies demonstrating DNA adducts in several tissues of male mice and male rats (263). In these studies, the N7-(2-hydroxyethyl)-guanine adduct is the most abundant representing about 90% of the alkylated DNA sites and reaching similar levels in DNA across all tissues examined (553). Additional studies in which calf thymus DNA was exposed to EtO also resulted in numerous DNA adducts (553). The N7-(2-hydroxyethyl)-guanine adduct was again the most abundant DNA adduct followed by O6-(2-hydroxyethyl)-guanine and N3-(2-hydroxyethyl)-adenine (463). The *in vitro* ratios for N7-(2-hydroxyethyl)-guanine, N3-(2-hydroxyethyl)-adenine, and O6-(2-hydroxyethyl)-guanine were 200:23:1. Two other DNA adducts, suspected to be N7-(2-hydroxyethyl)-adenine and N1-(2-hydroxyethyl)-adenine, were also observed. Metabolism studies have suggested that, in addition to direct reaction with DNA, EtO may produce DNA

adducts through a minor pathway leading to chloroacetaldehyde by way of 2-chloroethanol (263).

An additional consideration when evaluating EtO adducts is the contribution of endogenous sources of EtO. The N7-(2-hydroxyethyl)-guanine DNA adduct has been shown to occur endogenously as part of the background spectrum of DNA lesions present in cells (427). Accordingly, depurination of N7-(2-hydroxyethyl)-guanine adducts derived from endogenously produced EtO may be a source of both spontaneous DNA damage and mutation. Although the significance of this EtO-induced background damage is difficult to evaluate, it is important to consider these background lesions when interpreting results of adducts formed via exogenous exposure to EtO (553).

### ***5.2.3.2 Mutations/Chromosomal Damage – Ethylene Oxide***

The N7-(2-hydroxyethyl)-guanine adduct is not considered promutagenic in itself, as it does not cause base pair changes and has no mispairing properties during DNA replication. In contrast, the O6-(2-hydroxyethyl)-guanine adduct is promutagenic and is therefore considered more relevant, though less abundant than, N7-(2-hydroxyethyl)-guanine adducts (543). However, many EtO adducts, including N7-(2-hydroxyethyl)-guanine, are unstable and thought to form abasic sites by spontaneous or enzymatic depurination thus promoting base substitutions, frameshift mutations, or large deletions (427). Data suggests that depurination of N7-(2-hydroxyethyl)-guanine results largely in G -> T transversions, G -> A transitions and, to a lesser extent, G -> C transversions (299), whereas O6-(2-hydroxyethyl)-

guanine primarily causes G -> A transitions (555). In contrast, other studies in mice have shown most EtO-induced mutations to occur at AT base pairs (427).

#### **5.2.3.3 DNA Repair – Ethylene Oxide**

EtO has been shown to induce unscheduled DNA synthesis in human leukocytes, which is suggested to be the manifestation of repair of DNA lesions such as N7-(2-hydroxyethyl)-guanine (103). Additional studies have suggested that EtO may induce a concentration-dependent inhibition of DNA repair capacity in spermatids (103).

#### **5.2.3.4 Cell Cycle and Apoptosis – Ethylene Oxide**

EtO has been shown to have a distinct effect on cell cycle G1/S progression as studied in human fibroblasts (263). It was found that EtO induced G1 arrest within 6-18 hours after exposure. In addition, the mode of cell death in response to EtO treatment was necrosis, rather than apoptosis, as indicated by the lack of chromatin condensation and formation of apoptotic bodies (263).

#### **5.2.3.5 P53 and Ras – Ethylene Oxide**

One study looked at the effects of EtO exposure on p53 and ras (133). Ember *et al.* detected higher expression of the N-ras and p53 genes in lymphocytes of EtO-exposed hospital nurses as compared to their non-exposed counterparts. However, after 1 year post-exposure the p53 and N-ras expression levels had returned to normal (133).

## 5.2.4 Carcinogenicity – Ethylene Oxide

### 5.2.4.1 Human - Ethylene Oxide

The carcinogenicity of EtO was first reported in three Swedish cohort studies, which showed a significant increase in overall cancer mortality as well as an excess of stomach cancer and certain types of leukemia (especially chronic lymphatic leukemia and acute myeloid leukemia) (263). Subsequent studies have yielded mixed results. Several epidemiological studies have reported an association between EtO exposure and increased cancer, although other studies find no significant excesses in cancer risk (223). The most frequently reported association in exposed workers has been for lymphatic and hematopoietic cancer (223). A meta-analysis of 10 distinct cohort studies of EtO exposed workers found no association between exposure and increased risk of pancreatic or brain cancers; however, there was a suggestive risk for non-Hodgkin's lymphoma and stomach cancer (223). The largest study of U.S. workers exposed to EtO found an increase in mortality in males from hematopoietic neoplasms (500). Another study by Teta *et al.* showed that leukemia risk was increased in workers exposed to EtO for more than 10 years (435). In addition, of particular significance to the study in **manuscript one**, there have been a couple of studies reporting an increased incidence of breast cancer in EtO-exposed women (388, 501).

#### **5.2.4.2 Rodent - Ethylene Oxide**

The evidence that EtO is a human carcinogen is supported by studies in rodents that demonstrate carcinogenicity at multiple sites. In 2-year inhalation bioassays, EtO induced exposure-related increases in gliomas, peritoneal mesotheliomas, and mononuclear leukemias (MCL) in F344 rats and lymphomas and adenomas/carcinomas of the lung, Harderian gland, and mammary gland of B6C3F1 mice (223, 259, 390, 435, 487).

Qualitatively, there appears to be some degree of consistency between the types of EtO-induced tumors observed in mice (lymphomas, mammary carcinomas), rats (MCL), and those suspected in exposed people (leukemia and breast cancer); however, it should be noted that, although the target tissues identified may appear consistent, interpretation should be made with caution, as a direct comparison cannot always be made. For example, there are important differences between MCL and human leukemia with respect to the cell of origin and the background rates of formation. Because of these differences, MCL has been categorized by IARC as an unclassified leukemia with no known human counterpart (259).

### **5.3 THE ENVIRONMENT AND MAMMARY CARCINOGENESIS**

Mouse mammary carcinogenesis is strongly influenced by a variety of environmental agents as well as internal or physiological factors, such as steroid hormones. The environmental factors include traditional chemical carcinogens, such as polycyclic aromatic hydrocarbons; alkylating agents, such as nitrosureas and urethane; nitrosamines, fluorenylacetamides, and naphthylamines (342). As noted earlier, radiation is also an important carcinogen for the mouse mammary gland (539).

#### **5.3.1 Radiation - Mammary Carcinogenesis**

Ionizing radiation is an extremely potent agent for inducing genomic instability (365). It is also a complete carcinogen, able to both initiate and promote neoplastic progression and is a known carcinogen of human and rodent mammary gland (344). Tissue response to radiation is a composite of genetic damage, cell death, and induction of new gene expression patterns (28). With regard to radiation-induced mammary cancer, Ullrich *et al.* reported that the neoplastic changes arising from irradiated mouse mammary tissue most likely result from the induction of genomic instability (418). The rat has been widely used as a model for radiation-induced mammary carcinogenesis since the demonstration in the early 1950's that a single high dose of X-rays was able to induce an increased number of benign and malignant mammary tumors within 6 months of exposure (445). Several different types of radiation, including gamma rays, X-rays, and neutrons, have been shown to induce mammary tumor development (445). Although most studies have utilized whole-body irradiation, localized irradiation also induces mammary tumors in the rat. In general, studies utilizing fractionated

irradiation protocols have shown no decrease or increase in tumor latency or incidence when compared to animals exposed to a single dose of radiation (445). Sprague-Dawley and Lewis rats are the most susceptible to radiation-induced tumorigenesis. AxC, Fisher, Long-Evans, and Wistar/Furth are also susceptible but to a lesser degree. Likewise, mice have also shown strain dependent variations in radiation sensitivity. Studies have shown that Balb/c mice, in comparison to other mouse strains, are inefficient at repairing DNA double strand breaks. Epithelial cells from Balb/c mice demonstrate increased instability after radiation exposure as compared to other mouse strains such as C57BL/6 mice (393). This results in Balb/c mice being particularly susceptible to radiation induced mammary tumorigenesis (271, 418, 539).

#### **5.3.1.1 P53 and Radiation - Mammary Carcinogenesis**

P53 deficient mice are extremely susceptible to radiation-induced tumorigenesis (250). *In vitro* studies have shown that p53 mutations are associated with increased radioresistance (54, 339, 594). *In vivo*, the p53 status has proven to be an essential factor in determining the radiosensitivity of a variety of different tissues (53, 59, 200, 264, 305, 313, 350). Kemp *et al.* reported that a single dose of 4 Gy radiation dramatically decreased the latency for tumor development in p53 heterozygous mice (250). Ionizing radiation has also been shown to enhance tumorigenesis in mouse mammary outgrowth lines (344). Moreover, Minter *et al.* showed that susceptibility to radiation can vary depending on the proliferative state of the mammary tissue at the time of exposure (360). P53 is known to be an important player in radiation-induced apoptosis in many tissues and the mammary gland is no exception. Normal mammary glands and preneoplastic outgrowths with normal p53 exhibit radiation-induced apoptosis, however, preneoplastic outgrowths with deregulated p53 fail to demonstrate

radiation-induced apoptosis (344). Studies using knockout mice have shown that thymocytes lacking p53 do not undergo radiation-induced apoptosis but do undergo apoptosis in response to glucocorticoids. Similarly, radiation-induced apoptosis is blocked in the gastrointestinal tract of p53-null mice and in mammary preneoplasias with no or mutant p53 (344).

### **5.3.1.2 *Brca2 and Radiation - Mammary Carcinogenesis***

Both human and mouse Brca2 null cells are hypersensitive to irradiation (470, 605). In addition, Morimatsu *et al.* showed that cells lacking the Brca2 carboxy-terminus (like the mice used in **manuscript one**) exhibit hypersensitivity to gamma-radiation (366).

Furthermore, Tutt *et al.* showed that irradiation (4 Gy) had a disproportionate effect on animals homozygous for Brca2 disruption, inducing 3.4-fold more mutations as compared to wildtype animals (536).

### **5.3.2 *Chemically Induced - Mammary Carcinogenesis***

Experimental animal models have proven useful for answering specific questions on the biology of mammary cancer and for assessing the risk for breast cancer posed by toxic chemicals. The human population is exposed to a large number of environmental chemicals, such as polycyclic aromatic hydrocarbons, nitrosureas, and aromatic amines that have been demonstrated to be carcinogenic in experimental animal models and capable of inducing mutagenesis and neoplastic transformation of human breast epithelial cells *in vitro* (445). Several carcinogens that induce mammary tumors in rodents have been identified and extensively studied for more than 50 years in mice and more than 30 in rats (445).

To some extent, the susceptibility to particular carcinogens can be dependent on the particular strain used. Both the mouse (B6C3F1) and rat (Fischer 344) used by the National Toxicology Program (NTP) are relatively resistant to mammary gland tumorigenesis. Virgin mice of strains DBA/2 and Balb/C are typically more susceptible than C3H, C57BL, and FVB to chemical carcinogen-induced mammary tumorigenesis (342) and Sprague-Dawley rats are generally more susceptible than Fischer rats (32). Nonetheless, the NTP rodent bioassays have identified 42 chemicals that induce tumors in the mammary gland. The physical and chemical characteristics of the carcinogens vary, but epoxides (including chemicals metabolized to epoxides, such as benzene) and nitro-containing compounds are well represented (32). Mammary carcinomas have been induced in mice with 3,4-benzopyrene, 3-methylcholanthrene (MCA), 1,2,5,6-dibenzanthracene, 7,12-dimethylbenz (a)anthracene (DMBA), urethane, benzene, and ethylene oxide (435, 445). These mammary tumors often develop after a relatively long latency and their induction sometimes requires multiple applications. Enhanced tumorigenicity can be obtained with prolonged hormonal stimulation. The most frequently utilized rat mammary carcinogens are DMBA and N-methyl-N-nitrosourea (MNU), although MCA, 2-acetylaminofluorene, 3,4-benzopyrene, ethylnitrosourea, PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine), and butylnitrosourea have also been used (32, 445). The majority of rat mammary carcinomas induced by either DMBA or MNU are hormone-dependent. The susceptibility of the mammary gland to DMBA- or NMU-induced carcinogenesis is strongly age-dependent. Maximal susceptibility occurs when the carcinogens are administered to virgin females between the ages of 30 and 60 days, a time correlating with early sexual maturity and a high

density of proliferating TEB (445). Thus, the more differentiated the structure at the time of carcinogen exposure the more benign and organized is the lesion that develops (445).

Bennett *et al.* reported that 21 of the 42 known rodent mammary carcinogens are also human carcinogens including benzene, ethylene oxide, 1,3-butadiene, isoprene, chloroprene, C.I. basic red 9, and C.I. acid red 114 (32). In humans, most of these chemical exposures are associated with tumors in tissues other than mammary, however, there are associations with breast cancer for some of these chemicals (ethylene oxide) (501).

#### ***5.3.2.1 Ras - Chemically Induced Mammary Carcinogenesis***

Ras mutations are strongly associated with the carcinogen-induced mammary carcinomas in rodents (600). H-ras oncogene activation appears to be one of the earliest detectable genetic events in mammary carcinogenesis induced by nitrosomethylurea (NMU) in rats (600) and in DMBA-induced mammary carcinomas in mice (270).

## **5.4 RAS**

Ras research began in 1964 when Jennifer Harvey observed that a murine leukemia virus, taken from a leukemic rat, induced sarcomas in newborn rodents (319). Three additional retroviruses, later shown to carry ras oncogenes, were subsequently identified. Kirsten-MSV was obtained in 1967 by serial passage of murine leukemia viruses through Wistar-Furth rats. BALB-MSV was isolated in 1974 from a leukemic Balb/c mouse, and the Rasheed strain of rat sarcoma virus was generated in 1978 from cultured rat cells (319).

The ras superfamily of monomeric G proteins consists of more than 100 members (398). The highly homologous K-, H-, and N-ras proteins are ubiquitously expressed in mammalian cells, eliciting many similar, but not always identical, effects in normal and transformed cells (181). These proteins are small (21 kDa) membrane-localized GTPases that bind and hydrolyze guanosine triphosphate (GTP), cycling between a GTP bound active state and an inactive guanosine diphosphate (GDP) bound state. The general function of ras proteins in cells is to act as signal transduction molecules relaying external signals through tyrosine kinases and G-coupled receptors to both the cytoplasm and the nucleus (398). They regulate multiple signaling pathways involving a number of cellular processes including, cell proliferation, apoptosis, differentiation, senescence, cell adhesion, and migration (183).

Through interaction with its downstream effectors, such as the Raf family members or protein kinase B (PKB)/Akt, ras activates the Mek/mitogen-activated protein kinases. The mitogen-activated protein kinase (MAPK) cascade consists of a series of serine/threonine

kinases sequentially activated downstream of ras. Activation of the extracellular signal-regulated kinases 1 and 2 (Erk1 and Erk2) is required for mitogenesis. Accumulating evidence indicates that the extent and intensity of MAPK activation, as well as the cellular context, influence the biological outcome (323). MAPK activation subsequently regulates the activities of transcriptional factors, such as Elk-1 and AP-1 (312). MAPK signaling has also been linked to the cell cycle machinery via the regulation of cyclins (especially cyclin D) and tumor suppressors (Rb and p53) (312).

#### **5.4.1 Regulation – Ras**

All ras superfamily members share a common mechanism called the GTP/GDP cycle. Because these proteins have intrinsic GTP hydrolytic (guanosine triphosphatase) activity and a higher affinity for GTP than GDP, they cycle between GTP bound and GDP bound forms (398). However, the intrinsic GTPase activity of ras proteins is rather weak and not sufficiently effective for signal transduction pathways. In order to accelerate this low rate of hydrolysis and to enable a transient burst of signaling activity, regulatory proteins like GAPs (GTPase activating proteins) or NF1 (Neurofibromatosis Type 1), bind to the GTP-bound ras conformation and stimulate the GTPase activity more than 100-fold (337, 452). In a normal cell, GAPs help to keep most of the ras proteins in an inactive GDP-bound state. However, overexpression of ras protein can lead to saturation of these regulatory proteins, resulting in a constitutive, deregulated activation of ras proteins and oncogenic transformation. Another group of regulatory proteins involved in stimulating the transition of ras proteins from the inactive to the active GTP-bound state are designated Guanine Nucleotide Exchange Factors

(GEFs) or ras-GRFs (guanine nucleotide releasing factors) (337, 452). Normally, the release of GDP is regulated by the intracellular concentration of GTP. An increase in the GTP concentration leads to an enhanced dissociation of GDP. GEFs catalyze the dissociation of GDP (337, 452).

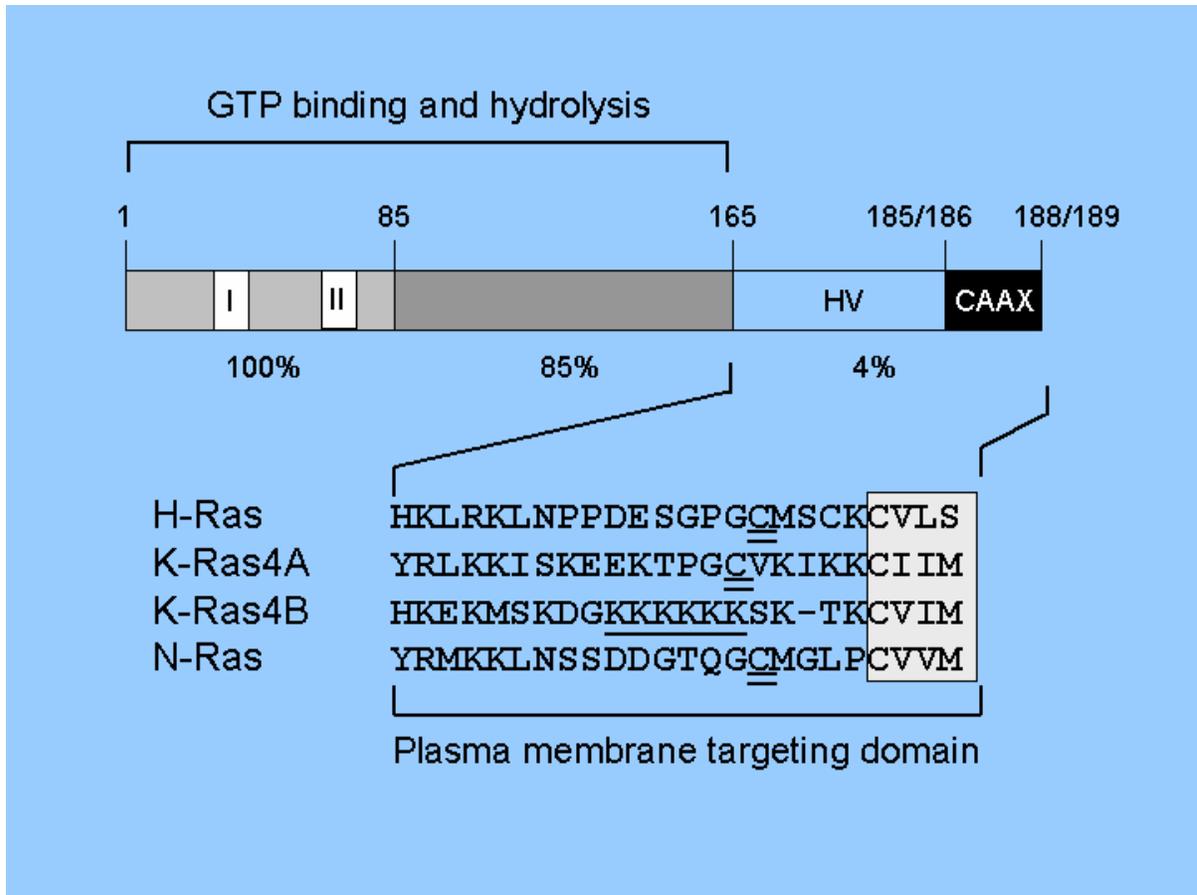
#### **5.4.2 Gene/Protein Structure – Ras**

**Alternative names (H-ras):** p21/H-ras-1, c-H-ras, HRAS1, RASH1, v-Ha-ras, Harvey rat sarcoma viral oncogene

In the mouse, H-, K-, and N-ras are located on chromosomes 7, 6, and 3, respectively. In humans, H-, K-, and N-ras are located on chromosomes 11, 12, and 1, respectively (394). Ras genes can differ in length due to differences in the size of their introns ranging from about 6 kb to 50 kb, but they each have four coding exons. Ras genes are expressed in all tissues and have a promoter region with multiple GC boxes, but lack a TATA- or CCAT-box (306). A comparison of human H-, K-, and N-ras nucleotide sequences with the corresponding regions in other mammalian ras genes reveals a remarkable sequence similarity (565).

Mammalian ras genes code for closely related, small proteins of 189 amino acids with a molecular weight of 21,000 Daltons (p21) (256). Alignment of ras amino acid sequences clearly indicates the presence of four domains within the ras molecules (Figure 2). The first domain includes 85 amino acids at the amino-terminus, which are found to be identical in H-, K-, and N-ras. This region constitutes the GTP/GDP binding domain. The following 80

amino acids form a second domain, showing less conservation (70-85%) within the ras proteins. The third domain spans the rest of the molecule and represents a hypervariable region. Only 10-15% amino acid conservation occurs in the hypervariable region (4% identity). The hypervariable region has been associated with different biological activities that may, in part, be related to role this region has in differentially modulating the intracellular trafficking of H- and K-ras. Starting from the carboxyl end, the hypervariable region can be divided into three regions. These include the CAAX box (C = cysteine, A = aliphatic amino acid, and X = methionine or serine), the secondary lipid association domain, and the linker region. Immediately following synthesis of ras proteins in the cytoplasm, the farnesyltransferase enzyme catalyzes the covalent addition of carbon farnesyl isoprenoid lipid to the CAAX motif. Additionally, in H-ras, cysteines 181 and 184 are palmitoylated, whereas in K-ras, there is a polylysine track (amino acids 176-181). These post-translational modifications as well as specific sequence determinants in the hypervariable region specify ras protein association with the inner surface of the plasma membrane (237, 256, 420, 421, 564).



**FIGURE 2.** Ras Protein Structure (474)

### 5.4.3 *Function – Ras*

#### 5.4.3.1 *Cell Cycle Regulation – Ras*

Many studies have demonstrated that ras is associated with a number of different, and sometimes opposing, cellular processes. However, whether the outcome of ras signaling is cell proliferation and differentiation, cell cycle arrest, cellular senescence, or apoptosis

ultimately depends on the cellular context, the presence or absence of other regulatory influences, and the persistence of the initial signal.

By activating the MAPK signaling cascade, ras plays a significant role in cell proliferation (5). Upon activation by Raf and Mek, Erk translocates to the nucleus where it phosphorylates transcription factors resulting in expression of genes involved in cell cycle progression (e.g. cyclin D1) (21, 180). In addition, Sears *et al.* showed that ras enhances the accumulation of myc by stabilizing the myc protein, which normally has a short half-life (292). Myc is a transcription factor that is also capable of promoting cell proliferation (462).

In contrast to promoting cell cycle progression, ras and its various effectors also have the capacity to elicit cell cycle arrest (341). Although initially described in cultured primary keratinocytes, the cell cycle inhibitory effects of ras have been described in a variety of cells and in transgenic mice (341). The central mechanism underlying this observation, is the ability of ras to induce the expression of cdk inhibitors of the INK4 or Cip/Kip family and tumor suppressors such as p53 (341). This is thought to act as a protective mechanism against hyperproliferation. In addition, oncogenic ras is able to induce a premature senescence program involving tumor suppressors such as p53, p16<sup>INK4a</sup>, p19<sup>ARF</sup>, p15<sup>INK4b</sup>, and the promyelocytic leukemia gene product (PML) (292, 466). The INK4a/ARF locus is a critical sensor of hyperproliferative signals produced by ras and other oncogenes (292). As mentioned previously, this locus encodes the two structurally unrelated tumor suppressor proteins p16<sup>INK4a</sup> and p19<sup>ARF</sup>. P16<sup>INK4a</sup> acts as a negative regulator of the cell cycle by binding to and inhibiting cyclin-dependent kinases 4 and 6 thus inhibiting the

phosphorylation of the Rb protein and inducing cell cycle arrest at the G1/S transition. In contrast, p19<sup>ARF</sup> activates p53 by interfering with the p53 antagonist Mdm2, leading to cell cycle arrest or apoptosis depending on cellular context. Both p16<sup>INK4a</sup> and p19<sup>ARF</sup> can be induced by expression of mitogenic oncogenes (292). Oncogenic ras induces p16<sup>INK4a</sup> and p19<sup>ARF</sup> through the MAPK cascade, suggesting that the process is directly coupled to the pro-mitogenic activity of oncogenic ras (292).

#### 5.4.3.2 *Apoptosis – Ras*

The role ras plays in apoptosis appears to vary depending on cellular context. Ras can exert an anti-apoptotic effects by activating the phosphatidyl inositol 3-kinase (PI3K) pathway (5). Activation of PI3K by ras inhibits apoptosis by activating protein kinase B (PKB/Akt) (249). PKB then phosphorylates and inactivates the pro-apoptotic proteins Bad and caspase-9 (67). Ras can also induce an anti-apoptotic effect by upregulating expression of Bcl-2 and other anti-apoptotic members of the Bcl-2 family (258). In contrast, excessive ras activity can lead to increased apoptosis in a p53-dependent manner. Transiently increased ras activity has been shown to induce expression of the pro-apoptotic protein Bax. Ras-mediated apoptosis can also be promoted by the c-jun amino-terminal protein kinase (JNK) pathway (150). In the JNK pathway, ras activates MEKK, which activates different MAPKK proteins that ultimately phosphorylate JNK. Substantial evidence has implicated JNK as an essential component of the apoptotic cascade (530). JNK activation can result in upregulation of FasL expression, thus promoting apoptosis (382).

## **5.4.4 Carcinogenesis – Ras**

### **5.4.4.1 Mutation - Ras**

Significant experimental and epidemiological evidence supports that aberrant ras activation plays a critical role in rodent and human tumorigenesis (183). While native ras is an essential growth-regulating component of eukaryotic cells, single point mutations are sufficient to convert the cellular protein into a tumor promoting oncoprotein (183). When chronically active, due either to activating mutations or to activation by growth factor receptors, ras proteins trigger diverse enzymatic cascades, which can lead to uncontrolled cell growth and cell transformation (181). Point mutations of the ras genes are found in approximately 30% of human tumors and thus constitute the most prevalent oncogenes in human carcinogenesis (398). Activating mutations of ras are found in nearly all human pancreatic cancers, one-half of colon and thyroid tumors, and one-third of lung tumors (49). Activating point mutations have been localized to codons 12, 13, 59, 61, 63, 116, 117, 119, and 146 (27). All of these alterations occur at or near guanine nucleotide binding sites. The effects of point mutations are either reduced GTPase activity (if amino acids 12, 13, 59, 61, 63 are involved), so that oncogenic ras mutants are locked in the active GTP-bound state, or decreased nucleotide affinity (if amino acids 116, 117, 119, or 146 are affected), resulting in increased exchange of bound GDP for cytosolic GTP. Each of these point mutations causes an accumulation of activated ras-GTP complexes, leading to continuous signal transduction (27, 564).

Mutations leading to amino acid substitutions at the positions 12, 13, and 61 are the most common in naturally occurring human neoplasms and experimentally induced animal tumors

(36, 321, 499). The glycine residue located in position 12 (Gly12) lies close to the “finger loop” of the GAP proteins. Any mutation at this position results in the incorporation of a residue with a side chain (Gly is the only amino acid without a side chain), which sterically interferes with GAP proteins preventing them from hydrolyzing GTP (319). The glutamine position 61 (Gln 61) has a vital role in catalysis as it forms a hydrogen bond with a specific residue (Arg 789) of the GAP p120 protein allowing for the nucleophilic attack of a water molecule crucial for GTP hydrolysis. Mutations of this residue results in impaired GTPase activity (319).

#### **5.4.4.2 *Overexpression – Ras***

In addition to point-mutational activation, overexpression of non-mutated ras genes can also have an oncogenic effect. Increased mRNA expression can be derived either from high transcriptional activity of heterologous promoters and enhancers or from amplified ras genes (73, 386). Unlike that seen in rodents, ras overexpression is a much more common event than mutation in human breast cancers (398).

#### **5.4.4.3 *Organ Specificity – Ras***

Most human and rodent cancers with ras activation are associated exclusively with only one isoform of the closely related ras family members (9, 58, 148, 210). For example, in humans, K-ras activation is associated with pancreatic and colon cancers; H-ras activation is associated with bladder cancers; and N-ras activation is found in myeloid leukemia (256). In rodents, K-ras is associated with colon and lung cancers, whereas H-ras is associated with

mammary and skin cancers (256). The molecular basis of this organ-specific ras activation is not completely understood. It has been shown that activated H-ras is ~10-fold more potent than activated K-ras in rat mammary carcinogenesis and that this organ specificity resides in the Ras protein itself and not in differential regulation of ras gene expression (256). Kim *et al.* showed that the membrane localization elements within the hypervariable region of H-ras are responsible for this organ specificity (256).

## CHAPTER SIX

### MANUSCRIPT TWO

(Manuscript in Preparation for Submission to *Toxicologic Pathology*)

## FREQUENT P53 AND H-RAS MUTATIONS IN BENZENE- AND ETHYLENE OXIDE-INDUCED MAMMARY CARCINOMAS FROM B6C3F1 MICE

### 6.1 ABSTRACT

Benzene and ethylene oxide are potent multi-site carcinogens in rodents and are classified as known human carcinogens by the National Toxicology Program. In 2-year mouse studies, both chemicals caused an increased incidence of mammary carcinomas. Spontaneous, benzene-, and ethylene oxide-induced mouse mammary carcinomas were examined for p53 protein expression, using immunohistochemistry, and for p53 (exons 5-8) and H-ras (codon 61) mutations using cycle sequencing techniques. P53 protein expression was detected in 42% (8/19) of spontaneous, 42% (6/14) of benzene-, and 67% of ethylene oxide-induced mammary carcinomas. Semi-quantitative evaluation of p53 protein expression revealed that both benzene- and ethylene oxide-induced tumors exhibited expression levels five- to six-fold higher than spontaneous tumors. P53 mutations were detected in 50% (7/12) of spontaneous, 57% (8/14) of benzene-, and 67% (8/12) of ethylene oxide-induced tumors. H-ras mutations were identified in 26% (5/19) of spontaneous, 50% (7/14) of benzene-, and 33% (4/12) of ethylene oxide-induced tumors. For both p53 and H-ras, there were significant differences in the mutational spectra between chemically induced and spontaneous tumors. These results demonstrate that p53 and H-ras mutations are common in mouse mammary

carcinomas. These results also show that both chemicals can alter the mutational spectra in these genes, providing a potential mechanism underlying their role as mouse mammary carcinogens. Furthermore, the frequent occurrence of p53 and H-ras mutations in the same tumor type suggests these genes may act cooperatively in the process of mammary tumorigenesis.

## **6.2 INTRODUCTION**

Benzene is used as an intermediate for the manufacturing of polymers, detergents, pesticides, drugs and other industrial chemicals (389, 590). It is also found in cigarette smoke, natural gas and coal tar and is used as an additive in gasoline (443). Benzene is a well-documented environmental pollutant that induces hematotoxicity and hematopoietic neoplasia in humans and is a multi-site carcinogen in rodents including the development of mammary carcinomas in mice (215, 223, 413, 494, 592). Several studies indicate that metabolism of benzene to its reactive metabolites is a prerequisite for its cytotoxic and genotoxic properties (2, 178, 496, 541). Benzene metabolism is complex and its toxicity is likely related to a variety of different factors including growth factor regulation (383), oxidative stress (275), DNA damage (278), cell cycle regulation (591), and apoptosis (364). Benzene itself is not highly reactive with DNA although several of its metabolites are genotoxic and mutagenic (2, 68, 70, 291, 493). Benzene is initially oxidized to benzene oxide by CYP2E1 (and to a lesser extent by CYP2B1) in the liver, and benzene oxide then gives rise to all subsequent metabolites, including phenol, catechol, hydroquinone, 1,2- and 1,4-benzoquinone, and the muconaldehydes (493). It has long been recognized that several metabolites of benzene can bind covalently to proteins and nucleic acids (92). The metabolites catechol and

hydroquinone can be oxidized by peroxidases resulting in the production of reactive oxygen species and DNA reactive quinones (138, 204). These quinones react with DNA to form predominantly depurinating adducts at the N7 of guanine and the N3 of adenine. In some cases, these adducts induce base substitutions (294, 375), as well as DNA strand breaks (204). It has been suggested that DNA lesions such as these may be important factors in the initiation of breast and other human cancers including those induced by benzene (69). Previous studies have suggested that p53 dysfunction is involved in benzene-induced toxicity (48, 203, 592). Yoon *et al.* (2003) showed that the cell cycle is still functional in benzene exposed p53 knockout mice, whereas wildtype mice undergo cell cycle arrest due to expression of checkpoint genes such as p53. This suggests that inactivation of p53 during benzene exposure may lead to an increased mutation frequency and therefore could be a potential factor leading to benzene-induced carcinogenesis. Further support for this comes from mouse studies showing that inhaled benzene is able to induce a high frequency of thymic lymphomas in p53 heterozygous mice (111). An additional study showed that nearly 90% of benzene-induced thymic lymphomas in C57BL/6-p53 haploinsufficient mice exhibited loss of the functional p53 locus (47).

Ethylene oxide (EtO) is used as an intermediate in the production of several industrial chemicals the most notable being ethylene glycol (223, 427). It is also used as a gas sterilant for medical equipment and can be found in cigarette smoke and automobile exhaust (223). In humans, EtO exposure has been associated with the development of leukemia, lymphoma, pancreatic, stomach, and breast cancers (42, 223, 501). In rodents, EtO is a multisite carcinogen capable of inducing mammary carcinomas in mice (223, 492). EtO is a direct

acting carcinogen that reacts with nucleophilic molecules to form a variety of different DNA adducts and is a powerful mutagen and clastogen at all phylogenetic levels (223, 349, 463, 554, 582). Inhaled EtO induces an increased frequency of *lacI* and/or *hprt* mutations in mice, rats and humans (302, 487, 522, 543, 556). Mouse bone marrow cells exposed to EtO exhibit *lacI* mutations with the distinction of only inducing an increased frequency of AT to TA transversions (427). EtO exposure in humans may also result in elevated expression of ras and p53 (as measured in white blood cells) (133).

Tumor formation is a multi-step process involving imbalance between growth stimulatory and growth inhibitory signals. P53 and ras have been implicated in the regulation of both of these conflicting cellular pathways. P53 and ras are the two most commonly altered genes in human cancers and it has been observed that mutations in both genes often occur in the same cancer cell (22, 51, 282, 537).

A number of lines of evidence support an interaction between these proteins in the process of tumorigenesis. P53 was initially described as an oncogene owing to its ability to cooperate with activated ras to transform cells (130). It was later found that this “transforming” p53 was in fact a mutated form and that wildtype p53 actually suppressed transformation, a finding that finally categorized p53 as a tumor suppressor protein (130). Since then, a number of *in vitro* and *in vivo* studies have supported a role for mutant p53 and ras cooperation. For example, wildtype p53 was shown to suppresses transformation by mutant ras, whereas mutant forms of p53 cooperated with mutant ras in the transformation of primary rat cells (221). In addition, oncogenic ras was shown to cooperate with p53 loss in the transformation

of primary keratinocytes (292) and in the promotion of urothelial tumorigenesis in mice (159). Furthermore, the combination of mutant p53 and activated H-ras was shown to efficiently transform primary-cultured fibroblasts while p53 mutation by itself could not (255, 598). Likewise, oncogenic ras is unable to transform primary cells due to the induction of cell cycle arrest related to the expression of tumor suppressors such as p53 (144, 320, 399, 406, 466). Moreover, p53 has been shown to directly counteract ras/MAPK signaling via caspase-mediated cleavage of Erk2/MAPK (323). Constitutively active ras/MAPK signaling stimulates the synthesis of p53 and can increase p53 protein stabilization via the induction of p19<sup>ARF</sup> (472, 568). Elevated levels of p53 provokes cell cycle arrest in some cells (298, 468, 578) and cellular senescence in others (466). It is thought that the mechanisms leading to these cellular responses must be circumvented in order to induce tumorigenesis (143, 341, 466). Primary mouse embryo fibroblasts and human keratinocytes lacking p53 are known to be highly susceptible to ras transformation (341, 567). In addition, MMTV-ras transgenic mice are highly susceptible to the development of mammary tumors and the incidence increases with the additional loss of p53. Tumors from these ras/p53 knockout mice exhibit higher histological grades, increased growth rates and extensive genomic instability (219, 220, 485)

Based on the high frequency of p53 mutations and the frequent activation of ras signaling in human mammary cancers, the primary goal of this study was to determine how these genes might be involved, either individually or combined, in spontaneous, benzene-, and ethylene oxide-induced mouse mammary carcinogenesis. An additional attempted was made to

identify chemical-specific and universal alterations common to mouse mammary tumors in general.

## **6.3 MATERIALS AND METHODS**

### **6.3.1 Mammary Carcinomas**

The benzene study included 50 female B6C3F1 mice exposed to 0, 25, 50, or 100 mg/kg of benzene via gavage 5 days per week for 103 weeks (389). The ethylene oxide study included 50 female B6C3F1 mice exposed to air containing 0, 50, or 100 ppm of ethylene oxide for 6 hours per day, 5 days per week for 102 weeks (390). Husbandry and experimental procedures complied with the requirements set forth by the Public Health Service's *Guide for the Care and Use of Laboratory Animals*. Representative benzene- and ethylene oxide-induced mammary carcinomas from these studies were examined for p53 protein expression and for p53 (exons 5-8) and H-ras (codon 61) mutations. For comparison, a number of spontaneous mammary carcinomas were also analyzed for p53 and ras alterations. The spontaneous mammary tumors were selected from female control mice in National Toxicology Program (NTP) studies conducted around the same time-period as the benzene and ethylene oxide studies. All studies had similar collection procedures, fixation methods, and tissue processing techniques. Briefly, mammary carcinomas were fixed in 10% neutral buffered formalin, routinely processed, and embedded in paraffin.

The following tumor sets were used for analysis of p53 and H-ras alterations: fourteen mammary carcinomas from benzene-exposed mice, twelve mammary carcinomas from EtO-exposed mice, and nineteen spontaneous mammary carcinomas from feed control mice.

### **6.3.2     *Immunohistochemistry***

Spontaneous, benzene-, and ethylene oxide-induced mammary carcinomas were screened for p53 protein expression by immunohistochemical analysis using the avidin-biotin-peroxidase detection system (Vectastain Rabbit Elite Kit; Vector Laboratories, Burlingame, CA). The immunohistochemical staining procedure was performed as previously described (533). A 1:500 dilution of the primary polyclonal rabbit antibody CM-5 (Vector Laboratories), which detects p53 protein in rodents, was used with a 30 minute incubation at room temperature. Normal rabbit serum (Vector Laboratories) was used as the negative control in place of the primary antibody. Tissue specimens from a known p53 positive tissue served as the positive control.

### **6.3.3     *Grading Criteria***

P53 protein expression was graded using the quickscore method (107). This method independently grades the proportion of cells stained using a 6-point scale (1 = 0-4%; 2 = 5-19%; 3 = 20-39%; 4 = 40-59%; 5 = 60-79%; 6 = 80-100%) and the intensity of the stain using a scale of 0 to 3 (0 = no expression; 1 = weak; 2 = intermediate; 3 = strong). The two

grades are multiplied together to arrive at the quickscore, which ranges from a minimum of 0 to a maximum of 18.

#### **6.3.4 DNA Isolation and Amplification**

DNA was extracted from paraffin-embedded sections of mammary carcinomas by previously described methods (191, 483). Briefly, unstained 10-micrometer thick sections were cut from paraffin blocks containing individual mammary neoplasms that had been dissected from surrounding tissue. After digesting with proteinase K, DNA was extracted using a DNeasy tissue kit (Qiagen Inc, Valencia, CA) according to the manufacturer's instructions. The extracted DNA was amplified using PCR-based techniques (448).

The second exon of H-ras was amplified using nested primers as previously described (108). In addition, PCR amplification of exons 5-8 of the p53 gene was done using PCR sequencing primers and annealing temperature profiles as reported previously (273). Positive and no-DNA controls for both H-ras and p53 mutations were run with all sets of reactions.

Amplified DNA was electrophoresed on 1% agarose minigels with ethidium bromide to test for proper product size and purity. The amplification bands were cut from the gel then purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

As indicated in Table 4, I was unable to amplify certain p53 exons from seven different tumors. Exon 5 provided the most difficulty with inadequate amplification in samples 7, 8,

16, 18, and 19. In addition to these, I was unable to amplify exon 7 from samples 4 and 10. This left a total of 12 spontaneous tumors with mutation data for all four p53 exons, which was comparable to the number of benzene- and EtO-induced mammary tumors that were evaluated.

### **6.3.5     *Cycle Sequencing***

To identify H-ras and p53 mutations, samples were sequenced with a cycle sequencing kit (US Biochemical, Cleveland, OH) that incorporates alpha-<sup>33</sup>P dideoxynucleotide terminators (A, C, G, and T) into the sequencing products. The amplification primers also served as the sequencing primers. The sequencing reaction products were analyzed by electrophoretic separation on an 8% acrylimade gel containing 8 M urea. Gels were dried and exposed to x-ray films overnight.

## **6.4        *RESULTS***

Both benzene and EtO have been shown to induce an increased incidence of mammary carcinomas in female B6C3F1 mice (389, 390) and this is illustrated in Table 1. Mammary carcinomas appeared histologically similar between chemically induced and spontaneous mammary carcinomas and there were no differences between dose groups or type of chemical exposure. Most tumors displayed small to large glandular patterns with occasional solid regions. Some tumors had a population of spindle shaped cells streaming around adjacent glands. A few also exhibited varying degrees of squamous differentiation and were

therefore diagnosed as adenosquamous carcinomas. Many tumors exhibited invasion into surrounding tissues although no metastases were observed.

#### **6.4.1 Benzene-Induced Mammary Carcinomas**

The incidence of altered p53 protein expression, p53 mutations, and H-ras mutations in benzene-induced mammary carcinomas is presented in Table 2. There were no clear differences in the frequency or pattern of p53 or H-ras mutations between the various benzene exposure levels. P53 protein expression (Figure 1A) was detected in 43 percent (6/14) of the tumors and was always localized to the nucleus. The average quickscore for all benzene-induced tumors was 3.57. P53 mutations (Figure 2) were detected in 57 percent (8/14) of the tumors and three of these exhibited two or more mutations per tumor. Mutations were identified at codons 147, 149, 150, 151, 199, 203, 207, 210, 212 (x2), 244, and 290. Overall, there were fourteen base pair substitutions including two double mutations at codons 150 (exon 5) and 290 (exon 8). Of the twelve mutant codons, nine were missense mutations and three were silent mutations. Of the fourteen p53 mutations, 71% involved either guanine or adenine bases. These mutations were most common in exons 5 (5/14) and 6 (6/14), which accounted for 79% of all p53 mutations.

Codon 61 H-ras mutations (Figure 3) were detected in 50% (7/14) of the benzene-induced mammary tumors and all were missense mutations. Six of the seven mutations (86%) involved the second base (A), whereas just one was found involving the first base (C). Five

of the seven H-ras mutations were A to G transitions (71%) resulting in an amino acid change from glutamine to arginine.

#### **6.4.2 Ethylene Oxide-Induced Mammary Carcinomas**

The incidence of altered p53 protein expression, p53 mutations, and H-ras mutations in ethylene oxide-induced mammary carcinomas is presented in Table 3. P53 protein expression (Figure 1B) was detected in 67% (8/12) of the ethylene oxide-induced mammary carcinomas and was always localized to the nucleus. The average quickscore for all EtO-induced tumors was 3.83. P53 mutations (Figure 4) were detected in 67% (8/12) of the carcinomas with five of the eight having two or more mutations. Mutations were identified at codons 188, 189, 191, 198, 241(x4), 246, 252, 255, and 264 (x3). Overall, there were fourteen base substitutions including eight silent mutations, five missense mutations, and one nonsense mutation. Of the fourteen mutations, nine (64%) involved guanine bases. P53 mutations were most common in exon 7 (7/14), 57 percent of which occurred at codon 241. In addition, there were no mutations in exon 5 and all of the mutations from exon 8 were localized to codon 264. The exposure level of ethylene oxide also appeared to have an influence on the incidence of p53 mutations. Three of the seven tumors from the 50-ppm dose group exhibited increased p53 protein expression, whereas all five of the tumors from the 100-ppm dose group exhibited p53 protein expression. Similarly, of the seven tumors from the 50-ppm dose group three p53 mutations were found as compared to nine mutations in the five tumors from the 100-ppm dose group.

Codon 61 H-ras mutations (Figure 5) were detected in 33% (4/12) of the ethylene oxide-induced mammary carcinomas. All of these were missense mutations and in every tumor the mutation was localized to the second base (A), although one tumor had a double mutation that also involved the first base (C). Overall, there were two amino acid changes from glutamine to arginine (A to G) and two from glutamine to leucine (A to T).

### **6.4.3 *Spontaneous Mammary Carcinomas***

The incidence of altered p53 protein expression, p53 mutations, and H-ras mutations in spontaneous mammary carcinomas is presented in Table 4. P53 protein expression was detected in 42% (8/19) of the spontaneous mammary carcinomas although expression levels were low (Figure 1C) with an overall quickscore of just 0.63 as compared to 3.57 and 3.83 for benzene- and EtO-induced tumors, respectively. In all cases, p53 expression was localized to the nucleus. All nineteen spontaneous tumors were analyzed for p53 mutations in exons 5 through 8; however, in certain tumors there were difficulties in amplifying all four exons. As a result, the evaluation of the p53 mutation incidence in spontaneous tumors only included the twelve tumors in which data were available for all four exons. Using these criteria, p53 mutations were identified in 58% (7/12) of the spontaneous mammary carcinomas. Mutations were found at codons 132, 136, 145, 146, 147 (x2), 180 (x3), 241, 262, and 265 (x2). Overall, thirteen base substitutions were demonstrated including seven missense mutations, five silent mutations, and one nonsense mutation. Of the thirteen mutations, seven were C to T base transitions. Nine of the thirteen mutations occurred in exon 5.

Codon 61 H-ras mutations were detected in 26% (5/19) of the spontaneous mammary carcinomas. All five of these were missense mutations the majority of which resulted in amino acid changes of glutamine to lysine (C to A).

Table 5 is a group summary of the genetic alterations identified in the spontaneous, benzene-, and ethylene oxide-induced mammary carcinomas.

## **6.5 DISCUSSION**

The high frequency of p53 and H-ras mutations in both chemically induced and spontaneous mammary carcinomas suggests that these are common alterations in mouse mammary tumors. However, the distinct shift in p53 and H-ras mutational spectra between chemically induced and spontaneous tumors suggests that benzene and EtO exposure can induce mammary specific genetic alterations that predispose female mice to mammary tumor development (Figure 8 and Figure 9). In addition, the frequent occurrence of both p53 and H-ras mutations in the same tumor type suggests that these two genetic alterations might cooperate in the development of these tumors.

The difference in mutation profiles between spontaneous and chemically induced neoplasms suggests that different mechanisms are likely involved in their development. A comparison of the alterations noted in spontaneous and chemically induced mammary carcinomas revealed several notable differences (Figure 8, Figure 9, and Figure 10). In chemically induced tumors, p53 and H-ras mutations predominantly involved purine bases (A or G),

whereas the majority of the spontaneous mammary tumors had mutations involving pyrimidine bases (all C). In addition, H-ras mutations in chemically induced tumors most frequently involved the second base of codon 61, whereas spontaneous mutations usually involved the first base. The majority of the spontaneous tumor H-ras mutations resulted in amino acid changes of glutamine to lysine, whereas ten of the eleven H-ras mutations in the chemically induced mammary tumors resulted in amino acid changes from glutamine to either leucine or arginine.

Although these experiments did not specifically address the cause of these genetic alterations, there are a number of potential mechanisms that may be involved. In the p53 gene, one of the most striking changes was a shift from predominantly C to T mutations in spontaneous tumors to a relatively high incidence of mutations involving guanine and adenine in benzene-induced tumors and guanine in EtO-induced tumors. This finding suggests that guanine is a primary target for mutation by both chemicals and that benzene also targets adenine. In codon 61 of the H-ras gene, the most prominent change was a shift from C to A mutations in spontaneous tumors to mutations predominantly involving adenine in the chemically induced tumors. This again suggests that benzene may target adenine but also suggests that EtO can as well.

G to A mutations following EtO exposure have been described by others and are frequently attributed to the most common EtO DNA adduct, N7-(2-hydroxyethyl)guanine (543). It should also be noted that, although benzene itself is not highly reactive with DNA, several

benzene metabolites can form DNA adducts and two of these are known to induce G to A mutations (161).

G to C transversions were the second most common p53 mutations in EtO-induced tumors but were rare in benzene-induced and spontaneous tumors. Similar mutations were found in 1,3-butadiene-induced mammary tumors and it was speculated that epoxide derived N7 guanine adducts were the cause (608). As mentioned above, these adducts are also common following EtO exposure suggesting that a similar mechanism may be involved. An additional consideration would be ROS-induced mutations considering that G to C transversions are one of the more common mutations generated by singlet oxygen (232).

In benzene-induced tumors, A to G transitions were the most common H-ras mutations and the second most common p53 mutations. H-ras A to G transitions were also one of the more common mutations identified in EtO-induced tumors. One major mechanism capable of inducing A to G mutations is adenine adduct formation (439, 593). Potential sources for these adducts would include the chemical itself or one of its metabolites. Previous studies have shown that EtO is capable of forming N1 and N3 adenine adducts (543). Another potential source for DNA adducts would be malonaldehyde, a naturally occurring product of lipid peroxidation. It reacts with DNA to form adducts to deoxyadenosine and deoxyguanosine (329). Considering the high lipid content of mammary tissue and the potential for redox cycling related to benzene metabolism (20) it is possible that this mechanism is involved in benzene-induced mammary tumorigenesis.

A to T transversions were one of the most common H-ras mutations found in EtO-induced mammary carcinomas. Previous studies have demonstrated that EtO exposure was capable of inducing A to T mutations (427). A number of other chemicals are also known to induce these mutations specifically in H-ras codon 61 (484, 550, 576). As with A to G mutations, one potential mechanism for inducing A to T mutations would be through the formation of adenine adducts particularly those that lead to depurination with insertion of an adenine opposite the apurinic site.

As mentioned previously, C to T transitions were particularly common p53 mutations in spontaneous mammary carcinomas. One mechanism for the formation of these mutations is spontaneous deamination of 5-methylcytosine (502). Transition mutations at methylated CpG sites are common in many cancers (412) and p53 has a number of potentially methylated CpG sites. Additional studies would be necessary to determine if this mechanism was related to the p53 mutations detected in this study. EtO can also form N3 cytosine adducts that could, through hydrolytic deamination, result in the C to T mutations seen in some of the EtO-induced tumors. In addition, oxidative damage to cytosine results in at least 40 modified species some of which have the potential to induce C to T mutations (232, 277).

C to A mutations were particularly common in the H-ras gene from spontaneous mammary tumors. Other investigators have also demonstrated a high incidence of H-ras C to A mutations in spontaneous mouse liver tumors suggesting that these are likely spontaneously derived (165, 403, 442). Endogenous mutagens such as ROS are also capable of causing

these mutations suggesting a possible mechanism whereby these mutations may arise spontaneously over time.

EtO-induced mammary carcinomas exhibited a high incidence of silent p53 mutations, which may suggest an overall increase in the general mutation rate. Others have suggested that the p53 gene can become hypermutable under certain conditions (510). Although the exact mechanism for this finding is uncertain, it has been shown that silent mutations are not necessarily benign in that they may alter translation efficiency (510) and have the potential to create or destroy splice sites (206, 510). Although the p53 mutations detected in these tumors did not directly involve any of the known splice sites in the mouse p53 gene, we cannot be certain about the possibility of new splice sites being created. Additional considerations for this finding would include sequencing artifacts or possibly rare polymorphisms.

Only the EtO-induced tumors exhibited a clear dose-related response in relation to the level of p53 protein expression and the number of p53 mutations (Figure 7). Interestingly, the tumor response *in vivo* did not show a dose-related increase suggesting that p53 mutational analysis may be a more sensitive method for identifying dose response with this chemical.

Benzene-induced tumors exhibited a wider range of mutations as compared to EtO-induced and spontaneous tumors (Figure 9). Of the twelve possible base substitutions eight were identified in benzene-induced tumors as opposed to six and five for EtO-induced and spontaneous tumors, respectively. This is perhaps not surprising considering the complex nature of benzene metabolism and the potential generation of a number of different genotoxic

metabolites. This may suggest that benzene-induced mutations are generated through a number of different mechanisms or that the predominant mechanism results in widespread random mutations such as might occur with some forms of oxidative damage (232).

The percentage of tumors exhibiting p53 protein expression was similar between benzene-induced and spontaneous mammary tumors (42-43%) but was slightly higher for EtO-induced tumors (67%). However, the overall p53 protein expression levels, as measured by the quickscore, were much higher in benzene- (3.57) and EtO-induced (3.83) tumors as compared to spontaneous tumors (0.63) (Figure 6). It should also be noted that although EtO-induced tumors more frequently exhibited p53 protein expression, the benzene-induced tumors tended to exhibit higher intensity staining when present. Interestingly, p53 expression in the EtO-induced tumors was often most intense in cells with a spindle cell morphology although the significance of this finding is uncertain. In certain instances, there was discordance between p53 protein expression and p53 mutation. This has been reported to occur in other tumor types although the exact mechanisms are not always understood (168). One possible consideration is that not all p53 mutations result in enhanced p53 protein stability. Another would be related to sampling variation particularly when many of these alterations are not universal changes present throughout the whole tumor. Additional sample variation could occur due to differing proportions of stroma and tumors cells. The presence of p53 protein expression without p53 mutation could be due to p53 mutations outside the regions examined or possibly due to alteration of other proteins downstream of p53 (168).

In conclusion, these results show that, like human breast cancers, multiple genetic pathways are altered in mouse mammary carcinomas. The results additionally demonstrate that p53 and H-ras mutations are relatively common in mouse mammary carcinomas, although both benzene and ethylene oxide are capable of shifting the mutational spectra of both genes in the mammary gland. This chemically induced shift in mutational spectra likely underlies the mammary carcinogenic properties of benzene and ethylene oxide in the mouse. Moreover, the concurrence of both p53 and H-ras mutations in the same tumor type, regardless of cause, suggests that these genes act cooperatively in the genesis of mouse mammary carcinomas.

**FIGURE 1**

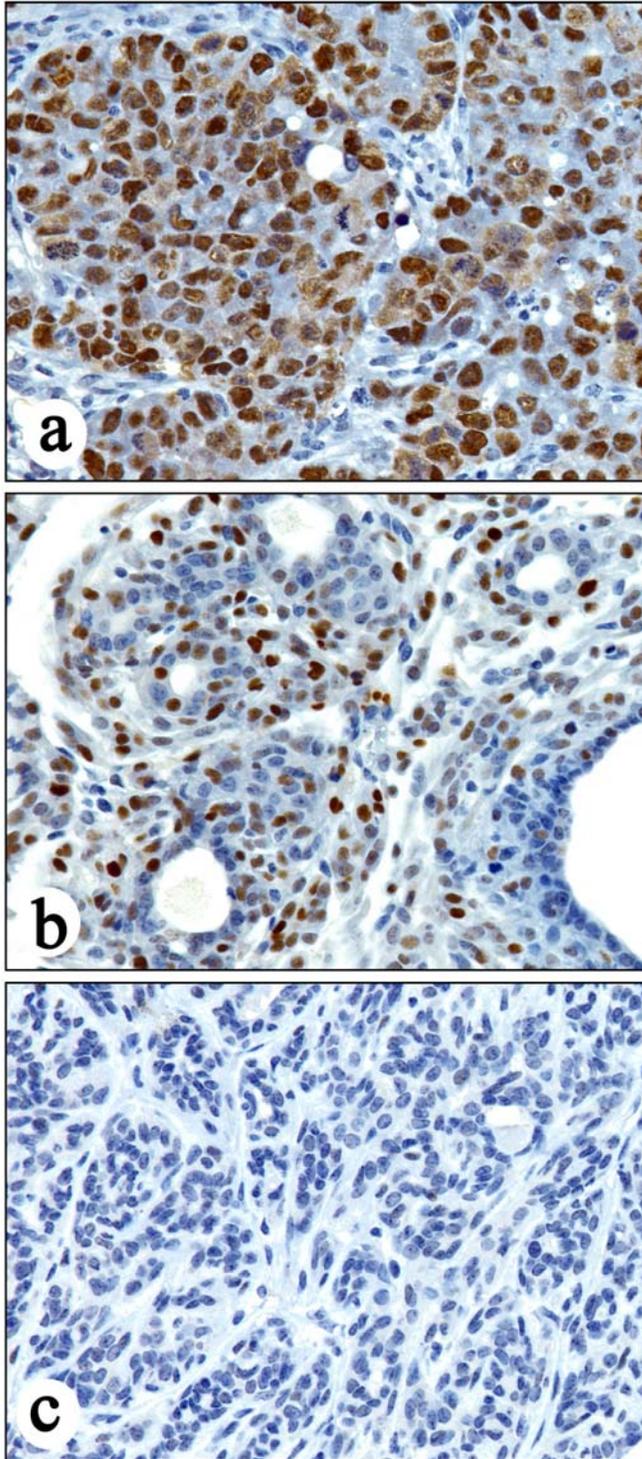


FIGURE 1. – p53 protein expression in mammary carcinomas by immunohistochemical analysis. Panels represent spontaneous (a), benzene - (b), and ethylene oxide -(c) induced mammary tumors from samples 10, 3, and 1, respectively.

**FIGURE 2**

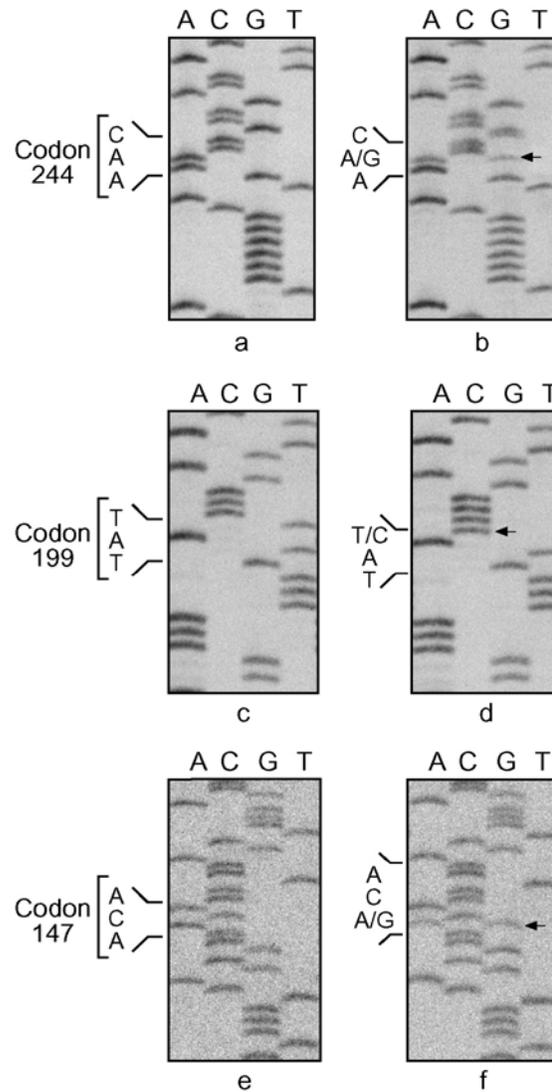


FIGURE 2. – *p53* mutations in benzene-induced mammary carcinomas from B6C3F1 mice by cycle sequencing analysis. A, C, G, and T represent the four nucleotides in DNA, adenine, cytosine, guanine, and thymine, respectively. Sequencing panels (a), (c), and (e) are normal *p53* sequences. Panels (b and d) are mutated sequences at codons 244 (exon 7) and 199 (exon 6) from tumor samples 2 and 3, respectively. Panel (f) is a mutated sequence at codon 147 (exon 5) from tumor sample 3. The wild-type alleles are also visible.

**FIGURE 3**

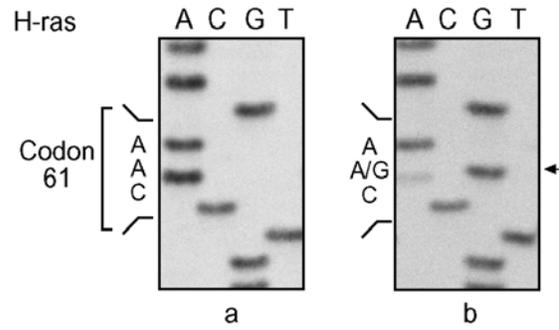


FIGURE 3. – *H-ras* mutations at exon 2 in benzene-induced mammary carcinomas from B6C3F1 mice by cycle sequencing analysis. A, C, G, and T represent the four nucleotides in DNA, adenine, cytosine, guanine, and thymine, respectively. Sequencing panels are from left to right: (a) Normal *H-ras* codon 61 sequence CAA from normal mammary gland. (b) Mutated sequence CGA at codon 61 from tumor sample 3. The wild-type alleles are also visible.

**FIGURE 4**

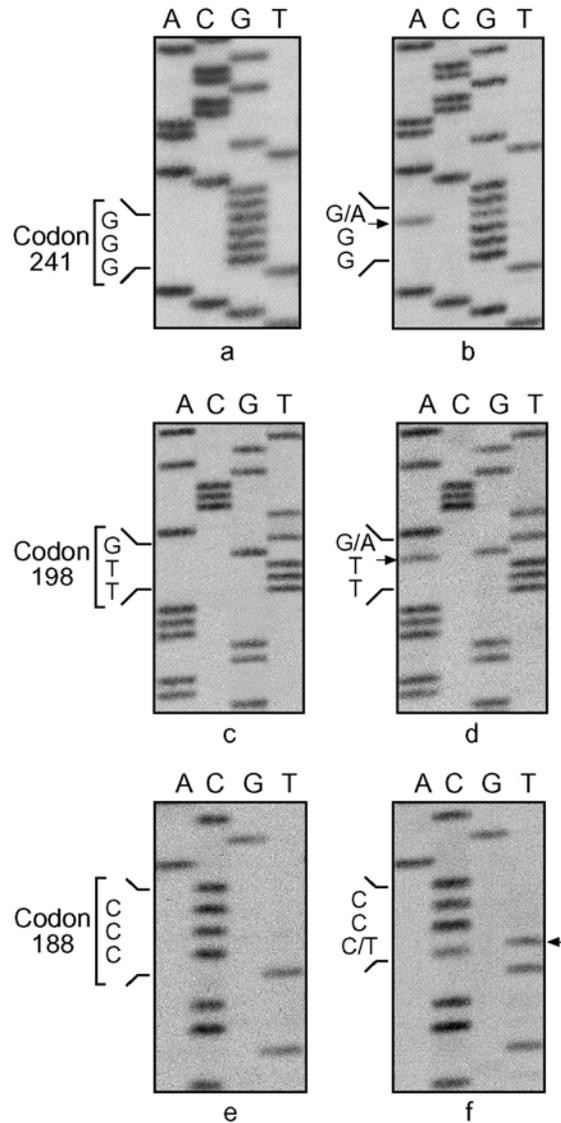


FIGURE 4. – *p53* mutations in ethylene oxide-induced mammary carcinomas from B6C3F1 mice by cycle sequencing analysis. A, C, G, and T represent the four nucleotides in DNA, adenine, cytosine, guanine, and thymine, respectively. Sequencing panels (a), (c), and (e) are normal *p53* sequences. Panel (b) is a mutated sequence at codon 241 (exon 7) from tumor sample 8. Panels (d) and (f) are mutated sequences at codons 198 (exon 6) and 188 (exon 6) from tumor samples 11 and 9, respectively. The wild-type alleles are also visible.

## FIGURE 5

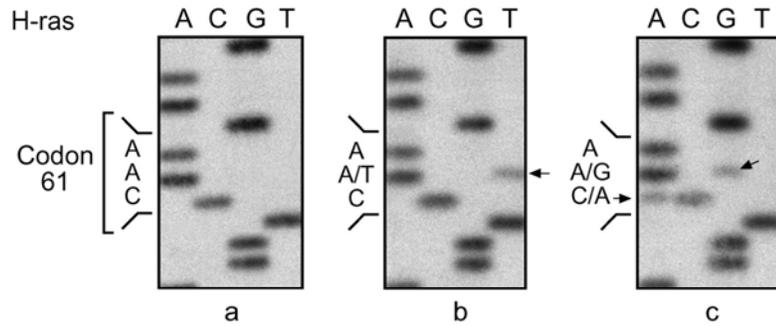


FIGURE 5. – H-ras mutations at exon 2 in ethylene oxide-induced mammary carcinomas from B6C3F1 mice by cycle sequencing analysis. A, C, G, and T represent the four nucleotides in DNA, adenine, cytosine, guanine, and thymine, respectively. Sequencing panels are from left to right: (a) Normal H-ras codon 61 sequence CAA from normal mammary gland. (b) Mutated sequence CTA at codon 61 from tumor sample 5. (c) Mutated sequence AGA at codon 61 from tumor sample 3. The wild-type alleles are also visible.

**FIGURE 6**

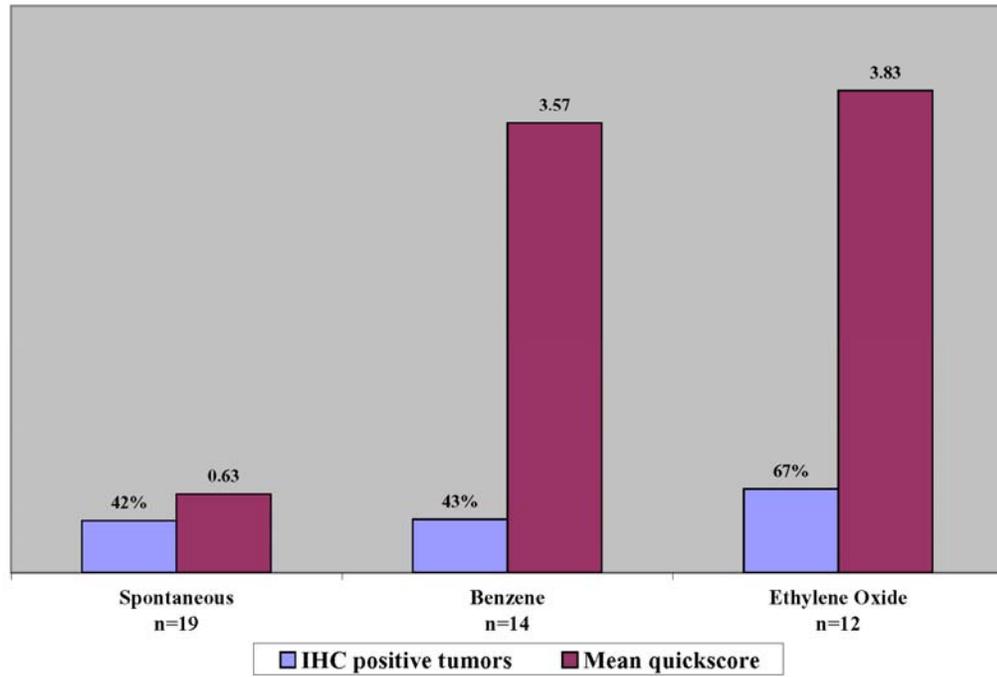


FIGURE 6. – P53 protein expression was evaluated using immunohistochemistry. Each tumor was first graded as either positive or negative based on the presence or absence of staining, regardless of the extent or intensity of the staining. These criteria were used to calculate the overall percentage of positive tumors (light/blue bars). In addition, each tumor was graded using the quickscore method (107), which takes into consideration the percentage of cells stained and the intensity of that staining. The mean quickscore for each group is represented by the dark/red bars.

**FIGURE 7**

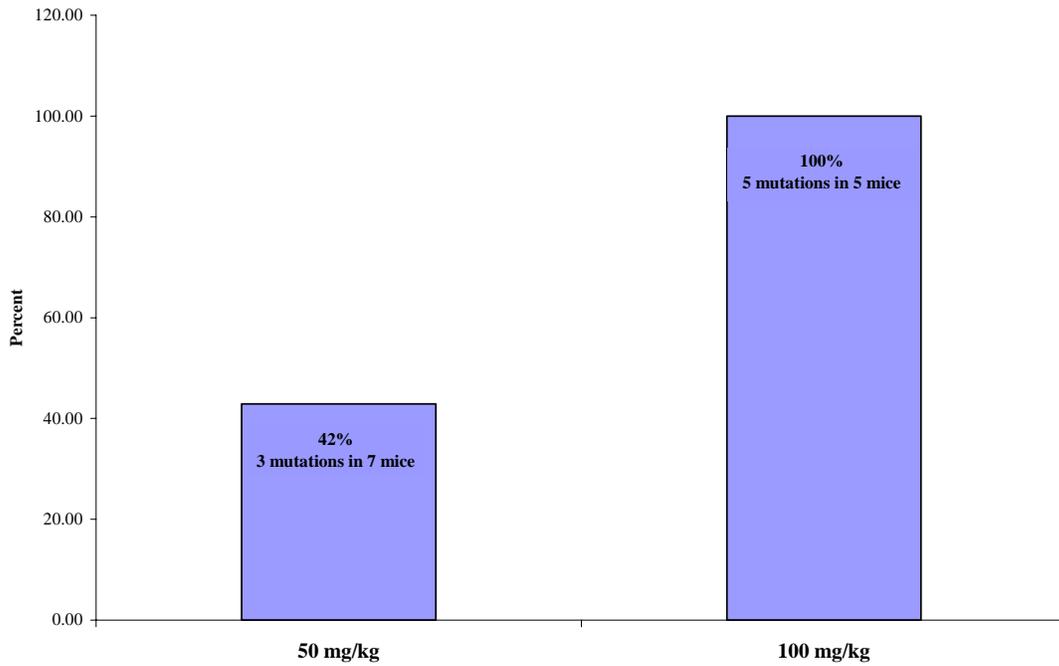


FIGURE 7. – The percentage of p53 mutations (exons 5-8) in ethylene oxide-induced mouse mammary carcinomas exhibited a positive dose related trend.

**FIGURE 8**

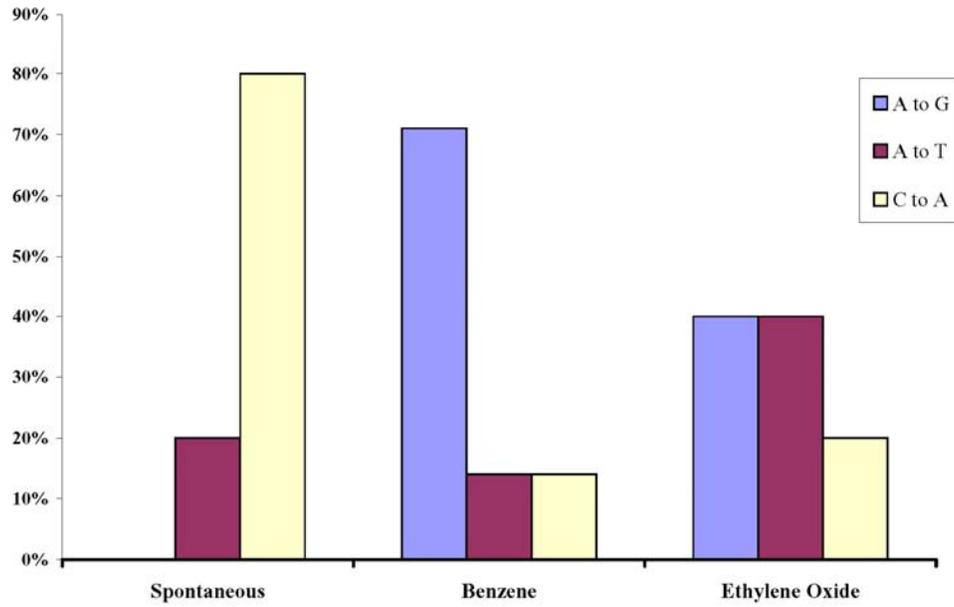


FIGURE 8. – Codon 61 (CAA) H-ras mutation spectrum in spontaneous, benzene-, and ethylene oxide-induced mouse mammary carcinomas.

**FIGURE 9**

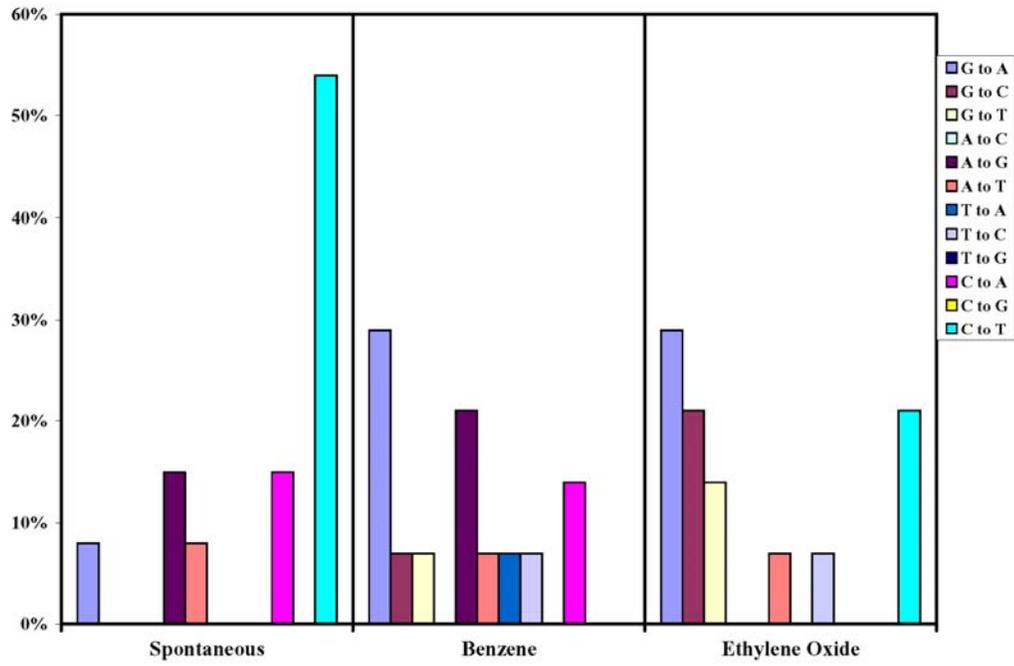


FIGURE 9. – P53 mutation spectrum in spontaneous, benzene-, and ethylene oxide-induced mouse mammary carcinomas (p53 exons 5-8).

**FIGURE 10**

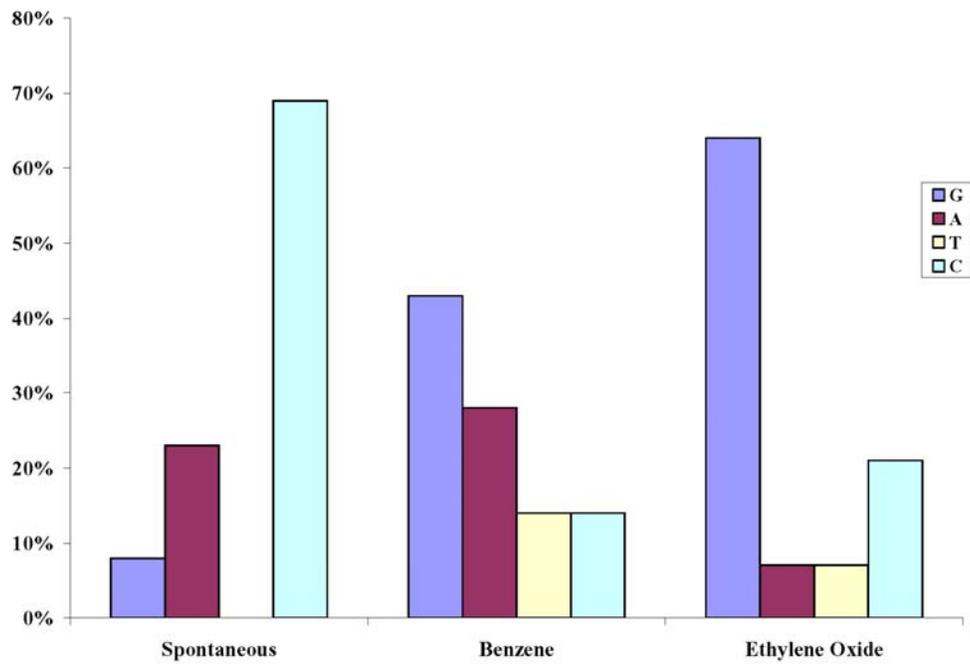


FIGURE 10. – P53 mutation base preference in spontaneous, benzene-, and ethylene oxide-induced mouse mammary carcinomas (exons 5-8).

## TABLE 1

Table 1. – Incidence of mammary carcinomas in B6C3F1 mice after benzene<sup>a</sup> or ethylene oxide exposure<sup>b</sup>

Exposure	Vehicle	Low Dose	Mid Dose	High Dose
Benzene	0% (0/50)	4% (2/50)	12% (6/50)	28% (14/50)
Ethylene oxide	2% (1/49)	17% (8/48)	–	12% (6/49)

<sup>a</sup>Female B6C3F1 mice exposed to 0, 25, 50, or 100 mg/kg of benzene via gavage 5 days per week for 103 weeks (NTP 1986).

<sup>b</sup>Female B6C3F1 mice exposed to air containing 0, 50, or 100 ppm of ethylene oxide for 6 hours a day, 5 days a week for 102 weeks (NTP 1987).

**TABLE 2**

**Table 2. – p53 protein expression and mutations in benzene-induced mammary carcinomas from B6C3F1 mice.**

Sample No.	Diagnosis	Dose (mg/kg)	p53 protein expression		H-ras mutations		p53 mutations		
			IHC quickscore	Codon 61 (CAA)	Exon 5	Exon 6	Exon 7	Exon 8	
1	Adenocarcinoma	25	0	NM	NM	Codon 207 CAG → CAA (Gln → Gln)	NM	NM	
2	Adenocarcinoma	50	0	CAA → CTA (Gln → Leu)	NM	NM	Codon 244 AAC → AGC (Asn → Ser)	NM	
3	Adenocarcinoma	50	1	CAA → CGA (Gln → Arg)	Codon 147 ACA → GCA (Thr → Ala)	Codon 199 TAT → TAC (Tyr → Tyr)	NM	NM	
4	Adenosquamous Carcinoma	100	8	NM	NM	NM	NM	NM	
5	Adenocarcinoma	100	0	CAA → CGA (Gln → Arg)	NM	Codon 203 CTG → CAG (Leu → Gln)	NM	NM	
6	Adenocarcinoma	100	0	NM	NM	NM	NM	NM	
7	Adenocarcinoma	100	1	CAA → AAA (Gln → Lys)	Codon 150 GCT → CAT (Ala → His)	Codon 212 AGC → AAC (Ser → Asn)	NM	NM	
					Codon 151 GGG → TGG (Gly → Trp)				
8	Adenosquamous Carcinoma	100	4	CAA → CGA (Gln → Arg)	NM	Codon 210 CGC → AGC (Arg → Ser)	NM	NM	
9	Adenocarcinoma	100	0	CAA → CGA (Gln → Arg)	NM	NM	NM	NM	
10	Adenocarcinoma	100	18	NM	NM	Codon 212 AGC → AAC (Ser → Asn)	NM	Codon 290 GGA → GAT (Gly → Asp)	
11	Adenocarcinoma	100	0	NM	NM	NM	NM	NM	
12	Adenocarcinoma	100	0	CAA → CGA (Gln → Arg)	NM	NM	NM	NM	
13	Adenocarcinoma	100	18	NM	NM	NM	NM	NM	
14	Adenocarcinoma	100	0	NM	Codon 149 CCA → CCG (Pro → Pro)	NM	NM	NM	
<b>Totals</b>			<b>3.57</b> average quickscore	<b>50%</b> 7/14 tumors	<b>57%</b> 8/14 tumors				

Abbreviations: NM = no mutation; IHC = immunohistochemistry

IHC quickscore = proportion of cells staining (1-6) x average stain intensity (0-3) as described in Detre et al., 1995

**TABLE 3**

**Table 3. – p53 protein expression and mutations in ethylene oxide-induced mammary carcinomas from B6C3F1 mice.**

Sample No.	Diagnosis	Dose (ppm)	p53 protein expression		p53 mutations			
			IHC quickscore	H-ras mutations Codon 61 (CAA)	Exon 5	Exon 6	Exon 7	Exon 8
1	Adenocarcinoma	50	0	NM	NM	NM	NM	NM
2	Adenocarcinoma	50	4	NM	NM	NM	Codon 255 GAA → GAT (Glu → Asp)	NM
3	Adenocarcinoma	50	9	CAA → AGA (Gln → Arg)	NM	NM	NM	Codon 264 CGG → CGT (Arg → Arg)
4	Adenocarcinoma	50	0	NM	NM	NM	NM	NM
5	Adenocarcinoma	50	0	CAA → CTA (Gln → Leu)	NM	NM	NM	NM
6	Adenosquamous Carcinoma	50	0	NM	NM	NM	NM	NM
7	Adenosquamous Carcinoma	50	4	CAA → CGA (Gln → Arg)	NM	Codon 189 CAG → CAA (Gln → Gln)	Codon 252 ATC → ATT (Ile → Ile)	Codon 264 CGG → CGC (Arg → Arg)
8	Adenocarcinoma	100	8	NM	NM	NM	Codon 241 GGG → AGG (Gly → Arg)	Codon 264 CGG → CGT (Arg → Arg)
9	Adenocarcinoma	100	6	NM	NM	Codon 188 CCC → ICC (Pro → Ser)	Codon 246 CGA → TGA (Arg → Stop)	NM
10	Adenocarcinoma	100	2	CAA → CTA (Gln → Leu)	NM	Codon 191 CTT → CTC (Leu → Leu)	Codon 241 GGG → GCG (Gly → Ala)	NM
11	Adenocarcinoma	100	9	NM	NM	Codon 198 TTG → TTA (Leu → Leu)	Codon 241 GGG → GGA (Gly → Gly)	NM
12	Adenocarcinoma	100	4	NM	NM	NM	Codon 241 GGG → GCG (Gly → Ala)	NM
<b>Totals</b>			<b>3.83</b> average quickscore	<b>33%</b> 4/12 tumors	<b>67%</b> 8/12 tumors			

Abbreviations: NM = no mutation; IHC = immunohistochemistry

IHC quickscore = proportion of cells staining (1-6) x average stain intensity (0-3) as described in Detre et al., 1995

**TABLE 4**

Table 4. – p53 protein expression and mutations in spontaneous mammary carcinomas from B6C3F1 mice.

Sample No.	Diagnosis	p53 protein expression		H-ras mutations		p53 mutations			
		IHC quickscore	Codon 61 (CAA)	Exon 5	Exon 6	Exon 7	Exon 8		
1	Adenocarcinoma	1	CAA → AAA (Gln → Lys)	Codon 146 GCC → GTC (Ala → Val)  Codon 180 TCC → TTC (Ser → Phe)	NM	NM	NM		
2	Adenocarcinoma	0	NM	NM	NM	NM	NM		
3	Adenocarcinoma	0	NM	Codon 180 TCC → TTC (Ser → Phe)	NM	NM	NM		
4	Adenocarcinoma	4	NM	NM	NM	Not amplified	NM		
5	Adenocarcinoma	0	CAA → AAA (Gln → Lys)	NM	NM	NM	NM		
6	Adenocarcinoma	0	NM	Codon 136 AAG → TAG (Lys → stop)	NM	NM	NM		
7	Adenocarcinoma	0	CAA → AAA (Gln → Lys)	Not amplified	NM	NM	NM		
8	Adenocarcinoma	0	NM	Not amplified	NM	Codon 241 GGG → GGA (Gly → Gly)	NM		
9	Adenocarcinoma	0	CAA → AAA (Gln → Lys)	NM	NM	NM	NM		
10	Adenocarcinoma	1	NM	Codon 145 AGC → AGT (Ser → Ser)	NM	Not amplified	NM		
11	Adenocarcinoma	1	NM	Codon 147 ACA → GCA (Thr → Ala)  Codon 180 TCC → TCT (Ser → Ser)	NM	NM	NM		
12	Adenocarcinoma	1	NM	Codon 132 TGC → TGT (Cys → Cys)	NM	NM	NM	Codon 265 GAC → GAA (Asp → Glu)	
13	Adenocarcinoma	0	NM	NM	NM	NM	NM		
14	Adenocarcinoma	1	CAA → CAT (Gln → His)	Codon 147 ACA → GCA (Thr → Ala)	NM	NM	NM		
15	Adenocarcinoma	1	NM	NM	NM	NM	NM		
16	Adenocarcinoma	0	NM	Not amplified	NM	NM	NM		
17	Adenocarcinoma	2	NM	NM	NM	NM	NM	Codon 262 CTG → TTG (Leu → Leu)  Codon 265 GAC → GAA (Asp → Glu)	
18	Adenosquamous Carcinoma	0	NM	Not amplified	NM	NM	NM		
19	Adenocarcinoma	0	NM	Not amplified	NM	NM	NM		
<b>Totals</b>		<b>0.63</b>	<b>26%</b>	<b>58%</b>					
		<b>average quickscore</b>	<b>5/19 tumors</b>	<b>7/12 tumors*</b>					

Abbreviations: NM = no mutation; IHC = immunohistochemistry  
 IHC quickscore = proportion of cells staining (1-6) x average stain intensity (0-3) as described in Detre et al., 1995  
 \* only includes tumors with data for all four exons

**TABLE 5****Table 5. – Summary of alterations in chemically induced and spontaneous mammary carcinomas from B6C3F1 mice**

<b>Exposure</b>	<b>p53 protein expression (average IHC quickscore)</b>	<b>p53 mutations (exons 5, 6, 7, and 8)</b>	<b>H-ras mutations (codon 61)</b>
Benzene	42% 6/14 tumors (3.57)	57% 8/14 tumors	50% 7/14 tumors
Ethylene Oxide	67% 8/12 tumors (3.83)	67% 8/12 tumors	33% 4/12 tumors
Spontaneous	42% 8/19 tumors (0.63)	58% 7/12 tumors*	26% 5/19 tumors

IHC = immunohistochemistry; quickscore = proportion of cells staining (1-6) x average stain intensity (0-3) as described in Detre et al., 1995

\* only includes tumors with data for all four exons

## CHAPTER SEVEN

### LITERATURE REVIEW FOR MANUSCRIPT THREE

#### 7.1 *OVARIAN CANCER*

Ovarian cancer is the fifth leading cause of cancer death in women in the United States and the most lethal gynecological malignancy (136). The incidence of ovarian cancer has been steadily increasing over the past 10 years, with an overall lifetime risk of 1.8%. Although ovarian cancer is nine times less common than breast cancer, it is approximately three times more likely to be fatal. In fact, 70-75% of women with ovarian cancer initially present with disseminated carcinomatosis, which corresponds with a 5-year survival of just 12%. The lethality of ovarian cancer is primarily attributable to the advanced stage of the disease at the time of initial diagnosis. Although family history of the disease is an important risk factor, most cases occur sporadically. The incidence of ovarian cancer rapidly increases after age 40 with a mean age of 60. Less than 15% of the cases occur in women less than 50 years of age (136).

##### 7.1.1 *Etiology – Ovarian Cancer*

The etiology of epithelial ovarian carcinomas is poorly understood. Environmental agents have been implicated, but not proven. These include talc, industrial pollutants, smoking, asbestos, and infectious agents (19). Epidemiological studies point to possible racial, geographic, social, and hormonal causative factors (19). Epidemiological factors associated with ovarian cancer are remarkably similar to those described for breast cancer and include

nulliparity, age, early menarche, and late menopause (19). It has also been suggested that inflammation may be a contributing factor in ovarian cancer development, because tubal ligation and hysterectomies act as protective factors, possibly by preventing passage of environmental initiators of inflammation (19). High parity and oral contraceptive use have been associated with reduced risk. These observations help support one of the first hypotheses proposed by Fathalla in 1971 (139). The theory, otherwise known as the “incessant ovulation” theory, suggests that frequent ovulation contributes to increased risk because of the repeated rupture and repair of the ovarian surface epithelium (OSE) at the sites of ovulation. Fathalla hypothesized that the repeated rupture/wounding of the ovarian surface followed by rapid proliferation of surface epithelial cells, as occurs with repetitious ovulation, drives the transformation of these cells. He noted that in contrast to most other species, where ovulation occurs only during the breeding season or as a reflex after copulation, the majority of ovulations in the human are purposeless. This markedly increases the cumulative trauma to the ovarian surface of humans. Fathalla also noted a brief report describing a high incidence of peritoneal carcinomatosis in domestic fowl forced to incessantly ovulate. This theory is further supported by the finding of an increased incidence of ovarian cancer among individuals subjected to hyper-ovulation as a treatment for infertility (19).

### ***7.1.2 Ovarian Surface Epithelium – Ovarian Cancer***

### ***7.1.2.1 Embryonic Ovarian Surface Epithelium – Ovarian Cancer***

Early in development, the future OSE forms part of the coelomic epithelium, which is the mesodermally derived epithelial lining of the intraembryonic coelom. It overlies the presumptive gonadal area and, by proliferation and differentiation, gives rise to part of gonadal blastema (19). The coelomic epithelium near the presumptive gonads invaginates to give rise to the Mullerian (paramesonephric) ducts. These ducts serve as the primordia for the epithelia of the oviduct, endometrium, and endocervix (19). Furthermore, the gonadal ridge lies near the region where invagination of the coelomic epithelium gives rise to the mesonephros and the ovarian rete. Accordingly, the coelomic epithelium in and near the gonadal area has the capacity to differentiate along many different pathways. It is this capacity that likely gives rise to the striking variety of phenotypes found among surface epithelial-derived ovarian carcinomas, and to their frequent differentiation along the lines of Mullerian duct derivatives. Histologically ovarian epithelial tumors tend to recapitulate normal structures derived from coelomic epithelium (19).

### ***7.1.2.2 Adult Ovarian Surface Epithelium – Ovarian Cancer***

In recent years, there has been a strong interest in the normal biology of OSE since it is the apparent source for most human ovarian cancers (19). OSE is modified pelvic mesothelium that covers the ovary (19). In the mature woman, OSE is a monolayer of squamous-to-cuboidal epithelium. It is known to express keratin types 7, 8, 18, and 19, which is a common expression pattern for many forms of simple epithelia. Intercellular contact and epithelial integrity of OSE are maintained by simple desmosomes, incomplete tight junctions, integrins,

and cadherins. OSE also has other classical epithelial features including cilia, apical microvilli, and a basal lamina and expresses mucin (MUC) and 17, 13-hydroxysteroid dehydrogenase (19). Despite the many epithelial features of OSE, there are indications that surface epithelial cell differentiation is not as firmly determined as that of many other epithelia. OSE cells constitutively co-express both epithelial (keratin) and mesenchymal (vimentin) intermediate filaments. In addition, cultured OSE has shown the capacity to express the connective tissue collagen types I and III. During postovulatory repair and in culture, OSE cells can exhibit a fibroblast-like form that reflects their close developmental relationship to ovarian stromal cells. OSE likely takes part in post-ovulatory repair of the ovarian cortex by synthesizing both epithelial and connective tissue components of the extracellular matrix and by using its contractile activity, which resembles the contractile capacity exhibited by connective tissue fibroblasts during wound healing (19). Like fibroblasts, which convert to myofibroblasts when engaged in tissue repair, OSE cells in culture contain smooth muscle actin (3). The exact mechanisms regulating this epithelial-mesenchymal conversion have not been completely defined although there is evidence that modulation of different cadherin types might be involved (details of this process will be provided in the section on cadherins). Similar epithelial-mesenchymal conversions occur in mesodermally derived cell types closely related to OSE, such as pleural mesothelial cells responding to injury and the cells of the developing Mullerian duct during regression in response to Mullerian inhibiting substance (170, 531). The capacity of OSE to undergo epithelial-mesenchymal conversion likely confers additional advantages during the postovulatory repair of the ovarian surface (19). Together, these characteristics show that OSE differentiation is not as firmly determined as in other adult epithelia and that it is better

described as being a pluripotential mesodermal embryonic precursor (19, 376). The OSE also differs from other epithelia in that it has a tenuous attachment to its basement membrane and can easily become detached by mechanical means. Whether this loose attachment has any physiological consequences is not known.

Unfortunately, there are few animal models suitable for the study of human ovarian epithelial neoplasms. There is a model in aging hens (151) although ovarian tumors in other species often do not arise from OSE but rather from follicular, stromal, or germ cells. Recently, a transgenic mouse model has been described (87). These mice express the transforming region of SV40 under control of the Mullerian inhibitory substance type II receptor gene promoter. Fifty percent of the female mice develop bilateral ovarian tumors. Other than these, however, there is a general lack of appropriate experimental models, and as a result, the etiology and mechanisms involved in ovarian carcinogenesis are poorly understood (19).

### ***7.1.3 Ovarian Carcinoma Pathology – Ovarian Cancer***

Most human ovarian tumors are classified into one of several categories based on presumed histogenesis and direction of differentiation (19). The three major categories include common epithelial tumors, sex cord-stromal tumors, and germ cell tumors (19). Epithelial ovarian carcinomas, which represent approximately 90% of all human ovarian malignant neoplasms, are thought to be derived from the surface epithelium of the ovary (461). Histopathologically and immunohistochemically, ovarian carcinomas are some of the most complex of all human malignancies (19). One of the most unusual aspects of ovarian carcinogenesis is the change

in differentiation that accompanies neoplastic progression. As OSE progresses to malignancy, it loses its stromal characteristics and acquires the characteristics of Mullerian duct-derived epithelia. This differentiation occurs in such a high proportion of ovarian carcinomas that it serves as the basis for classification of these tumors. Common epithelial tumors are divided into five main categories according to the type of cell into which they differentiate: 1) serous tumors, whose cells resemble those of the fallopian tube; 2) mucinous tumors, whose cells mimic those of the endocervix; 3) endometrioid tumors, whose cells are similar to those of the endometrium; 4) clear-cell tumors, whose cells resemble those of endometrial epithelium during pregnancy (or cells derived from the mesonephros); and 5) Brenner tumors, whose cells are urothelial in appearance (19). Serous adenocarcinomas are the most common comprising from 60 to 80% of all epithelial ovarian cancers with mucinous (10%), endometrioid (15%), and clear cell (5%) types being less common (521).

At the cellular level, Mullerian differentiation in these tumors is indicated by the appearance of altered cell shapes, E-cadherin expression, junctional complexes, epithelial membrane antigens, and secretory products including mucins (MUC1, MUC2, MUC3, and MUC4) and CA125 (19). Histologically, the tumors form polarized epithelia, papillae, cysts, and glandular structures. Unlike carcinomas in most other organs, ovarian epithelial carcinomas tend to become more differentiated than their cell of origin. Only in the late stages do these specialized epithelial features diminish, although they can persist even in advanced or metastatic tumors (243).

#### **7.1.4     *Metastasis – Ovarian Cancer***

Metastasis in general involves a complex cascade of interrelated genetic, molecular, and biochemical events involving a diverse range of biological processes including cellular adhesion, migration, extracellular matrix degradation, invasion, proliferation, and neovascularization (376). Ovarian carcinoma metastasis is no exception as it involves many of these mechanisms, however, the nature in which they are utilized may be different considering the unique patterns of metastasis that these tumors exhibit. Ovarian carcinoma can spread by direct extension to adjacent organs or exfoliated tumor cells can be transported throughout the peritoneal cavity by normal peritoneal fluid. This “seeding” of the peritoneal cavity is the most widely recognized behavior of ovarian carcinoma and is frequently associated with formation of ascites, which is rich in cytokines and growth factors. In contrast to most other types of carcinoma, dissemination of ovarian carcinoma through the vasculature is rare. However, the high frequency of pelvic and para-aortic lymph node involvement, particularly in advanced cases, indicates that lymphatic spread of ovarian carcinoma is also used as a method for systemic dissemination (109, 376).

## **7.2 CADHERINS - GENERAL**

Cadherins comprise a family of transmembrane calcium-dependent cell-cell adhesion glycoproteins that are components of a number of cellular junctions, including adherens junctions, desmosomes, cardiac junctions, endothelial junctions, and synaptic junctions (571). Cadherins are critical for normal development and alterations in cadherin function have been implicated in tumorigenesis (571). In humans, there are more than 80 members of the cadherin superfamily. Classic cadherins in mammals consist of two subgroups: vertebrate type I classic cadherins like epithelial (E)-, placental (P)-, neuronal (N)- and retinal (R)-cadherin and vertebrate type II classic cadherins such as vascular endothelial (VE)-cadherin (106). Cadherins are composed of three segments including an extracellular domain comprised of five well-conserved amino acid sequences (cadherin repeats) that mediate adhesion, a single pass transmembrane domain, and a conserved cytoplasmic domain that interacts with catenins, thus linking cadherins to the actin cytoskeleton (552). Cadherin adhesion is usually homotypic, although heterotypic interactions have been described (99).

### **7.2.1 Development and Metastasis - Cadherins**

Members of the cadherin superfamily have distinct expression patterns during embryonic development and in the adult (394). Changes in cadherin expression are often associated with changes in cellular morphology and tissue architecture. E-cadherin is first expressed at the preimplantation morula stage where it serves compaction, the earliest form of epithelial organization (394). Mice homozygous for E-cadherin mutations are not compatible with life;

these embryos exhibit severe abnormalities before implantation and the adhesive cells of the morula dissociate shortly after compaction resulting in abnormal polarization (394). In normal embryos, during gastrulation, cells start to migrate from the ectoderm as E-cadherin is downregulated in the primitive streak and N-cadherin is expressed (106). During this process, an epithelial-mesenchymal transition occurs. This switch is regulated by multiple growth and transcription factors (106). Interestingly, this process appears to be recapitulated during tumorigenesis and particularly during the metastatic process where it has been referred to as the “cadherin switch” (193).

#### **7.2.1.1 *Cadherin Switch - Cadherins***

Loss of the invasion-suppressor E-cadherin has been shown to result in upregulation of N-cadherin in several invasive tumor cell lines and tissues from breast and prostate cancers and melanomas (193). Transfection of N-cadherin induces a scattered morphology in squamous tumor cells and renders MCF-7 or BT 20 breast tumor cells more motile, invasive, and metastatic in nude mice. Conversely, exogenous expression of E-cadherin into the N-cadherin-expressing MDA-MB-435 cell line does not decrease N-cadherin levels or the invasive potential of the cells. Moreover, metastases derived from N-cadherin expressing MCF-7 tumors continue to express E- and N-cadherin at distant sites (193). Overall, these findings suggest that E-cadherin loss may not be as crucial an event for tumor progression, as previously suggested, and that additional cooperative alterations might also be necessary.

### **7.2.2     *Signaling – Cadherins***

Cadherins not only play an important role in mediating cell-cell adhesion but also are critical in modulating intracellular signaling (571). Probably the best-studied signaling pathway involving cadherins is the beta-catenin/Wnt pathway. The Wnt or wingless pathway plays a major role in developmental processes in vertebrates and invertebrates. Wnt is an extracellular matrix-associated growth factor that interacts with its receptor to initiate a signal transduction pathway that stimulates synthesis of proteins involved in cell growth, such as cyclin D1 and myc (571). The catenins were first identified as proteins that co-immunoprecipitated with cadherins and were termed alpha-catenin, beta-catenin, and gamma-catenin (plakoglobin). Either beta-catenin or gamma-catenin binds directly to the cadherin and to alpha-catenin, whereas alpha-catenin associates directly and indirectly with actin filaments (552). The ability of cadherins to simultaneously self-associate and link to the actin cytoskeleton allows strong cell-cell adhesion. Mutant cadherin molecules lacking the catenin-binding sites fail to interact with the actin filaments (205). Beta-catenin's role in this pathway is to bind to members of the TCF/LEF family of transcription factors and function as a co-activator. In the absence of a Wnt signal, beta-catenin that is not bound to cadherins in an adherens junction is ubiquitinated and degraded by the proteosomal pathway. When Wnt binds to its receptor, beta-catenin degradation is inhibited, allowing it to accumulate in the nucleus, where it binds to TCF/LEF. The Wnt pathway is tightly regulated during development, but its regulation may be disrupted during tumorigenesis.

In addition to catenins, p120<sup>ctn</sup>, which was originally identified as a Src substrate, binds to the cytoplasmic domain of cadherins and has been suggested to play a role in regulating the adhesive activity of cadherins (552). p120<sup>ctn</sup> binds to the juxtamembrane domain of cadherins, a domain that has been implicated in cadherin clustering and cell motility (552). It is thought that p120<sup>ctn</sup> influences the strength of cadherin-mediated adhesion, perhaps by influencing the organization of the actin cytoskeleton (423, 552). When p120<sup>ctn</sup> is phosphorylated, its binding to cadherins is increased, whereas loss of p120<sup>ctn</sup> can result in cadherin degradation (106). In fibroblasts, cytoplasmic p120<sup>ctn</sup> inhibits RhoA resulting in an increase in cell motility and activation of Rac1 and Cdc42 (106, 571).

### ***7.2.3 Cadherins and the Ovarian Surface Epithelium - Cadherins***

In the human, ovarian surface epithelium (OSE), granulosa cells, and extraovarian mesothelium are connected by N-cadherin, which characterizes adhesive mechanisms of mesodermally derived tissues (19). E-cadherin is constitutively present in human oviductal, endometrial, and endocervical epithelia and in mouse and porcine OSE (19). However, the pattern of expression of E-cadherin in normal human ovarian surface epithelia remains controversial. Some groups maintain that E-cadherin expression in normal ovarian surface epithelia is rare, while others demonstrate that E cadherin is present (19). It has been shown that human OSE that assumes a columnar shape frequently expresses E-cadherin (19) (similar results were found in the studies described in **manuscript three**). OSE cells tend to assume cuboidal to columnar shapes, especially within clefts and inclusion cysts. When this occurs it becomes apparent that the epithelium has developed phenotypic characteristics

consistent with Mullerian epithelium, including the expression of epithelial type markers such as E-cadherin (19). In addition to these metaplastic changes, OSE-lined clefts and inclusion cysts are also sites of early neoplastic progression (19). With age, the human ovary assumes increasingly irregular contours and forms OSE-lined surface invaginations and epithelial inclusion cysts in the ovarian cortex. It has been suggested that these inclusion cysts form from OSE fragments that become trapped in or near ruptured follicles at the time of ovulation (19).

#### **7.2.4 *Cadherins and Ovarian Carcinomas - Cadherins***

Studies looking at E-cadherin expression in human ovarian carcinomas have yielded conflicting results. In many studies, including that described in **manuscript three**, the results contrast what is typically found in other tissues. In the ovary, E-cadherin expression tends to increase as cells progress from normal OSE to metaplastic and dysplastic cells and eventually on to neoplastic cells (514). As with the study described in **manuscript three**, other investigators have shown that E-cadherin expression fails to decrease as ovarian carcinomas metastasize (514). Upregulation of E-cadherin during the later stages of ovarian tumorigenesis may contribute to the differentiation and migratory behavior of these cells, therefore understanding the regulation of E-cadherin in this tumor type is critical (376). It is possible that upregulation of E-cadherin in ovarian cancers contributes to the unique patterns of histological differentiation of these tumors. Another possibility is that ovarian cancer cells attach to one another through E-cadherin-mediated cell-cell adhesion, and if these cells acquire the ability to locally attenuate surface E-cadherin activity, they can migrate while

attached to one another. Moreover, E-cadherin might allow metastatic tumor cells to reattach to other sites or cells they encounter within the peritoneum and could therefore contribute to their ability to colonize new sites. These ideas are supported by studies from Burleson *et al.*, which found that a significant proportion of ovarian cancer cells in ascites of patients exist as multicellular aggregates, and that these aggregates show adhesiveness to mesothelial cells (376).

N-cadherin is reportedly expressed in multiple different stages of ovarian carcinomas, however, similar to the results described in **manuscript three**, at least one report mentioned that mucinous cystadenomas were N-cadherin negative (106). The cause for this variation in mucinous carcinomas is uncertain but perhaps signifies a different cell of origin. For the other ovarian carcinoma types, the increased N-cadherin expression is in keeping with its known role as an invasion promoter.

## **7.2      *E-CADHERIN***

E-cadherin is expressed early in development and is found in most embryonic and adult epithelia (19). It is encoded by the CDH1 gene and functions in multiple processes, including development, tissue integrity, cell migration, morphology, and polarity (423). E-cadherin is also a tumor suppressor whose expression is frequently reduced or silenced as tumorigenesis progresses (423). In fact, reduced E-cadherin expression is regarded as one of the main molecular events involved in dysfunction of the cell-cell adhesion system (394).

In addition to E-cadherin's well known function in cell adhesion, it has been shown to negatively regulate the activation of several receptor tyrosine kinases (RTKs) by inhibiting their ligand-dependent activation (423). EGFR and E-cadherin can form a complex that depends on the extracellular domain of E-cadherin, but is independent of beta-catenin or p120<sup>cas</sup> binding. E-cadherin is also able to induce ligand-independent activation of the EGF receptor and subsequent activation of MAP kinase (571). Furthermore, E-cadherin has been shown to transmit anti-growth signals via its cytoplasmic interaction with beta-catenin (29) or through its role in increasing expression of the cyclin-dependent kinase inhibitor p27 (63).

### **7.2.1      *Gene/Protein Structure – E-cadherin***

#### **Alternative names:**

Protein: E-cadherin (epithelial), uvomorulin, UVO, cadherin 1, ARC-1

Gene: CDH1, UVO, CDHE, ECAD, LCAM, Arc-1

The human E-cadherin gene was mapped to chromosome 16q22.1 and has 16 exons spanning approximately 100 kb of genomic DNA. The gene structure is similar to that of other cadherins (40, 136). The promoter of E-cadherin contains positive regulatory elements, a CCAAT-box, and GC-boxes, as well as two E-boxes (324).

In the mouse, E-cadherin is located on chromosome 8 (322). Sequence comparison between mouse and human E-cadherin shows over 80% identity in both nucleotide and amino acid sequences (322). E-cadherin is a single transmembrane domain protein whose amino-terminus is extracellular and carboxy-terminus is intracellular (423, 571). Calcium is required for E-cadherin to mediate its adhesive function, and the extracellular portion of E-cadherin contains several calcium-binding sites (423).

### **7.2.2 *Tumorigenesis and Metastasis – E-cadherin***

*In vitro* studies have shown that reduced expression of E-cadherin leads to altered phenotypes such as cell morphology changes, loose cell-to-cell contacts, cell dissociation, and enhanced cell motility. Inhibition of E-cadherin by function-blocking antibodies disrupts epithelial cell monolayers and induces a shift from an epithelial to a scattered/motile phenotype (193). In addition, a direct role for E-cadherin in the suppression of tumor invasion has been demonstrated by reversion of the invasive phenotype in malignant epithelial tumor cells transfected with E-cadherin cDNA (193). Invasiveness of the transfected cells could be restored by treatment with anti-E-cadherin antibodies or E-cadherin anti-sense RNA.

A transgenic mouse model of pancreatic beta cell carcinogenesis (Rip1Tag2) has provided strong support for E-cadherin's role in tumor development. In these mice, the SV40 large T antigen is expressed under the control of the rat insulin promoter, thus inducing neoplastic transformation from differentiated adenoma to invasive carcinoma in beta cells of the islets of Langerhans (324). Pancreatic beta cell adenomas and invasive adenocarcinomas occur in 74 and 26% of the mice, respectively. E-cadherin is downregulated in these tumors, however, forced re-expression arrests tumor development at the adenoma stage. Conversely, expression of a dominant-negative form of E-cadherin results in early invasion and metastasis (106). In another *in vivo* model, it was shown that cleavage of E-cadherin by targeted expression of stromelysin-1 in the mammary gland triggers an epithelial-to-mesenchymal conversion and stimulates mammary carcinogenesis (193).

Based on these clinical and experimental observations, it has been proposed that loss of E-cadherin-mediated cell-cell adhesion is a prerequisite for tumor cell invasion and metastasis formation, although others have suggested that loss of E-cadherin by itself is often not sufficient (71).

### **7.2.3     *Regulation - E-cadherin***

### **7.2.3.1 Positive Regulation - E-cadherin**

The Wilm's tumor 1 (WT1) protein can transactivate the E-cadherin gene (CDH1) directly through binding to the proximal GC-rich sequence in the promoter as evidenced by transfection of 3T3 fibroblasts (324). Rb and c-myc can also activate expression of the E-cadherin gene through interaction with the transcription factor Ap-2 (29).

### **7.2.3.2 Negative Regulation - E-cadherin**

Cano *et al.* found that transcription of mouse E-cadherin is under the control of Snai1, a strong repressor that specifically interacts with the mouse E-cadherin promoter (65). It was also found that abnormal expression of Snai1 could underlie the tumorigenic conversion of epithelia associated with the loss of E-cadherin expression (65). Transfection experiments with several epithelial cell lines, showed that Snai1 overexpression leads to a dramatic conversion to a fibroblastic phenotype and induced a tumorigenic and invasive phenotype (65). Likewise, Hajra *et al.* showed that in addition to Snai1, the closely related protein, Slug, could repress the expression of E-cadherin when transfected into human breast cancer cell lines (571). These authors suggested that Slug is likely to function as an E-cadherin repressor in the progression of breast cancer. SIP1, whose specificity partly overlaps with that of Snai1, is a Smad-interacting zinc finger protein that binds to the E-boxes in the E-cadherin promoter. Studies have shown that SIP1 silences E-cadherin expression in much the same manner as Snai1 and that expression of SIP1 in MDCK cells produces invasive cells with decreased expression of E-cadherin (571). Another transcriptional repressor, the E2A gene product has been shown to bind to the E-box region of the mouse E-cadherin promoter and

repress E-cadherin expression (409). Similar to expression by Snai1 and SIP1, expression of the E2A gene is inversely correlated with that of E-cadherin in invasive human carcinomas. Hypermethylation of promoter CpG islands is another method utilized to silence E-cadherin. This has been demonstrated in human carcinomas of the breast, prostate, bladder, colon, and oral cavity (205, 324). In hereditary diffuse gastric cancer, in which there is a mutation in one CDH1 allele, hypermethylation constitutes the second hit eliminating expression of E-cadherin. In a gastric cancer cell line, the expression of E-cadherin could be restored by treatment with the demethylating agent 5-azacytidine (205, 324).

#### **7.2.4 Adhesion Regulation – E-cadherin**

An alternative mechanism for inactivating E-cadherin is to disrupt its connection to the cytoskeleton, thus altering its adhesive properties. For example, mutations in beta-catenin that disrupt its binding to alpha-catenin results in a non-adhesive phenotype (571). In addition, mutations in the alpha-catenin gene effectively inactivate E-cadherin function by not allowing the cadherin complex to associate with the cytoskeleton (571). The adhesive strength of E-cadherin can also be altered by post-translational modification. E-cadherin has a number of serines and threonines within the binding domain that are putative phosphorylation sites for casein kinase I and II and glycogen synthase kinase-3 beta. It was shown that phosphorylation of these sites serves to modulate the affinity of E-cadherin for beta-catenin and thus determines the strength of the resulting cell-cell interactions (571). Furthermore, tyrosine phosphorylation has been implicated in the regulation of cadherin function. Receptor tyrosine kinases (RTKs) such as EGFR, c-met, and FGFR, and the non-

receptor tyrosine kinase c-src, can phosphorylate E-cadherin resulting in disassembly of the cytoplasmic adhesion complex, disruption of cadherin-mediated cell adhesion, and cell scattering (72). Tyrosine phosphorylated E-cadherin targets it for endocytosis and proteasome-mediated degradation (72).

### **7.2.5 Mutations - E-cadherin**

Mutations in CDH1 are the exception rather than the rule as they occur only in a diffuse type of familial gastric cancer, lobular breast cancer, and endometrial cancer (324). The low frequency of E-cadherin mutations is in contrast to the quite frequent disturbance of E-cadherin in invasive and even in preinvasive cancers. This suggests that alternative mechanisms of inactivation, as described above, provide an advantage over mutational inactivation. Although the reasons for this are not entirely clear, it may be related to the reversibility of many of these alternative mechanisms. Perhaps intermittent or periodic downregulation of E-cadherin is a more effective means of promoting tumorigenesis than permanent disablement of the protein.

### 7.3 *N-CADHERIN*

In the adult, N-cadherin is expressed in neural tissue, retina, endothelial cells, fibroblasts, osteoblasts, mesothelium, myocytes, limb cartilage, oocytes, spermatids, and Sertoli cells (106). N-cadherin is involved in cell-cell adhesion, differentiation, embryogenesis, migration, invasion, and signal transduction. Multiple *in vitro* and *in vivo* studies have shown that aberrant N-cadherin expression correlates with an epithelial-mesenchymal transdifferentiation leading to a more motile and fibroblastic phenotype (106). Additional studies have shown that N-cadherin can promote cell survival, particularly in cancers such as melanoma and prostate carcinoma. It does this by recruiting phosphatidylinositol-3-kinase (PI3K) which then activates Akt, resulting in inactivation of the pro-apoptotic molecule Bad (106). Furthermore, N-cadherin can promote survival by inhibiting apoptosis in normal ovarian granulosa cells using an FGFR-dependent mechanism (532). As with E-cadherin, N-cadherin can also have an inhibitory effect on cell proliferation. Overexpression of N-cadherin has been shown to suppress cell proliferation by prolonging the G2/M phase and inducing expression of the cdk inhibitor p21. Another cdk inhibitor, p27, is involved in N-cadherin-mediated contact inhibition of cell growth and cell cycle arrest in the G1 phase (106).

N-cadherin is known to activate several signaling pathways including those involving Rho GTPases and tyrosine kinase signaling via the fibroblast growth factor receptor. N-cadherin interacts directly with the FGF receptor and is thought to signal through this receptor by facilitating dimerization (106, 571). Moreover, as with other cadherins, the cytoplasmic

domain of N-cadherin is complexed with a number of signaling molecules, such as beta- and alpha-catenin (106).

### **7.3.1 Gene/Protein Structure – N-cadherin**

#### **Alternative names:**

Protein: N-cadherin (neuronal), cadherin 2, CDHN

Gene: CDH2, CDHN, NCAD

The N-cadherin gene in both humans and mice is located on chromosome 18 and consists of 16 exons dispersed over more than 200 kb of genomic DNA (394). Wallis *et al.*

demonstrated that the human N-cadherin gene sequence is highly similar to the mouse N-cadherin gene (96% identity) and to other cadherin genes (106, 394, 430). The promoter of N-cadherin does not contain CCATT or TATA boxes, but does have a high CpG dinucleotide content and several consensus Sp1 and Ap2 binding sequences (106). The N-cadherin gene encodes a 907 amino acid protein that has five extracellular cadherin repeats (EC1 to EC5), each with a transmembrane and cytoplasmic domain (106).

### **7.3.2 Tumorigenesis and Metastasis - N-cadherin**

Forced expression of N-cadherin in epithelial-like squamous cells causes downregulation of E-cadherin and the acquisition of a more fibroblastic and invasive phenotype (106, 192). N-cadherin expression in tumors may exhibit re-expression, increased expression, or decreased

expression depending on the cell of origin. For example, mesothelial cells, which already express N-cadherin, can exhibit elevated levels of N-cadherin once neoplastic (pleural mesothelioma) (106); cells such as melanocytes, which only express N-cadherin during embryogenesis, can exhibit re-expression during tumorigenesis (melanoma) (106); and certain cancer types such as osteosarcoma and astrocytoma, exhibit downregulation of N-cadherin during the tumorigenic process (106). N-cadherin expressing cells injected into the mammary fat pad of nude mice have been shown to metastasize widely to the liver, pancreas, salivary gland, omentum, lung, lymph nodes, and lumbar spinal muscle (192). Furthermore, the expression of both E- and N-cadherin was maintained in both the primary tumors and metastatic lesions (192). These data imply that N-cadherin expression during tumor progression might be equally necessary or sufficient to overcome E-cadherin-mediated cell-cell adhesion and to promote malignant tumor progression (45, 84).

### **7.3.3 Motility - N-cadherin**

The motile phenotype induced by N-cadherin appears to be independent of its role in cell-cell adhesion (106). N-cadherin's ability to promote an invasive phenotype results, in part, from its functional interaction with the FGF receptor (FGFR) (193). N-cadherin forms an extracellular complex with the FGFR-1, inhibiting its internalization, and resulting in sustained cell surface expression. This leads to a persistent MAPK-Erk activation, matrix metalloproteinase-9 (MMP-9) expression, and tumor invasion (106). The activated FGFR may also stimulate the activation of diacylglycerol leading to an increase in motility (324). Moreover, Cavallaro *et al.* presented evidence suggesting that N-cadherin can interact with

FGFR-4 and that this interaction is mediated by N-CAM, another cell-cell adhesion molecule of the Ig superfamily. Loss of N-CAM from the complex stimulates metastasis from pancreatic beta-cell tumors, possibly through alteration of FGFR-regulated cell-matrix adhesion (324).

In addition to signaling via the FGF receptor, N-cadherin may enable tumor cells to permeate secondary tissues using its homophilic adhesive mechanisms. It has been proposed that N-cadherin homophilic interactions between tumor cells and N-cadherin-expressing tissues, such as the stroma and the endothelium, might facilitate the transit and survival of tumor cells in distant organs (193). It is also possible that physical association of tumor cells with fibroblasts and/or endothelial cells by N-cadherin provides a juxtacrine mechanism by which tumor cells can induce host cells to produce growth factors and/or proteases that focally support the growth and invasion of tumor cells (193).

#### **7.4 TETRASPANINS - GENERAL**

The tetraspanin superfamily is a group of transmembrane proteins with greater than 30 members (155). Tetraspanins are widely expressed in eukaryotic organisms. Nearly all cell and tissue types contain multiple tetraspanins, often expressed at 30,000-100,000 or more copies per cell (50). Some tetraspanins are found in virtually all tissues (CD81, KAI1, CD9, CD63), whereas others are highly restricted, such as CD37 (B cells) or CD53 (lymphoid and myeloid cells) (315). The focus of this dissertation is on the role of tetraspanins in metastasis and at least three tetraspanins have been implicated in this process, including CD9, CD63, and KAI1 (300).

A major characteristic of tetraspanins in general, is their ability to form cell-surface complexes with other molecules, a characteristic that underlies their role in a number of known functions including cell adhesion, cell signaling, and cell motility (50). Hemler *et al.* reported that over 38 different proteins have been shown to physically associate with one or more tetraspanins (195). These include 11 different integrins, multiple Ig superfamily members (e.g., CD2, CD3, CD4, CDB, MHC class I, MHC class II, etc.), other tetraspanins, proteoglycans (syndecan, CD44), complement-regulatory proteins (CD21, CD46), growth factor receptors and their ligands (EGFR, c-kit, proTGF $\alpha$ , proHB-EGF), and other miscellaneous molecules (CD19, gamma-glutamyl transpeptidase, ADAM10, etc.) (195, 315, 370).

A fundamental role of tetraspanins appears to be organizing other proteins into a network of multi-molecular membrane microdomains, sometimes called the tetraspanin web (50).

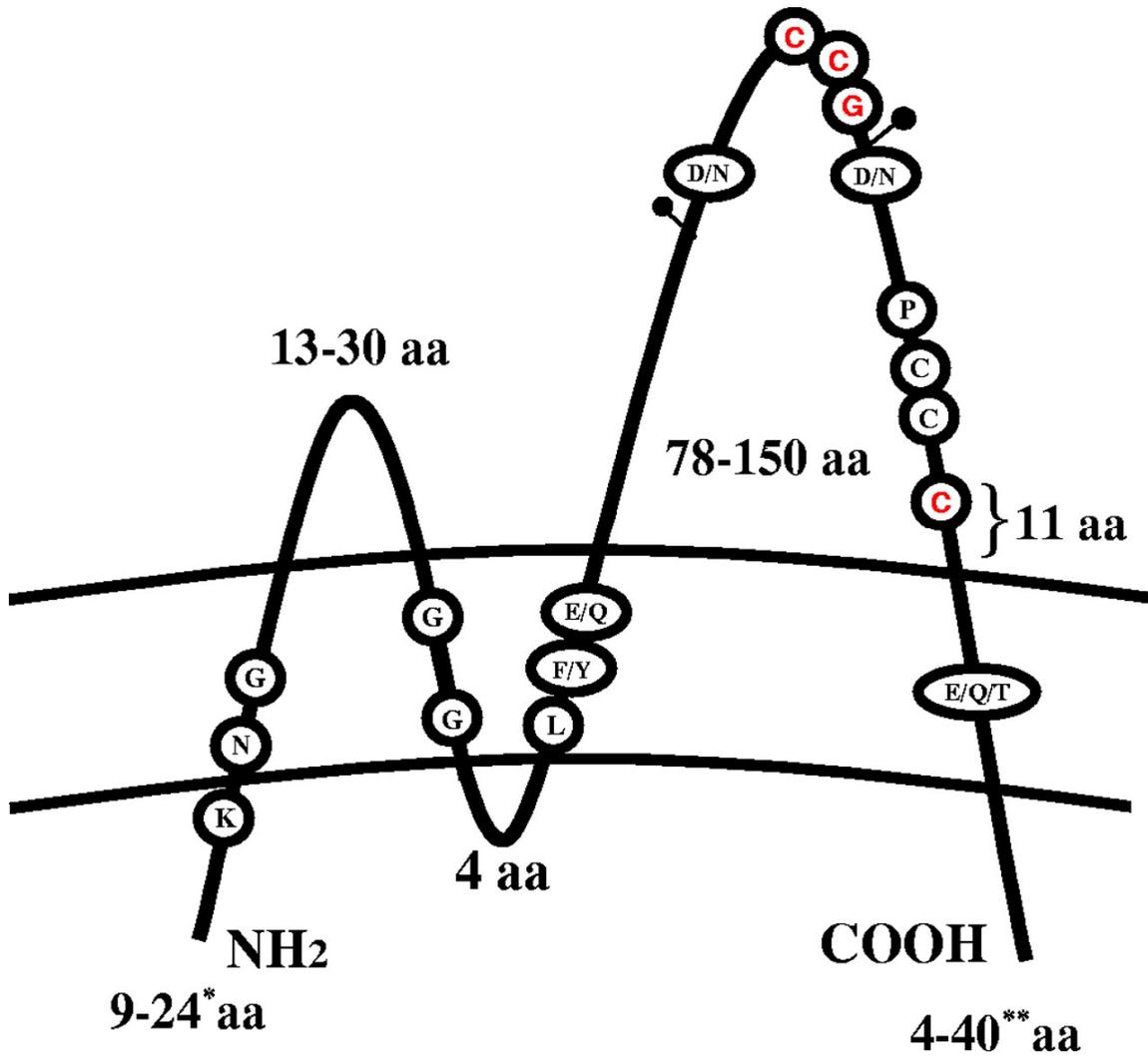
Within this web there are primary complexes in which tetraspanins show specific and direct lateral associations with other proteins (504). The strong tendency of tetraspanins to associate with each other also contributes to the assembly of a network of secondary interactions in which non-tetraspanin proteins are associated with each other using palmitoylated tetraspanins as linker proteins. The additional association of lipids, such as gangliosides and cholesterol, contributes to the assembly of even larger tetraspanin complexes (504). In addition to providing a site for proteins involved in cell-cell adhesion, it appears that the tetraspanin web also provides a scaffold by which membrane proteins can be laterally organized for cell signaling (504). The contribution of tetraspanins to adhesion-dependent signaling is thought to be linked with their ability to recruit certain signaling enzymes into the integrin complexes (37). Within the tetraspanin web, tetraspanin proteins associate with several signaling enzymes including phosphatidylinositol 4-kinase (PI4-kinase) and protein kinase C (PKC). In addition, clustering of alpha3beta1-tetraspanin complexes in breast carcinoma cells was shown to stimulate the PI3-kinase-dependent signaling pathway (37).

#### **7.4.1     *Protein Structure – Tetraspanins***

**Alternative names:** TM4SF (transmembrane-4 superfamily), 4TM, tetraspan

Tetraspanins are glycoproteins that have four hydrophobic, transmembrane domains (TM1-TM4), forming a small and a large extracellular loop (EC1 and EC2) with short intracellular

amino and carboxy cytoplasmic domains. The extracellular loop domains are susceptible to post-translational phosphorylation and/or N-linked glycosylation (37, 195, 315, 370) (Figure 1).



**FIGURE 1.** Schematic of a generic tetraspanin protein (37)

The transmembrane domains are highly conserved and participate in complex formation with other transmembrane molecules (315). The amino- and carboxy-termini of tetraspanins, although well preserved across vertebrate species, exhibit no similarities between the individual family members (37). A hallmark of the tetraspanin superfamily is the presence of a Cys-Cys-Gly sequence (the CCG motif) within the large extracellular loop (EC2) of the protein (37).

## 7.5 *CD9*

CD9 is a tetraspanin that interacts with integrins and other membrane proteins, and is postulated to participate in cell migration, adhesion, proliferation, and differentiation (213).

### 7.5.1 *Gene/Protein Structure – CD9*

**Alternative Names:** Motility related protein 1 (MRP1), MIC3, BA2, P24, DRAP-27, growth-inhibiting gene 2 protein

The human and mouse CD9 genes are located on chromosomes 12 (12p13) and 6, respectively (315, 394). The CD9 gene is composed of 8 exons spanning more than 20 kb of genomic DNA. There is no TATA or CAAT box in the 5' flanking domain of the CD9 gene, but a 120-bp region extremely rich in C and G (88%) contains several Sp1 binding sites and a consensus site for the binding of zinc-finger proteins of the Krox/EGR family (440). The CD9 protein is a 227 amino acid long, 24-kDa molecule with four hydrophobic domains and one N-glycosylation site. There is 89% homology at the amino acid level between the human and mouse CD9 proteins. Most of the differences (19 out of 24) are located in the large extracellular domain encoded by exons 5 and 6 (441).

### **7.5.2 Expression/Regulation/Function – CD9**

CD9 complexes with integrins at the cell membrane and many of its biological functions are thought to be mediated through integrin signaling (213). CD9 is expressed on the cell membrane of various cell types, including hematopoietic cells, endothelial cells, platelets, oocytes, smooth muscle cells, and developing B-lymphocytes (155, 315). It is also strongly expressed by a variety of different epithelial tissues, including the epithelium of the gastrointestinal tract, alveolar epithelium of the lung, and urothelium. However, expression is weak in the pituitary gland, spleen, and hepatocytes, and absent in the testes and spinal cord (214).

CD9 is involved in several activities related to immune system regulation. For example, it has a role in regulating neutrophil adhesion to endothelial cells and is reported to provide a co-stimulatory signal to T cells in the absence of antigen presenting cells (315). CD9 also plays a role as a receptor or co-receptor for certain viruses such as the canine distemper and feline immunodeficiency viruses and for diphtheria toxin (195, 315). CD9 enhances membrane fusion between muscle cells, plays a role in the adhesion and migration of Schwann cells, and is involved in platelet activation and aggregation (195, 315, 370). Kaji *et al.* showed that CD9 is critical in the gamete fusion process at fertilization (242) and Waterhouse *et al.* showed that CD9 is the receptor for the pregnancy-specific glycoprotein, psg17 (562). Pregnancy-specific glycoproteins are a family of highly secreted proteins produced by the placenta.

CD9 can associate with and modulate the function of several growth factor receptors. For example, the c-kit transmembrane receptor tyrosine kinase forms a complex with CD9 in steel factor-dependent cells, such that basal tyrosine phosphorylation is increased, but steel factor-stimulated tyrosine phosphorylation is decreased (195). In addition, CD9 associates with and modulates the functions of pro-TGF- $\alpha$ , pro-HB-EGF, and pro-amphiregulin, all membrane-bound agonists for the EGF receptor. CD9 association markedly increases the potency of these ligands during juxtacrine signaling (202, 229, 473). Although other tetraspanins can associate with HB-EGF, specific residues within CD9 are needed for it to stimulate mitogenic activity (373).

### **7.5.3 Tumorigenesis and Metastasis - CD9**

Experimental and clinical studies have demonstrated that CD9 has a functional role as a tumor metastatic suppressor. Transfection with human CD9 cDNA revealed that cell motility was suppressed in CD9-expressing cells (226). In addition, transfection with human CD9 cDNA suppressed pulmonary metastasis in a spontaneous metastatic model using immunodeficient mice (361). Many clinical studies have revealed that reduced CD9 expression is associated with a poor prognosis in various cancers, including head and neck, non-small-cell lung, breast, pancreatic, gastric, esophageal, and colon cancers (213, 370). CD9 expression in primary melanomas has also been inversely correlated with metastatic potential (315). On the other hand, some *in vitro* experiments have shown that CD9, much like N-cadherin, can play an important role in the process of invasion in some tumor cells (26). Upregulation of CD9 *in vivo* has been documented in aggressive gastric carcinomas (27) and

high-grade astrocytic tumors (155). Of particular relevance to the work described in **manuscript three**, Furuya *et al.* demonstrated that downregulation of CD9 in ovarian carcinoma cells was able to attenuate the expression of several beta1 integrins and rearrange junctional and cytoskeletal molecules (155). In this ovarian carcinoma cell line, integrins alpha2, alpha3, alpha5, and alpha6 but not integrin alpha4 were co-expressed with CD9 on the cell surface, and their expression was attenuated from 6 to 41% by CD9 silencing. These findings suggest that CD9 downregulation is an event involved in the process of ovarian carcinoma metastasis (155).

## **7.6      *KAI1***

KAI1 is a tetraspanin originally identified as a gene that suppressed metastasis of rat prostate tumor cells. Ichikawa *et al.* introduced specific human chromosomes into highly metastatic rat prostatic cells by use of chromosome transfer techniques (225). The introduction of human chromosome 11 resulted in suppression of metastatic ability without suppression of the *in vivo* growth rate or tumorigenicity of the hybrid cells. Dong *et al.* isolated the metastasis suppressor gene and designated it KAI1 for “kang ai” (Chinese for anticancer) (116).

### **7.6.1      *Gene/Protein Structure – KAI1***

**Alternative names:** CD82, kangai 1, ST6, C33 antigen, inducible membrane protein R2, IA4, 4F9

The human and mouse KAI1 genes are located on chromosomes 11 (11p11.2) and 2, respectively (233, 315, 394). The gene has 10 exons and 9 introns, and spans a total of about 80 kb of DNA (115). The 5' flanking region of the KAI1 gene lacks both TATA and CCAAT boxes, is associated with a CpG island, and has many putative binding motifs for various transcription factors including p53, Ap2, Ap1, and Sp1 sites (159, 233, 394).

KAI1 specifies an evolutionarily conserved protein of 267 amino acids, with four hydrophobic transmembrane domains and one large extracellular hydrophilic domain

containing three potential N-glycosylation sites (233, 394). The protein also has cytoplasmic amino- and carboxy-terminal domains and two extracellular loops (137). Due to glycosylation, KAI1 has a molecular mass of 46-60 kDa, rather than 28 kDa as would be expected based on amino acid content (233).

### **7.6.2 Expression/Regulation/Function – KAI1**

KAI1 was first identified as being strongly upregulated in mitogen-activated human T cells. However, expression of KAI1 is not restricted to leukocytes as it is also expressed at high levels on many other cell and tissue types including lung, liver, kidney, breast, prostate, gastrointestinal tract, and a variety of other epithelial tissues, as well as bone marrow, endothelial cells, and fibroblasts. Interestingly, KAI1 protein expression is absent in several CD9 positive tissues (e.g., smooth muscle, adrenal cortex, urothelium, myelin of peripheral nerves, and amniotic epithelium) but strongly expressed in certain CD9 negative cells (e.g. spinal cord gray matter) (159, 214, 233, 300). KAI1 interacts with a variety of other proteins including the integrin alpha4beta1, E-cadherin, other tetraspanins, and cell surface molecules including, CD4, CD8, CD19, CD21, and MHC class I and class II molecules on lymphocytes (233).

There have been occasional reports suggesting a role for KAI1 in regulating cell survival (606), although KAI1 is best known for its role as a metastasis suppressor protein. Reduced KAI1 expression is associated with altered adhesion to specific components of the extracellular matrix, reduced cell-cell interactions, and increased cell motility, leading to a

more invasive and metastatic phenotype. Numerous studies suggest that the mechanisms of decreased KAI1 expression does not involve promoter methylation, gene mutation, or allelic loss of the KAI1 gene (159). Therefore, the expression of this gene appears to be downregulated at the post-transcriptional level, presumably by the loss of an activator or gain of a suppressor (137, 332). The exact mechanism(s), however, remain to be elucidated. The recent identification of signaling pathways downstream of KAI1, and proteins that specifically interact with KAI1, are beginning to elucidate the biological pathways involving KAI1 (233).

Upstream factors regulating KAI1 transcription are not clearly defined, however, phorbol ester elevates KAI1 mRNA levels in prostate cancer cell lines suggesting a role for protein kinase C (7). Likewise, nerve growth factor upregulates KAI1 mRNA levels in prostate cancer cells (480). Mashimo *et al.* found that p53 could directly activate the KAI1 gene by interacting with the 5' upstream region (332). The p53 responding region is located approximately 860 bases upstream of the transcriptional initiation site, and contains a typical tandem repeat of the p53 consensus binding sequence. Mutations of this sequence abolish the responsiveness to p53 and the ability to bind to p53 protein. Immunohistochemical analysis of 177 samples of human prostate tumors showed that the expression of the KAI1 gene correlated strongly with that of the p53 gene and that the loss of these two markers resulted in poor survival of patients. The data indicated a direct relationship between the p53 and KAI1 genes and suggested that loss of p53 function leads to downregulation of the KAI1 gene (332).

KAI1 can directly interact with the epidermal growth factor receptor (EGFR) and attenuate EGFR-induced lamellipodia formation and migration, by regulating dimerization and internalization of the EGFR (233). This interaction may in part explain the tumor suppressor activity of KAI1 (195). In addition, KAI1 can suppress motility by decreasing signaling via the FAK-Lyn-p130CAS-CrkII pathway, which regulates organization of the actin cytoskeleton (233). One study found that KAI1 interactions with CD3 resulted in cytoskeletal changes that were dependent on Rho GTPase activity (285). KAI1 associated protein (KASP) and KAI1 carboxy-terminal interacting tetraspanin (KITENIN) are two newly identified KAI1-binding proteins likely involved in KAI1 regulation (233). KASP is a member of the immunoglobulin superfamily and inhibits cell migration. Binding of KAI1 to KASP enhances this effect in a synergistic manner. In contrast, KITENIN enhances invasion and metastatic behavior of tumor cells, and binding to KAI1 inhibits these effects (233).

### ***7.6.3 Tumorigenesis and Metastasis – KAI1***

Downregulation of KAI1 has been reported to be associated with progression, invasion, and metastasis of a large number of malignancies (455). These tumors tend to have an aggressive clinical behavior resulting in shorter patient survival time. KAI1 has been shown to inhibit pulmonary metastases in experimental metastasis models of prostate cancer and melanoma (455). KAI1 expression is decreased in the progression of lymphoid neoplasms and several solid epithelial tumors, including cancers of the lung, prostate, breast, esophagus, cervix, colon, endometrium, stomach, pancreas, skin, oral cavity, liver, urinary bladder, and now ovary (**manuscript three**) (137, 159, 300, 394, 455).

## CHAPTER EIGHT

### MANUSCRIPT THREE

(Published in *Gynecologic Oncology* 2002 Jul; 86 (1):69-78)

## LOSS OF EXPRESSION AND ALTERED LOCALIZATION OF KAI1 AND CD9 PROTEIN ARE ASSOCIATED WITH HUMAN EPITHELIAL OVARIAN CANCER PROGRESSION

### 8.1 ABSTRACT

Impairment of cell adhesion plays a vital role in tumor progression. E- and N-cadherin, CD9, and KAI1 are all adhesion molecules that have been implicated in the progression of several different tumor types. To help explain the potential role these adhesion molecules have in ovarian cancer, comparisons were made between expression patterns in normal ovary and various grades of primary and metastatic epithelial ovarian cancers. Thirty-two primary and eight metastatic human ovarian epithelial carcinomas and eighteen samples of normal ovarian tissue were examined for adhesion molecule expression using immunohistochemistry. KAI1 and CD9 revealed an inverse relationship between tumor grade and expression levels, characterized by high expression in low-grade tumors and low expression in high-grade tumors and metastases. KAI1 and CD9 also demonstrated a shift in cellular localization from the membrane in grade 1 tumors to the cytoplasm in grade 3 tumors. N-cadherin expression showed a positive trend between expression levels and tumor grade. E-cadherin expression varied little between different tumor grades and metastases. Inclusion cysts and surface invaginations often strongly expressed KAI1, CD9, and E-

cadherin. KAI1 expression was variable in ovarian follicles and corpora lutea depending on their stage of development. These findings suggest that progression of ovarian epithelial carcinomas is associated with downregulation and altered cellular localization of KAI1 and CD9. In addition, variable KAI1 expression during follicular and luteal development suggests it is hormonally regulated in the ovary. Further investigation will be needed to see if it is also regulated this way during progression of ovarian cancers.

## **8.2 INTRODUCTION**

Cell adhesion is mediated by a diverse group of cell surface glycoproteins that play critical roles in a wide variety of biologic processes including embryogenesis, maintenance of cell polarity, cell growth, cell differentiation, and inflammation (128, 518). Impairment of cell adhesion is a characteristic of neoplastic cells and plays a vital role in tumor progression particularly with regard to invasive and metastatic properties (128, 207). KAI1 (CD82), CD9, E-cadherin, and N-cadherin are four adhesion molecules that have been implicated in the progression of a number of different tumors (41, 70, 99, 115, 205, 212, 300, 385, 518, 538).

KAI1 and CD9 belong to the tetraspanin superfamily and are expressed in a wide variety of tissues (315, 580). Although the precise biological functions of tetraspanins are not fully elucidated, several studies have shown involvement in cell growth, adhesion, and motility (315, 477, 580). KAI1 and CD9 bind to each other and to a growing list of other proteins including other tetraspanins and several different types of integrins (primarily beta-1 integrins) (38, 315, 580). In addition, KAI1 interacts with E-cadherin (6). Previous studies have shown a correlation between decreased expression of either KAI1 or CD9 and

metastasis (212, 477, 538). KAI1, while originally shown to be involved in lymphocyte function (162) was later defined as a metastasis suppressor in prostate cancer (117).

Cadherins are a family of transmembrane glycoproteins that mediate calcium-dependent homotypic cell-cell adhesion (475, 518). Expression of each cadherin displays a certain degree of cell specificity, although cells can express more than one type (128). E-cadherin is expressed by most epithelial cells while N-cadherin is expressed in neural tissue, the lens of the eye, muscle, and kidney (128). Like KAI1 and CD9, cadherin-mediated adhesion is modulated in a variety of different tumor types during the acquisition of a malignant phenotype (518). E-cadherin is downregulated with malignant progression in several different epithelial tumor types (41). Elevated N-cadherin expression is reported to correlate with increased cell motility as well as invasive and/or metastatic phenotypes (60, 385).

The objective of this study was to examine expression of the cadherins, KAI1, and CD9 in normal and neoplastic ovarian tissue to better understand the biologic function of these molecules in the ovary and determine how they may be involved in ovarian tumorigenesis and progression.

## **8.3 MATERIALS AND METHODS**

### **8.3.1 Tissue Samples**

Samples of frozen ovarian tumors were obtained from the Southern Division of the Cooperative Human Tissue Network. These consisted of 32 patients with primary epithelial ovarian cancer and 8 patients with metastatic ovarian epithelial cancer. Histologic diagnoses

were initially made by an attending pathologist and submitted with the tissues. Hematoxylin and eosin stained slides of all samples were re-examined before performing immunohistochemistry, and the original histopathologic diagnoses were confirmed. The 32 primary ovarian tumors included 17 serous carcinomas, 8 endometrioid carcinomas, 4 mucinous carcinomas, and 3 clear cell carcinomas. The metastatic tumors included 4 serous carcinomas, 2 endometrioid carcinomas, 1 mucinous carcinoma, and 1 poorly differentiated carcinoma. Additional samples of normal ovarian tissue were obtained from 18 women who underwent ovariectomy under approved internal review board protocols.

### **8.3.2     *Immunohistochemistry***

Frozen ovarian tissues were sectioned (6 micrometer) on a cryostat and immediately fixed in Rapid Fixx™ (Shandon-Lipshaw, Pittsburgh, PA) for 7 seconds. The slides were immersed in 1X automation buffer (Biomedex, Foster City, CA) until all sections were cut. After the last section was cut, the slides were rinsed twice in 1X automation buffer for 5 minutes each. Endogenous peroxidases were blocked by immersion in 0.3% H<sub>2</sub>O<sub>2</sub> (Fisher Scientific, Fair Lawn, NJ) for 30 minutes at room temperature. Following a 5 minute rinse in 1X automation buffer, the slides for KAI1, E-cadherin, and N-cadherin were immersed in citrate buffer (Biocare Medical, Walnut Creek, CA) for heat induced epitope retrieval (HIER). CD9 slides required no antigen retrieval and were loaded directly onto an automated stainer (Nexes, Ventana Medical Systems, Tucson, AZ). The primary antibody for CD9 (monoclonal M31-15, a kind gift from Dr. Miyake (362) was applied at a dilution of 1:3000. For antigen retrieval, the KAI1 slides were placed into a steamer (Black & Decker, Towson, MD) for 30

minutes followed by a 20 minute cool down period. E-cadherin and N-cadherin slides were placed into a pressure cooker (Decloaker, Biocare Medical, Walnut Creek, CA) for 5 minutes followed by a 10 minute depressurization period. Following HIER, slides were rinsed twice in distilled water for 3 minutes each. E-cadherin and N-cadherin slides were then loaded onto an automated stainer (Nexes, Ventana Medical Systems, Tucson, AZ) in which the primary antibodies (monoclonal E-cadherin, Transduction Laboratories, Lexington, KY and monoclonal N-cadherin, Zymed Laboratories, San Francisco, CA) were both applied at a 1:100 dilution. KAI1 slides, which were stained manually, were placed into a humidified chamber and blocked with 5% normal horse serum (Jackson ImmunoResearch, West Grove, PA) for 20 minutes at room temperature. An avidin block (Vector Laboratories, Burlingame, CA) was then applied for 15 minutes followed by a quick rinse in 1X automation buffer and then a 15 minute biotin block (Vector Laboratories, Burlingame, CA). Immediately following the biotin block, slides were incubated with mouse anti-KAI1 primary antibody (monoclonal C33 antibody, a kind gift from Dr. Yoshie (227) using a 1:100 dilution for 1 hour at room temperature. After incubation with the primary antibody, slides were washed in 1X automation buffer for 5 minutes followed by application of the secondary antibody (biotinylated horse anti-mouse; Vector Elite Kit, Burlingame, CA) at a dilution of 1:200 for 30 minutes at room temperature. After a quick rinse in 1X automation buffer, the slides were incubated for an additional 30 minutes at room temperature with the ABC Elite complex (Vector Elite Kit, Burlingame, CA). The slides were then rinsed for 5 minutes in 1X automation buffer followed by application of the chromagen-substrate reagent 3, 3'-diaminobenzidine (DAB, DAKO, Carpinteria, CA) for 6 minutes at room temperature in the dark. Rinsing in tap water for 3 minutes stopped this reaction. All slides were counterstained

in Harris' hematoxylin (Fisher, Pittsburgh, PA), dehydrated through a graded series of ethanol and xylene washes and then cover-slipped in Permount (Fisher, Pittsburgh, PA). The staining procedure for CD9, E-cadherin, and N-cadherin followed a similar protocol adapted for use in an automated stainer. Tissue known to express the relevant antigens were used as positive controls (human colon for E-cadherin, CD9 and KAI1; rat brain for N-cadherin). Negative controls were performed for all samples by replacing the primary antibody with normal mouse serum (Jackson ImmunoResearch, West Grove, PA) diluted to a protein concentration equivalent to that of the primary antibody.

### **8.3.3 *Tumor Grading***

All tumors were classified as ovarian epithelial carcinomas and graded on a scale of 1 to 3 using hematoxylin and eosin stained sections. Tumor grading was based on the Gynecologic Oncology Group (GOG) grading system, which takes into consideration both architectural and cytologic feature of the tumor (30). Grading of immunohistochemistry slides was done independently by two pathologists (CDH, XYD) using the quickscore method described previously (107). In brief, this method evaluates both the average intensity of staining and the proportion of malignant cells staining. The average intensity was graded on a scale of 0 to 3 while the proportion of cells staining for each of the antigens was graded on a scale of 1 to 6. The two scores are multiplied to arrive at a quickscore. Quickscores for both membranous and cytoplasmic staining were determined.

### **8.3.4     *Statistics***

Differences in staining between tumor grades were analyzed by Wilcoxon Rank Sum analysis and post hoc analysis was done using the Student t test (JMP statistical software, SAS Institute Inc, Cary, NC). Statistical significance was set at  $p < 0.05$ .

## **8.4        *RESULTS***

### **KAI1, CD9, E-cadherin, and N-cadherin expression levels in ovarian tumors**

Forty ovarian carcinomas, including 22 serous, 10 endometrioid, 5 mucinous, and 3 clear cell, were examined for expression levels of KAI1, CD9, E-cadherin, and N-cadherin. Both cell membrane and cytoplasmic staining were graded and recorded semi-quantitatively as a quickscore. Comparisons were made between various tumor grades and tumor types.

A summary of expression levels between tumor types is shown in Table 1. E-cadherin expression levels were high in each of the four different tumor types. Moderate to strong KAI1 and CD9 expression was noted in serous, endometrioid and mucinous tumors. In contrast, CD9 expression was minimal and KAI1 expression was almost undetectable in clear cell carcinomas. N-cadherin expression levels were similar between serous and endometrioid carcinomas, although, little expression was apparent in mucinous or clear cell carcinomas. Overall, clear cell carcinomas expressed large amounts of E-cadherin, however, KAI1, CD9, and N-cadherin expression was minimal to absent.

A summary of membranous and cytoplasmic expression levels between tumor grades is shown in Figures 1 and 2, respectively. Of the forty tumors examined there were seven grade 1, twelve grade 2, ten grade 3, and eight metastases. Analysis of membrane expression among the various tumor grades revealed an inverse relationship between tumor grade and levels of KAI1 and CD9 (Figure 1 and Figure 3). Statistically significant differences were noted between high expression levels of KAI1 and CD9 in low-grade tumors and low expression levels in high-grade tumors and metastases (Figure 1). Although not statistically significant, N-cadherin membrane expression showed a positive trend correlating low expression levels in low-grade tumors and high expression levels in metastases (Figure 1 and Figure 4). Membrane expression levels of E-cadherin were relatively high with little variation between different tumor grades and metastases (Figure 1 and Figure 4).

Expression of each adhesion molecule was primarily localized to the cell membrane, however, variable amounts of cytoplasmic KAI1 and CD9 expression were also apparent (Figure 5). Cytoplasmic expression of KAI1 and CD9 was most prominent in grade 3 tumors (Figure 2). KAI1 and CD9 both revealed the same trend of low cytoplasmic expression in low-grade tumors and high cytoplasmic expression in high-grade tumors, however, only KAI1 showed statistical significance (Figure 2). KAI1 and CD9 staining was also noted in the stroma immediately adjacent to tumor cells.

#### **KAI1, CD9, E-cadherin, and N-cadherin expression levels in normal ovaries**

Normal ovarian surface epithelium often lacked expression for each of the four adhesion molecules, however, occasional focally intense regions of KAI1, CD9, and E-cadherin

expression were noted in areas that displayed a plump cuboidal morphology (Figure 7). Surface epithelial invaginations and epithelial inclusion cysts revealed intense expression of KAI1, CD9, and E-cadherin, although, N-cadherin expression was often weak to absent in these structures (Figure 6). Granulosa cells of small growing follicles expressed variable amounts of each adhesion protein, however, KAI1 in particular seemed to display a distinct pattern of expression in relation to follicular and luteal development (Figure 8). KAI1 expression was found in primordial follicles as well as in granulosa cells of variably sized small growing follicles. Differentiated granulosa cells in antral follicles lacked KAI1 expression, however, surrounding theca cells continued to express KAI1 protein. Early corpora lutea stained positively for KAI1 and CD9, although expression decreased as the corpora lutea aged (Figure 7 and Figure 8). Staining was most intense on the cell membranes of luteal cells. The cadherins were not expressed in any corpora lutea and corpora albicans completely lacked expression of any of the four adhesion molecules. KAI1 and CD9 staining was noted in the tunica media of most arterioles and small to medium sized arteries (Figure 9). KAI1 protein was particularly concentrated in the region of the tunica intima including the internal elastic lamina. CD9 expression was more diffuse throughout the tunica media and, as previously described, did show expression in smooth muscle cells (214). Also consistent with previous studies, small amounts of N-cadherin expression was noted on the cell membrane of endothelial cells (379).

## 8.5 *DISCUSSION*

These results suggest that the malignant progression of epithelial ovarian carcinomas is associated with downregulation and altered cellular localization of KAI1 and CD9. These findings are consistent with a recent report showing decreased expression of KAI1 in association with progression of ovarian epithelial carcinomas (295). In addition, this study shows that KAI1 and CD9 expression is low to absent in normal surface epithelium, then subsequently increases in surface epithelial modifications and low-grade tumors, followed by progressive loss of expression as tumors develop into a more malignant phenotype. Such a biphasic pattern of KAI1 expression is also noted in prostatic and pancreatic cancers (52, 175). In addition to downregulation, these results are the first to suggest that a shift in localization of KAI1 and CD9 from the membrane in grade 1 tumors to the cytoplasm in grade 3 tumors may be another mechanism by which tumor cells lose their adhesive properties as they evolve toward a metastatic phenotype.

In contrast to KAI1 and CD9, E-cadherin expression remained at high levels throughout all tumor grades and metastases. This finding is consistent with a previous report showing elevated levels of E-cadherin throughout all stages of ovarian cancer progression (514). This suggests that E-cadherin may not play a significant role in ovarian tumor metastases as it does in a variety of other epithelial cancers. However, this study did not investigate other aspects of E-cadherin, such as its interaction with catenins, and thus the functionality of this protein cannot be determined. N-cadherin expression, particularly in endometrioid and serous carcinomas, gradually increased with tumor grade and peaked in expression in metastases. Although not statistically significant, this trend suggests that increased expression of N-

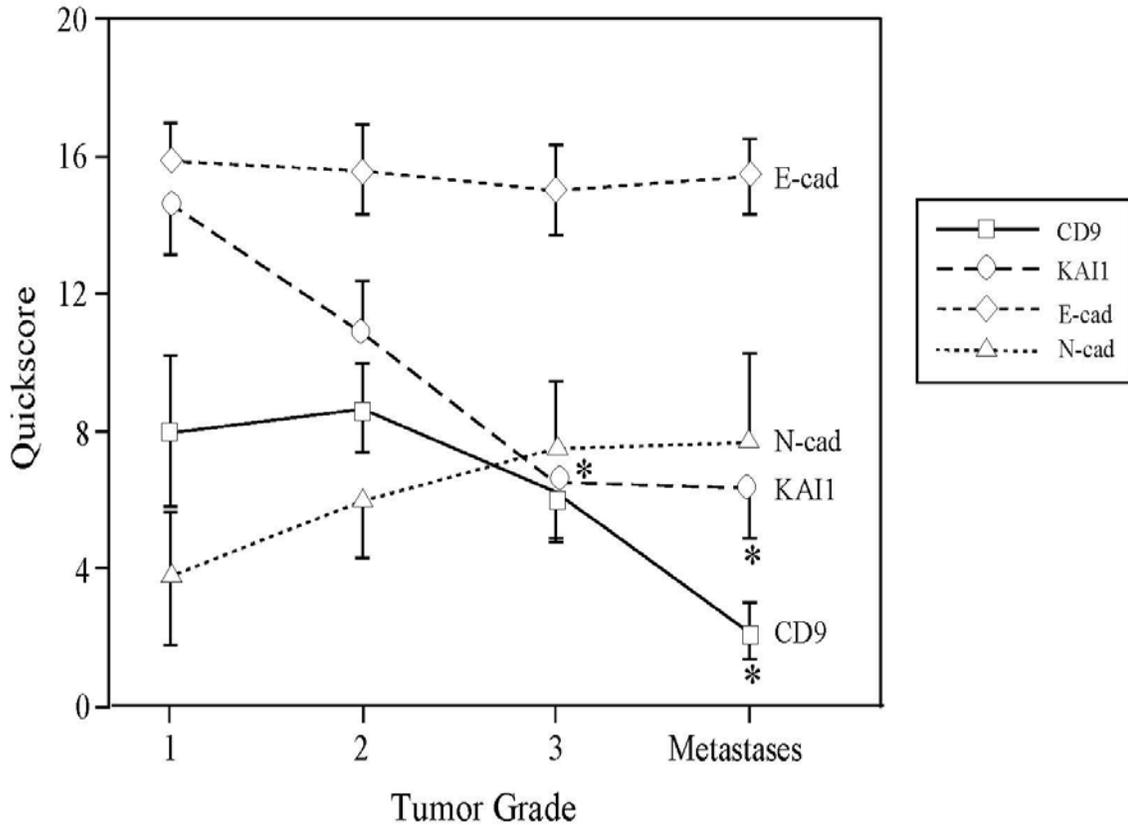
cadherin is associated with a more malignant phenotype in ovarian epithelial tumors. Similar findings have been reported for prostate and breast cancer cell lines in which elevated N-cadherin expression is associated with tumor cell invasion and motility (60, 385).

Expression of each adhesion molecule on normal surface epithelium was quite variable suggesting that they are differentially regulated as a part of normal ovarian physiology. Overall, a large part of the normal surface epithelium either lacked or had minimal expression of any of these markers except in areas where the surface epithelium displayed a plump cuboidal (epithelial) phenotype. This epithelial phenotype was most prominent in surface epithelial invaginations and epithelial inclusion cysts, which often showed intense expression for KAI1, CD9, and E-cadherin. Similar results have been reported previously for E-cadherin (514). A cause for this differential regulation is not certain, although it is quite possible that peptide hormones or growth factors mediate many of these effects. Further evidence to support potential hormonal modulation of these adhesion molecules was revealed in their differential expression in developing ovarian follicles. In particular, KAI1 was highly expressed in granulosa cells of growing follicles, clearly downregulated in pre-ovulatory follicles, but upregulated again as cells differentiated in the formation of corpora lutea. As KAI1 is an anchoring protein for cells, the distinct regulation of this protein in the periovulatory period in follicles suggests it plays a role during ovulation and release of cells and the egg during the process of ovulation. Moreover, KAI1 was downregulated in older corpora lutea and corpora albicans, again suggesting that this adhesion molecule plays a role in the processes of luteolysis, involution, and remodeling of the ovary.

Although KAI1 is well known as a prostate cancer gene, little is known about its function and control in other hormonally responsive tissues. These results provide evidence that KAI1 is differentially regulated during normal ovarian function and neoplastic progression.

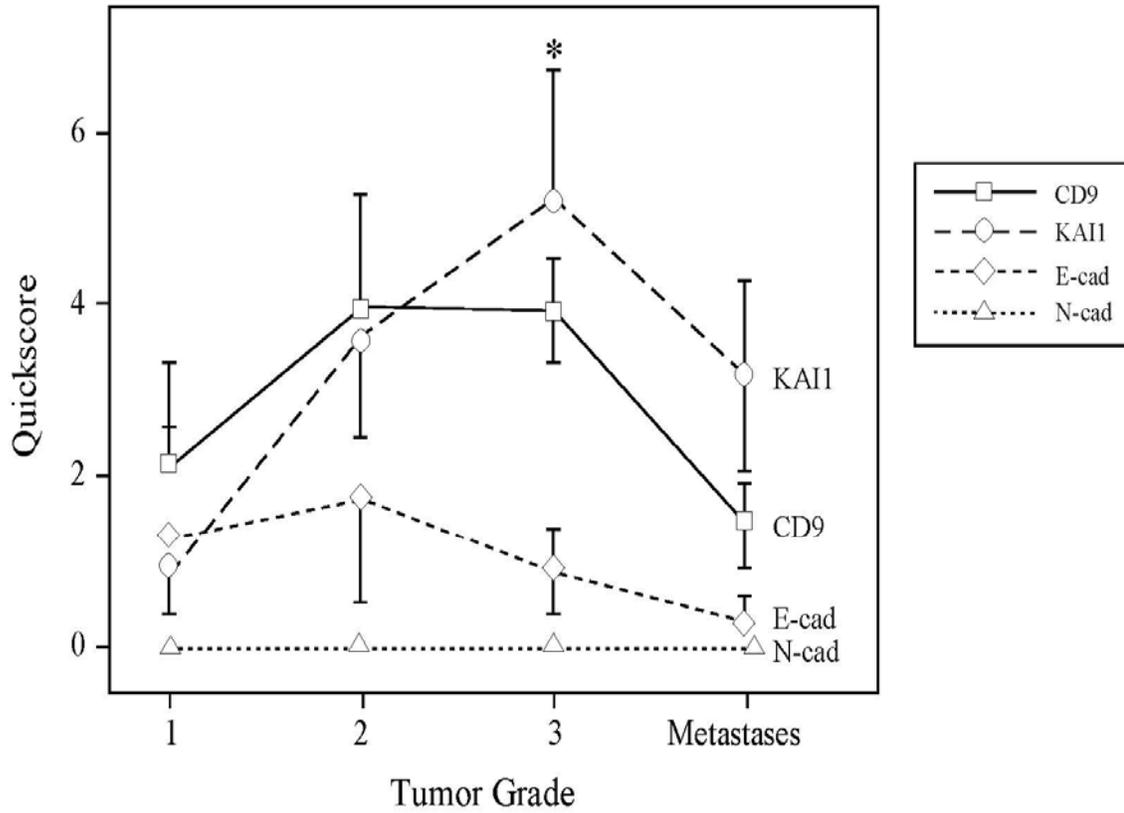
Certainly, a better understanding of the regulation and function of normal surface epithelium, particularly in regards to hormonal and growth factor regulation, would help further elucidate the role these adhesion molecules may play in the early stages of ovarian tumorigenesis.

Figure 1



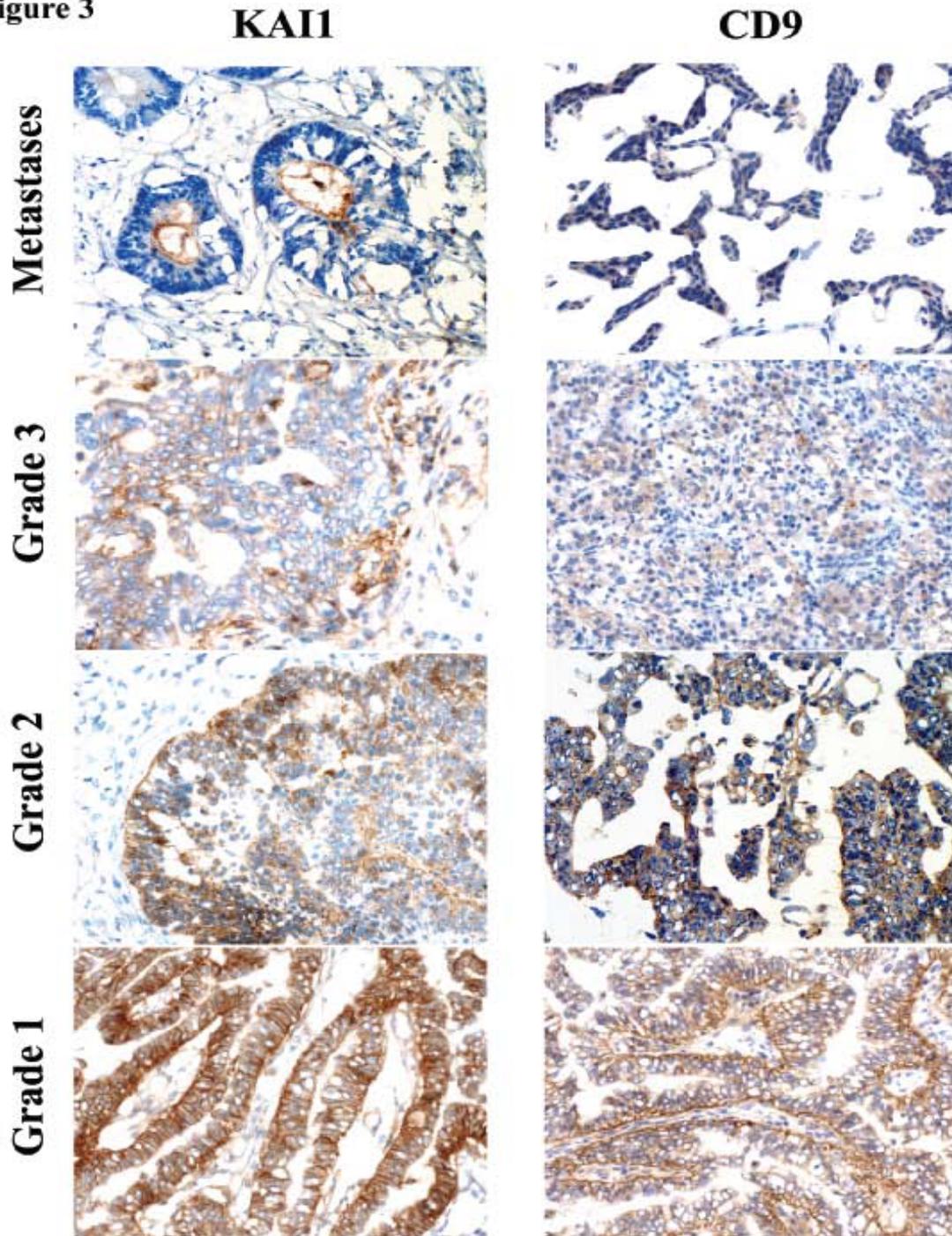
**Figure 1.** Correlation of membrane expression (quickscore) with tumor grade. Note the relative decrease of KAI1 and CD9 expression as tumors progress from low grade to high grade. Also, note the high level of E-cadherin expression throughout all tumor grades. The results shown represent the mean  $\pm$  SD of quickscores from all graded tumors including serous, endometrioid, and mucinous carcinomas. \* Significant differences between tumor grades were determined using the Student t test. KAI1 metastases versus grade 1 tumors ( $P = 0.002$ ) and KAI1 grade 3 tumors versus grade 1 tumors ( $P = 0.013$ ). CD9 metastases versus grade 2 tumors ( $P = 0.002$ ) and CD9 metastases versus grade 1 tumors ( $P = 0.024$ ).

Figure 2



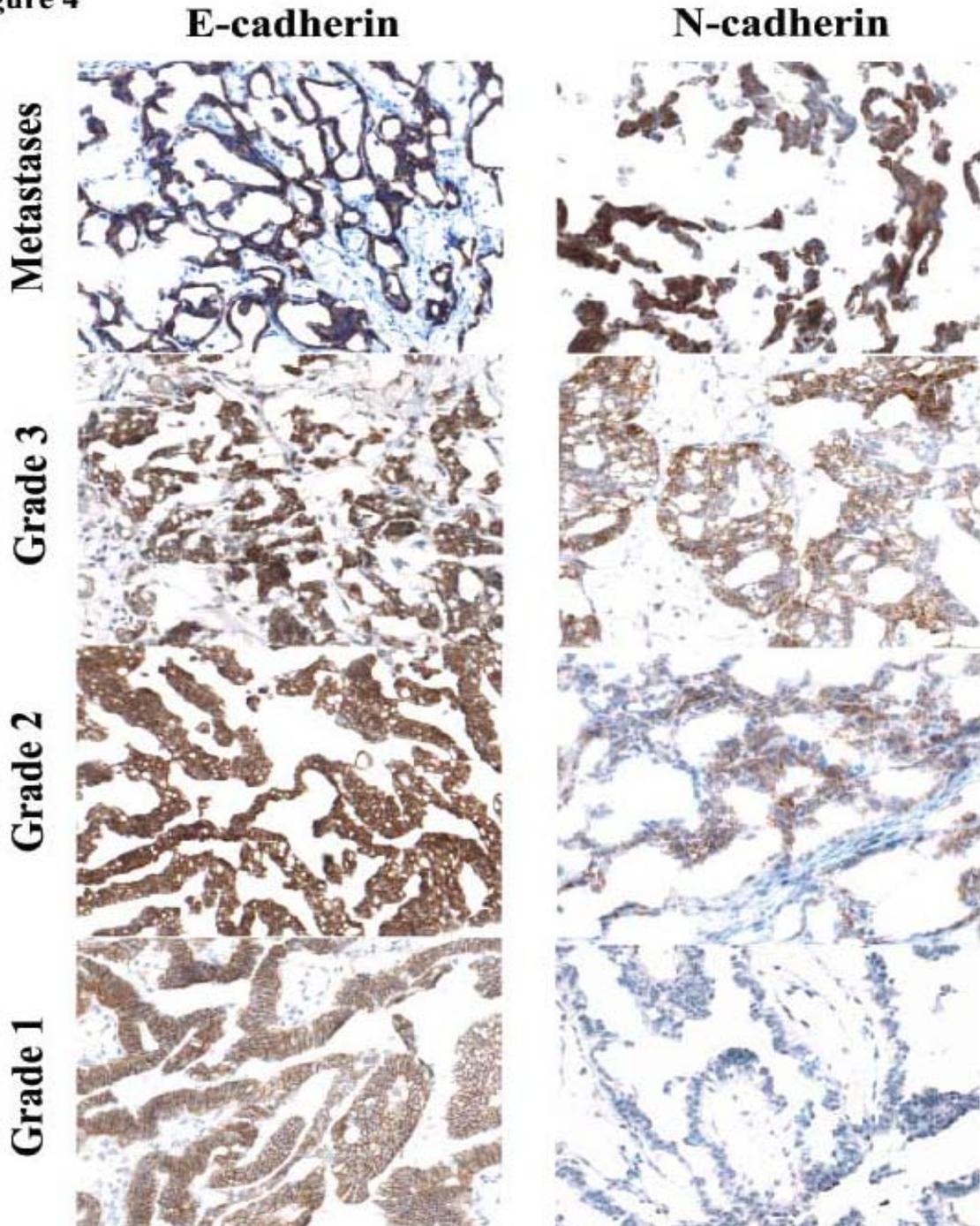
**Figure 2.** Correlation of cytoplasmic expression (quickscore) with tumor grade. Note the increase in cytoplasmic KAI1 protein as tumors progress from grade 1 to grade 3. CD9 reveals a similar but less dramatic trend. The results shown represent the mean  $\pm$  SD of quickscores from all graded tumors including serous, endometrioid, and mucinous carcinomas. \*Significant differences between tumor grades were determined using the Student t test. KAI1 grade 3 tumors versus grade 1 tumors (P = 0.035).

**Figure 3**



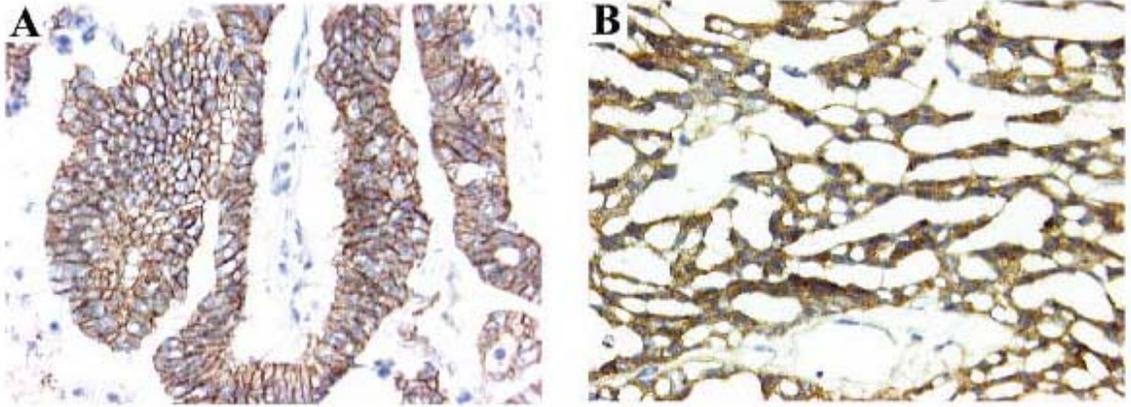
**Figure 3.** Immunohistochemical detection of KAI1 and CD9 in representative ovarian carcinomas. Note the relative decrease in expression as tumors progress from grade 1 to metastases. Original magnifications x200.

**Figure 4**



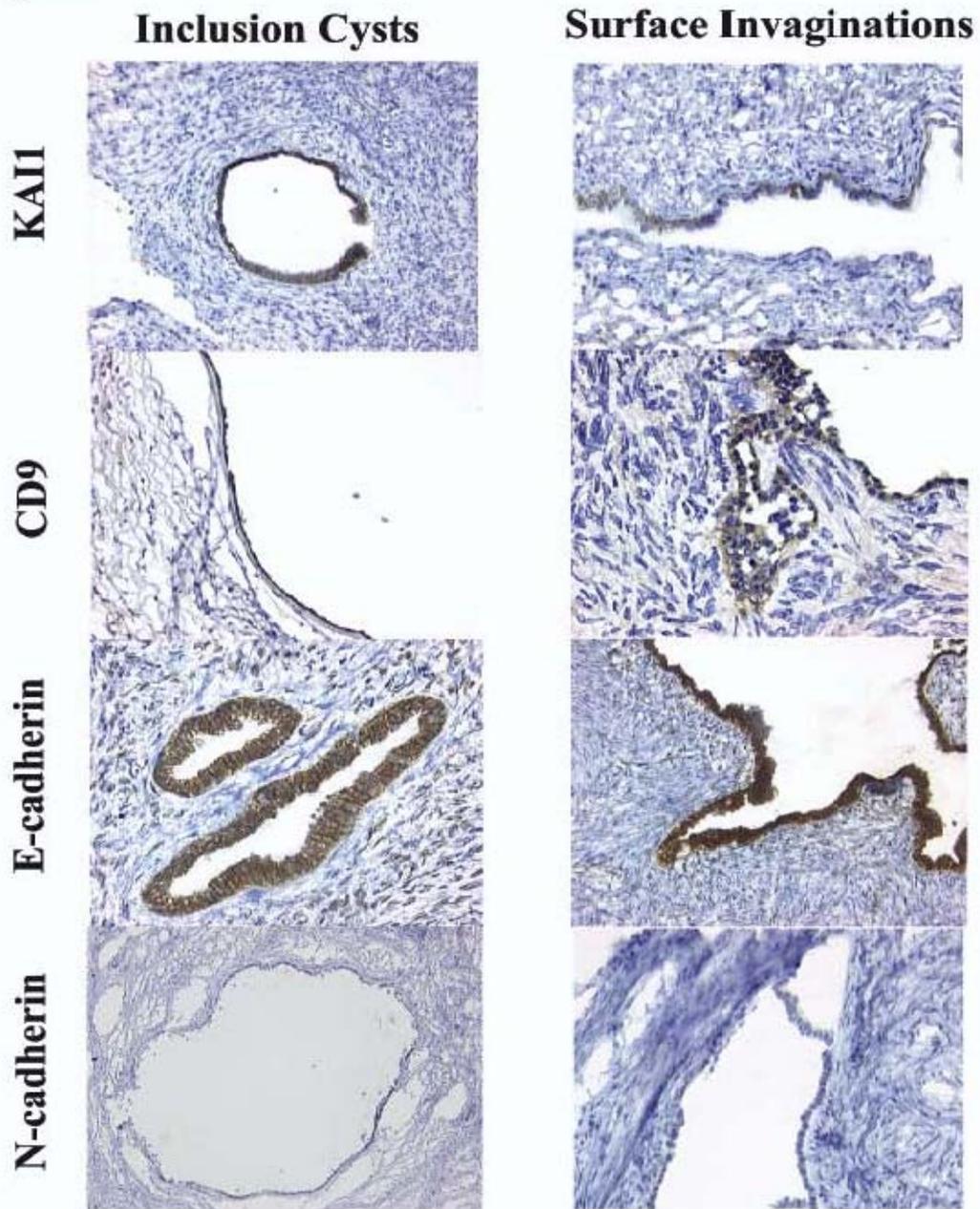
**Figure 4.** Immunohistochemical detection of E and N-cadherin in representative ovarian carcinomas. Note the relatively high levels of E-cadherin expression throughout all tumor grades. Also, note the relative increase in N-cadherin expression as tumors progress from grade 1 to metastases. Original magnifications x200.

**Figure 5**



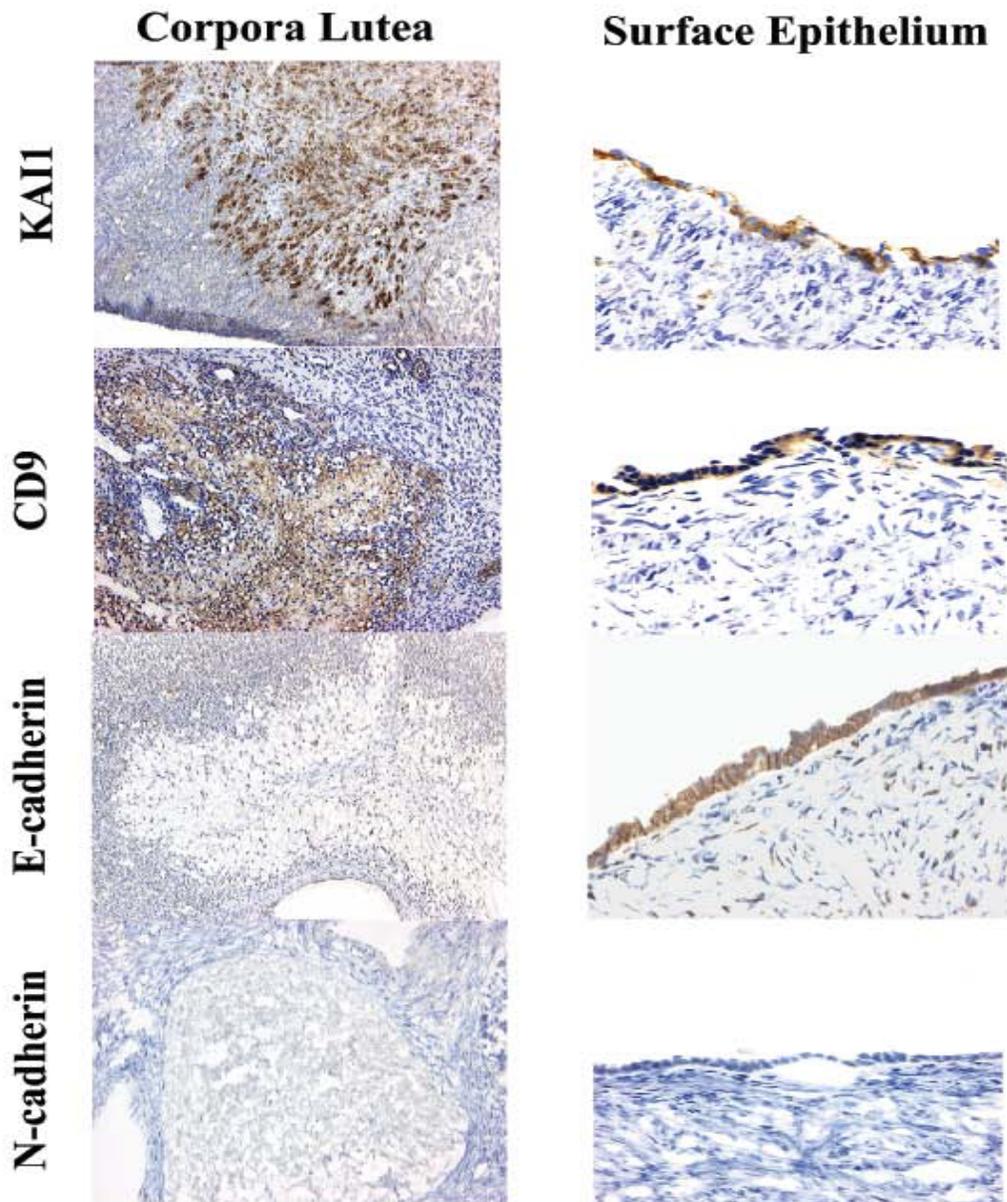
**Figure 5.** Immunohistochemical detection of KAI1 in representative ovarian carcinomas. Note the sharp outline of staining around cell membranes (A) as opposed to diffuse cytoplasmic staining (B). Original magnifications x400.

**Figure 6**



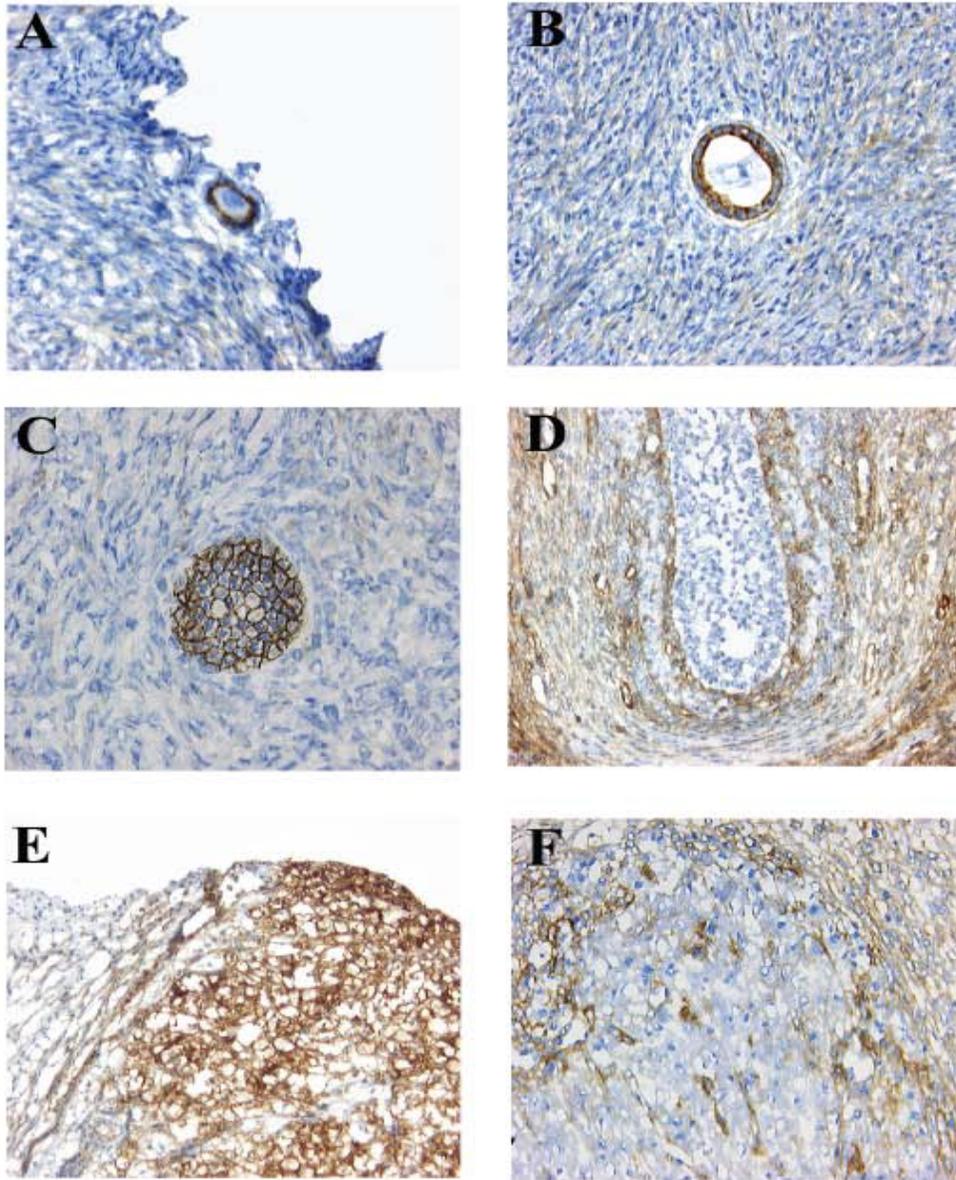
**Figure 6.** Immunohistochemical detection of all four adhesion molecules in ovarian cysts and surface invaginations. Both structures express high levels of KAI1, CD9, and E-cadherin although N-cadherin expression was often low to absent. Original magnifications x200

**Figure 7**



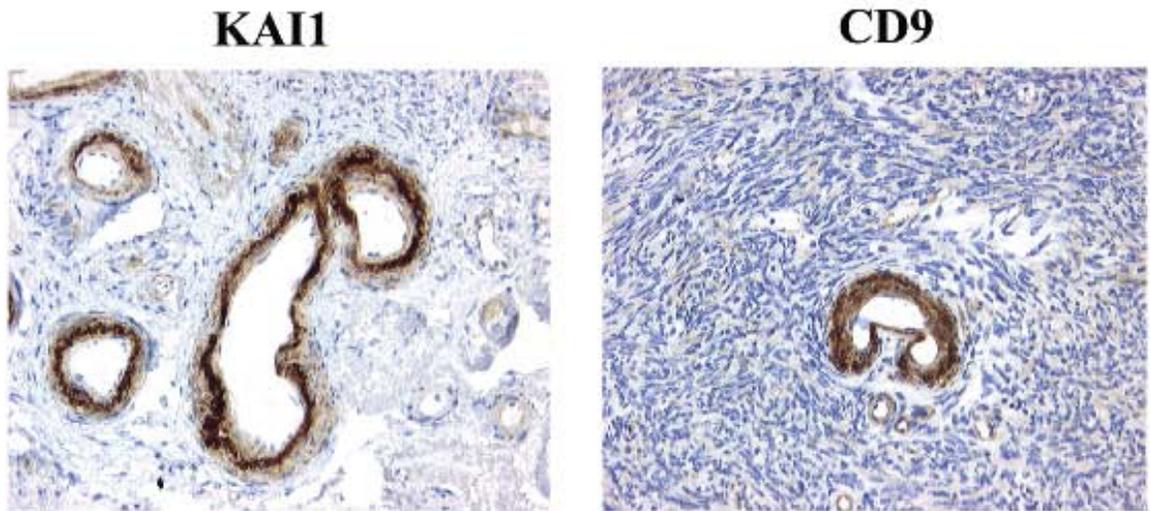
**Figure 7.** Immunohistochemical detection of all four adhesion molecules in corpora lutea and surface epithelium. KAI1 and CD9 expression was noted in early but not late corpora lutea. E- and N-cadherin expression was not detected in any corpora lutea. Surface epithelial expression was limited, although there were focally intense regions of KAI1, CD9, and E-cadherin expression. N-cadherin expression was generally weak or absent in surface epithelium. Original magnifications x200.

**Figure 8**



**Figure 8.** Immunohistochemical detection of KAI1 protein in various stages of follicular and luteal development. KAI1 expression was noted in primordial (A) follicles as well as variably sized small growing follicles (B, C). In mature antral follicles granulosa cells lack KAI1 expression, while surrounding theca cells continue to express KAI1. Early corpora lutea (E) also express large amounts of KAI1 protein although this expression was much reduced in older corpora lutea (F). Original magnifications x200.

**Figure 9**



**Figure 9.** Immunohistochemical detection of KAI1 and CD9 protein in ovarian blood vessels. Large amounts of KAI1 and CD9 protein were detected in the tunica media of small arteries and arterioles. Original magnifications x200.

**TABLE 1****Table 1. Average Quickscores Between Tumor Types**

	<b>KAI1</b>	<b>CD9</b>	<b>E-cadherin</b>	<b>N-cadherin</b>
Serous Carcinomas (17)*	10.7 ± 1.4	6.2 ± 1.0	15.6 ± 0.9	6.7 ± 1.3
Endometrioid Carcinomas (8)	6.8 ± 1.6	11.6 ± 1.6	13.9 ± 1.8	6.8 ± 2.3
Mucinous Carcinomas (4)	16.0 ± 2.0	6.0 ± 2.0	18.0 ± 0	1.5 ± 1.5
Clear Cell Carcinomas (3)	0.5 ± 0.5	3.2 ± 0.2	17.0 ± 1.0	0

\* number examined, (includes primary tumors only)  
± standard error

**Table 1.** Summary of adhesion molecule expression between epithelial ovarian carcinoma subtypes.

## CHAPTER NINE

### GENERAL SUMMARY AND CONCLUSIONS

This dissertation has explored cooperative gene/protein interactions that I hypothesized would be selected for during the tumorigenic process. Cooperation in this sense can be defined as working towards the common goal of promoting some aspect of the tumorigenic process. Various components of the tumorigenic process were examined including early alterations in growth control and later changes involving metastasis. **My overall hypothesis was that certain mutations or altered proteins would have a greater tumorigenic effect when acting cooperatively rather than independently.** Three distinct studies were conducted to provide support for this hypothesis and each was focused on the female reproductive system including the mammary gland and ovary.

The first study in support of this hypothesis evaluated potential cooperative effects between the tumor suppressor genes, Brca2 and p53. These genes were studied in the developing mouse mammary gland of genetically altered mice with and without exposure to ionizing radiation. The endpoints evaluated were cell proliferation and apoptosis, two processes frequently altered during tumorigenesis. The primary reasons for studying these genes in the developing mammary gland after irradiation was based on the known roles these genes play in IR-induced DNA damage repair and in breast cancer development in people. Additional factors influencing the decision were the known sensitivity of the developing mammary gland to carcinogens such as radiation and the high rate of cell turnover with abundant apoptosis and cell proliferation during this stage of mammary gland development. **My**

**hypothesis was that combined mutation of both genes would produce a more deleterious response than mutation of either gene alone. I also proposed that the harmful effects of Brca2 mutation would not be fully realized without concurrent p53 mutation.** A deleterious response in this case was defined as an effect opposite or significantly different than that demonstrated by the wildtype mice.

The role of p53 in response to radiation has been well characterized, however, few have specifically looked at the mouse mammary gland, and even fewer have evaluated its potential cooperative interaction with Brca2. Numerous studies outlined in the previous chapters have strongly implied that there is an interaction between these two genes, although the exact nature of this interaction is uncertain. Both Brca2 and p53 are known for their roles in DNA repair suggesting that their interaction may likely involve a repair pathway. However, p53 is also well known for its role in the acute response to carcinogens like radiation including the induction of cell cycle arrest and apoptosis. In contrast, Brca2's role in these acute responses is largely unknown. This study is one of the few to have investigated the potential for cooperative interaction between Brca2 and p53 during the acute response to irradiation.

Results from this study did demonstrate a cooperative interaction between Brca2 and p53 following irradiation, although not under normal physiologic conditions. The growth index in particular revealed that unirradiated mice were able to compensate for their genetic deficiencies even if both Brca2 and p53 were concurrently mutated. In contrast, irradiated mice apparently lose this compensating ability especially when both Brca2 and p53 are mutated. The growth index in irradiated mice revealed that individuals with only one mutant

gene were capable of responding with a wildtype-like response, however, mutation in both genes resulted in a particularly adverse response characterized by decreased apoptosis and increased cell proliferation. This finding supports my hypothesis by showing that combined mutation of both genes results in a more deleterious response than loss of either gene alone and also shows that the harmful effects of Brca2 mutation were not fully realized without concurrent p53 mutation. Furthermore, the type of response seen in these double mutant mice suggests that irreparably damaged cells might survive the genotoxic insult and therefore could potentially promote future tumorigenesis. Results described in **appendix three** have helped to confirm this assumption. Although the single gene mutant mice are also predisposed to tumorigenesis, mutation of both genes increased this tumorigenic effect.

Overall, these results provide support for my hypotheses and provide additional evidence as to why p53 mutations commonly occur in Brca2-associated cancers. In addition to the potential cooperative effects related to DNA repair, these results suggest that they also cooperate in regulating growth control, particularly in response to irradiation. This could have potential human health implications in determining whether exposure to certain forms of radiation, such as that used for routine mammography, would be safe for women carrying Brca2 mutations.

**Limitations:** One consideration when interpreting results from this study is that these mice lack only the terminal exon of Brca2 and presumably still produce a majority of the Brca2 protein. Despite loss of just one exon, the study here as well as others have continued to reveal the functional significance of this last exon. Unfortunately, mice with larger

truncations of the Brca2 gene often die *in utero* making the study of other regions of this gene problematic. In some cases, conditional knockouts have been used to circumvent this problem with success.

An additional consideration for any study utilizing mutant mice is the genetic background of the parent strains. As noted in previous chapters, genetic background can have a large effect on the response of an animal. In this study the genetic backgrounds of the Brca2- (C57BL/6) and p53- (Balb/cJ) mutant mice were different. This could have a potential influence on the results considering that the two respective mouse strains vary in their response to irradiation and in their susceptibility to mammary tumorigenesis. Balb/cJ mice are more sensitive to radiation and more commonly develop mammary tumors as compared to C57BL/6 mice (31).

Finally, because of the significant amount of redundancy that occurs in biological pathways, it is possible that alternative pathways could compensate for the reduced function of p53 and Brca2. For example, closely related homologous proteins such as p73 might be able to compensate for p53 deficiency. Whether or not this truly occurs *in vivo* is not certain.

**Future Studies:** Additional studies utilizing this model would involve a more detailed analysis of the molecular mechanisms involved in this interaction between Brca2 and p53. As outlined in **appendix three**, long-term studies in these mice have demonstrated enhanced tumorigenesis. However, the double mutant mice that exhibited the most pronounced effects in this study, developed lymphomas at such a young age that they do not serve as a good model for breast cancer, as was their original intent. Movement of these mutations into a

conditional model for tissue specific expression in the mammary gland would be a better method to evaluate their effects in the mammary gland. In addition, movement of both mutant genes completely onto a genetic background more susceptible to mammary tumors (such as Balb/c mice) would help improve this as a breast cancer model. Finally, additional studies to characterize the mouse Brca2 protein would also be informative. Unfortunately, a suitable antibody for identifying mouse Brca2 was not available at the time this study was conducted.

The second study in support of my hypothesis continued analysis of p53 by characterizing its involvement in ethylene oxide- and benzene-induced mammary tumors and explored how this was related to concurrent mutation of the oncogene H-ras. Ras is one of the more commonly altered genes in chemically-induced tumors, including those of the mammary gland, and functional studies outlined in previous chapters suggest that concurrent p53 mutation would be permissive to the tumorigenic effects of ras. Because of this, I hypothesized that chemically induced mouse mammary tumors with H-ras mutations would also possess p53 mutations due to the cooperative pro-tumorigenic effects noted between these two altered genes. Few studies have specifically looked for these particular mutations in ethylene oxide and benzene-induced mouse mammary tumors, although other chemicals have been shown to target these genes (608). In addition to the effects on mouse mammary tumorigenesis, this study also had the potential to provide important information on the general carcinogenic mechanisms of these chemicals even as they relate to human health. **My hypothesis was that both p53 and H-ras mutations would commonly occur together in**

**ethylene oxide- and benzene-induced mammary tumors as compared to spontaneously induced mammary tumors.**

Results from this study did indeed support the assumption that p53 and H-ras mutations would be common events in benzene- and ethylene-oxide induce mammary tumors, however, it was unexpected to find a similar number of these genetic alterations in spontaneous mammary tumors. Nonetheless, the distinct shift in mutational spectra between chemically induced and spontaneous tumors, suggested that benzene and ethylene oxide exposure could induce mammary specific genetic alterations predisposing female mice to mammary cancer. My initial thought process when forming the hypothesis for this study was that chemically induced mammary tumors would likely develop at a faster rate and at an earlier age than spontaneously induced mammary tumors and therefore they would have to employ a more potent or efficient combination of genetic events to accomplish this feat. The spontaneous mammary tumors would likely occur in aged animals, which have had a longer period of time to accumulate the number of genetic and epigenetic changes necessary for tumor formation. In addition, in these aged animals, the mutations would be expected to occur primarily as a stochastic process and therefore I speculated that the spontaneous tumors as a group would likely involve a wider variety of cancer-related genes. Interestingly, this did not appear to be the case as both spontaneous and chemically induced tumors targeted H-ras and p53. This suggests a strong tendency towards selection of alterations in these particular genes during the development of mouse mammary carcinomas, regardless of cause. This finding also supports my original hypothesis that these genes act cooperatively in the genesis of

mouse mammary tumors. Whether or not this same process occurs in benzene- and ethylene oxide-induced human tumors remains to be determined.

**Limitations:** Although both the benzene and ethylene oxide studies revealed a statistically significant increase in mammary carcinomas, the total number of tumors available for evaluation was somewhat limited. I believe that the results obtained in this study were still quite valuable and sufficient to identify general trends, however, a larger number of tumors would certainly help strengthen the conclusions. Additional limitations in this study stem from the inherently difficult process of extracting DNA from a heterogenous population of tumor cells. Although we make every effort to avoid large regions of necrosis, one can never be certain of the exact tissue composition of the sample used for examination. In addition, to the high degree of genetic variability in the tumor cells themselves, there are also many non-tumor cells potentially contributing to the DNA pool. These would include stromal cells, endothelial cells, inflammatory cells, and cells from adjacent normal tissues. All of these factors must be taken into consideration when interpreting these results.

**Future Studies:** In terms of future studies, I think it would be valuable to evaluate other chemically induced mammary tumors looking for similar alterations and common mechanisms involved in the induction of this tumor type. Considering some of the limiting issues described above, I think it would also be useful to use a technology such as laser capture microdissection in order to better characterize the cell types being analyzed. In addition, in keeping with the overall theme of this dissertation, I feel it would be helpful to utilize a method such as microarray analysis for identifying combinations of genes

abnormally expressed during mammary tumorigenesis. This would give valuable information towards identifying additional cooperative interactions and help to identify key pathways involved in the genesis of mammary tumors. In addition to genetic alterations, I think it would be valuable to evaluate epigenetic alterations in mammary tumors as well as other tumor types. For example, methylation plays an important role in regulating gene expression and methylation of certain bases can render them more susceptible to mutation. Identification of chromosomal aberrations in these tumors would also be of interest.

The third study in support of my hypothesis evaluated the protein expression of four different cell adhesion molecules (KAI1, CD9, E-cadherin, and N-cadherin) known to be involved in invasive and metastatic processes. Expression was compared between normal tissues, various grades of primary ovarian epithelial carcinomas, and metastatic ovarian epithelial carcinomas. This study was rather unique in that it was essentially a time course evaluating how adhesion molecules change throughout the entire spectrum of the tumorigenic process.

As in the early stages of tumorigenesis, the later stages also require the multistep accumulation of genetic modifications and it is quite likely that cooperative events also occur during the acquisition of a metastatic phenotype. The selective pressures that initially enable a cancer cell to form are different from those that later favor tumor cell invasion and metastasis. This stage of the process has a new set of challenges that a primary cancer cell must overcome in order to become metastatic. Success of the metastatic process requires tumor cells to possess decreased adhesive interactions with surrounding cells at some stages

and increased adhesive interactions at other times. The development of both of these antagonistic properties in the same cell would suggest that more than one adhesion molecule is likely involved in the process. In addition, developmental studies have shown cell migration is a process that requires the coordination/cooperation of a number of different proteins, many of which are cell adhesion molecules. Interestingly, the metastatic process mimics many of these developmental events and as a result, I speculated that similar cooperative interactions would also be necessary for successful metastasis. Considering all of these factors, **I hypothesized that more than one but not all of these adhesion molecules would be downregulated as tumors progressed towards a metastatic phenotype.**

Results from this study provided support for my hypothesis by showing that most but not all of these proteins were altered during the tumorigenic and metastatic processes of ovarian cancer. The tetraspanins, CD9 and KAI1, exhibited similar trends characterized by high levels of cell membrane expression in low-grade tumors followed by a drop in membrane expression as the tumor progressed towards the metastatic phenotype. This same trend has been detected for these proteins in several other tumor types, although this is one of the first to show a similar trend in ovarian epithelial tumors. In contrast, N-cadherin exhibited its lowest membrane expression in the low-grade tumors and then gradually increased membrane expression in correlation with tumor grade showing its highest membrane expression in grade three tumors and metastases. This correlated well with the known pro-migratory activities of N-cadherin suggesting that its expression is important in development of an invasive phenotype in ovarian epithelial carcinomas. Interestingly, E-cadherin membrane expression remained elevated in all tumor grades and metastases. Given E-

cadherin's known function in cell adhesion, this finding was somewhat unexpected. In many, if not most, tumor types, E-cadherin expression generally decreases with development of a metastatic phenotype. The findings in this study suggest that metastasis of ovarian epithelial carcinomas is an exception to this rule.

Although this study was not specifically designed to address the functional implications of these changes, these findings do suggest several potential areas of cooperation. For example, the increase in N-cadherin expression might promote an invasive or motile phenotype in these tumors as they progress even when E-cadherin expression is maintained. This could set a scenario whereby the reduction in CD9 and KAI1 reduces cell adhesiveness, the increased N-cadherin promotes invasion, and the maintenance of E-cadherin expression allows for tumor cell attachment at distant sites. Of course, such a conclusion cannot be proven by the results from this study, however, this type of cooperative interaction between cell adhesion proteins is what would be needed for the metastatic process to be successful.

An additional consideration with the tetraspanin proteins is that they have a potential role in regulating a number of different proteins on the cell surface. As mentioned in the preceding chapters, tetraspanins play a key role in helping to organize other proteins into a network on the cell surface. Because of this, we must also consider the possibility that downregulation of KAI1 and CD9 may disrupt the function of a number of different classes of cell adhesion and signaling molecules involved in these large multi-protein complexes.

**Limitations:** One major limitation in this study was the supply of adequate tissue specimens. Human tissue specimens such as these are difficult to obtain and as a result, limits the number of samples available for analysis. However, I do believe the results obtained in this study were adequate to identify general trends. Certainly, more samples would be helpful to confirm these findings and would allow for a more complete evaluation of tumor type specific changes.

An additional limitation was related to tumor heterogeneity. Not all cells in a particular tumor express the same amount of protein, which can lead to some difficulties when attempting to quantify the results. To help deal with this issue, a grading system was used that takes into account both the area and intensity of staining. In addition, the samples were independently graded by two pathologists in order to maintain consistency.

Finally, additional studies looking at gene expression and alternative methods for looking at protein expression would be helpful to confirm the findings of this study. Additional molecular analysis looking for mechanisms of these changes in expression would also be useful. For example, considering the known interaction between p53 and KAI1, it would be interesting to determine if p53 mutations were present in those samples exhibiting decreased KAI1 expression.

**Future Studies:**

In the future, I would like to perform a parallel study to this utilizing an animal model of metastasis. It would also be interesting to look for global changes in gene expression between

primary and metastatic tumors. This could provide valuable information as to the genes involved in the development of this deadly process. It would also be valuable to perform a similar study comparing differences in gene expression throughout the whole tumorigenic process, starting with normal and hyperplastic tissues and then moving on to later stages as neoplasia develops (adenoma, carcinoma, and metastatic carcinoma).

Overall, this dissertation evaluated multiple aspects of the dynamic process known as tumorigenesis. It involved the analysis of environmentally induced (radiation and chemical), spontaneous (mammary and ovarian epithelial carcinomas), and even some familial forms of cancer (germline mutations in Brca2 and p53-Li Fraumeni Syndrome). Topics such as DNA repair (Brca2 and p53), cell cycle regulation (p53 and ras), apoptosis (p53), cell signaling (ras), and cell adhesion (KAI1, CD9, E-cadherin, and N-cadherin) were explored providing a broad range of knowledge and a greater overall comprehension of the very complex and dynamic process known as tumorigenesis. Support for pro-tumorigenic cooperative interactions between several different classes of cancer-related genes and proteins were provided by three independent studies. Results from these studies generally supported my original hypothesis that certain mutations or altered proteins would have a greater tumorigenic effect when acting cooperatively rather than independently. In conclusion, this dissertation revealed how complex interactions between specific genes and proteins can lead to or promote the tumorigenic process and supports the idea of looking for similar interactions in future studies.

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## **APPENDICES**

## APPENDIX ONE

### CB6F1 PILOT STUDY

This pilot study was undertaken to determine the optimum timepoint for looking at apoptotic and proliferation parameters following exposure to 5 Gy of ionizing radiation. This information was used for designing the experiment described in **manuscript one**.

This particular strain of mouse (CB6F1) was chosen because they closely matched the genetic background of the mutant Brca2/p53 mice that were used in the primary study. CB6F1 mice are a cross between a BALB/c female and C57BL/6J male mouse. Thirty, 5 week old, female CB6F1 mice were divided into ten groups of three. Five of the groups were irradiated (5 Gy) and the other five (controls) were not (see study design below). Following irradiation ( $^{137}\text{Cs}$  source) mice were sacrificed at various timepoints including 0 hours, 6 hours, 12 hours, 24 hours, and 48 hours. Control mice were treated identically to the irradiated mice (including being loaded into an irradiation holding device) however the actual irradiation step was not done. Prior to sacrifice each mouse was injected with BrdU (50 mg/kg) followed by a 1 hour incubation time. After sacrifice, a partial necropsy was performed on each mouse removing the left fourth mammary gland, which was fixed for 24 hours in 10% neutral buffered formalin then routinely processed to paraffin block. Two sections from each block were taken and stained for BrdU and TUNEL using the same procedures described in the primary study (**manuscript one**). Images were taken of the epithelial structures in each gland from both the TUNEL and BrdU stained slides. The number of epithelial cells in each image was estimated by counting pixels using color

thresholding techniques. TUNEL and BrdU positive cells were manually counted. A relative apoptotic and proliferation index was generated by dividing the number of TUNEL or BrdU positive cells by the total area measured in pixels (Table A1.1). Although this method did not provide an apoptotic or proliferation index based on absolute cell numbers it did provide relative indices adequate for comparing differences between groups. These results revealed that the maximal effects of 5 Gy of irradiation, in terms of the degree of change in both apoptosis and proliferation, occurred around 6 hours post-irradiation (Figure A1.1).

### ***A1.1 STUDY DESIGN***

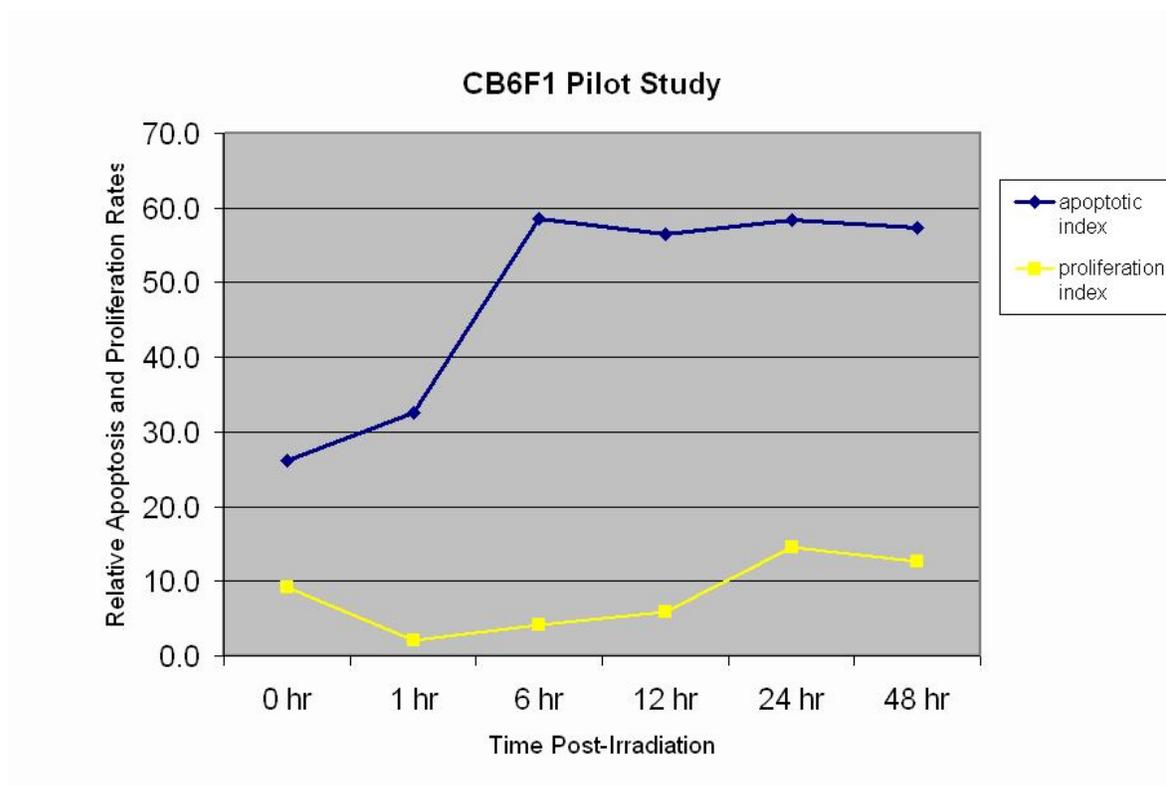
Time 0 hours: 3 irradiated mice and 3 control mice

Time 6 hours: 3 irradiated mice and 3 control mice

Time 12 hours: 3 irradiated mice and 3 control mice

Time 24 hours: 3 irradiated mice and 3 control mice

Time 48 hours: 3 irradiated mice and 3 control mice



**FIGURE A1.** CB6F1 post-irradiation time course study (apoptosis and proliferation)

CB6F1 Pilot Study							
CB6F1 mice	total area (pixels)	TUNEL positive cells	BrdU positive cells	relative apoptotic index*	graph data	relative proliferation index*	graph data
0 hr	877149	23	8	0.0000262	26.2	0.0000091	9.1
1 hr	2493828	81	5	0.0000325	32.5	0.0000020	2.0
6 hr	1453557	85	6	0.0000585	58.5	0.0000041	4.1
12 hr	1011533	57	6	0.0000564	56.4	0.0000059	5.9
24 hr	548349	32	8	0.0000584	58.4	0.0000146	14.6
48 hr	1811502	104	23	0.0000574	57.4	0.0000127	12.7

\* decimal place moved 6 spaces to the right to arrive at the graph data

**TABLE A1.** Image analysis based pixel counts used to estimate the relative apoptotic and proliferation indices.

## APPENDIX TWO

The following manuscript involved an analysis of the Brca2 deficient mice utilized in **manuscript one** of this dissertation. This study demonstrated that these mice were indeed tumor prone. My part in this study involved histopathological examination of all tumors and interpretation of the final data. The manuscript was published in 2002 (McAllister *et al.* (2002) *Cancer Research* 62, 990 –994). A copy of this manuscript is included after **appendix three**.

## APPENDIX THREE

### COOPERATIVE EFFECT OF BRCA2 AND P53 GERMLINE MUTATION ON SPONTANEOUS AND RADIATION-INDUCED TUMOR SUSCEPTIBILITY, LATENCY, AND SURVIVAL

(Manuscript in preparation for submission to *Cancer Research*)

To help further characterize the effects noted in **manuscript one**, Brca2/p53 deficient mice were followed throughout their lifespan looking for the effect of genotype and/or radiation on tumorigenesis. As noted in **appendix two**, the Brca2 deficient mice had already been characterized as to their tumor susceptibility; however, this study incorporates the additional effects conferred by radiation exposure and p53 deficiency. My role in this study involved the histopathological examination of a full screen of tissues as well as all tumors and other gross lesions identified at necropsy.

The Brca2/p53 deficient mice used in this study were generated as previously described in **manuscript one**. In addition to the four genotypes examined in **manuscript one** (WT, Brca2<sup>-/-</sup>, p53<sup>-/-</sup>, and dKO), five other heterozygous combinations were also examined:

1. Brca2<sup>+/+</sup>:p53<sup>+/+</sup> (WT in **manuscript one**) - wildtypes
2. Brca2<sup>-/-</sup>: p53<sup>+/+</sup> (Brca2<sup>-/-</sup> in **manuscript one**) – p53 wildtypes or Brca2 nulls
3. Brca2<sup>+/+</sup>:p53<sup>-/-</sup> (p53<sup>-/-</sup> in **manuscript one**) – Brca2 wildtypes – p53 nulls
4. Brca2<sup>-/-</sup>:p53<sup>-/-</sup> (dKO in **manuscript one**) double nulls
5. Brca2<sup>+/-</sup>:p53<sup>-/-</sup> Brca2 heterozygotes: p53 null
6. Brca2<sup>+/-</sup>:p53<sup>+/-</sup> double heterozygotes
7. Brca2<sup>+/-</sup>:p53<sup>+/+</sup> Brca2 heterozygotes
8. Brca2<sup>-/-</sup>:p53<sup>+/-</sup> Brca2 null:p53 heterozygote
9. Brca2<sup>+/+</sup>:p53<sup>+/-</sup> p53 heterozygotes

(Genotypes 3, 4, and 5 not evaluated in this study)

Approximately 1200 virgin female mice were either irradiated with a single 5 Gy dose of radiation at five weeks of age or observed as unirradiated virgin female mice. Most mice were necropsied at 18 months of age but some were done earlier if found within a moribund state. A subset of mice was observed for 24 months. All tissue and tumor samples were removed post mortem and fixed in 10% neutral buffered formalin. Specimens were processed for routine histology, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The tissues examined histopathologically included, adrenal gland, brain, cervix, heart, kidney, liver, lung, lymph node, mammary gland, ovary, pancreas, pituitary gland, spleen, stomach, thymus, urinary bladder, uterus, vagina, and Zymbal's gland. When possible, the classification of mammary tumors followed the guidelines and recommendations established

at the Annapolis meeting (Cardiff, *et al.* (2000) *Oncogene*. Feb 21; 19(8):968-88). The subtypes of mammary carcinomas were based on characterizing the predominant histological pattern in the tumor, although if more than one pattern was found to predominate then the tumor was classified as mixed.

In general, survival of the p53 null genotypes was dramatically reduced and the frequency of lymphoma was very high. Unfortunately, the high incidence and rapid onset of lymphomas in this subset of mice precluded their use for characterizing tumorigenesis in other tissues. As a result, details on the p53 null mice will not be provided other than to say that the majority of these mice succumbed to lymphomas, predominantly of thymic origin. This outcome was also unfortunate considering that the most dramatic effects noted in **manuscript one** were found in dKO mice. Although data from **manuscript one** suggests the dKO mice would be susceptible to mammary carcinogenesis, it appears that they are even more susceptible to tumorigenesis elsewhere. A mammary specific conditional knockout mouse model would likely be necessary to identify the true effects of this genotype on mammary tumorigenesis.

### ***A3.1 RESULTS SUMMARY***

#### ***A3.1.1 Unirradiated Mice***

##### *A3.1.1.1 Brca2 mutant mice (wildtype p53) - unirradiated*

In unirradiated mice, a significantly higher incidence of squamous cell carcinomas of the forestomach was observed in  $Brca2^{-/-}$  animals as compared to  $Brca2^{+/-}$  or  $Brca2^{+/+}$  littermates.

In many instances, these gastric squamous cell carcinomas were preceded by variable

degrees of squamous cell hyperplasia. Squamous cell carcinomas in other locations were almost exclusively represented in the  $Brca2^{-/-}$  genotype. In addition, carcinomas of all types were significantly higher in the  $Brca2^{-/-}$  mice as compared to  $Brca2^{+/-}$  or  $Brca2^{+/+}$  littermates.  $Brca2^{-/-}$  mice also exhibited a significantly higher overall tumor incidence and a higher incidence of multiple tumors as compared to  $Brca2^{+/-}$  or  $Brca2^{+/+}$  animals on the  $p53^{+/+}$  background.

#### *A3.1.1.2 Brca2 mutant mice (heterozygous p53) - unirradiated*

Osteosarcomas and lymphomas were significantly increased for  $Brca2^{-/-}$  mice on a  $p53^{+/-}$  background as compared to  $Brca2^{+/-}$  or  $Brca2^{+/+}$  littermates on the same  $p53^{+/-}$  background.

#### *A3.1.1.3 P53 mutant mice (wildtype Brca2) - unirradiated*

A significantly higher incidence of osteosarcomas, lymphomas, and mammary carcinomas were found in the  $p53^{+/-}$  mice as compared to  $p53^{+/+}$  mice. The presence of the single  $p53$  mutation particularly affected the incidence of mammary tumors as few mammary tumors occurred on the  $p53$  wildtype background. Six distinct subtypes of mammary carcinomas were noted, the most common of which were the glandular types comprising the majority of all mammary carcinomas found in this study. Additional subtypes included adenosquamous, solid, spindle cell, papillary, and cribriform types. In addition to mammary carcinomas, hemangiosarcomas, myoepitheliomas, lymphomas, and undifferentiated sarcomas were also noted in the mammary gland. While a significantly increased incidence of mammary tumors

was observed in the p53<sup>+/-</sup> mice, no correlations between histological subtype and genotype were apparent.

### **A3.1.2 Irradiated mice (5 Gy)**

#### *A3.1.2.1 Brca2 mutant mice (wildtype p53) – irradiated*

Osteosarcomas were significantly increased in irradiated Brca2<sup>-/-</sup> mice as compared to Brca2<sup>+/-</sup> mice. In addition, squamous cell carcinomas were significantly increased in the Brca2<sup>-/-</sup> mice as compared to Brca2<sup>+/+</sup> mice and lymphomas were significantly increased as compared to both Brca2<sup>+/-</sup> or Brca2<sup>+/+</sup> mice.

#### *A3.1.2.2 Brca2 mutant mice (heterozygous p53) – irradiated*

Both the Brca2<sup>+/-</sup> and Brca2<sup>-/-</sup> mice on a p53<sup>+/-</sup> genetic background exhibited a significantly higher incidence of lymphomas as compared to those mice on a wildtype p53 background. In addition, Brca2<sup>-/-</sup> mice on a p53<sup>+/-</sup> background exhibited a significantly higher incidence of mammary carcinomas as compared to those on a p53<sup>+/+</sup> background.

#### *A3.1.2.3 P53 mutant mice (wildtype Brca2) – irradiated*

A higher incidence of lymphomas and mammary carcinomas were found in the p53<sup>+/-</sup> mice as compared to p53<sup>+/+</sup> mice, which was similar to that seen in unirradiated mice of the same

genotypes. The irradiated mice, however, did not exhibit an increase in osteosarcomas as was seen in unirradiated mice, although this was likely attributed to the higher occurrence of lymphomas in irradiated mice.

Table A3 (A and B) provides an overall summary of the results obtained from this study. Figure A3 show representative tumors found in these mice.

### **A3.2 DISCUSSION**

This experiment revealed results in support of the hypothesis that Brca2 and p53 cooperate during the process of tumorigenesis. Brca2 deficiency was associated with a significantly higher overall tumor incidence as well as decreased tumor latency and decreased overall survival (data not shown for survival). In addition, loss of Brca2 correlated with an increased incidence of osteosarcomas, lymphomas, and carcinomas, particularly squamous cell carcinomas of the forestomach. Combined Brca2 and p53 deficiency resulted in an even higher tumor incidence and had a further influence on overall survival (data not shown for survival). It also increased the incidence and decreased the latency of certain tumor types. These findings are some of the most extensive *in vivo* data demonstrating a cooperative interaction between a germline Brca2 and p53 mutation on tumor development. The observation that specific tumor types are enhanced with the combination of both genetic mutations provides important insight into the molecular tumorigenesis pathway mediated by disruptions in these two key cancer susceptibility genes.

**TABLE A3 (A): Summary of Tumor Incidence by Genotype**

(A)	<b>P<sup>+/+</sup> B<sup>-/-</sup> 0 Gy</b>	<b>P<sup>+/+</sup> B<sup>+/-</sup> 0 Gy</b>	<b>P<sup>+/+</sup> B<sup>+/+</sup> 0 Gy</b>	<b>P<sup>+/+</sup> B<sup>-/-</sup> 5 Gy</b>	<b>P<sup>+/+</sup> B<sup>+/-</sup> 5 Gy</b>	<b>P<sup>+/+</sup> B<sup>+/+</sup> 5 Gy</b>
Lymphoma	4/36 (11)	13/101 (13)	15/55 (27)	24/48 (50)	37/104 (36)	19/45 (42)
Mammary	0/33 (0)	5/97 (5)	2/50 (4)	1/45 (2)	12/97 (12)	0/45 (0)
Osteosarcomas	4/36 (11)	1/101 (1)	2/55 (4)	4/48 (8)	0/104 (0)	2/45 (4)
Reproductive tumors (malignant)	0/36 (0)	2/95 (2)	0/55 (0)	2/48 (4)	30/104 (29)	15/45 (33)
Carcinoma (squamous or other)	12/36 (33)	15/101 (15)	7/55 (13)	7/48 (15)	25/104 (24)	1/45 (2)
Stomach tumors (squamous cell carcinomas)	4/35 (11)	0/99 (0)	0/53 (0)	3/47 (6)	1/99 (1)	0/45 (0)
Animals with multiple tumors	10/36 (28)	13/101 (13)	11/55 (20)	12/48 (25)	41/104 (39)	18/45 (40)
Total incidence of tumors	22/36 (61)	46/101 (46)	30/55 (55)	33/48 (69)	91/104 (88)	38/45 (84)

Numbers in parentheses are percentages.

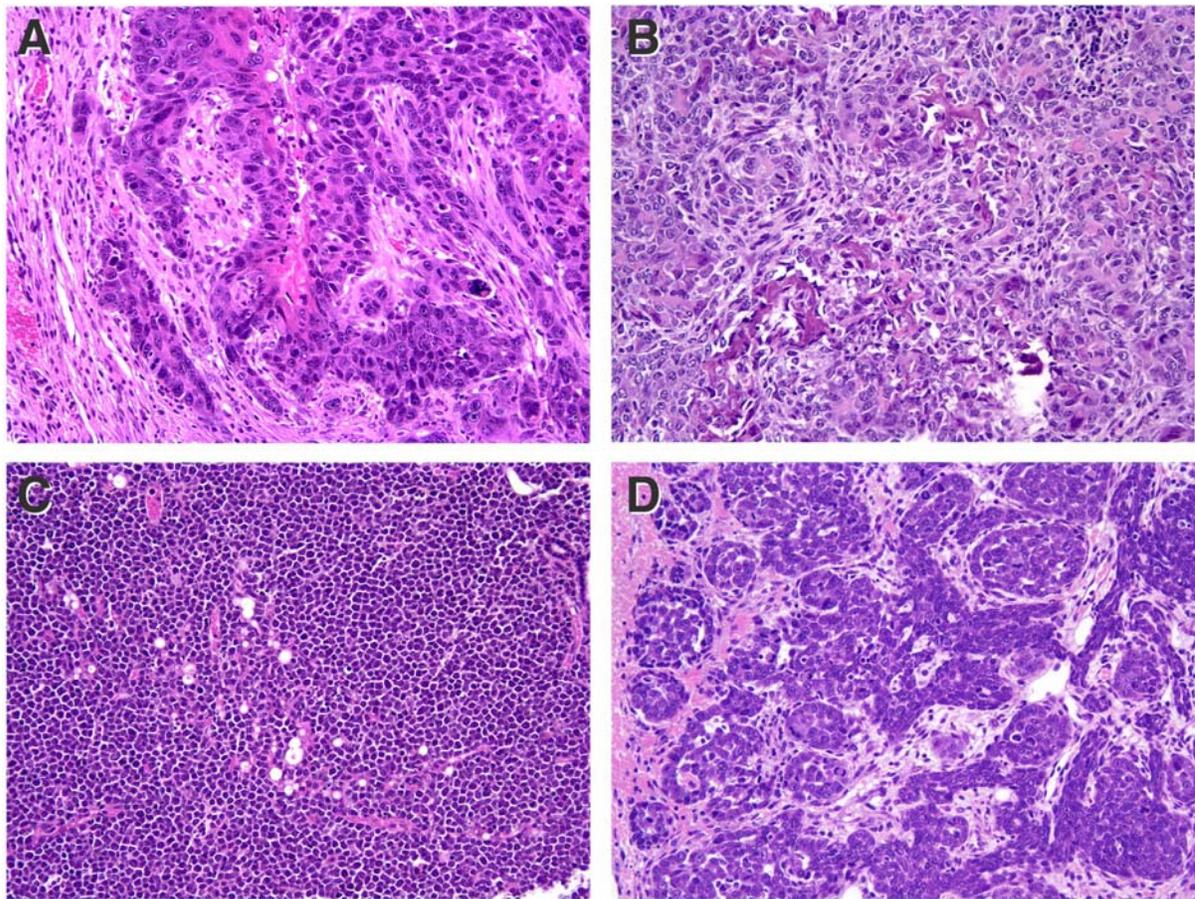
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**TABLE A3 (continued) (B): Summary of Tumor Incidence by Genotype**

(B)	P <sup>+/-</sup> B <sup>-/-</sup> 0 Gy	P <sup>+/-</sup> B <sup>+/-</sup> 0 Gy	P <sup>+/-</sup> B <sup>+/+</sup> 0 Gy	P <sup>+/-</sup> B <sup>-/-</sup> 5 Gy	P <sup>+/-</sup> B <sup>+/-</sup> 5 Gy	P <sup>+/-</sup> B <sup>+/+</sup> 5 Gy
Lymphoma	12/48 (25)	26/147 (18)	21/75 (28)	27/41 (66)	52/91 (57)	24/42 (57)
Mammary	11/47 (23)	29/138 (21)	17/72 (24)	11/41 (27)	43/86 (50)	16/42 (38)
Osteosarcomas	20/48 (42)	32/147 (22)	14/75 (19)	3/41 (7)	4/91 (4)	1/42 (2)
Reproductive tumors (malignant)	1/48 (2)	6/147 (4)	2/75 (3)	2/41 (5)	20/91 (22)	5/42 (12)
Carcinoma (squamous or other)	17/48 (35)	35/147 (24)	22/75 (29)	10/41 (24)	43/91 (47)	16/42 (38)
Stomach tumors (squamous cell carcinomas)	8/48 (17)	0/143 (0)	0/73 (0)	0/40 (0)	0/88 (0)	0/42 (0)
Animals with multiple tumors	18/48 (38)	37/147 (25)	16/75 (21)	9/41 (22)	41/91 (45)	14/42 (33)
Total incidence of tumors	39/48 (81)	113/147 (77)	56/75 (75)	33/41 (80)	84/91 (92)	39/42 (93)

Numbers in parentheses are percentages.

**TABLE A3 (A and B).** The number of mice with a particular tumor type is shown over the total number of mice examined for the p53 wildtype (A) and p53 heterozygous (B) groups in irradiated and unirradiated mice of all three Brca2 genotypes. Also shown is the number of animals that had multiple tumors and the overall number of animals of each group with tumors.



**FIGURE A3** Photomicrographs of representative tumors observed in mice with germline Brca2 and p53 mutations.

- A. **Squamous cell carcinoma.** Irregular cords of atypical squamous cells infiltrate into surrounding tissues. A few cells exhibit keratinization (200x magnification).
- B. **Osteosarcoma.** Irregularly arranged polygonal to spindle shaped cells occasionally surround small islands of osteoid (200x magnification).
- C. **Lymphoma.** A uniform population of small neoplastic lymphocytes effaces the architecture of this lymph node (200x magnification).
- D. **Mammary carcinoma.** Glandular pattern (200x magnification).

## Cancer Susceptibility of Mice with a Homozygous Deletion in the COOH-Terminal Domain of the *Brca2* Gene<sup>1</sup>

Kimberly A. McAllister,<sup>2</sup> L. Michelle Bennett, Chris D. Houle, Toni Ward, Jason Malphurs, N. Keith Collins, Carol Cachafeiro, Joseph Haseman, Eugenia H. Goulding, Donna Bunch, E. Mitch Eddy, Barbara J. Davis, and Roger W. Wiseman

Laboratory of Women's Health [K. A. M., C. D. H., T. W., J. M., N. K. C., B. J. D., R. W. W.], Biostatistics Branch [J. H.], and Laboratory of Reproductive Development and Toxicology [E. H. G., E. M. E.], National Institute of Environmental Health Sciences, NIH, Research Triangle Park, North Carolina 27709; Biology and Biotechnology Research Program, Lawrence Livermore National Laboratory, Livermore, California 94551 [L. M. B.]; Integrated Laboratory Systems, Inc., Durham, North Carolina 27713 [C. C.]; and Department of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599 [D. B.]

### Abstract

Inherited mutations of the human *BRCA2* gene confer increased risks for developing breast, ovarian, and several other cancers. Unlike previously described *Brca2* knockout mice that display predominantly embryonic lethal phenotypes, we developed mice with a homozygous germ-line deletion of *Brca2* exon 27 that exhibit a moderate decrease in perinatal viability and are fertile. We deleted this *Brca2* COOH-terminal domain because it interacts directly with the *Rad51* protein, contains a nuclear localization signal, and is required to maintain genomic stability in response to various types of DNA damage. These homozygous *Brca2*-mutant mice have a significantly increased overall tumor incidence and decreased survival compared with their heterozygous littermates. Virgin female mice homozygous for this *Brca2* mutation also display an inhibition of ductal side branching in the mammary gland at 6 months of age. Given their substantial viability and cancer predisposition, these mutant mice will be useful to further define the role of the COOH-terminal *Brca2* domain in tumorigenesis both *in vivo* and *in vitro*.

### Introduction

Women who inherit germ-line defects of the *BRCA2* breast cancer susceptibility gene have up to an 85% risk of breast cancer development by 70 years of age (1, 2). *BRCA2* mutation carriers also have increased risks for a variety of cancers including ovarian, pancreatic, colon, prostate, stomach, laryngeal, thyroid, male breast cancer, and ocular melanoma (3). Substantial evidence from studies of human and mouse cells indicates that the *BRCA2* protein is an important component in DNA damage response pathways, and loss of this function is considered a major factor in cancer predisposition (4, 5). *Brca2*-mutant cells and embryos are hypersensitive to ionizing radiation and other DNA-damaging agents and develop numerous spontaneous chromosomal abnormalities (6–10). In addition, several domains of the *BRCA2* protein appear to interact directly with *RAD51*, a protein having distinct roles in normal meiotic and mitotic recombination, DNA damage repair, and chromosome segregation. These *BRCA2* domains include the BRC repeats in exon 11 and a highly conserved *RAD51* binding domain in exon 27 (6, 11–13). The interaction of *Brca2* with *Rad51* is pivotal for the role *Brca2* plays in the activation of double-strand break repair and/or homologous recombination, and disruption of these processes may predispose individuals to cancer development.

Functional studies of *Brca2* in adult tissues have been hindered by

the embryonic lethal phenotypes of *Brca2* knockout mice reported to date (14). We and others previously described mutant mice with disruptions in the 5' half of *Brca2* that result in embryonic lethality during early to mid-gestation (6, 15–17). The few homozygous mutant *Brca2* mice that have been reported to survive to adulthood display gross developmental abnormalities, are infertile, and develop thymic lymphomas by 5 months of age (7, 8). Here, we describe the creation and initial phenotypic characterization of mutant mice that carry a homozygous germ-line deletion of exon 27 of the *Brca2* gene. We chose to target exon 27 because a *Rad51* binding domain has been identified between *Brca2* amino acids 3196–3232 in yeast two-hybrid studies (6, 11). In addition, this COOH-terminal domain of the *Brca2* protein contains nuclear localization signals that have been highly conserved among species (18, 19). Finally, cells lacking this COOH-terminal domain are hypersensitive to  $\gamma$ -radiation (10), and they have recently been shown to be deficient in error-free, homology-directed DNA repair (20, 21). The *Brca2*-mutant mice described in this report exhibit a low penetrance of perinatal lethality and an increased susceptibility to spontaneous tumorigenesis in a variety of tissues.

### Materials and Methods

**Targeting Construct Design.** A targeting construct was generated that contained exon 27 of the *Brca2* gene flanked by *loxP* sites (Fig. 1). The 5' targeting fragment consisted of a 4-kb *EcoRI* fragment containing exons 25, 26, and 27, and this was subcloned from a genomic mouse bacterial artificial chromosome clone (18). Likewise, a 3-kb *NsiI* fragment distal to the 3' untranslated region of *Brca2* was also subcloned. Double-stranded *loxP* oligonucleotides flanked by appropriate restriction sites were inserted into a *MunI* site in intron 26 of the 5' targeting fragment and a *BamHI* site of the 3-kb *NsiI* targeting fragment. Both fragments were then inserted into a previously described pgkNeoTK targeting vector (17). After linearization with *SalI*, this targeting vector was introduced into 129/Ola-derived BK-4 ES<sup>3</sup> cells by electroporation as described previously (17). Electroporated cells were subjected to positive and negative selection with geneticin (250  $\mu$ g/ml; Life Technologies, Inc., Rockville, MD) and gancyclovir (2  $\mu$ M; Roche, Hertfordshire, United Kingdom). A properly targeted ES cell clone with the "floxed" *Brca2* allele (*Brca2*<sup>Fllox27</sup>) was identified by Southern analyses with unique probes outside the *Brca2* targeting construct as well as PCR analyses using *loxP*-specific primers.

**Generation of Germ-Line Mutant Mice.** A Cre expression plasmid (generously provided by Dr. Robert Sobol, National Institute of Environmental Health Sciences) was transiently electroporated into ES cells carrying a single floxed *Brca2* allele. This *Brca2* allele was successfully deleted in ~10% of the floxed ES cells as determined by PCR using primers that flanked the 5' and 3' *loxP* sites (Fig. 1). The *Brca2*<sup>Wildtype</sup> and *Brca2*<sup>Fllox27</sup> alleles were amplified using the following PCR primers: B2F1, 5'-GGAGGAGGAGGAGTTGT-TGA-3' and B2R1, 5'-ATCTCGTCTCTCCACTCCA-3', whereas the *Brca2* <sup>$\Delta$ 27</sup> allele was detected using primers B2F1 and B2R2, 5'-

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<sup>2</sup> To whom requests for reprints should be addressed, at Laboratory of Women's Health, National Institute of Environmental Health Sciences, NIH, MD C4-06, 111 T. W. Alexander Drive, Research Triangle Park, NC 27709. Phone: (919) 541-3229; Fax: (919) 541-3720; E-mail: mcalls2@niehs.nih.gov.

<sup>3</sup> The abbreviation used is: ES, embryonic stem.

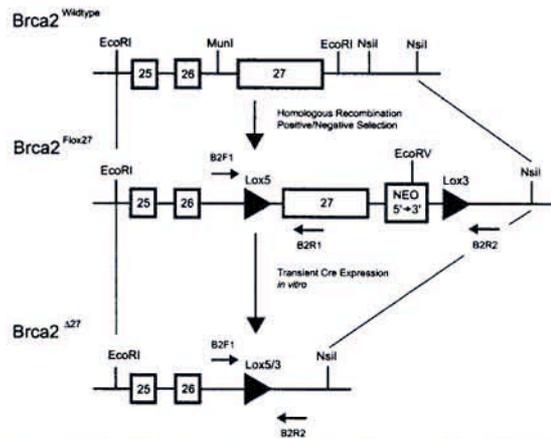


Fig. 1. Generation of *Brca2*<sup>Floxed27</sup> and *Brca2*<sup>Δ27</sup> ES cells. Homologous recombination between the endogenous *Brca2* locus (*Brca2*<sup>Wildtype</sup>) and a targeting vector carrying a pair of *LoxP* sites flanking *Brca2* exon 27 and a neomycin resistance cassette yielded a "floxed" *Brca2* locus (*Brca2*<sup>Floxed27</sup>). ES cells containing the properly targeted *Brca2*<sup>Floxed27</sup> allele were isolated by positive/negative selection and confirmed by Southern blot and PCR analyses. Conversion of the *Brca2*<sup>Floxed27</sup> allele to the *Brca2*<sup>Δ27</sup> allele was accomplished *in vitro* by transient electroporation of *Brca2*<sup>Floxed27</sup> ES cells with a Cre expression plasmid. The Cre recombinase induces site-specific recombination between the 5' and 3' *LoxP* sites, resulting in the deletion of exon 27 and the neomycin gene. Germ-line transmission of the desired *Brca2*<sup>Δ27</sup> allele was obtained by injection into blastocysts, followed by standard breeding techniques.

CAAAAAAGCCCAGATGATGAG-3'. *Brca2*<sup>Δ27</sup> ES cells were then injected into C57BL/6N blastocysts and transplanted into pseudopregnant CD-1 females. Eight chimeras were generated, and germ-line transmission of their *Brca2*<sup>Δ27</sup> allele was obtained by standard breeding techniques. Genomic DNA from pups was isolated from tail biopsies at weaning for genotyping by PCR.

**Mice and Tissues.** Mice carrying the *Brca2*<sup>Δ27</sup> mutation were backcrossed from a targeted 129-derived chimeric founder onto the C57BL/6N background for one to three generations, followed by intercrosses to generate mice homozygous for the *Brca2*-exon 27 deletion. Both virgin and multiparous females as well as males carrying this *Brca2*<sup>Δ27</sup> mutation were group housed in plastic cages on pressed wood-chip bedding. Animals had access to an NIH-31 diet (18% protein, 4% fat, and 5% fiber; Zeigler Bros., Gardeners, PA) and water *ad libitum*. Mice were monitored for 17 months and were sacrificed when palpable tumors developed or when signs of morbidity became apparent. Terminal sacrifices were performed on all surviving animals between 17 and 19 months of age. All tissue and tumor samples were removed postmortem and fixed in 10% neutral buffered formalin. Specimens were processed for routine histology, embedded in paraffin, sectioned, and stained with H&E. Histological examination was performed by veterinary pathologists (C. H. and B. D.). In all female mice, the right #4 and #5 mammary glands were fixed with neutral buffered formalin on the pelts for 24 h, dissected, fixed, and then stained with carmine as described previously for whole-mount analysis (22). Routine H&E staining was performed on the left #4 and #5 mammary gland of each animal, followed by histopathological analysis.

**Statistical Analysis.** A  $\chi^2$  goodness-of-fit test was performed for segregation analysis of the three genotypic classes from heterozygous intercrosses. The overall difference in survival between *Brca2*<sup>Δ27/Δ27</sup> and *Brca2*<sup>Δ27/+</sup> virgin females was compared with a life table test. Both a Peto analysis (23) and a Fisher's exact test were used to test for a difference in tumor rates between genotypic classes. Both procedures produced similar results.

**Results**

**Generation of Mice with a Homozygous Germ-Line Deletion of *Brca2* Exon 27.** ES cells carrying a targeted *Brca2* allele with *loxP* sites flanking exon 27 were generated by homologous recombination (Fig. 1). After transient Cre expression *in vitro*, ES clones with a *Brca2*<sup>Δ27</sup> allele were identified. PCR products generated with the

B2F1 and B2R2 primers that flank the 5' and 3' *loxP* sites (Fig. 1) were sequenced to confirm the expected exon 27 deletion. Germ-line transmission of the *Brca2*<sup>Δ27</sup> allele was identified by standard breeding techniques.

As expected, reverse transcriptase-PCR analysis with primers specific for exon 27 does not yield detectable messages in testes RNA from *Brca2*<sup>Δ27/Δ27</sup> mice, although this reverse transcription-PCR product is easily detectable for *Brca2*<sup>Δ27/+</sup> mice. Assuming a mutant transcript is expressed, it could give rise to a truncated protein product of 3142 amino acids compared with the wild-type murine *Brca2* protein that contains 3329 amino acids. Unfortunately, we are unable to confirm the presence or relative levels of this putative mutant *Brca2* protein because of the unavailability of specific antibodies directed against the murine gene product.

**Viability of *Brca2*<sup>Δ27/Δ27</sup> Mice.** Although the *Brca2*<sup>Δ27/Δ27</sup> animals are viable compared with previous *Brca2* germ-line mutants, survival analysis indicates an overall decrease in viability of homozygous mutants compared with heterozygous and wild-type littermates:

First, cumulative genotyping at weaning from *Brca2*<sup>Δ27/+</sup> intercrosses revealed a significant ( $P < 0.008$ ) deficit of *Brca2*<sup>Δ27/Δ27</sup> mice from the expected 1:2:1 Mendelian ratio. When both sexes are combined, only 47 of 278 animals analyzed at weaning were of the homozygous mutant genotype. Thus, there are ~33% fewer *Brca2*<sup>Δ27/Δ27</sup> mice than expected, and both male and female offspring were under represented. To further characterize the decreased overall viability of *Brca2*<sup>Δ27/Δ27</sup> mice during development, we examined embryonic fibroblasts from *Brca2*<sup>Δ27/+</sup> intercrosses at 13.5 days of gestation. Analysis of 39 such embryos shows that the genotypic distribution does not deviate from the expected Mendelian ratio. Thus,

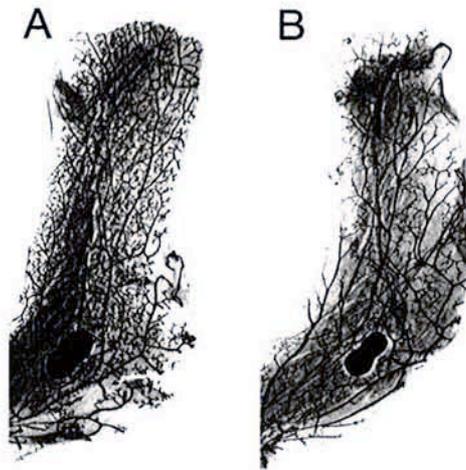


Fig. 2. Altered mammary ductal morphology in *Brca2*<sup>Δ27/Δ27</sup> mice. Representative mammary gland whole mounts stained with carmine are illustrated from 6-month-old virgin female mice. A, *Brca2*<sup>Δ27/+</sup> mammary gland. B, *Brca2*<sup>Δ27/Δ27</sup> mammary gland. Ductal side branching is clearly reduced in *Brca2*<sup>Δ27/Δ27</sup> mammary tissue compared with *Brca2*<sup>Δ27/+</sup> littermates.

Table 1. Incidence of spontaneous tumor development in *Brca2*-deficient mice

Group	<i>Brca2</i> <sup>Δ27/Δ27</sup>	<i>Brca2</i> <sup>Δ27/+</sup>	<i>Brca2</i> <sup>+/+</sup>
Virgin females	14/25 (56%) <sup>a,b</sup>	9/30 (30%)	3/11 (27%)
Multiparous females	5/7 (71%)	0/1 (0%)	
Males	6/9 (67%) <sup>b</sup>	2/12 (17%)	0/4 (0%)
Total	25/41 (61%) <sup>c</sup>	11/43 (26%)	3/15 (20%)

<sup>a</sup> Number of tumor-bearing mice/number of mice at risk (% incidence).  
<sup>b</sup> Significantly greater than *Brca2*<sup>Δ27/+</sup> littermates ( $P = 0.05$ , Fisher's exact test).  
<sup>c</sup> Significantly greater than *Brca2*<sup>Δ27/+</sup> littermates ( $P = 0.01$ , Fisher's exact test).

Table 2 Spectrum of spontaneous tumor development in *Brca2*-deficient mice

	<i>Brca2</i> <sup>Δ27/Δ27</sup>	<i>Brca2</i> <sup>Δ27/+</sup>	<i>Brca2</i> <sup>+/+</sup>
Virgin females			
Carcinomas			
Mammary adenocarcinoma	1		
Gastric adenocarcinoma	1		
Gastric squamous cell carcinoma	1		
Endometrial carcinoma	1		
A/B lung carcinoma	1 (63 weeks) <sup>a</sup>		
Adenomas			
Pituitary adenoma	1	1	
A/B lung adenoma			1
Ovarian cystadenoma	1		
Adrenal cortical adenoma		1	
Mammary adenomyoepithelioma		1	
Sarcomas			
Histiocytic sarcoma	3 (65 weeks)	2 (64 weeks)	
Hemangiosarcoma	1 (62 weeks)		
Lymphomas			
Mediastinal lymphoma	5 (50 weeks)		1
Nodal lymphoma	1	4	2
Total	17 tumors/14 mice	9 tumors/9 mice	4 tumors/3 mice
Multiparous females			
Carcinomas			
Gastric squamous cell carcinoma	2 (69 weeks)		
Gastric adenocarcinoma	1		
Lymphomas			
Mediastinal lymphoma	2 (70 weeks)		
Total	5 tumors/5 mice	0 tumors/1 mouse	0 tumors/0 mice
Males			
Carcinomas			
Squamous cell carcinomas	2 (70, 71 weeks)		
A/B lung carcinoma	1		
Adenomas			
Adrenal cortical adenomas	2		
Hepatic adenoma	1		
Sarcomas			
Leiomyosarcoma	1		
Lymphomas			
Mediastinal lymphoma	1 (68 weeks)	1	
Nodal lymphoma		1	
Total	8 tumors/6 mice	2 tumors/2 mice	0 tumors/4 mice

<sup>a</sup> Tumor latency (weeks) for mice sacrificed because of morbidity prior to terminal sacrifices between 73 weeks (17 months) and 86 weeks (19 months) of age.

a subset of *Brca2*<sup>Δ27/Δ27</sup> offspring appear to either die during late gestation or shortly after birth, suggesting that loss of *Brca2* function impacts overall viability.

Secondly, the overall survival of *Brca2*<sup>Δ27/Δ27</sup> compared with *Brca2*<sup>Δ27/+</sup> virgin female mice is significantly decreased ( $P < 0.05$ ) by a life table test, providing additional support that lifetime survival is affected by this *Brca2* mutation. Interestingly, test matings of both adult *Brca2*<sup>Δ27/Δ27</sup> males and females with wild-type mice do not reveal apparent infertility or difficulty in raising litters.

**Inhibition of Mammary Side Branching in *Brca2*<sup>Δ27/Δ27</sup> Mice.** Alterations in normal growth and differentiation of mammary tissue from *Brca2*-mutant virgin females were determined using whole-mount analysis. At 6 months of age, mammary tissue from three *Brca2*<sup>Δ27/Δ27</sup> animals exhibited a dramatic lack of side branching and a much lower density of ductules compared with that observed for three heterozygous and wild-type littermates, which were indistinguishable (Fig. 2). Although the ducts reached the limits of the mammary fat pad in the homozygous mutant animals, a substantial lack of side branching was observed in these *Brca2*<sup>Δ27/Δ27</sup> females. These observations were confirmed by examining histological sections from these mice. This general trend of inhibited side branching observed in *Brca2*<sup>Δ27/Δ27</sup> females was maintained in the nine animals of all three genotypic classes that were examined subsequently at 9 months of age.

**Predisposition of *Brca2*<sup>Δ27/Δ27</sup> Mice to Spontaneous Tumor Development.** *Brca2*<sup>Δ27/Δ27</sup> mice display an increased incidence of a wide variety of solid tumors compared with their *Brca2*<sup>Δ27/+</sup> and *Brca2*<sup>+/+</sup> littermates (Tables 1 and 2). We observed untreated animals on a mixed 129 × C57BL/6N genetic background for tumor devel-

opment or signs of morbidity. Prior to 17 months, 9 spontaneous tumors were detected in *Brca2*<sup>Δ27/Δ27</sup> mice, whereas only a single tumor was observed in the *Brca2*<sup>Δ27/+</sup> animals, and no tumors were observed in *Brca2*<sup>+/+</sup> animals during this time period (Table 2).

Terminal sacrifices of the remaining animals were performed between 17 and 19 months of age. This enabled us to develop a comprehensive tumor spectrum for mice from each genotype (Table 2). With the combined data from all of the moribund and terminal sacrifices, the *Brca2*<sup>Δ27/Δ27</sup> mice exhibited a >2-fold increase in overall tumor incidence compared with their *Brca2*<sup>Δ27/+</sup> and *Brca2*<sup>+/+</sup> littermates. In 41 *Brca2*<sup>Δ27/Δ27</sup> mice, 30 tumors were observed, compared with only 11 tumors in 43 *Brca2*<sup>Δ27/+</sup> animals and 4 tumors in 15 *Brca2*<sup>+/+</sup> animals. Overall, 25 tumor-bearing animals were observed among 41 *Brca2*<sup>Δ27/Δ27</sup> mice for a tumor incidence of 61% (Table 1). In contrast, only 11 of 43 *Brca2*<sup>Δ27/+</sup> animals were tumor bearing (26% incidence), and 3 of 15 animals (20% incidence) were tumor-bearing in the *Brca2*<sup>+/+</sup> littermates (Table 1). These data from all mice combined show a highly significant difference ( $P < 0.01$ ) in the overall tumor rates between the *Brca2*<sup>Δ27/Δ27</sup> and *Brca2*<sup>Δ27/+</sup> animals. These results were also analyzed separately for virgin females alone, virgin plus multiparous females, and males only. The tumor response patterns and incidences for each of these smaller subgroups are similar to the tumor response of all animals combined. Statistically significant differences ( $P < 0.05$ ) in tumor incidences between the *Brca2*<sup>Δ27/Δ27</sup> and *Brca2*<sup>Δ27/+</sup> animals are observed when the data for each subgroup is considered independently. Because a smaller number of *Brca2*<sup>+/+</sup> mice were examined, there was not enough statistical power to distinguish tumor rates between the wild-

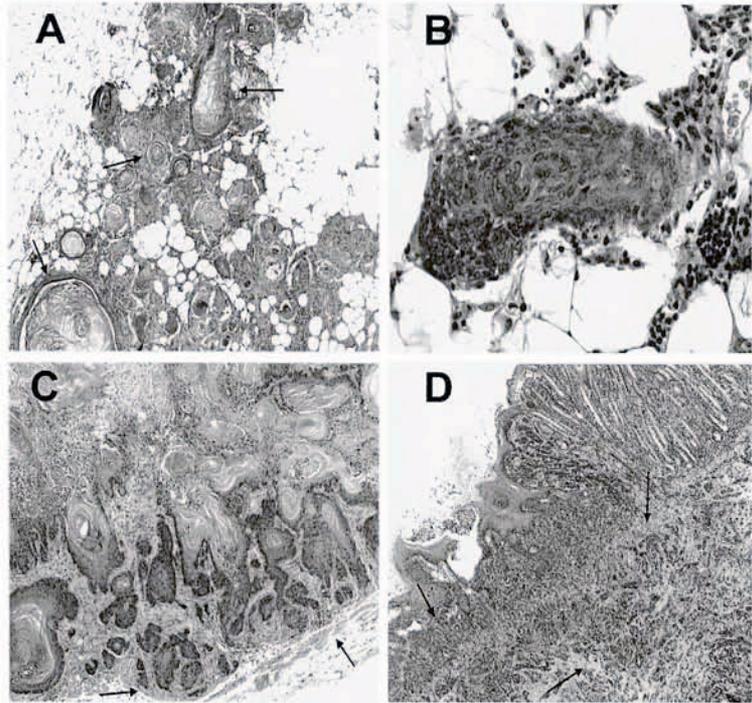


Fig. 3. Photomicrographs of carcinomas from *Brca2*<sup>Δ27/Δ27</sup> mice. *A*, mammary gland adenosquamous carcinoma characterized by irregular islands of neoplastic squamous cells admixed with small and large cysts filled with keratin debris (arrows) and inflammatory cells.  $\times 40$ , H&E stain. *B*,  $\times 400$  disorganized island of neoplastic squamous cells in mammary adenosquamous carcinoma pictured in *A*. *C*, gastric squamous cell carcinoma demonstrating islands of keratin-forming neoplastic cells invading into the submucosa and muscle wall (arrows).  $\times 40$ , H&E stain. *D*, gastric adenocarcinoma demonstrating various-sized acini and tubules separated by fine fibrovascular stroma and invading into the submucosa and wall of the stomach (arrows).  $\times 40$ , H&E stain.

type and other genotypic classes. We also did not observe a significant difference in overall tumor incidence between males and females for the *Brca2*<sup>Δ27/Δ27</sup> genotype with a 67% (6 of 9) incidence observed in males compared with a 59% (19 of 32) incidence observed for virgin and multiparous females combined (Table 1).

The tumor spectrum is diverse and includes carcinomas, adenomas, lymphomas, and sarcomas (Table 2). Of particular interest is the exclusive appearance of various types of carcinomas in the *Brca2*<sup>Δ27/Δ27</sup> mice. These include one mammary adenosquamous carcinoma (Fig. 3), five squamous cell carcinomas (three of gastric origin), two gastric adenocarcinomas, one endometrial carcinoma, and two A/B lung carcinomas. Fig. 3 illustrates representative histological sections for the two types of gastric tumors that were observed. Interestingly, a single mammary adenomyoepithelioma was detected in one *Brca2*<sup>Δ27/+</sup> mouse. The sarcoma incidence also appears to be increased in the *Brca2*<sup>Δ27/Δ27</sup> mice compared with their littermates. Three histiocytic sarcomas, along with a hemangiosarcoma and a leiomyosarcoma, arose in *Brca2*<sup>Δ27/Δ27</sup> mice, whereas only two histiocytic sarcomas were identified in their *Brca2*<sup>Δ27/+</sup> and *Brca2*<sup>+/+</sup> littermates. We found a wide variety of tumor types in the *Brca2*<sup>Δ27/Δ27</sup> mice that were sacrificed before 17 months of age because of morbidity (Table 2). Therefore, no obvious differences in latency between distinct tumor types were observed.

## Discussion

Here we describe germ-line *Brca2* mutant mice with a predisposition for tumor development. In contrast to previous reports of mice with homozygous germ-line mutations in the *Brca2* gene (6–8, 15–17), the *Brca2*<sup>Δ27/Δ27</sup> mice exhibit only a 33% decrease in expected viability during perinatal development. *Brca2*<sup>Δ27/Δ27</sup> mice of both sexes are fertile and lack gross developmental abnormalities. Mice harboring this germ-line mutation in the COOH-terminal domain of *Brca2* have an increased susceptibility for a wide spectrum of solid tumors. The fact that

9 of 10 spontaneous tumors that developed prior to 17 months arose in *Brca2*<sup>Δ27/Δ27</sup> mice indicates that not just the incidence but also the survival, because of tumor development, is significantly affected by the presence of this *Brca2* mutation. Overall, the tumor spectrum we observed is similar to that reported for knockout mice eliminating the COOH-terminal region of the *Brca1* gene, where an increased incidence of a wide variety of carcinomas, sarcomas, and lymphomas was found (24). One unique finding of our study is the exclusive presence of carcinomas in *Brca2*<sup>Δ27/Δ27</sup> mice and specifically a substantial number of stomach cancers, both adenocarcinomas and squamous cell carcinomas. We have shown previously that *Brca2* expression in adult mouse tissues correlates with cell proliferation and is relatively high in the glandular mucosa of the stomach (25). Interestingly, stomach cancers are among the various tumor types that have been associated with BRCA2 mutations in humans, with a 2.59 relative risk (3).

This report supports a strong correlation between the *Brca2* mutation position and the resulting *Brca2* mouse knockout phenotype. We and other laboratories have shown previously that mice homozygous for targeted *Brca2* mutations 5' of the BRC repeats in exon 11 exhibit early embryonic lethal phenotypes (6–8, 15–17). These BRC repeats interact with Rad51 (12, 13) and have been highly conserved in evolution (18, 26), which suggests that they are important functional domains of the *Brca2* protein. A few viable *Brca2*-mutant mice have been generated that retain at least some of these BRC repeats, and these animals display multiple severe developmental abnormalities, infertility, and early thymic lymphoma development (7, 8). The *Brca2*<sup>Δ27/Δ27</sup> mice lack only the COOH-terminal domain and could produce a truncated *Brca2* gene product that preserves all eight BRC repeats. In contrast to all other *Brca2*-null mice reported to date, these *Brca2*<sup>Δ27/Δ27</sup> mice display a modest loss of viability and have no gross developmental abnormalities or obvious infertility. Thus, full retention of the BRC repeats and other functional domains may direct a genotype-phenotype correlation.

This study extends previous findings by several laboratories that have demonstrated the functional significance of the COOH-terminal domain of both human and rodent BRCA2. Morimatsu *et al.* (10) demonstrated that mouse ES cells and embryonic fibroblasts lacking exon 27 are hypersensitive to  $\gamma$ -radiation and undergo premature senescence. Recent reports indicate that the COOH-terminus of Brca2 is required for error-free, homology-directed repair of DNA double strand breaks and the ability to facilitate the induction of Rad51 nuclear foci after ionizing radiation (20, 21). Thus, disruption of the exon 27 Brca2-Rad51 interaction appears to lead to defective repair of DNA damage and generalized chromosomal instability with a subsequent increased risk of neoplastic progression in *Brca2*-deficient cells (4, 5). The human COOH-terminal region of BRCA2 also appears to be critical because a truncating mutation (9808delCC) at position 3195, which occurs just upstream of the predicted Rad51-interacting domain in human BRCA2 exon 27, is associated with an elevated breast cancer risk (27).

Despite the increased susceptibility of *Brca2* <sup>$\Delta$ 27/ $\Delta$ 27</sup> mice to tumorigenesis, these animals are not highly susceptible to mammary tumorigenesis. Several factors may account for this observation, but the most likely reason is the fact that the *Brca2* <sup>$\Delta$ 27</sup> mutation was examined on a mixed C57BL/6N and 129 genetic background. C57BL/6N and 129/SvEvS6 mice are both extremely resistant to spontaneous as well as radiation-induced mammary carcinogenesis.<sup>4</sup> Thus, we are currently using microsatellite marker-assisted breeding techniques to transfer this *Brca2* <sup>$\Delta$ 27</sup> mutation onto inbred strains such as BALB/cJ that are susceptible to mammary carcinogenesis.

The reduced ductal branching phenotype seen in *Brca2* <sup>$\Delta$ 27/ $\Delta$ 27</sup> virgin female mice may be associated with increased mammary tumor risk in combination with other environmental or genetic interactions. We cannot exclude the possibility that the subtle mammary gland phenotype we have observed may be attributable to hormonal differences in the *Brca2* <sup>$\Delta$ 27/ $\Delta$ 27</sup> mice compared with their littermates rather than a more direct *Brca2* effect. However, Deng and Brodie (14) and Xu *et al.* (28) described a blunted ductal branching phenotype in the mammary glands of mice with a mammary-specific targeted *Brcal* mutation that was associated with subsequent tumor development after a long latency. In addition, a high incidence of mammary adenocarcinomas was reported recently in mice carrying a mammary tissue-specific mutation that completely disrupts the *Brca2* gene (29). Given their substantial viability and a cancer predisposition, *Brca2* <sup>$\Delta$ 27/ $\Delta$ 27</sup> mice and cells derived from them will be useful to further define the role of *Brca2* in tumorigenesis.

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