

**THE ROLE OF EPIGENETICS IN THE RAT MAMMARY GLAND**

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## **DEDICATION**

This thesis is dedicated to my family: my loving husband, Igor Koturbash; my ever-supportive parents, Wendy and Joseph Kutanzi; and my siblings, Heather, Cheryl, and Curtis Kutanzi.

## **ABSTRACT**

Epigenetics plays an important role in carcinogenesis with heritable changes in DNA methylation and histone modifications intricately linked to the initiation, promotion, and progression of cancer. Evidence shows that a number of chemical and physical agents can induce epigenetic changes during carcinogenesis. Two such agents, estrogen and ionizing radiation, are generally recognized as being carcinogenic. Yet the epigenetic repercussions of these carcinogens remain relatively unknown. More importantly, the combined effect of these carcinogens has never been addressed *in vivo* from an epigenetic standpoint. Therefore, we focused on the effect of estrogen and ionizing radiation applied separately or in conjunction. We have found that the exposure to estrogen, either alone or in combination with radiation, induced pronounced morphological alterations, which was paralleled by modifications to the epigenomic landscape in the mammary gland. The results obtained from these rodent models can potentially be extrapolated to humans.

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## LIST OF ABBREVIATIONS

[<sup>3</sup>H]-dCTP – tritiated cytidine triphosphate  
α – alpha  
β – beta  
4+8 – group receiving E<sub>2</sub> for 4 weeks, followed by 8 weeks without  
8-oxo-dG – 8-oxo-7,8-dihydro-2'-deoxyguanosine  
ACB – Alberta Cancer Board  
ACI – August Copenhagen Irish  
AHFMR – Alberta Heritage Foundation for Medical Research  
APE1 – apurinic/aprimidinic endonuclease 1  
BER – base excision repair  
CA – California  
COMT – catechol-O-methyltransferase  
CpG – cytosine-phosphate-guanine  
CT – control  
DCIS – ductal carcinoma *in situ*  
DNA – deoxyribonucleic acid  
DNMT – DNA methyltransferase  
DPM – disintegrations per minute  
DSB – double stranded DNA break  
dUTP – 2'-deoxyuridine 5'-triphosphate  
E<sub>2</sub> – estrogen; 17β-estradiol  
ECL - enhanced chemiluminescence  
EMCA – estrogen-induced mammary cancer allele  
ER – estrogen receptor  
ERE – estrogen response element  
ERK – extracellular signal-regulated kinase  
EZH2 – enhancer of zeste homologue 2  
GST – glutathione S-transferase  
Gy – gray  
h – homologue  
H&E – hematoxylin and eosin  
H3 – histone 3  
H4 – histone 4  
HAT – histone acetyltransferase  
HDAC – histone deacetylase  
HpaII – restriction enzyme  
HR – homologous recombination  
HRP – horseradish peroxidase  
i – inhibitor  
IHC – immunohistochemistry  
IN – Indiana  
IR – ionizing radiation  
JNK – c-jun N-terminal kinase  
K – lysine  
kV – kilovolt

LET – linear energy transfer  
LINE – long interspersed nuclear element  
MA – Massachusetts  
mA – miliamper  
MAPK – mitogen-activated protein kinase  
MBD – methyl-CpG binding domain  
MCS – mammary cancer susceptibility alleles  
MD – Maryland  
Mdm2 – mouse double minute 2  
Me3 – trimethylation  
µl – microliter  
MO –Missouri  
MeCP – methyl-CpG binding proteins  
miRNA – microRNA  
NHEJ – Non-homologous end joining  
NIEHS – National Institute of Environmental Health Sciences  
NSERC – Natural Sciences and Engineering Research Council of Canada  
ON – Ontario  
p – phosphorylated  
p42-44 – extracellular signal-regulated kinase 1/2  
PA - Pennsylvania  
PBS – phosphate buffered saline  
PCNA – proliferating cell nuclear antigen  
PCR – polymerase chain reaction  
PFA – paraformaldehyde  
PI3K – phosphatidyl inositol 3-kinase  
Pol.β – DNA polymerase β  
PRMT1 – protein arginine N-methyltransferase  
PVDF – polyvinylidene fluoride  
RNA – ribonucleic acid  
ROS – reactive oxygen species  
S – serine  
SAM – S-adenosyl-methionine  
SAPK – stress-activated protein kinase  
SDS – sodium dodecyl sulfate  
SEM – standard error of the mean  
SERM – selective estrogen receptor modulator  
Src – sarcoma  
SSB – single stranded DNA break  
ST – sulfotransferase  
Suv – suppressor of variegation  
SWI/SNF – switch/sucrose non-fermentable remodeling complex  
TdT – Terminal deoxynucleotidyl transferase  
TEB – terminal end bud  
TMA – tissue microarray  
TUNEL – terminal uridine deoxynucleotidyl transferase  
TX – Texas

UHRF1 – ubiquitin-like, containing PHD and RING finger domains 1  
UK – United Kingdom  
USA – United States of America  
VA – Virginia  
WI – Wisconsin  
X-ray – Rontgen ray

# 1. GENERAL INTRODUCTION

## 1.1. RISK FACTORS FOR BREAST CANCER

Breast cancer is the most commonly diagnosed malignancy in women and the leading cause of death among women between the ages of 35 to 55 years (Schairer et al., 2004). Currently, over one million cases occur worldwide each year, with a predicted 50% increase in cancer rates by the year 2020 (World Health Organization, 2003).

Although breast cancer mortality rates have started to decline in developed countries, due to improvements in early detection and treatment (World Health Organization, 2003), there remain serious gaps in understanding the underlying epidemiological factors. It is estimated that only 5% of new breast cancer cases are attributed to susceptibility genes (Ronckers et al., 2005), while the etiology for the remaining 95% of cases are rather obscure.

Breast cancer arises through a multi-step process that involves the de-regulation of growth regulatory pathways, resulting in uncontrolled proliferation and impaired apoptosis (Simpson, 2005; Feinberg, 2004). Carcinogenesis begins with a single cell which obtains a mutation in a critical gene (Simpson, 2005; Feinberg, 2004). When this happens the cell is said to be “initiated” – an event which is largely considered to be irreversible (Lo and Sukumar, 2008; Russo and Russo, 2006). Clonal expansion of the initiated cell is stimulated by promoting agents, including growth factors and hormones, and can be observed as focal proliferation (Yager and Davidson, 2006; Asch and Barcellos-Hoff, 2001). This is referred to as the promotional stage of carcinogenesis. Importantly, the clonal expansion of initiated cells observed at this stage was found to be, to some extent, reversible upon removal of the stimulating agent (Yager and Davidson,

2006; Farber, 1984; Tatematsu et al., 1983). These cells tend to exhibit genome instability and acquire further mutations over time that permit the malignant transformation to carcinoma cells (Lo and Sukumar, 2008; Chen et al., 2007; Hanahan and Weinberg, 2000). Unregulated growth of cancerous cells is regarded as the progression of cancer. These malignant cells may possess the capacity to invade surrounding tissue and metastasize to other parts of the body (Hanahan and Weinberg, 2000). It is important to note that there may be considerable time – several years or even decades – between the initiation of a single cell and the detection of cancer (Yager and Davidson, 2006; Ronckers et al., 2005).

It has been suggested that initiating events must occur relatively early in life because exposure to genotoxic agents later in life has been shown to not significantly increase subsequent breast cancer risk (Willett et al., 2000). This is supported by numerous studies that show the most susceptible targets in the mammary gland for transformation is the hormonally-responsive subpopulations of epithelial cells within the undifferentiated terminal end buds (TEBs) (Hilakivi-Clarke, 2007; Warri et al., 2007; Russo et al., 2005; Russo et al., 1982). These terminal end buds are highly proliferative by nature and have a reduced capacity to repair DNA damage (Russo and Russo, 1982). This damage may then be passed on as TEBs proliferate and differentiate into ductules and alveolar buds during mammary gland development (Russo and Russo, 1996A; Russo and Russo, 1982), giving rise to ductal and lobular carcinomas.

A number of intrinsic and extrinsic factors have been identified to play a causative role in breast carcinogenesis (Park et al., 2000; Ishibe et al., 1998; Helzlsouer et al., 1995). Different physical and chemical carcinogens acting at various stages of breast

cancer development affect a wide spectrum of cellular processes, including proliferation, apoptosis, DNA repair, and cell signaling (Kovalchuk et al., 2007; Loree et al., 2006; Shan et al., 2005; Fritz et al., 2003). The exposed cells are often subject to increased levels of DNA damage that, if left unrepaired, give rise to permanent mutations (Hasty, 2005). The accumulation of mutations over time contributes to genome instability and the formation of tumor cells (Simpson et al, 2005; Feinberg, 2004). Interestingly, growing evidence indicates many of these changes already occur in histologically normal breast epithelial cells from healthy women, which renders them more susceptible to malignant transformation (Ding et al., 2006; Crawford, et al., 2004; Li et al., 2004A).

Clearly, the importance of identifying breast cancer risk factors is many fold. Firstly, the assessment of susceptibility genotypes could be used to identify women at higher risk for breast cancer, which can be used to implement diagnostic and early treatment strategies. Secondly, studies of identical twins have demonstrated the importance of lifestyle factors, of which only 20% concordance was observed for breast cancer (Hamilton and Mack, 2003; Lichtenstein et al., 2000). This suggests that cancer is, at least in part, a preventable disease that may require lifestyle changes, including healthier food choices and physical activity (Anand et al., 2008). Several lines of evidence also suggest that lifestyle and other environmental agents modulate changes in the epigenome, thereby influencing gene expression patterns without altering the underlying DNA sequence (Anand et al., 2008; Pogribny et al., 2008; Nardone and Compare, 2008; Pogribny et al., 2007). The identification of epigenetic markers which regulate gene expression may be a promising field of research to identify new targets for therapy. Furthermore, genetic and epigenetic profiling has been used to identify cancer

and their subtypes, providing an important diagnostic tool that can be used to tailor treatment and predict response (Figuerola et al., 2010; Shen et al., 2007; Nigro et al., 2005; van Delft et al., 2005; Bucca et al., 2004; Downing, 2003; Yeoh et al., 2002). Future studies are needed to address the complex interplay between genetic, epigenetic, and environmental factors.

### **1.1.1. Role of Genetic Changes in Breast Cancer**

An individual's genome, which influences phenotypic outcomes, is determined by the maternal and paternal contributions during fertilization. Inheriting a mutation in a single gene, for example, can increase the probability of developing diseases (Klein and Schlossmacher, 2006; Schilsky and Fink, 2006; Peto and Houlston, 2001; Russo et al., 2000; Gatti, 1993). Genetic testing is an important tool for identifying individuals who are genetically predisposed to certain health problems, providing an opportunity to intervene with preventative treatments and for early detection.

In the past, identification of genetic factors linked to increased risk largely came from familial studies in which specific diseases were found to occur at high frequencies (Lichtenstein et al., 2000; Peto and Mack, 2000). For example, the observation that some families exhibit high frequencies of breast cancer has led to the identification of two major breast cancer genes, BRCA1 and BRCA2 (Tavtigian et al., 1996; Wooster et al., 1995; Futreal et al., 1994; Miki et al., 1994). It was found that mutations in the germline gave rise to these high penetrance mutant alleles, in which 60-85% of carriers develop the disease over their lifetime (van der Kolk et al., 2010; Brose et al., 2002; Easton et al., 1995). This represents a major predisposing genetic risk factor in 10-25% of familial

breast cancer cases (Bradbury and Olopade, 2007; King et al., 2003). Similarly, a number of low-penetrance susceptibility genes have also been identified, including FGFR2, TOX3, MAP3K1 which control cell proliferation and are associated with chromatin structure, however they account for only a small portion of familial breast cancer genes (Paglia et al., 2010; Easton et al., 2007; Tchatchou et al., 2007; Dumitrescu et al., 2005; Iau et al., 2001).

Studies of mutations in rare syndromes have also been associated with a wide variety of cancers, including increased risk of breast cancer in female carriers, suggesting a common theme of instability (Birch et al., 2001; Nichols et al., 2001). For example, in the Li-Fraumeni syndrome, mutations in the p53 tumor suppressor gene impairs the cell's ability to respond to stress signals, including radiation and some DNA-damaging drugs (Malkin et al., 1993). As the guardian of the genome, p53 functions in a number of anti-cancer mechanisms, including the activation of DNA repair proteins, cell cycle arrest, and induction of programmed cell death (Efeyan and Serrano, 2007; Chumakov, 2000). All of these processes are impaired by mutations in p53 (Nichols et al., 2001; Greenblatt et al., 1999; Selvanayagam et al., 1995). Interestingly, a variant of Li-Fraumeni syndrome was attributed to mutations in CHEK2, a kinase which activates p53 activity (Lee et al., 2001). Similarly, particular alterations in the ATM gene are associated with compromised p53 function and increased *in vitro* chromosomal sensitivity to radiation (Broeks et al., 2007; Gutierrez-Enriquez et al., 2004; Broeks et al., 2000). Since then, numerous associations have been reported between breast cancer and genes involved in pathways critical for genomic stability (Paglia et al., 2010; Walsh and King, 2007; Tchatchou et al., 2007).

One pivotal biological process implicated in maintaining genomic integrity is DNA repair. The number of spontaneous errors arising due to intrinsic causes alone, including the misincorporation of nucleotides by DNA polymerase during replication and in response to damage caused by reactive oxygen species (ROS), can result in up to  $1.9 \times 10^5$  DNA modification events per cell per day (Shapiro, 1981). Moreover, the majority of etiological factors implicated in breast cancer, such as ionizing radiation (IR), estrogen, and diet, are capable of generating reactive oxygen radicals, which may cause the formation of oxidized DNA bases, bulky DNA adducts and DNA strand breaks (Loree et al., 2007; Mobely and Brueggemeier, 2004; Kang, 2002; Chen et al., 1998; Oakley et al., 1996). If the rate of DNA damage exceeds the capacity of the cell to repair it, damage sentinels, such as p53, may initiate events leading to cell cycle arrest, early senescence and apoptosis (Chen et al., 2007; Hanahan and Weinberg, 2000). If these sentinels are inactivated and the damaged cells are allowed to divide, these mutations become permanent (Chen et al., 2007; Hanahan and Weinberg, 2000). If the mutation occurs within a critical gene responsible for maintaining genome integrity, a snowball effect is observed, with subsequent generations acquiring additional mutations leading to the development of cancer (Hanahan and Weinberg, 2000). Therefore, it would appear that women who carry defective DNA repair genes are at higher risk for developing breast cancer (Andrieu et al., 2006; Bell et al., 1999; Fan et al., 1999; Roberts, 1999; Shen et al., 1998). Although the majority of DNA repair genes have low-penetrance (Smith et al., 2008; Chang-Claude et al., 2005), their resulting phenotypes follow a common theme of genome instability and may serve as markers of breast cancer susceptibility.

The clinical benefit of the identification of such susceptibility genes is in assessing the risk of developing breast cancer in carriers. Ultimately, it is hoped that knowledge of an individual's genetic profile in relation to these genes may be used in targeted preventative and treatment modalities for maximal efficacy. Understanding the effects of these genetic factors is crucial in choosing an appropriate form of treatment. For example, studies have shown that germline mutations in BRCA1, BRCA2, ATM or CHEK2 may double the risk of radiation-induced secondary malignancies in the contralateral breast following radiotherapy to treat the first breast cancer (Broeks et al., 2007). In the future, clinical approaches should take into consideration the underlying genetic factors, tailoring treatment modalities to a patient's genetic background to improve long-term health.

### **1.1.2. Role of Epigenetics in Breast Cancer**

Classic genetics alone cannot account for the differences in disease susceptibility. Indeed, a number of studies have found discordance in the incidence of neurological and neoplastic diseases between monozygotic twins, despite their identical DNA sequences, that raise questions regarding the importance of the epigenome and the influence of environmental factors in the development of diseases, including cancer (Ballestar, 2009; Haque et al., 2009; Singh and O'Reilly, 2009; Wilson, 2008; Poulsen, 2007; Fraga et al., 2005A).

The term "epigenetics" is used to describe the study of meiotically and mitotically stable regulation of gene expression patterns that is not encoded in the DNA sequence itself. The epigenome, which encompasses the overall epigenetic state of a cell, is

primarily comprised of two interconnected markers by which mammalian cells modify the expression of their genomes without altering the DNA sequence – DNA methylation and covalent histone modifications (Fraga et al., 2005B; Jaenisch and Bird, 2003; Ehrlich, 2002; Robertson et al., 2002). More importantly, unlike DNA, epigenetic changes are reversible, allowing genes to be turned “on” or “off” as need be (Reik, 2007; Jaenisch and Bird, 2003).

The epigenetic state of the genome is largely established early during development (Reik, 2007). Epigenetic changes are responsive to a number of endogenous and environmental cues, that regulate differentiation of cells – transforming a single fertilized egg into an organism with very distinct cell types, such as neurons, muscle and epithelial cells, blood vessels, and so on - each exhibiting very different gene expression patterns (Reik, 2007). Transcription of these genes are regulated by methylation and histone patterns in the developing embryo giving rise to functionally and morphologically very diverse cells and tissues (Reik, 2007).

Over the years, the role of epigenetics in the etiology of diseases, including breast cancer, has become increasingly recognized (Feinberg and Tycko; 2004, Feinberg, 2004; Jones and Baylin, 2002; Widschwendter and Jones, 2002; Feinberg and Vogelstein, 1983). The identification of DNA hypomethylation in diseased tissue compared to adjacent, histologically normal tissue in cancer patients was one of the first epigenetic markers linked to human disease (Feinberg and Vogelstein, 1983A). Since then, a number of other epigenetic alterations have been found to mediate inappropriate gene expression linked to increased genome instability (Salisbury, 2001). Furthermore, errors in establishing “normal” epigenomic patterns during preneonatal development have been

correlated with increased cancer predisposition (Sparago et al., 2007; DeBaun et al., 2002; Debaun and Tucker, 1998).

Inappropriate silencing of critical regulatory genes, such as the p16, MGMT and hMLH1 tumor suppressors, has been shown to lead to cancer (Starland-Davenport et al., 2010; Esteller, 2005; Brown and Strathdee, 2002; Esteller and Herman, 2002; Harris, 1996). In adopting Knudson's two-hit hypothesis (Knudson, 1996; Knudson, 1971) in the field of epigenetics, the concept that tumor suppressor genes must be inactivated on both alleles can be interpreted not only in the sense of genetic mutations, but also due to epigenetic modifications. For example, DNA hypermethylation of both alleles, or in combination with genetic mutations, was shown to effectively prevent genes from being expressed, thereby providing certain pro-survival and growth advantages during carcinogenesis (Esteller et al., 2001; Grady et al., 2000). Silencing of genes, such as those involved in DNA repair and cell cycle regulation, appears to escalate during the progression towards malignancy (Schultz et al., 2009). Furthermore, the silencing of genes regulating cell adhesion and motility enables tumor cells to break away from the primary tumor and metastasize (Schultz et al., 2009). Interestingly, however, the loss of such gene products is not always advantageous, and may in fact interfere with the tumor's ability to survive in a new location (Domann and Futscher, 2004). Exploiting the reversible nature of the epigenome can provide a selective advantage to tumor cells, allowing re-expression of genes previously silenced by epigenetic mechanisms (Domann and Futscher, 2004). For example, following colonization of cancer cells at distant sites, the malignant tumor may reactivate genes whose products suppress motility and invasion

to create an environment to sustain the growth of a secondary tumor (Welch, 2006; Yates et al., 2005; Domann and Futscher, 2004; Jawhari et al., 1999).

Importantly, epigenetic alterations can also be observed prior to tumor development (Pogribny and Beland, 2009; Pogribny et al., 2008; Kovalchuk et al., 2007). According to the epigenetic hypothesis of cancer initiation, it is the interaction between genetic and epigenetic components that determine the effect of initial genetic insults (Feinberg and Tycko, 2004). It is believed that these alterations can predispose cells to genomic instability in which the initiated cells acquire a growing number of genetic changes during carcinogenesis (Baylin and Ohm, 2006; Gould et al., 1996). It is also now generally accepted that epigenetic instability is a hallmark of cells progressing towards malignancy (Jaenisch and Bird, 2003; Jones and Baylin, 2002).

#### **1.1.2.1. DNA Methylation**

Cytosine DNA methylation was the first epigenetic mark to be identified, and is one of the best studied epigenetic mechanisms for regulating expression of the genome (Feinberg and Tycko 2004; Jones and Baylin 2002; Jones and Laird 1999; Feinberg and Vogelstein, 1983A). DNA methylation regulates important cellular processes, such as differentiation, proliferation and apoptosis during normal development (Baylin and Ohm, 2006; Jaenisch and Bird, 2003; Feil et al., 1994; Holliday and Pugh, 1975), giving rise to cell- and tissue-specific gene expression patterns (Illingworth et al., 2008; Domann and Futscher, 2004; Raiche et al., 2004; Futscher et al., 2002). DNA methylation has also been implicated in regulating X chromosome inactivation, genomic imprinting, and silencing of foreign DNA, acting as a repressor of gene expression to maintain genome

stability (Raiche et al., 2004; Bird, 1993; Li et al., 1993; Singer-Sam and Riggs, 1993; Zhang et al., 1993).

Cytosine DNA methylation is a chemical modification that results from the transfer of a methyl group from the cofactor S-adenosylmethionine (SAM) to the 5 carbon position of cytosine residues by DNA methyltransferases (DNMTs) (Chen and Li, 2006; Chiang et al., 1996). In mammalian cells, DNA methylation patterns are primarily established by DNMT3a and DNMT3b during embryogenesis, with DNMT1 largely being responsible for ensuring that these set patterns are faithfully maintained (Chen and Li, 2006; Goll and Bestor, 2005; Robertson, 2002; Okano et al., 1999; Turker and Bestor, 1997). The presence of methyl groups on cytosine residues can recruit proteins, known as methyl-CpG-binding proteins (MBDs), to the genomic region in which it is localized to assist in transcriptional repression (Kimura and Shiota, 2003; Jones et al., 1998; Nan et al., 1998). In this way, with the help of such proteins as the methyl-CpG-binding protein 2 (MeCP2), DNMT1 is able to bind to hemimethylated DNA immediately after replication, adding methyl groups to unmethylated cytosine residues on the nascent strand opposite to the template (Jones et al. 1998; Nan et al. 1998). However, since many genes should only be expressed during certain developmental stages or in response to changes in the cellular environment, unnecessary genes can be inactivated by the addition of methyl groups to previously unmethylated CpG sites by the *de novo* methyltransferases, DNMT3a and DNMT3b (Okano et al., 1998; Robertson et al., 1999).

Typically methylation occurs at CpG islands, which are regions of the genome possessing greater than 60% CG content (Takai and Jones, 2002; Gardiner-Garden and Frommer, 1987). These regions occur most frequently within promoter regions of genes,

and provide an opportunity to regulate transcriptional activity by methylation (Bird, 2002; Takai and Jones, 2002; Gardiner-Garden and Frommer, 1987). About 60% of human genes are transcribed from CpG-rich promoter sequences, with the majority of genes being unmethylated (Clouaire et al., 2010; Weber et al., 2007; Bird, 1993). The presence of methyl groups on DNA may physically impede the binding of transcriptional activation factors and RNA polymerase, thereby effectively preventing gene expression (Weber et al., 2007; Weber and Schuebeler, 2007; Schuebeler et al., 2000). Methylated DNA may also indirectly hinder transcription when bound by MBDs, which recruit chromatin remodelling complexes to modify the packaging of the DNA, resulting in a more compact and inaccessible chromatin structure termed heterochromatin (Fuks et al., 2003; Schuebeler et al., 2000; Jones et al., 1998; Nan et al., 1998). This link between DNA methylation and chromatin structure is very important for the regulation of gene expression.

Abnormal DNA methylation patterns have been associated with a number of developmental defects and human diseases, including cancer (Jaenisch and Bird, 2003; Ehrlich, 2002; Robertson, 2002; Baylin et al., 2001). Indeed, global hypomethylation, as well as regional hyper and hypo-methylation of specific genes has been shown to be very important in breast cancer etiology and pathogenesis (Ronckers et al. 2005; Szyf et al., 2004; Yang et al. 2001; Bernardino et al., 1997).

The loss of genomic DNA methylation was the first epigenetic abnormality detected in cancer cells, and is linked with the activation of oncogenes and transposable elements, leading to increased genome instability, including chromosome breakage and aneuploidy (Pogribny et al., 2007; Weber and Schuebeler, 2007; Kovalchuk et al., 2004; Pogribny et al., 2004; Raiche et al., 2004; Robertson and Wolffe, 2000; Feinberg and

Vogelstein, 1983B; Gama-Sosa et al., 1983). The overall loss of DNA methylation has been attributed to a number of factors, including decreased DNA methyltransferase activity (Dudley et al., 2008; Dodge, 2005). Low levels of DNMT1, by virtue of the nature of replication which produces daughter strands that are unmethylated, would, over time, lead to passive demethylation (Dudley et al., 2008). Recent data suggest the inactivation of *de novo* methyltransferases also contributes to hypomethylation of DNA and chromosome instability (Dodge, 2005). Alternatively, active removal of DNA methyl groups by demethylating enzymes, such as RNA-dependent 5-methylcytosine glycosylase, can considerably influence global genomic methylation with an inverse relationship observed in cancer cells (Szyf, 2000). DNA methylation patterns can also be altered by chemical and physical agents (Pogribny et al., 2009; Koturbash et al., 2005; Christman, 1993; Szyf, 2003). DNA damage, such as that induced by carcinogens, can interfere with the methylating ability of DNA methyltransferases by stalling them at the site of the lesions (Panayiotidis et al., 2004; Smith, 1998; Turk et al., 1995). Furthermore, during repair, DNA polymerases incorporate cytidine but not methylcytidine, thus the presence of DNA lesions and activation of DNA repair mechanisms may also contribute to DNA hypomethylation (Pogribny et al., 2005). Regardless of the mechanism involved, loss of genomic DNA methylation can be observed early in tumor development (Kovalchuk et al., 2007; Pogribny and Beland, 2009) and may be a cause, not merely a consequence, of malignant transformation (Gaudet et al., 2003).

Similarly, genome instability can also arise from the hypermethylation of critical genes, such as tumor suppressors, by *de novo* methyltransferases. The transfer of methyl groups to previously unmethylated CpG islands, such as with the hypermethylation of

promoter regions of tumor suppressor genes, affect a number of growth regulatory molecular networks and leads to increased risk of mammary cancer (Fernandez et al., 2010; Berg and Steigen, 2008; Esteller, 2002; Yang et al., 2001).

#### **1.1.2.2. *Histone Modifications***

Tightly coordinated interactions between DNA and histone proteins determine the level of DNA packaging and thus transcriptional regulation (Cedar and Bergman, 2009; Esteller, 2006). The positive charge of histone proteins interact with negatively charged DNA (Hong et al., 1993). An octamer of histones – two molecules each of H2A, H2B, H3, and H4 – wrap around 146 base pairs of DNA (Luger et al., 1997; Fletcher and Hansen, 1995). Histone H1 locks the DNA into place, linking the nucleosomes together, ultimately leading to the formation of a higher-order chromatin structure (Sato et al., 2001). Modifications to histone proteins affect the degree to which they can bind to DNA to regulate chromatin packaging (Grant, 2001).

Histone modifications, including acetylation, methylation, phosphorylation and ubiquitination, are important in the regulation of transcription and overall genome stability (Moss and Wallrath, 2007; Weidman et al. 2007; Pogribny et al., 2006; Tryndyak et al., 2006; Jenuwein and Allis, 2001). It has been proposed that patterns of histone modifications form a “histone code” that is read by post-translational complexes to regulate gene expression (de la Cruz et al., 2005; Turner, 2002; Jenuwein and Allis, 2001; Strahl and Allis, 2001). These modifications are thought to occur in a hierarchical fashion, with present markers at the same or nearby sites influencing further post-translational modifications (Stewart et al., 2005; Strahl and Allis, 2000; Jenuwein and

Allis, 2001). Some studies have shown, for example, that acetylation of histone H3 lysine 9 or lysine 14, which is associated with a more relaxed chromatin structure, is able to restrict histone 3 lysine 9 methylation, a repressive modification (Carbone et al., 2006; Nakayama et al., 2001; Rice and Allis, 2001). In this way, histone modifications can lead to either transcriptional activation or repression depending on the pattern of methylation, acetylation, or phosphorylation of amino acid residues on histone tails.

#### **1.1.2.2.1. HISTONE METHYLATION**

Histone methylation is the modification of certain amino acids in a histone protein by the addition of one to three methyl groups (Bannister and Kouzarides, 2004; Schotta et al., 2004; Rice and Allis, 2001). Several protein residues can be methylated, most notably the positive groups of lysine and arginine (Rice and Allis, 2001). Methylation at these sites is used to regulate the binding of proteins to nucleic acids, without altering the positive charge of histone tails (Bannister and Kouzarides, 2004; Rice and Allis, 2001).

Histone methylation is associated with both transcriptional activation and repression, depending upon the residues methylated and to which extent. For example, trimethylation of histone H3 lysine 9 (H3K9me<sub>3</sub>), histone H3 lysine 27 (H3K27me<sub>3</sub>), and histone H4 lysine 20 (H4K20me<sub>3</sub>) are associated with transcriptional repression, whereas methylation of arginine residues on H3 and H4 is associated with transcriptional activation (Bannister and Kouzarides, 2004; Schotta et al., 2004; Rice and Allis, 2001).

Post-translational modifications to these histone tails serve as recognition motifs for the binding of chromatin-associated proteins, which can influence other epigenetic-mediated mechanisms of gene regulation (Zeng et al., 2009; Schlesinger et al., 2007; Vire

et al., 2006; Freitag and Selker, 2005). For example, two well-studied histone methyltransferases (HMT), enhancer of zeste drosophila homologue 2 (EZH2) and the suppressor of variegation 3-9 homologue 1 (Suv39h1), has been shown to mark genes for DNMT1-mediated DNA hypermethylation by trimethylating H3K27 and H3K9, respectively (Schlesinger et al., 2007; Vire et al., 2006; Lehnertz et al., 2003), thereby reinforcing long-term gene silencing (Cedar et al., 2009; Esteller, 2008; Vire et al., 2006).

In a normal cell, the recruitment of chromatin-modifying complexes plays an important role in maintaining heterochromatic regions, suppressing repetitive elements, and actively partaking in the correct assembly of telomeric chromatin (Benetti et al., 2007). However, in cancer cells, the inappropriate recruitment of these complexes can lead to an altered chromatin state (Esteller and Herman, 2002; Baylin, 1997). In general, cancer formation is characterized by two different types of alterations in histone lysine methylation patterns: loss of global histone H3K9, H3K27, and H4K20 trimethylation and an increase of these marks at gene promoter regions (Van Den Broeck et al., 2008; Wei et al., 2008; Fraga et al., 2005B; Kondo et al., 2004). Furthermore, it was recently demonstrated that the loss of trimethylation of H3K9 and H4K20, paralleled by the diminished expression of the HMT Suv4-20h2, is associated with the formation of a more aggressive phenotype in human breast cancer cell lines (Tryndyak et al., 2006).

#### **1.1.2.2. HISTONE ACETYLATION**

Histone residues can also be acetylated and deacetylated on lysine residues in the  $\epsilon$ -N-terminal tail as part of gene regulation. Typically, these reactions are catalyzed by

enzymes with histone acetyltransferase (HAT) or histone deacetylase (HDAC) activity, respectively (Esteller, 2008).

The addition of an acetyl group to histone tails neutralizes the positive charge on the lysine residue, thereby reducing the electrostatic attraction for the negatively charged nucleic acids (Hong et al., 1993). This results in a more relaxed chromatin state, thereby increasing accessibility for the transcription machinery to bind DNA. Conversely, histone deacetylation strengthens the associations between histone proteins and DNA to induce a repressive heterochromatic state (Jenuwein and Allis, 2001).

Imbalances between histone acetylation and deacetylation have been detected in various tumors, including human breast cancer (Blanca et al., 2008; Maass et al., 2002; Archer and Hodin, 1999). While hypoacetylation has been associated with silencing of tumor suppressors (Fukushige et al., 2009; Kondo et al., 2005; Mielnicki et al., 1999), hyperacetylation has been linked to a more active transcriptional state that may be associated with uncontrolled proliferation and the activation of oncogenes (Yasuda et al., 2007; Verdone et al., 2006). It is widely believed that the abnormal state of histone acetylation is associated with the downregulation of HATs and overexpression of HDACs reported in breast cancer (Kawai et al., 2003). Furthermore, it was shown that the overexpression of HDAC1 contributed to the progression of breast carcinogenesis by down-regulating estrogen receptor alpha (ER $\alpha$ ), a critical growth regulatory gene (Kawai et al., 2003; Macaluso et al., 2003).

### **1.1.2.2.3. HISTONE PHOSPHORYLATION**

Phosphorylation is perhaps the most important chemical modification of proteins, inducing structural changes that affect molecular interactions. A phosphate group can be attached to serine, threonine and tyrosine residues by kinases, thereby adding a negative charge at that site (Wei et al., 1999; Wei et al., 1998). The addition of a phosphate group to serine 10 on the histone protein H3 (H3S10) has gained a lot of attention for its role in the maintenance of chromosome condensation in actively dividing cells during mitosis (Wei et al., 1999; Wei et al., 1998). The precise mechanism by which increased levels of pH3S10 affects genome stability is still under debate (Johansen et al., 2006; Dyson et al., 2005; Zhong, 2000; Wei et al., 1999; Wei et al., 1998) and may depend on the status of histone modifications on nearby residues. Moreover, phosphorylation of H3S10 can activate immediate-early genes during interphase which may be linked with the induction of proto-oncogenes (Thomson et al., 1999; Mahadevan et al., 1991).

### **1.1.2.3. *Clinical Application of Epigenetics***

Unlike genetic alterations, epigenetic changes need not be permanent in tumor cells (Jaenisch and Bird, 2003). Thus when the cellular epigenome is altered, giving rise to a new gene expression pattern, the cell and its progeny possess the capability to revert back to previous gene expression patterns (Jaenisch and Bird, 2003). The ability to reverse these cancer-related changes makes epigenetic machinery an ideal target for therapeutic intervention by inducing transcriptional reprogramming. This plasticity would provide an important mechanism for re-regulating genes, such as cell-adhesion, that have been silenced during the course of selective processes in cancer progression,

which provides the tumor with the necessary phenotypic characteristics to metastasize (Futscher and Domann, 2006; Conacci-Sorrell et al., 2002; Jawhari et al., 1999).

An important point to consider is that genes with tumor suppressor function, rarely, if ever, are mutated – rather they are often silenced by aberrant DNA methylation (Domann and Futscher, 2004; Hamai et al., 2003; Futscher et al., 2002; Cairns et al., 2001; Esteller et al., 1999). Therefore the use of pharmacological inhibitors of DNA methylation, such as 5-azacytidine and 5-aza-2'-deoxycytidine, has great potential to reactivate genes that have been inappropriately silenced (Egger et al., 2004; Christman, 2002). Like the nucleotide cytosine, they pair with guanine during DNA synthesis. After they are incorporated, they block DNMT enzymes from acting, thereby inhibiting DNA methylation (Egger et al., 2004; Christman, 2002).

However, considering that DNA methylation changes are often paralleled by histone modifications associated with inappropriate gene expression during carcinogenesis (Taipale et al., 2005; Bannister and Kouzarides, 2004; Geiman and Robertson, 2002), it is likely that treatment strategies need to target multiple types of epigenetic modifications. Many studies have indicated that deacetylation of histone residues can also lead to silencing of tumor suppressor genes (Kondo et al., 2005; Hamai et al., 2003; Schubeler et al., 2000). To reactivate genes that have been inappropriately silenced, histone deacetylase inhibitors are used (Nebbio et al., 2005; Peart et al., 2005; Shetty et al., 2005; Michaelis et al., 2004). For example, two HDAC inhibitors (HDAC-i), Zolinza (Vorinostat) and Istodax (Romidepsin), which can induce differentiation, cell-cycle arrest, and apoptosis have recently been approved by the USA Food and Drug Administration for the treatment of cutaneous T cell lymphoma (Wang and Dymock,

2009). Clinical trials are underway to determine if HDAC inhibitors can also be used in the treatment of human breast cancer. Indeed the results of numerous studies have provided positive support for targeting epigenetic reprogramming during breast carcinogenesis, indicating that epigenetic therapy may be a promising new approach to treating cancer (Stearns et al., 2007; Arce et al., 2006).

Furthermore, since these epigenetic changes can be detected prior to tumor development they may serve as biomarkers for early detection of disease, prognosis, and response to treatment (Kristensen and Hansen, 2009; Esteller, 2008; Van Den Broeck et al., 2008; Ting et al., 2006; Dunn et al., 2003; Cairns et al., 2001). Investigations regarding the potential value of epigenomic profiling is currently underway using samples collected from tissue biopsies or from less intrusive methods, such as blood and urine samples, to evaluate the DNA-methylome and to map histone modifications (Esteller, 2008; Ting, 2006; Cairns et al., 2001). The information collected can then be used to predict which treatment would be most effective and then, after treatment, follow-up tests can be performed to see if the epigenetic modifications were successfully reversed to allow re-expression of tumor suppressors.

The importance of epigenetics, especially in our understanding of susceptibility to disease, is reflected in the huge undertaking to establish an international human epigenome (Jones et al., 2008). This can be used to identify individuals at higher risk for developing diseases, so that preventative strategies can be implemented to reduce the number of incidences and improve patient survival and quality of life (Mack, 2006). Clearly, future studies are needed to further address the potential for epigenetic profiling as a diagnostic and therapeutic tool.

### 1.1.3. Estrogen

Estrogen is the primary female sex hormone, which is naturally produced by developing follicles in the ovaries, corpus luteum, and placenta and plays an important role in the development of the human breast during sexual maturation (Russo and Russo, 2004; Russo and Russo, 1982). There are three major naturally occurring estrogens, with estradiol ( $E_2$ ) being the most predominant circulating ovarian steroid (Russo and Russo, 2006; Miettinen et al., 2000). By circulating in the bloodstream, this hormone targets receptors located on the cell surface, cytosol or within the cell nucleus to promote proliferation (Shang, 2007; Acconcia and Kumar, 2006). The binding of estrogen to its receptor induces a conformational change that activates subsequent receptor dimerization and interaction with coactivator molecules (Shang, 2006; Osborne et al., 2003).

There are two types of estrogen receptors (ER): ER-alpha ( $ER\alpha$ ), encoded by the ESR1 gene and ER-beta ( $ER\beta$ ), encoded by ESR2. Both have similar binding affinities for  $17\beta$ -estradiol, however they are thought to act through different mechanisms, which may, in part, explain the controversy regarding estrogen action in the mammary tissue (Kass et al., 2004; Routledge et al., 2000; Paech et al., 1997).

In the classical model,  $ER\alpha$  plays an important role in regulating mammary gland growth and differentiation, acting as either a positive or a negative gene regulator (Bourdeau et al., 2008; Carling et al., 2004; Hilakivi-Clarke et al., 1997; Panda and Runer, 1966). The binding of estrogen to its receptor can act directly or indirectly to activate gene expression, either by relocating to the nucleus to associate with estrogen response elements (EREs) or by initiating a signaling cascade (Shang, 2006; Acconcia and Kumar, 2006; Edwards, 2005; Filardo, 2002; Simoncini et al., 2000). Cross-talk

between these genomic and nongenomic pathways is what makes estrogen and its receptor a potent regulator of cellular activity.

#### **1.1.3.1. *Role of Estrogen in Breast Carcinogenesis***

A common thread linking the main risks for developing breast cancer in women is cumulative, excessive exposure to estrogen. Numerous studies have identified a strong correlation between the dose and length of estrogen exposure and increased breast cancer risk (Murray et al., 2007; Li et al., 2002; Clemons and Gross, 2001; Kabuto et al., 2000; Cauley et al., 1999; Toniolo et al., 1995; Blankenstein et al., 1977). Longer life-time exposures to endogenous ovarian estrogens, as well as chronic exposure to synthetic and environmental estrogens are factors contributing to breast carcinogenesis (Yager and Davidson, 2006; Ibarluzea et al., 2004; Clemons and Goss, 2001).

Early menarche and late menopause, for example, are associated with greater breast cancer risk, largely due to prolonged exposure of the mammary tissues to estrogens produced by the ovaries throughout the extended period of reproductive viability (Singletary, 2003; Bernstein and Ross, 1993; Kelsey et al.; 1993). Supporting this assertion are data indicating that an ovariectomy prior to menopause markedly reduces breast cancer risk (Kramer et al., 2005; Haber, 2002). Further evidence comes from rodent models, which also exhibit estrogen-dependent tumor formation that regresses upon removal of the ovaries (Russo, 1990). Interestingly, estrogen treatment was shown to restore tumor development in these ovariectomized animals to approximately that observed in ovary-intact animals (Russo, 1990). Similarly, studies have demonstrated that, in premenopausal women with advanced breast cancer, tumor size increases and

decreases in correlation with estrogen levels during the menstrual cycle (Ramakrishnan et al., 2002; Saad et al., 1994; Cooper, 1836).

Elevated circulating levels of endogenous estrogen are associated with an increased breast cancer risk in a dose-dependent fashion among pre-menopausal women (Eliassen et al., 2006). This has been linked to increased levels of androgens, which are precursors for estrogen biosynthesis (Eliassen et al., 2006; Onland-Moret et al., 2003). Increased activity of aromatase, the enzyme responsible for catalyzing this process, has also been shown to increase serum E<sub>2</sub> levels (Subramanian et al., 2008; Probst-Hensch et al., 1999). This effect is augmented by other factors, such as alcohol (Sarkola et al., 1999) and obesity (Key et al., 2003; Wu, 1999; Adami et al., 1995). Importantly, high local levels of estrogen have been shown to increase telomerase production and activity that is sufficient to transform human cells into immortalized cell lines (Vera et al., 2008; Friedman, 2007; Ouellette et al., 2000). Moreover, estrogen has been shown to promote breast cancer progression by stimulating proliferation of cells possessing genotoxic instability (Platet et al., 2004).

With the classification of steroidal estrogen as a known human carcinogen by the National Institute of Environmental Health Sciences (NIEHS) in 2002 (U.S. Department of Health and Human Services, 2002), this brings to light a serious health concern for the increasing prevalence of estrogen exposure in the environment. Moreover, it is estimated that 70% of breast cancers are ER-alpha-positive and estrogen-dependent, in which two thirds of advanced ER-positive breast cancers respond to therapy with antiestrogens (Ding et al., 2004; Katzenellenbogen and Frasor, 2004; Clarke et al., 2004; Clarke et al., 2003; Khan et al., 1998).

Although it is well-acknowledged that estrogen is involved in the etiology of breast cancer, there remains ambiguity in the precise role of estrogen in the biology of breast cancer induction. The effects of estrogen on the target cells in breast tissue are believed to be mediated through a number of mechanisms. The most generally accepted mechanism is through receptor-mediated interactions leading to the transactivation of specific genes regulating a wide variety of cellular processes in favor of cellular proliferation (Jensen et al., 2008; Bjornstrom and Sjoberg, 2005; Hall et al., 2001; Parker et al., 1997). In addition, estrogen decreases the time spent in the cell cycle, such that a spontaneous mutation that may arise during DNA synthesis could be passed on to daughter cells without being repaired, and could ultimately lead to a malignant phenotype (Luo et al., 2008; Yager and Davidson, 2006; Castoria et al., 2001; Epifanova, 1966).

As a strong promoting agent, estrogen-driven proliferation not only increases the number of estrogen-responsive cells, but also potentially stimulates clonal expansion of pre-cancerous cells. This increase in proliferation is termed hyperplasia and is one of the first morphological changes identified during carcinogenesis (Starland-Davenport et al., 2010; Allred et al., 2001). It appears that excessive signaling through the estrogen receptor may be one of the primary mechanisms for breast carcinogenesis, in which modest increases in ER-alpha were shown to lead to mammary hyperplasia (Frech et al., 2005). Promotion of initiated cells leads to selection of those cells with survival advantages, which acquire additional mutations, leading to progressively more malignant epithelial growth (Simpson et al, 2005; Clarke et al., 1994; Platlet et al., 2004; Adami et al., 1995).

Interestingly, the promotion of these initiated cells requires continuous exposure to estrogen to progress preneoplastic lesions to a state of transformation (Yager and Davidson, 2006). Removal of the promoting agent has been shown to reverse the changes, both at the level of gene expression and at the cellular level (Clarke et al., 1994; Pitot and Dragan, 1991). The regression of preneoplastic lesions upon withdrawal of the promoting agents has been linked to the “redifferentiation” or remodeling of the tissue (Hikita et al., 1999; Tatematsu et al., 1983). Knowing that operational reversibility during the promotion stage of carcinogenesis provides a window for intervention, the potential to reverse the effects of elevated levels of estrogen prior to tumor development may prove to be a promising avenue to explore. The development of strategies to reverse these pre-malignant changes depends on our ability to detect the early molecular and cellular events.

The estrogen receptor can also influence cellular proliferation indirectly by interacting with other signaling pathways, such as the phosphatidyl-inositol-3-kinase (PI3K) pathway (Acconcia and Kumar, 2006; Simoncini et al., 2000; Castoria et al., 2001). Activation of this pathway may be one of the steps leading to imbalances in proliferation and apoptosis, as PI3K was shown to inhibit the ATR pathway controlling cell cycle checkpoints (Pedram, 2009), as well as abrogate apoptosis through inactivation of the pro-apoptotic protein, BAD (Fernando and Wimalasena, 2004). Moreover, estrogen has been shown to upregulate BCL6 (Kovalchuk et al, 2007), a known proto-oncogene, which reportedly prevents mammary epithelial apoptosis and differentiation (Alenzi, 2008; Logarajah et al., 2003). Estrogen also stimulates Src which increases

aromatase activity, resulting in an autocrine feedback loop which may reinforce the initial response to estrogen during breast tumorigenesis (Catalano et al., 2009).

The estrogen receptor, itself, can be subject to modifications that influence cellular kinase signaling. For example, ER-alpha is methylated in the majority of epithelial cells in the healthy breast and is hypermethylated in a subset of breast cancers (Le Romancer et al., 2010). After E<sub>2</sub> treatment, an arginine methyltransferase, PRMT1, transiently methylates arginine 250 of ER-alpha's DNA-binding domain, promoting the activation of Akt signaling cascades and proliferation (Le Romancer et al., 2010). Hyperactivation of this pathway gives selective survival advantage for primary tumor cells even in the presence of anti-estrogens (Le Romancer et al., 2010).

Clearly a number of cellular signal transduction pathways can potentially be involved in response to E<sub>2</sub>, thereby influencing the responsiveness to ER signaling. The complex network and interactions of genomic and nongenomic ER pathways makes it difficult to unravel the mechanisms of estrogen-induced breast cancer and to define treatment modalities.

Of course, it is important to note that in order for DNA binding proteins, such as ER, to be able to access their recognition elements, they may be required to first recruit chromatin remodeling complexes to decrease the level of chromatin packaging. Studies have shown that ER recruits the SWI/SNF complex to estrogen-responsive promoters in a cooperative manner with HATS in order to affect transcriptional activation and stimulate proliferation (Belandia et al., 2002; DiRenzo et al., 2000).

ER-alpha can also interact with other epigenetic components, such as HDAC6, to promote the rapid deacetylation of tubulin which potentially contributes to cell migration

and to the aggressiveness of ER-alpha-positive breast cancer cells (Azuma et al., 2009). Moreover, exposure to estrogen is known to change DNA methylation patterns which may contribute to inappropriate gene expression (Cheng et al., 2008).

In the past, estrogen was primarily studied for its role as a promoting agent, stimulating the clonal expansion of initiated cells. More recently, estrogen has also been acknowledged as an initiating agent, with the formation of genotoxic metabolites that can directly damage DNA, thereby disrupting normal cell processes such as apoptosis, proliferation, and DNA repair (Cavalieri et al., 2006; Mailander et al., 2006; Chen et al., 2005; Huhr, et al., 2004; Mobley et al., 1999; Fishman et al., 1995).

Experimental studies of estrogen metabolism, which occurs actively in breast tissue as well as in liver (Jeffcoate et al., 2000; Williams and Phillips, 2000), have found evidence of the formation of DNA adducts and cell transformation by estrogen metabolites (Fernandez et al., 2005; Russo et al., 2003; Newbold and Liehr, 2000; Cavalieri et al., 1997). These studies suggest that phase I metabolism of estrogen into catecholestrogens and their derivatives by cytochrome P450 possess complete carcinogenic potential (Santen et al., 2009; Zhang et al., 2007; Cavalieri et al., 2006). Of these estrogen metabolites, 2-hydroxycatechol estrogen and 4-hydroxycatechol estrogen, are considered the most potent carcinogenic agents capable of damaging DNA (Cavalieri et al., 2006; Yagi et al., 2001; Cavalieri et al., 1999). One of the best studied is the production of 8-oxo-7,8-dihydro-2' deoxyguanosine (8-oxo-dG), which is mutagenic, causing G-T transversion, which after a round of replication become fixed mutations in daughter DNA molecules (Mobley et al., 1999). These metabolites also affect

chromosomal stability, including gains and losses of DNA segments, as well as the loss of heterozygosity (Santen et al., 2009).

More importantly, the effects of these metabolites were not abrogated by simultaneous treatment of anti-estrogens (Russo et al., 2003). Furthermore, studies have shown that estrogen metabolites are capable of inducing transformation in an ER-alpha-negative human breast epithelial cell line, suggesting that they act via ER-independent pathways to stimulate cell proliferation and exert their invasive capabilities (Fernandez et al. 2006; Fernandez et al., 2005; Russo et al., 2003). However, it was noted that transgenic mice harboring a knock-out of ESR1 delayed tumor onset and reduced the number of tumors formed (Santen et al., 2009; Yue et al., 2003). It appears that these metabolites can act through both ER-independent and ER-dependent mechanisms, as several estrogen metabolites have been shown to possess similar or higher binding affinities to the human ER as does estradiol, inducing ER-dependent gene expression (Zhu et al., 2006; Kuiper et al., 1997; Fishman and Martucci, 1980).

In human studies, women with breast cancer were frequently observed to have an imbalance in estrogen metabolism compared to women without breast cancer (Rogan et al., 2007; Rogan et al., 2003; Liehr and Ricci, 1996). This has been associated with increased biosynthesis of estrogens and their biotransformation into tumorigenic metabolites in the breast (Rogan et al. 2003; Simpson 2003; Jefcoate et al., 2000; Miller and O'Neill, 1987).

Catechol estrogens can be further oxidized to form electrophilic catechol estrogen quinones that can react with DNA to form depurinating adducts, which generate mutations, such as abasic sites, to initiate breast cancer (Cavalieri et al. 2006; Li et al.,

2004; Yue et al., 2003; Cavalieri et al., 1997). Analysis of depurinating estrogen-DNA adducts in urine demonstrates that women at high risk of, or diagnosed with, breast cancer have high levels of these adducts, indicating a critical role for adduct formation in breast cancer initiation (Gaikwad et al., 2008; Cavalieri et al., 2006; Tagesson et al., 1995).

Reduction of these estrogen quinones back to hydroquinones and catechols provides an opportunity for redox cycling to produce reactive oxygen species (ROS) and oxidative damage to macromolecules, including lipids and DNA (Sanchez et al., 2003; Jefcoate et al., 2000; Mobley et al., 1999; Seacat et al., 1997). ROS induces strand-breaking lesions and smaller amounts of base oxidation and abasic sites in DNA. If this depurination occurs on a single-stranded DNA molecule undergoing replication, DNA polymerase  $\beta$  (Pol. $\beta$ ) in the base excision pathway (BER) can add an incorrect base at the apurinic site in the absence of information from the complementary strand, thereby inducing mutations (Cavaleiri et al., 2006; Cabelof et al., 2004; Posnick and Samson, 1999; Miller and O'Neill, 1987). These mutations, if left unrepaired, are passed along to subsequent cell generations, and may account for the mutation of several important cell regulatory genes, including p53, in breast cancer patients (Amir et al., 2010; Schmutte et al., 1995; Greenblatt et al., 1994).

Increased oxidative DNA damage, along with the corresponding upregulation of repair-related genes, has been detected in target tissues after estrogen treatment in animal model systems (Miyamoto et al., 2006). One of the best-studied markers of oxidative damage, 8-oxo-dG, which is predominately repaired by the short-patch BER, has also been found to be elevated in tumor tissue of patients with breast cancer (Musarrat et al., 1996). More generally, higher levels of oxidative stress have been detected in human

breast cancer relative to women with histologically normal tissue, suggesting a direct association between DNA repair deficiencies and cell transformation (Ming-Shiean et al., 2010; Nowsheen et al., 2009; Yager, 2000).

Detoxification of estrogen and its metabolites are mediated by subsequent conjugation reactions involving methylation, glutathione or sulfation, via catechol-O-methyltransferase (COMT), glutathione S-transferase (GST), or sulfotransferases (ST), respectively (Raftogianis et al., 2000). Among these, studies revealed an important role for COMT in protecting the cell from genotoxic damage by transferring a methyl group to 2-hydroxy estradiol to form 2-methoxyestradiol, which is known to be protective against tumor formation (Schumacher and Neuhaus, 2001; Zhu and Conney, 1998). This anti-tumorigenic metabolite was shown to possess anti-angiogenic activity through a direct apoptotic effect in rapidly dividing tumor cells (Schumacher and Neuhaus, 2001; Zhu and Conney, 1998). The presence of estrogen at pharmacological doses has been shown to alter the expression of these detoxifying enzymes, suggesting that E<sub>2</sub> induces compensatory mechanisms to protect against oxidative stress via elevation of these anti-oxidant enzymes (Sanchez et al., 2003; Hudson et al., 1998).

It must also be noted that studies have shown differentiation of the mammary gland reduces the number of target cells, with full-term pregnancies and lactation at an early age decreasing the risk of breast cancer incidence by nearly half (Macmahon et al., 1970; Rosner et al., 1994). Moreover, studies in rodents have shown that estrogen and progesterone, at levels sufficient to mimic the effect of pregnancy, reduce the incidence of carcinogen-induced mammary tumors in rats (Cabanes et al., 2004; Sivaraman et al., 1998; Grubbs et al., 1985). These studies contrast the aforementioned findings as well as

several other studies which indicate that concentrations of  $17\beta$ -estradiol within the low picogram range in blood serum and breast tissue increased the risk of sporadic breast cancer (Chetrite et al., 2000; Thijssen et al., 1986; Vermeulen et al., 1986). Therefore, it is essential to gain a better understanding of how  $E_2$ , at physiologically relevant concentrations, elicit their effects in susceptible target tissues.

### **1.1.3.2. *Estrogen and Treatment Modalities***

Perhaps one of the strongest links between  $E_2$  and breast cancer can be demonstrated using estrogen antagonists in breast tissue for the treatment and prevention of breast cancer. Studies have clearly shown that tamoxifen, a selective estrogen receptor modulator (SERM), reduces the risk of recurrence of breast cancer for women, as well as the risk of developing new breast cancer in the contralateral breast (Early Breast Cancer Trialists' Collaborative Group, 2005). Tamoxifen also reduces the risk of breast cancer in healthy women who are considered to be at high risk for developing breast cancer by over a third, although the protective effects of tamoxifen are largely limited to ER-positive cells (Cuzick et al., 2003).

Taking into consideration that  $E_2$  has been shown to exert its carcinogenic effects through both ER-dependent and ER-independent mechanisms, treatment modalities for breast cancer with a strong link to estrogen should address both pathways. Aromatase inhibitors, for example, decrease the conversion of androgens to estrogen which can then be biotransformed into genotoxic metabolites, with promising results (Brodie and Njar, 1998; Perez and Borja, 1992). Women treated with aromatase inhibitors had superior

outcomes and a lower incidence of breast cancer in the contralateral breast than women who received tamoxifen (Goss et al., 2005; Howell et al., 2005).

#### **1.1.4. Radiation**

Radiation is a term used to describe energy transmitted as rays or waves through space, and is largely divided into two general categories based on the amount of energy it possesses. Radiation with sufficient energy to ionize particles, known as ionizing radiation (IR), due to its nature, possesses more severe biological effects than lower energy forms, which are capable only of excitation (Elgazaar and Kazem, 2006).

Humans are constantly exposed to background radiation, which is emitted from a variety of natural sources, including solar radiation, cosmic rays, and radioactive elements in the earth's crust (Elgazaar and Kazem, 2006; Charles, 2001; Schultz, 1985). Advances in technology have also enabled humans to utilize radiation for medicinal and industrial purposes.

Radiation has been used in the medical field for over 100 years for its ability to produce accurate imaging of internal organs and structures. These procedures provide a relatively non-invasive and quick assessment of trauma or physiological impairments. Similarly, radiation exposures given at higher doses have been used in the treatment of diseases, such as cancer.

##### **1.1.4.1. *Radiation Carcinogenesis and its Importance as a Treatment Modality***

The discovery of x-rays in 1895 by Wilhelm Rontgen (Rontgen, 1896) was closely followed by the experimental application of x-rays to shrink tumors (Grubbe,

1933; Freund, 1904). However, radiation proved to be a double-edged sword, with the appearance of secondary treatment-related malignancies within years of radiation exposure (Verhoeff and Bell, 1914). The carcinogenic properties of ionizing radiation have since been documented in a number of other tissues and organisms (Bernstein et al., 2010; Inskip et al., 1994; Russ, 1925; Warren and Whipple, 1922; Bovie, 1918). Notably, the female breast is one of the tissues with the highest sensitivity to radiation-induced carcinogenesis (Preston et al., 2007; Ronckers et al., 2005; Thompson et al., 1994).

Although the mechanisms of radiation-induced carcinogenesis are rather complex, the underlying principle is based on the ability of radiation to penetrate and deposit energy in body tissue, which can cause cell damage or death (Elgazaar and Kazem, 2006). The extent of the damage depends on a number of factors, including the total amount of energy absorbed and the exposure regime – dose rate, acute versus fractionated, and the time period of the exposure (Elgazaar and Kazem, 2006; Charles, 2001).

Chronic exposure is typically described as continuous or intermittent exposure to low doses of radiation, usually from background radiation, over long periods of time. Acute exposure, on the other hand, refers to a single relatively large dose of radiation, typically resulting from accidental exposure, such as the nuclear powerplant disaster at Chernobyl, or specific medical procedures (Prysyazhnyuk et al., 2007; Preston et al., 2007; Ronckers et al., 2005; Thompson et al., 1994). Therapy exploits the fact that higher doses of radiation can be used to destroy harmful cells (Elgazaar and Kazem, 2006; Charles, 2001). These findings led to the widespread practice of medical radiology, in

which radiation is currently used as a treatment modality in nearly half of cancer cases, either alone or in combination with surgery and chemotherapy (Choi et al., 2006; Van Lanschot et al., 1999; De Lena et al., 1981). The most common forms of external radiation therapy use gamma and x-rays in combination with radiosensitizing drugs like Cisplatin, Nimorazole, and Cetuximab to maximize damage to tumor cells (Choi et al., 2006; Van Lanschot et al., 1999; De Lena et al., 1981).

Despite the obvious benefits of radiotherapy, the risk of secondary radiation treatment-related malignancies is a clinical problem (Leone et al., 1999; Storm et al., 1992; Boice et al., 1992). For example, radiation treatment for Hodgkin's disease increased the development of breast cancer and a number of other secondary neoplasms (Crump and Hodgson, 2009; Bhatia et al., 1996). The exact mechanisms of radiation-induced secondary cancers, particularly the contributions of epigenetic mechanisms, remain unknown. Over the years, it has become generally accepted that tumors arise from stem-like cells, however the specific identity and location of the cells is poorly understood (Charles, 2001). An acute high dose of radiation is thought to kill most mammary stem cells, but those that remain exhibit mutations that continue to be passed along to daughter cells, whereby mutations accumulate over the life leading to malignancy (Charles, 2001; Kinzler and Vogelstein, 1996).

In general, the degree to which radiation affects a tissue is considered to be proportional to the rate of proliferation and inversely proportional to the degree of cell differentiation (Hilakivi-Clarke, 2007; Warri et al., 2007; Russo et al., 2005; Ulrich et al., 1996; Russo et al., 1982). Breast tissue, for example, is thought to be particularly sensitive to ionizing radiation prior to sexual maturity, when the gland is largely

comprised of rapidly dividing undifferentiated terminal end buds (Hilakivi-Clarke, 2007; Russo and Russo, 2006; Russo et al., 1982). The risk of developing radiation-induced breast cancer is highest among women exposed in childhood and adolescence and is among the highest known radiation-related risks for any cancer type (Charles, 2001). Radiation exposure doses linked to breast cancer development range widely between 0.2 and 20 Gy (Ronckers et al., 2005) with a linear dose-effect relationship observed with doses greater than 0.2 Gy (Broerse et al., 1986; Bond et al, 1960). Sublethal doses of different types of radiation, including x-rays and neutrons, were shown to induce mammary tumor development within a year (Broerse et al., 1986; Bond et al, 1960).

At doses of low linear energy transfer (LET) radiation, including x-ray and gamma-rays, each cell nucleus is likely to be traversed by more than one sparsely-ionizing track (Charles, 2001). In mammalian cells, low-LET radiation generates both single- and double-stranded DNA breaks, reactive oxygen species, base damage, and DNA-protein cross-links (Little, 2000; Pouget et al., 1999; Ward, 1988). Radiation affects a variety of processes in the exposed cell, including proliferation and apoptosis. Damage sentinels, such as p53 are activated in response to radiation to give the cell time to repair the DNA damage (Hartwell et al., 1994; Lowe et al., 1993). If the cell fails to restore even a single DNA double-strand break, the cytotoxic effects can be lethal (Clarke et al., 1993; Lowe et al., 1993). Moreover, incorrect repair of DNA damage leads to mutations and chromosome aberrations that are passed on to subsequent generations and, along with other acquired mutations, can lead to carcinogenesis (Hasty, 2005; Simpson et al, 2005; Feinberg, 2004).

There appears, however, to be some sort of damage threshold for the enhancement of DNA repair (Amundson et al., 1999), which reduces the number of cells undergoing an alternate pathway of apoptosis to deal with the damage (Feinendegen, 2005). Very low radiation doses were shown to be insufficient to stimulate cellular repair processes, resulting in high cell lethality, whereas doses above this threshold trigger repair processes, leading to increased radioresistance and cell survival (Singh et al., 1994; Wouters and Skarsgards, 1994).

Likewise, there is also a risk of overwhelming DNA repair systems, especially if DNA repair proteins become mutated (Russell et al., 1995; Arlett et al., 1980). This is an important point to consider in women receiving radiotherapy with mutations in DNA repair genes, such as BRCA1 and BRCA2, as they tend to have a very high risk of developing a second breast tumour (Frank et al., 2002; Haffty et al., 2002). Incorrect repair of DNA damage leads to genomic instability, one of the first events identified in radiation-induced cancer (Selvanayagam et al., 1995). This initial instability puts all genes at risk for mutation, but its major impact on carcinogenesis occurs when critical genes, such as the tumor suppressor p53 and DNA repair proteins, are mutated as a secondary consequence of the radiation exposure. The GENE-RAD-RISK project, a large European study, is currently underway to evaluate whether carriers of pathogenic alleles in DNA repair and damage recognition genes may confer an increased risk of breast cancer following medical irradiation (<http://generadrisk.iarc.fr/index.php>).

The role of epigenetic changes in the etiology of radiation-induced breast cancer has not been addressed until recently. One of the best-studied short-term epigenetic responses to radiation exposure is the phosphorylation of H2AX, which binds to the ends

of double-strand breaks and initiates DNA repair processes (Sedelnikova et al., 2003). The initial damage was shown to be repaired within 96 hours after IR exposure, however long-term repercussions were observed up to 7 months later in a rodent model (Koturbash et al., 2008). The activation of DNA repair processes was shown to be linked, at least in part, to a global loss of methylcytosine in exposed mammary tissue (Loree et al., 2006), which may contribute to increased genome instability. Furthermore, the loss of DNA methylation was associated with significant decreases in DNA methyltransferases responsible for both the maintenance and creation of methylation sites.

These epigenetic changes were observed in parallel with alterations in apoptosis and proliferation (Loree et al., 2006), suggesting IR alters cell cycle control mechanisms and signaling pathways in mammary tissue. These epigenetic and molecular parameters were predicted to early markers of radiation-induced oncotransformation (Loree et al., 2006).

#### ***1.1.4.2. Clinical Intervention to Minimize Radiation-Induced Carcinogenesis***

The primary focus of radiotherapy is to maximize damage in tumor cells, while minimizing disruption to nearby healthy tissue. Although reactive oxygen species produced during radiotherapy treatment are effective in killing tumor cells, they do cause painful side effects that require palliative treatment (Cheng and Lee, 2010; Borek, 2004; Kennedy et al., 2001). To offset the damage caused by free radicals, anti-oxidants have been used to reduce both short- and long-term tissue injury after radiation exposure, promoting the recovery of healthy cells (Wan et al., 2006; Borek, 2004). For example, taking green tea polyphenols which is known for its anti-oxidant, anti-inflammatory, and

immune modulating activity was found to reduce secondary treatment-related malignancies (Mantena et al., 2005). Under a similar principle, class III histone deacetylases, known as sirtuins, are currently being explored in animal models as a defense against radiation. This special class of HDACs was shown to be required for the maintenance of genomic integrity by directly aiding the repair of DNA damage and by reducing ROS-induced cellular changes following exposure to genotoxic stressors (Hasegawa et al., 2008; Blander and Guarente, 2004). However, much controversy has been raised with the finding that sirtuins and antioxidants increase radio- and chemo-resistance in both normal and malignant cells (Prasad et al, 2002; Conklin, 2000), which raises the question as to whether or not the mitigating effect of these forms of palliative care negatively influence the overall outcome of treatment for the primary tumor.

#### **1.1.5. Exposure to Multiple Carcinogenic Agents**

Living organisms are exposed to numerous natural and man-made agents that interact with molecules, cells, and tissue causing deviations from homeostatic equilibrium and irreversible damage. Many aspects of age-related diseases are thought to stem from accumulated effects of exogenous and endogenous deleterious agents acting on key components of cells within the body (Madia et al., 2007; Hasty et al., 2005). Therefore, clinical studies are, in fact, a study of combined exposures and must take into consideration a wide variety of factors – from dietary and lifestyle choices to environmental and occupational exposures. This is an important consideration to make, especially because risk assessment is largely performed with the simplifying assumption that the agent under study acts largely independently of other substances. Studies are

needed to look at the effects of combined exposures to make sure that contributing factors are recognized and taken into account in risk assessment.

The use of model organisms and cell culture has identified a number of factors that play a causative role in the induction of cancer. To determine the mechanism underlying the mode of action of these agents, studies have largely focused on the effect of one carcinogen at a time to make the cause-effect linkage easier. Although these studies have been instrumental in uncovering the contributions of a single agent to carcinogenesis, they are unable to demonstrate real-life scenarios whereby people encounter exposure to multiple carcinogenic agents (Charles, 2001).

More recently, cancer has become recognized as a complex, multi-step process that often arises as a result of exposure to many classes of agents, both endogenous and environmental (Mauderly, 1993; Streffer and Muller, 1987; Steel and Peckham, 1979; Warren and Brown, 1978; Loewe, 1953). Ionizing radiation, for example, which is generally accepted as a genotoxic agent, is thought to predominantly act as an initiator of carcinogenesis (Ronckers et al., 2005; Land et al., 2003; Shellabarger, 1971; Bond et al., 1960), while hormones, such as estrogen, which stimulates the proliferation of these initiated cells, are largely considered to be promoting agents (Russo et al., 2006; Ronckers et al., 2005; Clemons and Goss, 2001; Inano et al., 1993).

Of course, it must be recognized that some physical and chemical carcinogens are capable of inducing tumor formation alone (Cavalieri et al., 2006; Russo et al., 2003; Todorovic et al., 2001; Liehr, 2000; Cavalieri et al., 1997; Little and Vetrovs, 1988), with different carcinogens inducing different types of morphological changes. For example, in the breast tissue, chemical carcinogens tend to induce adenocarcinomas (Russo, 1990),

whereas physical carcinogens, such as IR tend to induce benign mammary tumors, known as fibroadenomas (Shellabarger et al., 1960). However, in some cases, IR has been shown to induce adenocarcinomas, and these malignancies share the same pathologic features as those induced by chemical carcinogens (Russo, 1990).

Interestingly, gene expression profiling of different chemical carcinogens and radiation, as well as spontaneous arising mammary cancers, were found to share a number of similarities in gene expression patterns between cancers of different etiological origins (Imaoka et al., 2009; Imaoka et al., 2008). It appears that although genetic alterations may be different between radiation and chemical carcinogenesis models, the resulting alterations in gene expression may be similar. This evidence points to epigenetics as a promising area of research, with the prediction that these expression patterns might share a common epigenetic origin.

#### **1.1.5.1. *Interactions Between Carcinogens***

Agents that are deleterious on their own often combine to produce an effect not directly predictable from the exposure of each carcinogen applied separately. The nature or severity of a single agent may be modified by another agent, producing an effect that may be smaller than anticipated, as with negative synergism, or with greater effect, as in the case of supra-additivity or synergism. Deviations from an expected outcome of additivity need to be considered to help elucidate the mechanism of interactions.

A plethora of studies have been initiated to address the combined potential of chemical and physical carcinogens (Kantorowitz et al., 1995; Kantorowitz et al., 1993; Watanabe et al., 1993; Borek et al., 1986; Kennedy and Weichselbaum, 1981).

Specifically, combined exposure to radiation and elevated levels of hormones has indicated that the carcinogens were reported to exert either an additive (Broerse et al., 1987) or a synergistic effect (Shellabarger et al., 1983; Holtzman et al., 1981; Holtzman et al., 1979) in breast tissue. In these studies, hormone administration considerably reduced the latency period of tumor formation and increased the number of malignant tumors per rat during IR-induced mammary tumorigenesis. This effect was similar for hormone administration one week prior to or over 28 weeks after IR exposure (Broerse et al., 1987). Age was shown to be a factor, with the administration of estrogen increasing the radiation sensitivity of the mammary gland in young animals considerably (Bartstra et al., 1998). There is, however, a limit to the detection of these supra-additive effects, especially when one carcinogen is given at levels that may be too high to properly assess the effect of the other.

From a clinical perspective, these interactions must be taken into consideration, particularly as radiotherapy may be combined with hormone therapy, as well as chemotherapeutic agents, which are, by nature, often carcinogenic. Secondary cancers, which are typically unrelated to the first cancer that was treated, may occur months or even years after the initial treatment (Bertelsen et al., 2009; Brown et al., 2007; Boice et al., 1992). These cancers are more frequent in patients who receive both radio- and chemotherapy compared to either treatment alone (Wong et al, 2003; Araujo et al., 1991). Clearly, further studies need to be conducted to elucidate the interacting mechanisms of combined modality treatments leading to secondary complications and malignancies.

Understanding how endogenous and exogenous agents interact is important for making medical decisions regarding diagnostic and treatment strategies. For example,

women with elevated estrogen levels are considered to be at higher risk for breast cancer development (Subramanian et al., 2008; Yager and Davidson, 2006) and would likely be exposed to diagnostic IR procedures on a more frequent basis. Similarly, many women with estrogen-induced breast cancer that undergo the IR treatment are exposed to relatively high X-ray doses to the healthy breast (Bernstein et al., 2010; Boice et al., 1992). Moreover, estrogen influences the response to acute and fractionated exposure, increasing the carcinogenic potential of an acute dose or radiation by up to 15 times greater than the fractionated exposures (Bartstra et al., 2000). Treatment with an ER antagonist reduces the incidence of IR-induced cancer (Welsch et al., 1981). Knowing that these factors interact to influence the risk of breast cancer, alternative strategies may need to be considered.

Interestingly, pre-menopausal women were considered to be the most susceptible to secondary treatment-related malignancies (Bertelsen et al., 2009; Brown et al., 2007). This increased sensitivity was also observed in nulliparous rodent models in response to a number of carcinogenic agents. Furthermore, early full-term pregnancy is thought to be protective (Lynch et al., 1984s; Russo and Russo, 1980) and that IR exposure at ages younger than 19 (McGregor et al., 1977), but not after pregnancy and lactation (Russo and Russo, 1987), strongly suggests that in the human female, the period between menarche and first-full term pregnancy might be critical for the initiation of breast cancer.

## 1.6. USE OF RODENT MODELS IN STUDIES OF CARCINOGENESIS

Due to the limitations of human studies, animal models have been extensively utilized to provide information when epidemiologic evidence is lacking or complicated by confounding factors.

In particular, these studies are invaluable for exploring the effects of carcinogens and therapeutic agents, since experimental studies in humans are often impractical and, in most cases, unethical (Ye et al., 2004). These studies are necessary to obtain information required to estimate cancer risks and establish exposure limits, using dose-response modeling and risk characterizations (Russo and Russo, 1996A). By simplifying the variables, studies can look for cause and effect relationships that will allow for a more conclusive link to be drawn between the carcinogen and the mechanism of action. Since cancer is a complex, multi-step process, this approach provides an important opportunity to determine the effects of a carcinogen at different stages of tumorigenesis. The identification of windows of opportunity for clinical intervention will allow therapeutic strategies to target specific molecular pathways to slow down, reverse, or potentially prevent the development of cancer.

The use of animal models exploits the conservation of genetic material, as well as metabolic and developmental pathways over the course of evolution to extrapolate these results to humans (Szpirer and Szpirer, 2007; Russell, 2003; Storer et al., 1988; Tomatis, 1979). Some species and strains more closely resemble their human counterpart in specific features than others, making them a choice model organism for a particular developmental process or disease. Rats, for example, are considered to be a more appropriate model to study diseases related to human physiology and metabolism than

mice (Russell, 2003). In fact, mammary gland carcinogenesis in the rat was found to share remarkably similar etiology as human breast cancer with respect to histopathology, responsiveness to ovarian hormones, and the apparent protective effect of a full-term pregnancy (Shepel and Gould, 1999; Isaacs, 1986; Russo and Russo, 1980; Thompson and Singh, 2000).

Clearly, studies utilizing rodent models can be informative, but care must be taken when generalizing from one organism to another. Further complexities arise from the very nature of breast cancer, which is a multifactorial disease that is heterogeneous at the morphological, genetic, and molecular levels. Any given animal model could not mimic the spectrum of human breast cancers - at best they could only model major subsets and pathways. Moreover, these studies can not account for the numerous confounding factors, such as the wide spectrum of environmental and lifestyle factors that exist in human populations.

The results of numerous studies have clearly shown that the observed differences in susceptibility to mammary cancer among rat strains cannot be accounted for by differences in the number of target cells or by systemic or cellular influences on progression alone (Shull, 2007; Ullrich and Pnnaiya, 1998; Broerse et al., 1987; Isaacs, 1986; Shellabarger et al., 1978; Ullrich et al., 1996; Bittner, 1952). Rather, mammary epithelial cells from strains with low tumor incidence appear to be inherently more resistant to the transforming effects of a carcinogen, such as ionizing radiation, when compared with mammary cells from susceptible strains (Ulrich and Pnnaiya, 1998; Ulrich et al., 1996). It has been suggested that genetic and epigenetic backgrounds may play an important role in determining susceptibility (Shull, 2007; Milsted et al., 1998).

### **1.6.1. Genetic Determinants of Mammary Cancer Susceptibility in Rat Models**

Evidence of genetic differences has been increasingly explored with the sequencing of the rat genome, shedding much light on the genetic determinants of mammary cancer susceptibility. Mammary cancer susceptibility (Mcs) is a polygenic trait, but not all strains control mammary carcinogenesis by the same genetic loci, suggesting that the mechanism determining susceptibility is different (Lella et al., 2007; Samuelson et al., 2005; Laes et al., 2001; Shepel and Gould, 1998; Hsu et al., 1994). Copenhagen and Wistar-Kyoto rat strains are resistant to the spontaneous development of mammary cancer as well as hormonally and chemically induced mammary cancer (Lella et al., 2007; Korkola and Archer, 1999; Gould et al., 1996). It has been shown that the resistance observed in Copenhagen rats is a dominant phenotype associated with the Mcs gene, which has been shown to inhibit the development of mammary cancer by modulating later events in the carcinogenic process (Korkola and Archer, 1999; Gould et al., 1996). Other strains, such as the Wistar-Kyoto rat, block the carcinogenic process at an early stage, preventing the development of preneoplastic lesions (Lella et al., 2007).

Recent publications have identified a subset of important alleles involved in the genetic susceptibility to estrogen-induced mammary cancer (Emca). The August Copenhagen Irish (ACI) rat, which is commonly used in studies of estrogen-induced mammary cancer, was shown to carry several alleles conferring susceptibility to estrogen-induced mammary cancer (Adamovic et al., 2007; Schaffer et al., 2006; Gould et al., 2004). This may explain the relatively high incidence rate of mammary cancer induced by elevated levels of  $17\beta$ -estradiol in this model. When the alleles were traced back to the parental strains, it appeared that the Copenhagen rat possessed alleles

conferring resistance, whereas the August rat carried alleles which increased susceptibility (Adamovic et al., 2007; Gould et al., 2004; Shull et al., 2001).

Furthermore, the Emca loci were found to control both latency and tumor incidence (Schaffer et al., 2006; Gould et al., 2004; Shull et al., 2001). These sensitivity genes, however, are not necessary for the development of mammary tumors, nor are they, by themselves, sufficient for cancer formation.

### **1.6.2. Epigenetic Determinants of Mammary Cancer Susceptibility in Rat Models**

Interestingly, several investigated strains expressed the Emca loci in a different manner (Shull, 2007; Schaffer et al., 2006; Gould et al., 2004; Shull et al., 2001).

Furthermore, candidate genes on the Emca loci include DNA methyltransferases, suggesting a role of epigenetics in determining mammary cancer susceptibility (Schaffer et al., 2006). As the evidence linking epigenetic alterations with the genesis of cancer grows, the focus has shifted towards elucidating the underlying epigenetic phenotype that predisposes individuals to this pathological state.

The inheritance of specific epigenetic patterns, which is associated with establishing gene expression patterns that modify the contributions of maternal and paternal alleles during embryogenesis (Reilly et al., 2004; Hawes et al., 2001; Latham, 1999; Latham, 1994; Surani, 1990), is believed to control not only normal growth and development but also disease susceptibility (Reilly et al., 2004). Maternal imprinting, for example, was found to reduce DNA methylation at specific genes, resulting in increased expression (Allen et al., 1990; Surani, 1990). Evidence that “inappropriate” DNA methylation patterns can be passed on to subsequent generations suggests an epigenetic-

mediated mechanism for inherited genome instability (Chan et al., 2006; Suter et al., 2004; Buiting et al., 2003), a feature associated with carcinogenesis (Coleman and Tsongalis, 1999).

Epigenetic-mediated genome instability also occurs postnatally in response to numerous lifestyle and environmental factors. Importantly, several studies have demonstrated that interstrain differences in the sensitivity to carcinogens are inversely related to the capacity to maintain normal patterns of DNA methylation (Bachman, 2006; Counts et al., 1997; Counts and Goodman, 1995). Furthermore, higher levels of trimethylation of specific histone residues, as well as a greater degree of DNA methylation in repetitive elements, was correlated with increased resistance to carcinogenesis (Pogribny and Beland, 2009; Pogribny et al., 2009; Bagnyukova et al., 2008), suggesting a more tightly regulated genome may be important in reducing cancer incidence.

These findings open up a whole new perspective for cancer predisposition that needs to be explored in other tissues and diseases. Further studies to address the role of epigenetic-mediated predisposition in the etiology of estrogen-induced breast cancer will be important for identifying women with increased risk.

### **1.6.3. The ACI Rat as a Model of Human Breast Cancer**

The ACI rat provides a unique opportunity for studying human breast carcinogenesis, as estrogen-induced mammary carcinogenesis in the female ACI rat exhibits remarkably similar histopathological features and hormone-responsiveness as observed in human breast cancer (Ruhlen et al., 2009; Li et al., 2002; Harvell et al., 2000;

Shull et al., 1997; Tomatis, 1979). Other common features include acquired genomic imbalances and the bioactivation of mutagenic estrogen metabolites (Yager and Davidson, 2006; Li et al., 2004; Russell, 2003; Li et al., 2002; Harvell et al., 2000). These events occur at physiological serum E<sub>2</sub> concentrations (Li et al., 2004).

The ACI rat is unique in that females exhibit high sensitivity to elevated levels of estrogen with a remarkably reduced latency of tumor development (Shull, 2007; Shull, et al., 2001; Harvell et al., 2000). Considering that few other rat strains exhibit this propensity to develop mammary cancer in response to continuous E<sub>2</sub> treatment, it has been suggested that the ACI rat possesses a particular genetic and epigenetic background that underlies its susceptibility to elevated estrogen levels (Schaffer et al., 2006; Gould et al., 2004; Shull et al., 2001; Harvell et al., 2000). Moreover, ACI rats have a low incidence of spontaneous mammary cancer, which allows studies utilizing this model to more clearly delineate the role of elevated levels of E<sub>2</sub> in carcinogenesis (Shull, 2007; Dunning et al., 1948).

#### **1.6.3.1. *Inducing Mammary Cancer in the Female ACI Rat***

The general protocol for the induction of estrogen-induced mammary tumors is the use of slow-release mini-pellets implanted subcutaneously into the shoulder region to maintain continuously elevated levels of circulating estrogen (Shull, 2007). Serum levels modulated by mini-pellets containing doses as low as 1 mg and as high as 27.5 mg of 17 $\beta$ -estradiol between 6 and 9 weeks of age were found to induce mammary tumors within a relatively short latency period (Li et al., 2004; Turan et al, 2004; Shull et al., 1997), with the majority of studies focusing on maintaining circulating E<sub>2</sub> at levels

observed during pregnancy, a period when breast cancer risk is elevated in humans (Shull, 2007).

Estrogen was shown to induce morphological changes that progressed from increased proliferation in mammary gland lobules and alveoli, which was followed by focal atypical epithelial hyperplasia preceding the development of multiple independently arising mammary tumors in female ACI rats (Kovalchuk et al., 2007; Weroha et al., 2006; Harvell et al., 2000). These mammary cancers possessed similar characteristics of ductal carcinoma *in situ* (DCIS) and primary invasive ductal breast cancers, including overexpression of c-myc and Aurora A kinase during the early stages, chromosome instability, and aneuploidy (Weroha et al., 2006; Li et al., 2004; Li et al., 2003; Li et al., 2002; Arnerlov et al., 2001; Liao and Dickson, 2000; Leal et al., 1995).

Most remarkably, the nonrandom loss of rat chromosome 5 during estrogen-induced mammary cancer in the ACI rat is similar to the loss of human chromosomes 1p and 9p in human breast cancer (Adamovic et al., 2007; Miller et al., 2003; Kleivi et al., 2002; Hoggard et al., 1995). Several known tumor suppressor genes reside within RNO5, including several cyclin dependent kinases (Schaffer et al., 2006). Furthermore, estrogen-mediated amplification of RNO7 and RNO10 in the ACI rat, which encodes the proto-oncogenes c-myc and ErbB2, respectively, is also frequently amplified and overexpressed in human breast cancers (Adamovic et al., 2007; Yu and Hung, 2000; Munzel et al., 1991).

These changes have been associated with the bioactivation of estrogen into genotoxic metabolites. Phase I metabolism of estradiol is mediated by cytochrome P450, of which 2-hydroxyestradiol (2-OHE<sub>2</sub>) and 4-hydroxyestradiol (4-OHE<sub>2</sub>) are the primary

metabolites formed in both the ACI rat and in humans (Singh et al., 2009; Zhang et al., 2007; Rogan et al. 2003). These compounds undergo metabolic redox cycling which generates reactive oxygen species and chemically-reactive quinones, leading to an increased number of gene mutations and ultimately mammary cancer (Park et al., 2009; Singh et al., 2009; Zhang et al., 2007; Cavalieri et al., 2006; Fernandez et al., 2006; Cavalieri et al., 1997). Production of these mutagenic metabolites by localized metabolism in breast tissue was also shown to induce the malignant transformation of breast epithelial cells (Rogan et al., 2003; Russo et al., 2003).

Based on the extensive literary evidence, the ACI rat model of estrogen-induced mammary carcinogenesis should be regarded as a highly relevant research tool for defining the molecular mechanisms underlying subsets of breast cancer development, predominantly hormonally-responsive DCIS and some invasive adenocarcinomas.

## 1.7. PRELIMINARY EVIDENCE OF EPIGENETIC DYSREGULATION IN THE RAT MAMMARY GLAND

In the course of my undergraduate and graduate degree, I was involved in the analysis of the molecular and epigenetic repercussions of exposure to elevated levels of estrogen in the mammary gland of female ACI rats. This study was conducted by Dr. Olga Kovalchuk's laboratory in collaboration with Dr. Igor Pogribny's laboratory (Kovalchuk et al., 2007). We also investigated the epigenetic mechanisms underlying the response to ionizing radiation (Loree et al., 2006). The two papers arising from this work is summarized below to provide additional insight as to the epigenetic nature of these two carcinogens of choice in my Ph.D thesis.

### **1.7.1. Estrogen-induced Rat Breast Carcinogenesis is Characterized by Alterations in DNA Methylation, Histone Modifications and Aberrant MicroRNA Expression**

To study the effect of elevated levels of  $17\beta$ -estradiol on the epigenome, eight week old ACI rats received a single pellet containing 25 mg of 90-day release mini-pellets ( $E_2$ ) which was surgically implanted in the shoulder region. Epigenetic parameters were correlated with morphological alterations and markers of genome instability in the mammary gland after 6, 12, and 18 weeks of treatment.

Constant exposure to elevated level of  $E_2$  produced significant temporal histopathological changes in the mammary gland. Normal appearing alveolar and ductal hyperplasia was observed at 6 weeks and progressed to focal atypical hyperplastic changes by 12 weeks. Atypical hyperplasia, which represents a putative precursor lesion to mammary carcinoma in this model (Li et al, 2004; Shull et al., 1997), suggests clonal

expansion of epithelial cells with aberrant growth which progressed in severity by 18 weeks. Lobular involution was also evident in all mammary glands examined at 18 weeks, which may be attributed to the depletion of estrogen in the 90-day release mini-pellet. Interestingly, E<sub>2</sub>-treated rats retained alveolar atypical hyperplastic foci in the mammary gland at 18 weeks, suggesting two possibilities: (1) that the regression of atypical hyperplastic lesions, after discontinuation of E<sub>2</sub> treatment, lagged behind involution of typical lobular hyperplasia of the mammary gland; or (2) some atypical foci no longer required E<sub>2</sub> stimulation for maintenance and/or support of further growth. Additional studies will be needed to examine these possibilities.

These morphological alterations were paralleled by significant increases in the incorporation of [<sup>3</sup>H]dCTP into HpaII-digested DNA isolated from the mammary tissue of E<sub>2</sub>-treated rats, which indicated an increase in global DNA hypomethylation. In view of studies suggesting that the genome-wide loss of DNA methylation in cancer cells is associated mainly with large repetitive elements (Rollins et al., 2006), methylation analysis of the long interspersed nucleotide elements (LINE1), which constitutes 23% of the rat genome, was found to decrease at 18 weeks.

A sustained decrease in DNMT1/PCNA ratio, beginning after 6 and 12 weeks of E<sub>2</sub>-exposure, may be one of the contributing factors to the observed loss of DNA methylation. Although the DNMT1/PCNA ratio in mammary glands of E<sub>2</sub>-treated rats at 18 weeks increased compared to 6 and 12 week values in the estrogen-treated groups, the levels still remained lower than the control values.

Recent studies have indicated that the global loss of DNA methylation may also be associated with alterations in the methylation of histones, specifically with a decreased

trimethylation of H4 lysine 20, and that these changes may be indicative of the tumorigenic process (Fraga et al., 2005B). To determine if the interrelationship between changes of DNA methylation and histone H3 and H4 trimethylation during mammary gland carcinogenesis may be mediated by elevated levels of estrogen, we targeted antibodies against these epigenetic modifications using western blotting techniques. Our findings demonstrate that exposure to E<sub>2</sub> leads to a rapid and sustained loss of H3K9me<sub>3</sub> and H4K20me<sub>3</sub> in breast tissue. Interestingly, these changes were paralleled by a prominent increase in the histone methyltransferase Suv39h1, while the expression of Suv4-20h2 did not change significantly.

Several possible mechanisms can be proposed for the loss of DNA and histone methylation, such as estrogen-induced DNA damage, increased cell proliferation, altered expression of DNA methyltransferases, and interference or influence of preexisting modifications in the amino-terminal tails of histones H3 and H4. Any or all of these mechanisms can severely impact heterochromatin organization, thereby predisposing the cells to genomic instability and neoplastic cell transformation.

Recent studies have indicated that overexpression of Aurora-A kinase is a crucial event during early mammary gland development, leading to chromosomal instability and centrosome amplification (Marumoto et al., 2005; Li et al., 2004). In order to determine whether the observed epigenetic changes are mechanistically related to genome destabilization and malignant transformation, we measured the level of Aurora-A kinase in the rat mammary gland. Our data demonstrated that increased levels of Aurora-A can be detected after 18 weeks of E<sub>2</sub>-treatment, just 6 weeks after the first appearance of atypical hyperplasia, a precursor lesion to mammary gland tumors. A comparison of the

dynamic changes in Aurora-A expression with morphological changes in the mammary gland clearly demonstrates that over-expression of Aurora-A is associated with the progression of the neoplastic process rather than the initiation of malignancy.

The results of this study show that deregulation of cellular epigenetic processes plays a crucial role in the mechanism of E<sub>2</sub>-induced mammary carcinogenesis in ACI rats, especially in the tumor initiation process. As mentioned above, there is a major gap in our understanding of the role of epigenetic dysregulation in carcinogenesis, particularly a lack of knowledge about specific epigenetic changes that may be mechanistically related to neoplastic transformation, and the precise timeline of epigenetic alterations occurring in the transition of a normal cell to a tumor cell. By comparing the emergence of epigenetic alterations with the observed morphological changes in mammary tissue, we were able to provide evidence that epigenetic changes precede formation of preneoplastic lesions. The observed changes in DNA and histone methylation, may lead to the emergence of epigenetically reprogrammed cells with a tumor-specific phenotype leading to subsequent malignant transformation.

### **1.7.2. Radiation-induced Molecular Changes in Rat Mammary Tissue: Possible Implications for Radiation-induced Carcinogenesis**

The aim of this study was to analyze the epigenetic changes induced by a high dose of IR in the rat mammary gland. A well-established rat model (Russo and Russo 1996B; Ronckers et al., 2005) was utilized to analyse the molecular and epigenetic changes induced in mammary gland tissue upon exposure to ionizing radiation. Six

month old, sexually mature female Long Evans rats were exposed to 5 Gy of X-rays (90 kV, 5 mA) and epigenetic changes were analysed 6 and 96 hours after exposure.

Using a methylation-sensitive cytosine extension assay we observed a significant and persistent loss of genomic cytosine methylation in the exposed mammary tissue. This study is the first to report on the loss of DNA methylation in radiation-exposed rat mammary tissue.

A number of mechanisms may be responsible for the loss of methyl-groups, including altered activity of DNA methyltransferases and methyl-binding proteins in the rat mammary gland. In this study, global DNA hypomethylation was paralleled by a reduction in the levels of maintenance (DNMT1) and *de novo* (DNMT3a and 3b) DNA methyltransferases and methyl-binding protein, MeCP2. Importantly, the changes became even more pronounced 96 hours after exposure.

The observed DNA hypomethylation was also linked, at least in part, to the DNA repair processes activated in response to the radiation-induced DNA strand breaks. Radiation exposure led to the significant upregulation of Rad51, Ku70 and DNA Pol.β protein levels 6 and 96 h after exposure, indicative of the profound activation of DNA repair by homologous recombination (HR), non-homologous end joining (NHEJ), and base excision repair (BER) pathways, respectively. Of particular importance is the persistent up-regulation of the Rad51 protein, which has been shown to mediate recombination between misaligned sequences, resulting in loss of heterozygosity, a known feature of cancer cells (Richardson et al., 2004).

To address if the observed changes were reflected on the cellular level, we analysed cellular proliferation and cell death in the control and exposed mammary tissue.

We have noted that exposure to 5 Gy of X-rays has led to a statistically significant elevation of apoptosis after 6 hours followed by a slight elevation in the number of (PCNA)-positive cells 96 hours after irradiation. Concurrently, we observed a delayed increase in the levels of phosphorylated extracellular signal-regulated kinase (p-ERK1/2) and phosphorylated AKT kinase (p-AKT), as well as elevated levels of cyclin D1 and cyclin D3, suggesting IR alters pro-survival signaling pathways and cell cycle control mechanisms in mammary tissue.

In conclusion, the results of this study have demonstrated that a single exposure to 5 Gy of X rays leads to noticeable epigenetic changes in the rat mammary gland that occurred in the context of activation of DNA damage and repair and alterations in the pro-survival growth-stimulatory cellular signaling pathways. Of special importance was the fact that all the aforementioned changes occurred in a delayed fashion and manifested 96 h after exposure, when the initial damage was repaired, while significant epigenetic changes still persisted. The roles that global DNA hypomethylation, elevated recombination, and altered signaling play in radiation-induced breast carcinogenesis must be further elucidated as these changes might appear to be early markers of radiation-induced oncotransformation.

## 1.8. SUMMARY

**In summary**, from the literature and our preliminary data, we have learned that epigenetic mechanisms are induced in response to carcinogenic agents. However, relatively few studies have evaluated the response to a combined insult of carcinogens. Furthermore, the role of epigenetic changes in the early stages of carcinogenesis have never been addressed *in vivo*. More importantly, the persistence and long-term repercussions of these epigenetic modifications upon removal of the carcinogen need to be discerned.

## 1.9. HYPOTHESES

### **Rationale:**

The epigenome responds to a dynamic environment to establish flexible control over gene expression. In this way, epigenetics regulate a wide spectrum of cellular processes, ranging from normal development and differentiation to the malignant transformation of breast epithelial cells. The epigenetic response to chemical and physical carcinogens in the breast tissue has yet to be discerned. This phenomenon has only recently been identified in the epigenetic domain. The mechanisms of epigenetic-mediated susceptibility have yet to be explored.

### **We hypothesized that:**

- 1. Epigenetic changes occur early, prior to tumor development. We predicted that these changes could be detected very early after the exposure to elevated levels of estrogen. Furthermore, we hypothesized that these epigenetically-mediated changes may be reversible upon removal of the estrogen mini-pellet.**
- 2. Exposure to two known human breast carcinogens, estrogen and ionizing radiation, would induce morphological changes that would be greater than expected from the exposure to either carcinogen alone. We hypothesized that these changes would be mediated by imbalances in proliferation and apoptosis.**
- 3. Exposure to elevated levels of estrogen and radiation would induce alterations in DNA methylation patterns and histone markers in the rat mammary gland. We expect these changes would be correlated with alterations in morphology, DNA repair, and cellular signaling. Furthermore, we predict that the combined**

**exposure to both carcinogens would produce an epigenetic response that is more pronounced than their separate application.**

Several experiments were designed to test the proposed hypotheses. These experiments will be further described as chapters of this thesis.

## **2. REVERSIBILITY OF PREMALIGNANT ESTROGEN-INDUCED EPIGENETIC CHANGES**

<sup>1</sup>Chapter 2 accepted for publication in its entirety:  
Kutanzi K, Koturbash I, Kovalchuk O. Reversibility of pre-malignant estrogen-induced epigenetic changes. *Cell Cycle*, in press. (Submission number 2010CC2639)

## 2.1. ABSTRACT

The development of early detection and prevention strategies of breast cancer relies on defining molecular and cellular events that characterize progressive alterations underlying preneoplastic changes in the mammary epithelium. Studies have shown that estrogen exerts its carcinogenic effects through both genetic and epigenetic pathways to promote imbalances in proliferation and apoptosis, genomic instability and cancer.

The purpose of this study was to identify the earliest epigenetic changes that could be detected in response to estrogen treatment. More importantly, having detected these early pre-malignant epigenetic changes, a follow-up study was designed to address the potential to reverse these estrogen-induced alterations. Using a well-established ACI rat model, morphological and epigenetic changes were identified in the mammary gland tissue as early as 2 days after exposure to constitutively elevated estrogen levels produced by continuous release estrogen mini-pellets.

Progressive hyperplastic changes were paralleled by epigenetic disturbances, including the upregulation of DNA methyltransferases and hyperacetylation of histone residues. These changes could be detected early, and they continued to persist if estrogen was maintained within a high physiological range. Epigenetic features of short-term estrogen exposure were strikingly similar to hallmarks of cancer promotion and progression. Yet, importantly, these changes exhibited a degree of reversibility if a source of elevated levels of estrogen was removed.

Knowing that operational reversibility during the promotion stage of carcinogenesis provides a window for intervention, the potential to reverse the effects of

elevated levels of estrogen prior to tumor development may prove to be a promising avenue to explore.

## 2.2. INTRODUCTION

The development of early detection and prevention strategies of breast cancer relies on defining the molecular and cellular events that characterize progressive alterations underlying preneoplastic changes in the mammary epithelium. The identification of factors that increase the incidence of breast cancer is an important step forward in identifying high risk individuals. For a number of years, epidemiological and experimental evidence has collectively implicated the natural occurring hormone, estrogen, in breast cancer development (Russo and Russo, 2006). These studies have found evidence that elevated levels of estrogen exposure, as well as the duration, is intricately linked to breast cancer risk (Sante et al., 2009; Russo and Russo, 2006). With the classification of steroidal estrogen as a known human carcinogen by NIEHS in 2002 (U.S. Department of Health and Human Services, 2002), this brings to light a serious health concern for the increasing prevalence of estrogen exposure. Not only is there increased evidence of negative health effects due to environmental exposure, but millions of women turning to hormonal therapy as a method of regulating fertility and controlling menopausal symptoms.

Although it is well-accepted that estrogen is involved in the etiology of breast cancer, there remains ambiguity in the precise role of estrogen in the biology of breast cancer induction. The effects of estrogen on the target cells in breast tissue are believed to be mediated through a number of mechanisms (Jensen et al., 2009). The most generally mechanism uses receptor-mediated interactions leading to transactivation of specific genes regulating a wide variety of cellular processes in favor of cellular proliferation (Terasaka et al., 2004). In addition, estrogen shortens the cell cycle (Prall et

al., 1998; Clarke et al., 1994; Brunner et al., 1989), such that a spontaneous mutation that may arise during DNA synthesis could be passed on to daughter cells without being repaired. Furthermore, estrogen metabolism produces genotoxic by-products that can directly damage DNA (Zhang et al., 2001), thereby disrupting normal cellular processes such as apoptosis, proliferation, and DNA repair (Pedra et al., 2009; Mense et al., 2008; Okobia and Bunker, 2006; Russo and Russo, 2006). As a strong promoting agent, estrogen-driven proliferation not only increases the number of estrogen-responsive cells, but also potentially stimulates clonal expansion of pre-cancerous cells (Cheng et al., 2008; Yager and Davidson, 2006; Yoshidome et al., 2000).

Interestingly, the promotion of these initiated cells requires continuous exposure to estrogen to progress pre-neoplastic lesions to a state of transformation. Removal of the promoting agent has been shown to reverse the changes, both at the level of gene expression and at the cellular level (Clarke et al., 1994; Pitot and Dragan, 1991). The regression of preneoplastic lesions upon withdrawal of promoting agents has been linked to the “redifferentiation” or remodeling of the mammary gland (Meidna et al., 2003; Hikita et al., 1999; Tatematus et al., 1983). Knowing that operational reversibility during the promotion stage of carcinogenesis provides a window for intervention, the potential to reverse the effects of elevated levels of estrogen prior to tumor development may prove to be a promising avenue to explore. For example, tamoxifen, an anti-estrogen chemopreventive agent was shown to effectively inhibit or reduce breast carcinogenesis in BRAC1 and BRCA2 mutation carriers (King et al., 2001; Narod et al., 2000). Clearly the use of biomarkers to identify high-risk women who would benefit from similar chemopreventive measures constitutes an important step forward in cancer prevention.

The development of strategies to reverse these premalignant changes, however, depends on our ability to detect early molecular and cellular events.

Although many studies have addressed the contributions of genetic factors in the early stages of breast carcinogenesis, the role of epigenetic mechanisms remains unclear. Recent studies have demonstrated that epigenetic dysregulation leads to changes in gene expression, including the activation of oncogenes, and suppression of tumor suppressors that can produce similar effects as genetic mutations (Moss and Wallrath, 2007; Ehrlich, 2002). Considering the important role of epigenetic regulation of gene expression, it is becoming increasingly clear that understanding the effect of carcinogens on the epigenome is key to unraveling the complex multistage process of carcinogenesis.

This importance is further underlined by the fact that the global loss of DNA methylation, which is one of the best studied epigenetic markers, is a known hallmark of cancer (Feinberg and Tycko, 2004; Baylin et al., 2001; Feinberg and Vogelstein, 1983A). Evidence also suggests that this epigenetic mechanism is altered prior to tumor development, and may serve as an early marker for increased predisposition of cancer development (Kristensen and Hansen, 2009; Pogribny and Beland, 2009). Combined with the knowledge that estrogen can change methylation patterns (Cheng et al, 2008), this suggests that elevated levels of estrogen may induce carcinogenesis through epigenetic mechanisms.

Previously, we have shown that estrogen-induced epigenetic dysregulation occurs prior to tumor development (Kovalchuk et al., 2007), yet the earliest point at which these epigenetic changes can be detected in response to elevated levels of estrogen remains

unknown. More importantly, the potential reversibility of these epigenetic changes upon removal of the stimulating agent has yet to be explored.

In the present study we have used the well-established ACI rat model of estrogen-induced breast carcinogenesis to address these questions. The ACI rat shares remarkably similar etiology as human breast cancer, with the developing mammary gland of a young adult being the most susceptible for chemical and physical agents to exert their carcinogenic potential (Ariazi et al., 2005; Russo and Russo, 1982). By examining the influence of estrogen exposure on epigenetic mechanisms that parallel the histological progression of premalignant changes in mammary gland, we hope to characterize the sequence of early epigenetic markers that can be detected prior to tumor development. Furthermore, we hypothesize that the removal of the promoting agent will lead to the regression of pre-malignant alterations that may be related to “redifferentiation” and remodeling of the epigenomic landscape. Understanding the epigenetic mechanisms that underlie this window of operational reversibility may allow chemopreventive strategies to be implemented to revert or halt these early estrogen-induced changes prior to the development of cancer.

## 2.3. MATERIALS AND METHODS

### 2.3.1. Early Effects of Estrogen Exposure:

#### 2.3.1.1. *Animal Treatment*

To evaluate the specific epigenetic characteristics that mark the early stages of carcinogenesis, intact, female ACI rats were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). The animals were housed 2 per cage in a temperature-controlled (24°C) room with a 12 hour light-dark cycle, and given *ad libitum* access to water and NIH-31 pelleted diet. Handling and care of animals was in strict accordance with the recommendations of the Canadian Council for Animal Care and Use. The procedures have been approved by the University of Lethbridge Animal Welfare Committee.

At 6 weeks of age, the rats were anaesthetized with ketamine and xylazine. Estrogen mini-pellets containing 7.5 mg 17 $\beta$ -estradiol released over a 90 day period were implanted subcutaneously, while control groups received a placebo pellet (Innovative Research of America Inc., Sarasota, FL.). Five rats per group were humanely euthanized at 2 days, 1 week, 2 weeks, and 4 weeks.

The paired caudal inguinal mammary glands were excised. One gland was frozen immediately in liquid nitrogen and stored at -80°C for subsequent analyses. The contralateral gland was fixed in 4% PFA for 48 h, embedded in paraffin, sectioned at 4 microns, and mounted on glass slides.

## **2.3.2. Persistence of Estrogen-induced Changes**

### **2.3.2.1. *Animal Treatment***

Continuous estrogen treatment was induced in young adult female ACI rats of 7-8 weeks in age using the same dose of estrogen as described above for either a 4 or 12 week time period. To determine the persistence of estrogen-induced changes, after 4 weeks of exposure, the estrogen pellet was replaced with a placebo pellet for an additional 8 weeks (4+8 group). Ten animals per group were humanely euthanized at each of the 4 and 12 week time points, including control groups implanted with a placebo pellet. The mammary gland was then processed as detailed above.

### **2.3.3. Histopathological Evaluation**

The sections were stained with hematoxylin and eosin to evaluate the estrogen-induced morphological alterations. The mammary gland tissue was graded as follows: simple epithelium (1); mild hyperplasia (2); moderate hyperplasia (3); moderate-severe hyperplasia (4); severe hyperplasia (5). Each tissue was graded according to the most disrupted morphological pattern observed within it. The histological assessment was independently confirmed by two trained pathologist blinded to the treatments.

### **2.3.4. Global DNA Methylation Analysis**

Genomic DNA was isolated from rat mammary tissue by using Qiagen Dneasy<sup>TM</sup> Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. The extent of the global DNA methylation was evaluated with a well-established radiolabeled [<sup>3</sup>H]-dCTP extension assay (Pogribny et al., 1999). In brief, 1 µg of genomic DNA was digested

with 20 U of methylation-sensitive HpaII restriction endonuclease (New England Biolabs, Beverly, MA) for 16-18 h at 37°C. A second DNA aliquot (1 µg) of undigested DNA served as background control. The single nucleotide extension reaction was performed in a 25 µl reaction mixture containing 1.0 µg DNA, 1X PCR buffer II, 1.0 mM MgCl<sub>2</sub>, 0.25 U AmpliTaq DNA polymerase, and 0.1 µl of [<sup>3</sup>H]dCTP (57.4 Ci/mmol), and incubated at 56°C for 1 h. Samples were applied to DE-81 ion-exchange filters and washed three times with 0.5 M Na-phosphate buffer (pH 7.0) at room temperature. The filters were dried and processed for scintillation counting. The [<sup>3</sup>H]dCTP incorporation into DNA was expressed as mean disintegrations per minute (dpm) per µg of DNA after subtraction of the dpm incorporation in undigested samples (background). Two technical repeats of each experiment were conducted to ensure consistency of the data.

### **2.3.5. Western Blot Analysis of Protein Expression**

Mammary tissue was sonicated in 1% sodium dodecyl sulfate (SDS), followed by centrifugation at 10,000 x g at 4°C for 10 min. The supernatant was collected and boiled for 10 min. Equal amounts of proteins (20 µg) were separated by SDS-polyacrylamide electrophoresis (PAGE) on 8% polyacrylamide gels and transferred to PVDF membranes (Amersham, Baie d'Urfé, Québec). Membranes were probed with primary antibodies against DNMT1 (1:1000 Santa Cruz Biotechnology, Santa Cruz, CA), MeCP2 and β-Actin (1:1000 Abcam, Cambridge, MA). Horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and the ECL Plus immunoblotting detection system (Amersham, Baie d'Urfé, Québec) were used to reveal antibody binding. Chemiluminescence was detected by Biomax MR films (Eastman

Kodak, New Haven, CT). All membranes were stained with Coomassie Blue (BioRad, Hercules, CA) to confirm equal protein loading. Signals were quantified using NIH ImageJ 1.63 Software and normalized to loading controls. Images are representative of two independent immunoblots.

### **2.3.6. Immunohistochemical Analysis**

Tissue microarrays comprised of 2.5 mm representative cores from each treatment were mounted on positively charged slides, for immunohistochemical (IHC) analysis. The tissue was fixed to the slides by baking at 60°C for 1 hour, deparaffinized, rehydrated, steamed in antigen retrieval citrate buffer (pH 6.0) (DAKO, Carpinteria, CA), endogenous peroxidase activity was quenched in 3% H<sub>2</sub>O<sub>2</sub> and blocked in 3% goat serum (Santa Cruz Biotechnology, Santa Cruz, CA). Tissues were probed with primary antibodies against DNMT3a (1:400, Santa Cruz Biotechnology, Santa Cruz, CA) and AcH4K12 (1:300; Cell Signaling Technology, Beverly, MA). Binding was detected by avidin-biotinylated horseradish peroxidase and visualized with DAB (ABC Staining System, Santa Cruz Biotechnology, Santa Cruz, CA). Tissues were counterstained with hematoxylin (Santa Cruz Biotechnology, Santa Cruz, CA).

Staining for histone modifications were scored semi-quantitatively in at least 5 high power fields per animal in each group. The sections were evaluated by the percentage of positive cells in each field of view.

### **2.3.7. Statistical Analysis**

Results are presented as mean  $\pm$  S.E.M. Statistical analyses were conducted using the students *t*-test. P-values  $<0.05$  were considered significant.

## 2.4. RESULTS

### 2.4.1. Influence of Short-term Exposure on Mammary Gland Histopathology

Morphological alterations were one of the first changes that could be detected after short term exposure to elevated levels of exogenous estrogen (Table 2.1; Figure 2.1). Mild hyperplastic changes were detected in 4/5 animals in the estrogen-treated group at 2 days, which progressed in severity upon continuous exposure to estrogen. By 4 weeks, all but one animal exposed to constitutively elevated estrogen exhibited severe hyperplasia, giving an average score of  $4.6 \pm 0.5$ . Importantly, the removal of the estrogen pellet at 4 weeks, followed by 8 weeks without, led to the regression of hyperplastic changes. Only a mild grade of hyperplasia was observed in 3/8 animals in the 4+8 group, averaging a score of  $1.4 \pm 0.5$  (Table 2.2; Figure 2.4).

### 2.4.2. Effects of Elevated Levels of Estrogen on DNA Methylation

Estrogen is known to influence DNA methylation (Cheng et al., 2008), which coordinates both gene expression profiles of epithelial cells and the architecture of the mammary gland (Plachot and Lelievre, 2004). To determine if the observed alterations in mammary gland morphology was correlated with changes in methylation, we used the global HpaII-based cytosine extension assay to measure the levels of global DNA methylation. Interestingly, we did not detect any significant changes in global DNA methylation levels within 4 weeks of exposure to elevated levels of estrogen (Figure 2.2). However, by 12 weeks of continuous exposure to  $17\beta$ -estradiol, we observed a 1.3-fold loss of global methylation ( $p < 0.01$ ) (Figure 2.5).

Regardless of global methylation levels, past research has shown that the dysregulation of DNA methylation machinery is an early event that precedes tumor development (Belinsky et al., 1996), and is paralleled by the dysregulation of DNA methylation machinery during hyperplasia (Kovalchuk et al., 2007). In this study, we found evidence for the induction of both DNMT1, and DNMT3a, which are typically designated as maintenance and *de novo* methyltransferase, respectively (Figure 2.5). The percentage of DNMT3a-positive cells increased by 1.3-fold as early as 2 days, and remained greater than control levels by up to 3.3-fold during the 12 weeks of estrogen exposure ( $p < 0.05$ ). Interestingly, DNMT3a levels remained significantly elevated even after the estrogen pellet was removed (1.2 fold,  $p < 0.05$ ), although these changes were significantly less than in the rats exposed to continuously elevated levels of estrogen. In contrast, the 20% increase in DNMT1 observed between week 1 and 4 of estrogen-treatment was diminished upon the removal of the estrogen pellet in the 4+8 week group.

Research indicating a positive correlation between DNMT1 function and the progression of hyperplastic changes towards malignancy suggests an important role for MeCP2 in its capacity to maintain DNMT1's methyltransferase activity (Tryndyak et al., 2006; Kimura and Shiota, 2003; Saito et al., 2003). We noted that the induction of DNMT1 at 1 week of estrogen treatment was paralleled by a 20% increase in MeCP2 ( $p < 0.05$ ), which continued to be upregulated 12 weeks after exposure (Figure 2.2; Figure 2.5). However, removal of the estrogen pellet at 4 weeks, followed by 8 weeks without an exogenous estrogen source, resulted in the return of MeCP2 levels to that of age-matched controls.

### **2.4.3. Effects of Elevated Levels of Estrogen on Histone H4 Lysine 12 Acetylation**

Several reports have been published showing that estrogen hormones recruit mechanisms regulating histone acetylation to bring about their proliferative effects (Gunin et al., 2005; Margueron et al., 2004, Sun et al., 2001). The acetylation of histone H4 lysine 12 (H4K12), in particular, is required for the proper formation of the newly synthesized chromatin during proliferation (Gunin et al., 2005). To determine if the estrogen-induced hyperplastic changes were paralleled by altered levels of H4K12 acetylation in the virgin mammary gland, we used immunohistochemical methods to detect acetylated H4K12 (Figure 2.3). A slight, but significant increase in the number of AcH4K12-positive cells was observed after 2 weeks of exposure to constitutively elevated levels of estrogen relative to age-matched control (10% increase;  $p < 0.05$ ). By 4 weeks, the levels of acetylation were more pronounced and remained higher than control levels for up to 12 weeks of exposure (Figure 2.6). Although the level of histone acetylation remained higher in the 4+8 group than in age-matched controls, it was less than both the 4 and 12 week groups exposed to continuously elevated levels of estrogen (Figure 2.6).

## 2.5. DISCUSSION

The role of estrogen in the mammary gland remains a topic of much debate. Short-term exposure to pregnancy levels of estrogen was shown to have protective effects (Russo et al., 2005; Rajkumar et al., 2001), whereas long-term exposure resulted in promotion of initiated cells and ultimately the development of cancer (Clemons and Goss, 2001; Henderson et al., 1982).

The mammary gland is a dynamic tissue that is very sensitive to the hormonal milieu, with transient proliferation of epithelial cells occurring in parallel with the estrous cycle (Schedin et al., 2000). Therefore, in discussing mammary gland morphology, one must take into consideration the natural developmental processes. The fact that, during the first few weeks of the experiment, several cases of slight hyperplasia were found in the control groups likely represents the effect of natural hormones in stimulating the maturation of the mammary gland in young adult female rats. Although this may also, in part, explain the observation of mild hyperplasia in 4/5 animals in the estrogen-treated group at 2 days, the observed progression to moderate hyperplasia 5 days later, implies involvement of the exogenous source of estrogen. This positive correlation between estrogen exposure and morphological alterations provides further evidence of the carcinogenic potential of estrogen, which has been proposed to be capable of stimulating the clonal expansion of initiated cells (Yager and Davidson, 2006; Yoshidome et al., 2000). Alternatively estrogen has been shown to reduce the amount of time spent in the cell cycle (Prall et al., 1998; Clarke et al., 1994; Brunner et al. 1989), thereby increasing the probability of unrepaired errors.

Importantly, we have shown that the removal of the estrogen pellet at 4 weeks, followed by 8 week period without, was necessary for the regression of hyperplastic changes in the mammary gland. The observation of mild proliferation in the 4+8 group may be attributed to the process of restructuring in the mammary gland. Interestingly, studies have also shown that excess estrogen may be stored in adipocytes and released over time, providing weak proliferative effects in nearby tissue (Feher et al., 1982).

Exposure to estrogen is known to change DNA methylation patterns (Cheng et al., 2008), allowing for increased proliferation (Prall et al., 1998). Estrogen-driven proliferation increases DNA synthesis by recruiting cells into the cell cycle (Prall et al., 1998), thereby increasing the proportion of cells with nascent, unmethylated DNA strands. Although this would likely lead to the activation of methylation machinery to maintain the faithful replication of methylation patterns, several studies have shown that these hyperplastic changes are paralleled by the global loss of methylation (Pogribny and Beland, 2009; Kovalchuk et al., 2007; Arens et al., 2005; Bernardino et al., 1997).

The fact that we did not detect significant changes in global methylation during short-term exposure to estrogen, suggests that the changes induced by estrogen-driven hyperplasia and DNA methyltransferase activity are balanced in such a way that no apparent differences in global DNA methylation levels are detected. It is possible that this observation is attributed to the maintenance of normal methylation, which is thought to prevent the malignant transformation of mammary epithelial cells (Pogribny and Beland, 2009). Alternatively, the induction of DNA methyltransferases may establish *de novo* regional hyper- and hypomethylation, resulting in the silencing of tumor suppressors and activation of oncogenes (Cheng et al., 2008). In this study, we have

found evidence for the induction of both maintenance and *de novo* DNA methyltransferases within a week of exposure to elevated estrogen levels. Furthermore, DNMT1, which is predominately classified as a maintenance methyltransferase, was also shown to have *de novo* activity (Yoder et al. 1997), and increases of DNMTase activity has been associated with neoplastic development (Belinsky et al., 1996). The positive correlation between estrogen-induced hyperplastic changes and DNMT1 was further supported by the early and long-term induction of MeCP2 in the present study, which has been shown to stabilize DNMT1 function in methylation-mediated gene silencing (Kimura and Shiota, 2003).

Alternatively, at some point the maintenance methyltransferase activity may become overwhelmed resulting in global hypomethylation, a well-characterized marker of carcinogenesis, that tips the scale in favor of the malignant transformation. The observed loss of global methylation at 12 weeks may be indicative of an overload of the methyltransferase machinery. Indeed, the fact that DNMT1 levels have returned to control levels, despite the increased levels of proliferation in the estrogen-treated group, seems to support this hypothesis. Improper maintenance of methylation patterns, especially in an estrogen-driven environment, would likely lead to genomic instability and ultimately cancer.

The removal of the exogenous source of estrogen, prior to this point, is thought to involve the reversible enhancement or repression of gene expression (Medina et al., 2003). In support of this notion of reversibility, the induction of DNMT1 and MeCP2 observed after 4 weeks of continuous estrogen exposure was diminished to control levels. Only the levels of DNMT3a remained significantly elevated even after the estrogen pellet

was removed. Research showing that DNA methylation regulates tissue organization and epithelial differentiation in the mammary gland (Plachot et al., 2004) may indicate that regression of preneoplastic changes requires DNMT-mediated epigenetic remodeling or “redifferentiation”. We therefore hypothesize that DNMT3a may play a role in returning methylation patterns to a more tightly regulated epigenetic profile.

A second epigenetic mechanism involved in regulating gene expression and cellular differentiation in the mammary epithelium is the acetylation of lysine residues on histone tails (Wang et al., 2001; Loidl, 1988). The addition of acetyl groups decreases the interaction between core histone proteins and DNA, thereby allowing greater access to transcription factors, which underlies the positive correlation documented between histone acetylation and mitotic index (Kininis et al., 2007; Gunin et al., 2005). Several studies have shown that by recruiting mechanisms controlling histone acetylation, estrogen is effectively able to stimulate proliferation (Kininis et al., 2007; Gunin et al., 2005). In support of the literary evidence, we have shown that hyperacetylation of H4K12, which is required for loading newly synthesized histone proteins onto replicated DNA (Groth, 2009; Sobel et al., 1995), parallels increases in estrogen-induced proliferation. Moreover, the reduction in H4K12 hyperacetylation upon removal of the estrogen pellet, relative to the groups receiving continuous estrogen treatment, correlates with the reduced proliferation observed in the 4+8 week group.

In summary, our findings show that short-term estrogen exposure induces hyperplastic changes in the mammary gland that are paralleled by changes in epigenetic mechanisms. Exposure to elevated levels of estrogen alters components of the DNA methylation machinery that can be detected even at the very early stages. Although these

changes did not result in significant difference in global levels of methylation, the persistent upregulation of DNMT3a and hyperacetylation of H4K12 indicate that further studies are required to determine if changes are induced in specific genes and, if so, their role in signaling pathways linked to breast carcinogenesis.

Importantly, we have shown that the majority of epigenetic parameters that were measured returned to similar levels as in the age-matched controls upon removal of the estrogen pellet. Although there was a persistent upregulation of DNMT3a and hyperacetylation of H4K12, these changes were significantly lower after 8 weeks without exposure to the exogenous source of elevated estrogen compared to 4 and 12 week groups receiving constitutively elevated levels of estrogen. We hypothesize that the regression of preneoplastic changes upon withdrawal of the promoting agent may, in part, be due to epigenetic redifferentiation. Further understanding of the epigenetic mechanisms that underlie this window of operational reversibility is required to implement strategies targeted to halt these changes prior to the development of cancer. Moreover, the observed epigenetic changes should be explored as potential biomarkers for breast cancer predisposition, by which high risk individuals who would benefit most from chemopreventive treatments could be identified.

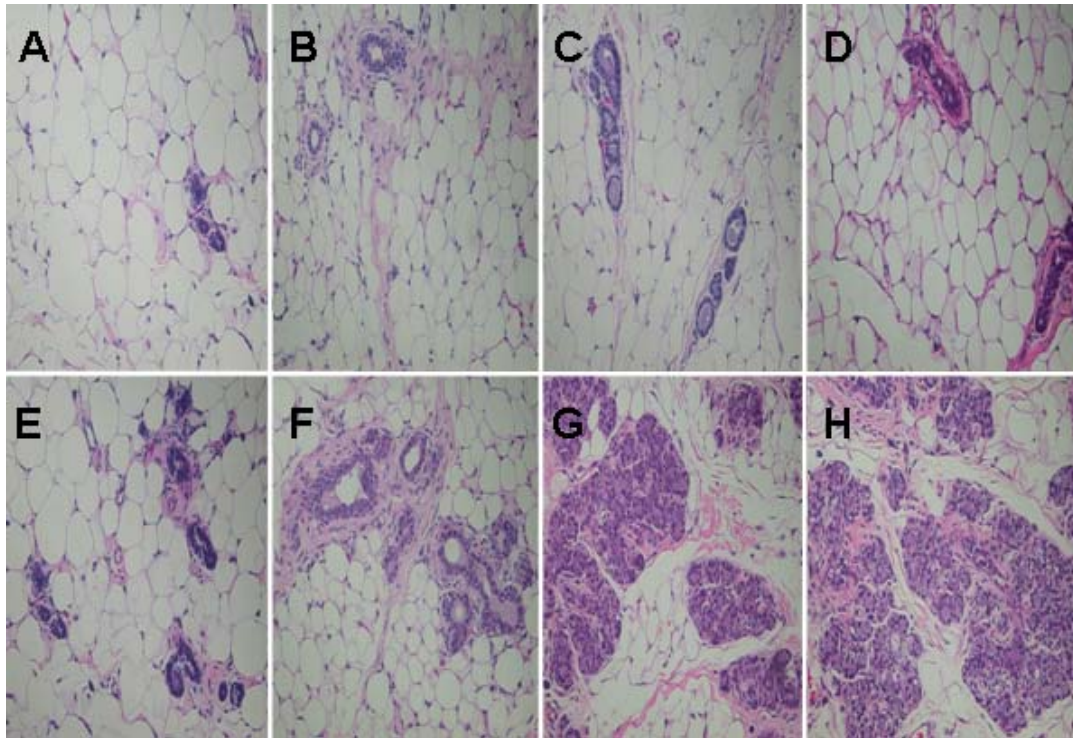
## 2.6. FIGURES AND TABLES

**Table 2.1. The progression of morphological changes in the mammary gland of female ACI rats after exposure to constitutively elevated levels of estrogen.**

Morphological changes in the mammary gland after exposure to sham or estrogen (E<sub>2</sub>) treatment at 2 days, 1 week, 2 weeks, and 4 weeks. Data presented as means ± S.E.M.

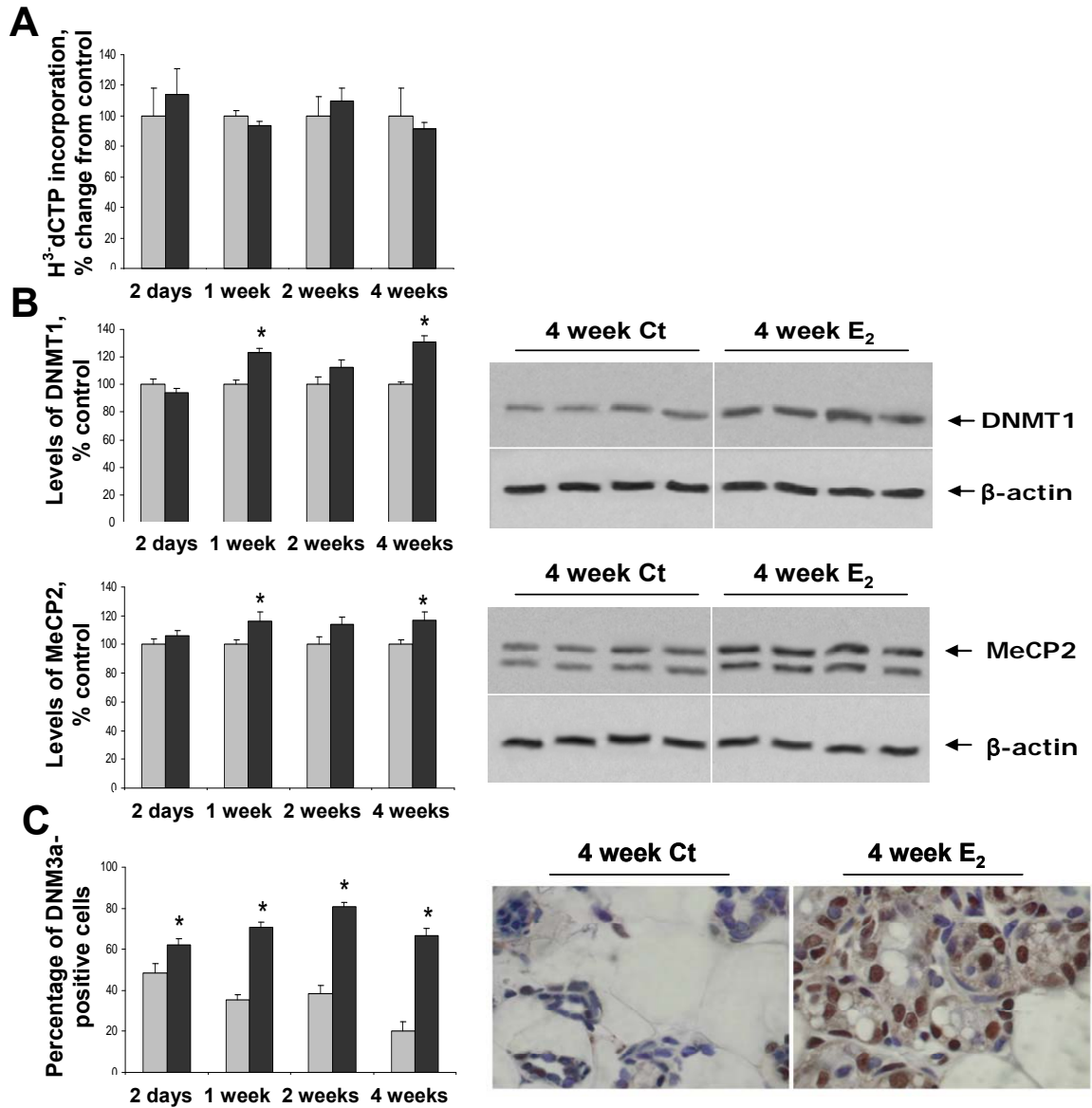
(n=5). \*Significantly different from the age-matched control. Scoring: 1 – histologically normal; 2 – mild hyperplasia; 3 – moderate hyperplasia; 4 – moderate/severe hyperplasia; 5 – severe hyperplasia.

<b>Group</b>		<b>Histopathology score</b>
2 days	Control	1.2 ± 0.4
	E <sub>2</sub>	1.8 ± 0.4
1 week	Control	1.4 ± 0.5
	E <sub>2</sub>	2.6 ± 0.8*
2 weeks	Control	1.2 ± 0.4
	E <sub>2</sub>	4.0 ± 0.0*
4 weeks	Control	1.0 ± 0.0
	E <sub>2</sub>	4.6 ± 0.5*



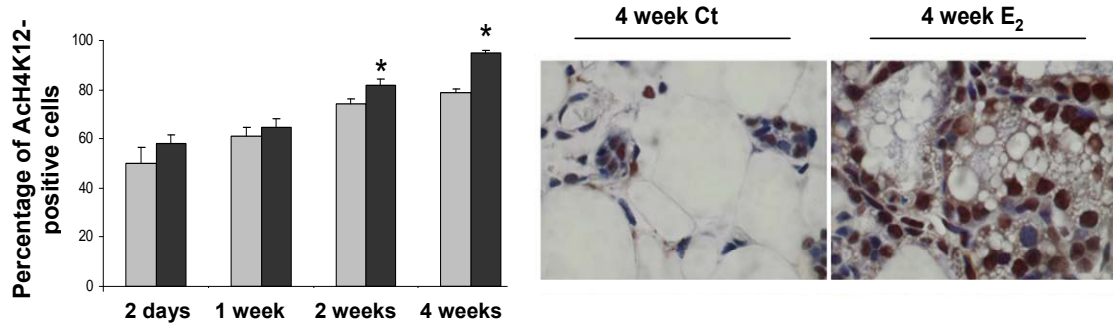
**Figure 2.1. The progression of morphological changes in the mammary gland of female ACI rats after exposure to constitutively elevated levels of estrogen.**

Representative images of the rat mammary gland after 2 days, 1 week, 2 weeks, and 4 weeks of sham-treatment (A-D respectively) and after estrogen-treatment (E-H respectively) (Original magnification x10).



**Figure 2.2. Effect of constitutively elevated levels of estrogen on DNA methylation machinery.**

(A) Global levels of DNA methylation in response to short-term estrogen exposure. (B) Western blot analysis of DNMT1 and MeCP2 protein levels. (C) Percentage of DNMT3a-positive cells. \*Significantly different from the age-matched control. Grey bars – control-treatment; black bars – estrogen-treatment. The right panel shows representative images from two independent technical repeats.



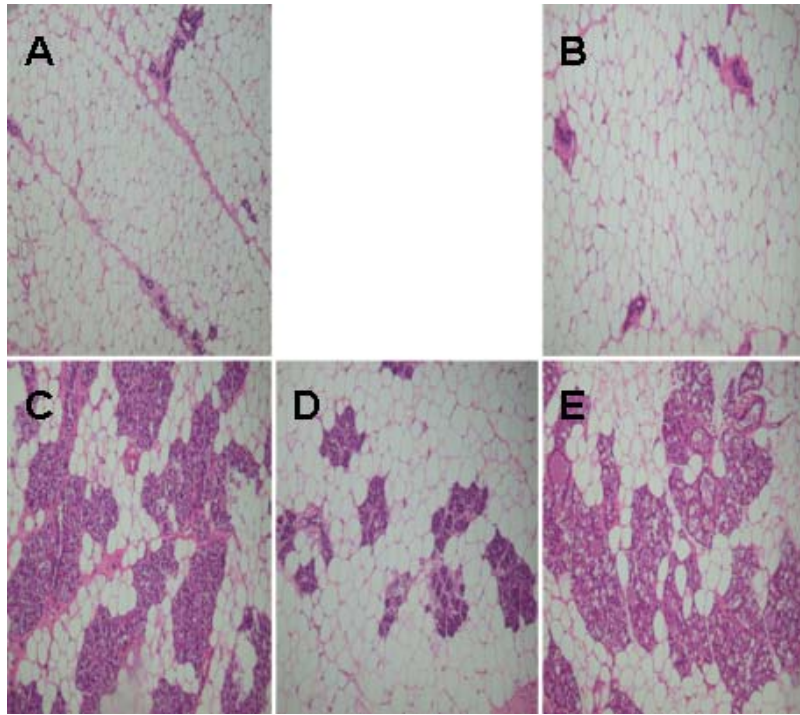
**Figure 2.3. Immunohistochemical analysis of acetylated histone levels induced in response to elevated levels of estrogen.**

Percentage of cells stained positive for the acetylated histone H4 lysine 12 residue (AcH4K12). \*Significantly different from the age-matched control. Grey bars – control-treatment (Ct); black bars – estrogen-treatment (E<sub>2</sub>). The right panel shows representative immunohistochemical images from two independent technical repeats (Original magnification x100)

**Table 2.2. Progressive morphological changes in the mammary gland after continuous exposure to estrogen (E<sub>2</sub>) treatment, followed by the regression of these changes after removal of the estrogen pellet.**

Data presented as means ± S.E.M.; n=10. \*Significantly different from the age-matched control. Scoring: 1 – histologically normal; 2 – mild hyperplasia; 3 – moderate hyperplasia; 4 – moderate/severe hyperplasia; 5 – severe hyperplasia.

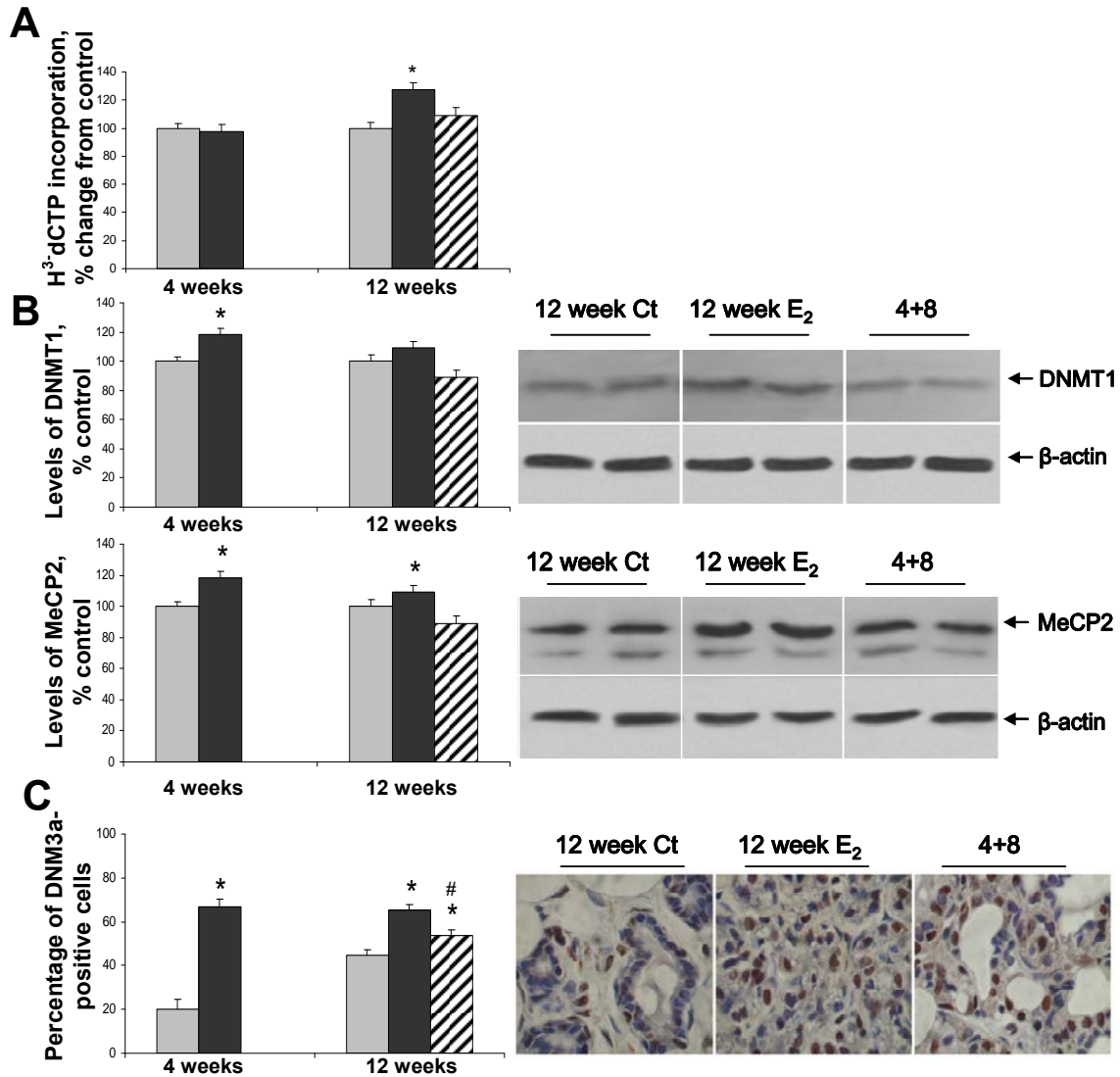
<b>Group</b>		<b>Histopathology score</b>
4 weeks	Control	1.0 ± 0.0
	E <sub>2</sub>	3.1 ± 0.6*
12 weeks	Control	1.0 ± 0.0
	E <sub>2</sub>	2.6 ± 0.5*
	4+8	1.4 ± 0.5



**Figure 2.4. Morphological changes in the mammary gland of female ACI rats after exposure to constitutively elevated levels of estrogen.**

Representative images of the rat mammary gland after 4 weeks and 12 weeks of sham-treatment (A, B respectively) and after continuous estrogen-treatment (C-E respectively).

The estrogen-induced morphological changes regressed 8 weeks after the 4 week estrogen treatment (D) (Original magnification x10).



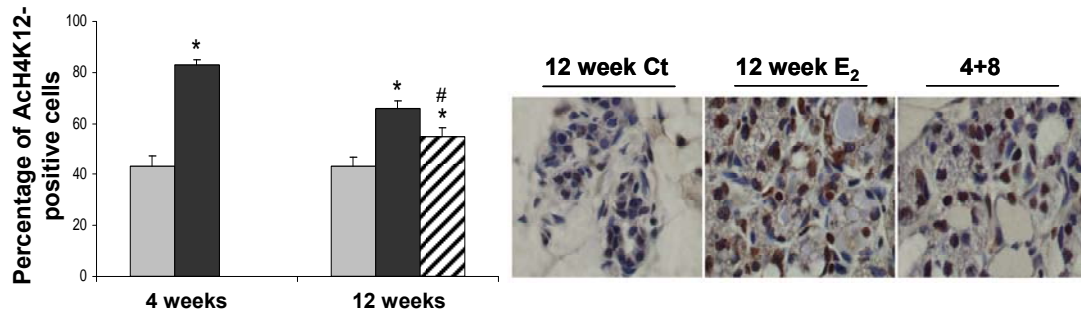
**Figure 2.5. Effect of constitutively elevated levels of estrogen on DNA methylation machinery.**

(A) Global levels of DNA methylation in response to estrogen exposure.

(B) Western blot analysis of DNMT1 and MeCP2 protein levels. (C) Percentage of DNMT3a-positive cells. \*Significantly different from the age-matched control.

#Significantly different from the rats exposed to continuously elevated levels of estrogen.

Grey bars – control-treatment (Ct); black bars – estrogen-treatment (E<sub>2</sub>), striped bars – 8 weeks after the removal of the estrogen pellet following 4 weeks of continuous estrogen treatment (4+8). The right panel shows representative images from two independent technical repeats.



**Figure 2.6. Immunohistochemical analysis of acetylated histone levels induced in response to elevated levels of estrogen.**

Percentage of cells stained positive for the acetylated histone H4 lysine 12 residue (AcH4K12). \*Significantly different from the age-matched control. #Significantly different from the rats exposed to continuously elevated levels of estrogen. Grey bars – control-treatment (Ct); black bars – estrogen-treatment (E<sub>2</sub>). The right panel shows representative immunohistochemical images from two independent technical repeats (Original magnification x40).

**3. IMPAIRED P53-DEPENDENT APOPTOSIS AND CELL PROLIFERATION  
DURING EARLY STAGES OF MAMMARY GLAND CARCINOGENESIS IN  
ACI RATS**

<sup>1</sup>Chapter 3 submitted in its entirety:  
Kutanzi K, Koturbash I, Bronson R, Pogribny I, Kovalchuk O. Impaired p53-dependent apoptosis and cell proliferation during early stages of mammary gland carcinogenesis in ACI rats. *Mutat Res.* (Submission MUT-D-10-00052)

### 3.1. ABSTRACT

Estrogen and ionizing radiation are well-documented human breast carcinogens, yet the exact mechanisms of their deleterious effects on the mammary gland remain to be discerned. Here we analyze the balance between cellular proliferation and apoptosis in the mammary glands of rats exposed to estrogen and X-ray radiation and the combined action of these carcinogenic agents. We show that combined exposure to estrogen and radiation has a synergistic effect on cell proliferation in the mammary glands of ACI rats, as evidenced by a substantially greater magnitude of cell proliferation, especially after 12 and 18 weeks of treatment, when compared to mammary glands of rats exposed to estrogen or radiation alone. We also demonstrate that an imbalance between cell proliferation and apoptosis, rather than enhanced cell proliferation or apoptosis suppression alone, may be a driving force for carcinogenesis. Our studies further suggest that compromised functional activity of p53 may be one of the mechanisms responsible for the proliferation/apoptosis imbalance.

In sum, the results of our study indicate that evaluation of the extent of cell proliferation and apoptosis before the onset of preneoplastic lesions may be a potential biomarker of breast cancer risk after exposure to breast carcinogens.

### 3.2. INTRODUCTION

Breast cancer is the most common malignancy in women. Despite advances in understanding the molecular biology of breast cancer and improvements in early detection and treatment, the incidence of invasive breast cancer, the most serious form of breast cancer, in the United States was estimated to increase to 192,370 new cases in 2009 compared to 182,460 in 2008 (Jermal et al., 2009). This clearly illustrates the importance of elucidation of underlying molecular mechanisms associated with breast carcinogenesis and the identification of intermediate cellular and molecular biomarkers that precede mammary carcinogenesis and are indicative of an increased risk of breast cancer (Rose-Hellekant et al., 2006).

The results of numerous studies have identified estrogen and radiation exposure as two main causative factors for human breast cancer development (Pedraza-Fariña, 2006; Hanahan and Weinberg, 2000). Until recently, most studies on estrogen- or radiation-induced breast cancers have largely focused on the propagation of transformed cells and progression of breast cancer. Much less attention has been given to molecular changes occurring between the transition of a normal cell to a tumor cell and for defining how these specific molecular changes may be related mechanistically to neoplastic cell transformation. Investigating these molecular mechanisms in humans is often impractical and, in most cases, unethical (Rose-Hellekant et al., 2006; Tamm et al., 2001). In contrast, relevant animal models of mammary gland carcinogenesis provide an opportunity for the study of breast cancer initiation and progression. In this respect, the Augustus Copenhagen Irish (ACI) rat model of estrogen-induced mammary cancer is unique and the most relevant animal model for studying molecular mechanisms of human

sporadic breast cancer (Jagani and Khosravi-Far, 2008; Jager, 2007; Yager and Davidson, 2006; Land et al., 2003). This model and human sporadic breast cancer share many morphological and molecular features, including estrogen-dependence, chromosomal instability, aneuploidy, deregulation of the cell cycle, and epigenetic abnormalities (Jager, 2007; Yager and Davidson, 2006; Ronckers, 2005).

One of the fundamental features in tumorigenesis is deregulation of cell proliferation and apoptosis (Ye et al., 2004). The perturbations in these two processes lead to a disruption in the balance between cell proliferation and apoptosis, which may occur either through excessive sustained proliferation or failure of programmed cell death. This imbalance represents a pro-tumorigenic principle in tumorigenesis (Shull, 1997; Li and Li, 2003) and is considered one of the hallmarks of cancer (Ye et al., 2004). It is well established that suppression of apoptosis or impaired apoptosis is a fundamental event in tumor formation (Mense et al., 2008; Weroha et al., 2006; Ye et al., 2004); however, the role of these processes in the initiation of breast carcinogenesis remain inconclusive and contradictory (Mense et al., 2008; Rose-Hellekant, 2006).

In our previous studies on estrogen induced mammary carcinogenesis in ACI rats, we demonstrated that epigenetic alterations in general (Kovalchuk et al., 2007), and gene-specific epigenetic alterations of tumor suppressor genes, including *Rassf1a*, *Socs1*, and *p16<sup>INK4a</sup>*, critical regulators of the cell cycle, cell proliferation and apoptosis, in particular (Starland-Davenport et al., 2010), preceded formation of putative preneoplastic mammary gland lesions. The transcriptional inhibition of these genes may cause alterations in the functioning of cell proliferation and apoptosis leading to neoplastic transformation. Based on these considerations in the present study, we hypothesize that breast

carcinogenesis in rats induced by estrogen and/or X-ray exposure is associated with deregulation of cell proliferation and apoptosis, and these changes occur during the early stages of the tumorigenic process preceding formation of preneoplastic and neoplastic lesions in mammary tissue.

### 3.3. MATERIALS AND METHODS

#### 3.3.1. Animal Treatment and Tissue Preparation

Intact, female ACI rats were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). The animals were housed 2 per cage in a temperature-controlled (24°C) room with a 12 hour light-dark cycle, and given *ad libitum* access to water and NIH-31 pelleted diet. Handling and care of animals was in strict accordance with the recommendations of the Canadian Council for Animal Care and Use. The procedures have been approved by the University of Lethbridge Animal Welfare Committee.

At 8 weeks of age, the rats were randomly allocated into 4 groups of 18 rats each: i) sham treated controls; ii) E<sub>2</sub> treated; iii) IR treated; or iv) E<sub>2</sub> and IR treated. The estrogen treated groups received a single pellet, containing 7.5 mg of 90-day release 17β-estradiol (Innovative Research of America, Sarasota, FL) that was implanted subcutaneously in the shoulder region under ketamine and xylazine-induced anesthesia. Animals from the irradiated groups were exposed to a single dose of 3 Gy of X-rays 1 week later (90 kVp, 5 mA). Six rats per group were humanely euthanized after 6, 12, and 18 weeks of treatment. All animal experimental procedures were carried out in accordance with animal study protocols approved by the University of Lethbridge Animal Care and Use Committee.

The paired caudal inguinal mammary glands (and fat pad) were excised from the overlying skin. One gland was frozen immediately in liquid nitrogen and stored at -80°C for subsequent analyses. The contralateral gland and fat pad were carefully spread onto a 5x8 cm glass slide and excess fat and other tissue were trimmed. The gland was then placed flat in a cassette *in toto*. This provided a histological specimen with frontal plane

orientation, in which the gland profile is comparable to that of a mammary whole mount. This orientation allows visualization of the arborizing pattern of the duct system and associated alveoli more clearly and completely than is possible using a transverse section of the gland. The specimens were then fixed in 10% neutral buffered formalin for 48 h, processed, embedded in paraffin, sectioned at 4 microns, and mounted on glass slides. The sections were stained with hematoxylin and eosin for histopathological examination.

### **3.3.2. Immunohistochemistry**

The extent of cell proliferation, apoptosis, and expression of p53 and Mdm2 proteins was determined in mammary gland sections after 6, 12, and 18 weeks of experiment. Formalin-fixed paraffin-embedded mammary gland sections were deparaffinized and rehydrated.

#### **3.3.2.1. Cell Proliferation**

For evaluation of proliferative activity, the mammary gland sections were stained for Ki-67 protein. Endogenous peroxidases were inhibited by incubation with freshly prepared 3% hydrogen peroxide with 0.1% sodium azide for 10 min at room temperature. The tissue sections were then placed in an antigen retrieval solution (10 mM citrate buffer, pH 6.0) for 15 min in a microwave oven at 100°C at 600 W. After incubation with normal rat 10% serum, mouse monoclonal anti-rat Ki-67 (clone MIB-5, DAKO, Carpinteria, CA) antibody was applied to the sections at the dilution of 1:50 (3.8 µg/ml) for 1 h at room temperature. After incubation with primary antibody, tissue sections were incubated with biotinylated rat anti-mouse IgG (Jackson ImmunoResearch, West Grove,

PA) at a dilution of 1:400 for 30 min at room temperature and later with streptavidin-conjugated horseradish peroxidase (ExtrAvidin Kit, Sigma, St. Louis, MO) at a dilution of 1:30 for 30 min at room temperature. Staining was developed with 3,3'-diaminobenzidine. For a negative control, 3.8 µg/ml mouse IgG (Jackson ImmunoResearch, PA) or phosphate-buffered saline replaced the primary antibody.

### **3.3.2.2. Apoptosis**

Apoptotic bodies in mammary gland sections were detected by terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labeling (TUNEL) of DNA fragments using an ApopTag Peroxidase *in situ* Apoptosis Detection Kit obtained from Serologicals Corporation (Norcross, GA). The TUNEL assay was performed according to the manufacturer's suggestions. Briefly, endogenous peroxidase was quenched as described above and mammary gland sections were incubated with proteinase K (20 µg/ml) for 15 min at room temperature. The permeabilized tissue sections were enzymatically labeled with digoxigenin-nucleotide via TdT and subsequently exposed to horseradish peroxidase-conjugated anti-digoxigenin antibody. Staining was developed with 3,3'-diaminobenzidine.

### **3.3.2.3. p53 and Mdm2 Expression**

Staining mammary gland sections for p53 was conducted by using a polyclonal anti-p53 antibody (CM1, Novocastra Reagents; Leica Microsystems, Bannockburn, IL) as described previously (Koturbash et al., 2008).

The number of cells positively stained for Ki-67, p53, and Mdm2, as well as the number of apoptotic cells, was counted per 100 cells in five randomly chosen areas.

### **3.3.3. Western Blot Analysis**

Protein levels of c-myc and  $\beta$ -Actin were determined by Western immunoblot analysis as described previously (Starland-Davenport et al, 2010).

### **3.3.4. Statistical Analysis**

Results are presented as mean  $\pm$  S.E.M. Statistical analyses conducted by one- or two-way ANOVA, as appropriate, with pair-wise comparisons being conducted by Dunnett's test.

### 3.4. RESULTS

#### **3.4.1. Effect of 17 $\beta$ -estradiol (E<sub>2</sub>), X-ray Radiation (IR), or E<sub>2</sub> Plus IR Exposure on Cell Proliferation in the Mammary Glands of ACI Rats**

Table 3.1 shows that continuous exposure of ACI rats to E<sub>2</sub> or combined exposure to E<sub>2</sub> and IR resulted in a substantial increase in epithelial cell proliferation in mammary glands with a magnitude being greater after 6 weeks of exposure. In contrast, IR alone did not induce changes in the rate of cellular proliferation at the time points analyzed. These changes in cellular proliferation were confirmed by measuring the expression of Ki-67 protein in the mammary gland tissue sections. In the mammary glands of ACI rats continuously exposed to E<sub>2</sub>, the level of Ki-67 protein significantly increased after 6 weeks of exposure (Figure 3.1). At that time the number of Ki-67-positive cells in the mammary glands of E<sub>2</sub>-exposed rats was 7.7 times greater than in the age-matched control animals. At later times (12 and 18 weeks) the extent of cell proliferation in the mammary glands of ACI rats continuously exposed to E<sub>2</sub> decreased slightly compared to the 6 week value, but still remained 3.1-4.1 times greater than the control values. The number of Ki-67-positive cells in the mammary glands of IR-exposed rats did not change significantly over the 18 weeks of the experiment. Interestingly, combined exposure of rats to E<sub>2</sub> and IR caused profound increases in cellular proliferation in the mammary gland after 6 and 12 weeks, and especially after 18 weeks. At that time, the level of Ki-67 expression in the mammary glands of rats exposed to E<sub>2</sub> plus IR was, respectively, 17.3 and 4.2 times greater than in the age-matched control animals and rats continuously exposed to E<sub>2</sub> only (Figure 3.1).

### **3.4.2. Effect of E<sub>2</sub>, IR, or E<sub>2</sub> Plus IR Exposure on Apoptotic Cell Death in the Mammary Glands of ACI Rats**

Continuous exposure of ACI rats to E<sub>2</sub> resulted in a time-dependent increase in apoptotic cell death, as evidenced by the progressive increase of apoptotic bodies in the mammary glands (Figure 3.2). In contrast, ACI rats exposed to IR or E<sub>2</sub> plus IR exhibited increased levels of apoptosis in the mammary gland after 6 and 12 weeks only (Figure 3.2). Surprisingly, there was no difference in the extent of apoptotic cell death in the mammary glands in these two groups of rats after 18 weeks of experiment.

The observed differences in the level of cell proliferation and apoptosis in the mammary glands of ACI rats exposed to E<sub>2</sub>, IR or E<sub>2</sub> plus IR prompted us to investigate whether or not these exposures impaired the balance between cell proliferation and programmed cell death. Figure 3.3 shows that there are no changes in the ratio between cell proliferation and apoptosis in the mammary glands of rats exposed to E<sub>2</sub> or IR over 18 weeks of the experiment, except in rats continuously exposed to E<sub>2</sub> at 6 weeks. In contrast, the combined exposure to E<sub>2</sub> and IR caused a prominent increase in the Ki-67/apoptosis ratio in the mammary glands.

### **3.4.3. Expression of p53, Mdm2, and c-Myc Proteins in the Mammary Glands of ACI Rats Exposed to E<sub>2</sub>, IR, or E<sub>2</sub> and IR**

In order to elucidate mechanisms associated with alterations in apoptosis and cell proliferation in the mammary glands of ACI rats exposed to E<sub>2</sub>, IR or E<sub>2</sub> plus IR, we determined the levels of p53 tumor suppressor protein, mouse double minute 2 (Mdm2), and c-myc oncoproteins in mammary gland of control and experimental rats. Figure 3.4

shows that the level of p53 in the mammary glands of ACI rats exposed to E<sub>2</sub> or IR did not change over an 18 week period. In contrast, in mammary glands of rats exposed to E<sub>2</sub> plus IR, p53 became significantly up-regulated after 12 weeks. At that time, the level of p53 protein in the mammary glands of ACI rats exposed to E<sub>2</sub> plus IR was 7 times greater than in the age-matched control rats. At a later time (18 weeks) the level of p53 continued to increase further (Figure 3.4).

The exposure of ACI rats to E<sub>2</sub> or E<sub>2</sub> plus IR resulted in a progressive increase in expression of Mdm2 protein, with differences being significant starting after 6 weeks (Figure 3.4). At later times (12 and 18 weeks) the level of Mdm2 in the mammary glands of ACI rats continuously exposed to E<sub>2</sub> or E<sub>2</sub> plus IR increased significantly compared to the 6 week values; however, the up-regulation of Mdm2 in ACI rats exposed to E<sub>2</sub> plus IR were more pronounced as compared to rats exposed to E<sub>2</sub> only. In contrast, the level of Mdm2 protein in the mammary glands of IR exposed rats did not change over 18 weeks.

Additionally, exposure of ACI rats to E<sub>2</sub>, IR, or E<sub>2</sub> plus IR resulted in up-regulation of c-myc protein with a magnitude being greater in mammary glands of rats exposed to both E<sub>2</sub> and IR (Figure 3.5).

### 3.5. DISCUSSION

Accumulated evidence during recent years indicates that dysregulation in cell proliferation and apoptosis are indispensable features of the tumorigenic process, including breast carcinogenesis. The results of previous studies on breast carcinogenesis have documented elevated proliferative levels in mammary glands precedes the development of preneoplastic lesions (Imaoka et al., 2006; Rose-Hellekant et al., 2006). Indeed, in the present study we demonstrated similar enhanced epithelial cell proliferation in the mammary glands of ACI rats exposed to two major human breast carcinogens, estrogen and X-ray radiation (Pedraza-Fañña, 2006; Hanahan and Weinberg, 2000). More importantly, we demonstrate that combined exposure to E<sub>2</sub> and IR had a synergistic effect on cell proliferation in the mammary glands of ACI rats, which was evidenced by a substantially greater magnitude of cell proliferation, especially after 12 and 18 weeks of experiment, than in mammary glands of rats exposed to E<sub>2</sub> or IR separately (Figure 3.1).

It has been suggested that neoplastic transformation of mammary epithelial cells is not associated with an increase in cell proliferation but with a decrease in apoptotic cell death (Shilkaitis et al., 2000), which is also considered a fundamental event in tumorigenesis (Ye et al., 2004; Shull et al., 1997). In the present study, we demonstrate that imbalance between cell proliferation and apoptotic cell death, rather than enhanced cell proliferation or suppression of apoptosis alone, is a driving force leading to neoplastic transformation. This was evidenced by the profound dysbalance between cell proliferation and apoptosis in mammary glands of ACI rats exposed to E<sub>2</sub> plus IR, whereas in rats exposed to E<sub>2</sub> or IR alone these processes remained unaffected.

To elucidate the mechanisms responsible for this disassociation between cell proliferation and apoptotic cell death, we investigated whether or not this cell proliferation/apoptosis dysbalance is associated with deregulation of critical genes controlling these processes. The p53 tumor suppressor protein, often referred to as “guardian of the genome” (Meulmeester and Jochemsen, 2008; Efeyan and Serano, 2007), is the most important protein involved in the regulation of the decision of cell proliferation and cell death (Hallstrom and Nevins, 2009; Oren, 2003; Chumakov, 2000). Surprisingly, in the present study we detected a profound up-regulation of the p53 protein in the mammary tissue of ACI rats exposed to E<sub>2</sub> and IR together. Interestingly, this was the only experimental group that exhibited dysregulation between cell proliferation and apoptosis (Figure 3.3). This may suggest compromised functional activity of p53 induced by E<sub>2</sub> and IR exposure. Indeed, this suggestion was supported by data demonstrating the sustained up-regulation of Mdm2 in the mammary glands of ACI rats continuously exposed to E<sub>2</sub> and especially to E<sub>2</sub> and IR (Figure 3.4). It is well-established that Mdm2 is one of the primary negative regulators of p53 *in vivo* (Coutts and La Thangue, 2007; Toledo and Wahl, 2006; Haines, 1997) and is often up-regulated in breast cancer (Murray et al., 2005; Saji et al., 2001; Saji et al., 1999). Recent evidence has indicated that Mdm2 does not have to be grossly over-expressed to contribute to tumor development; even a moderate increase in Mdm2 expression has been linked to accelerated tumorigenesis (Bond and Levine, 2007). Mdm2, an E3 ubiquitin ligase, regulates p53 by controlling both the stability of the p53 protein and the activity of p53 as a transcription factor (Bouska and Eischen, 2009A; Toledo and Wahl, 2006). It has been shown that up-regulation of Mdm2 may lead to uncontrolled cell proliferation through its

ability to physically associate with the p53 tumor suppressor protein and block the growth suppressive, cell cycle arrest, and apoptotic functions of p53 (Haines, 1997). In this respect, our results demonstrating an accelerated cell proliferation accompanied by the eminent up-regulation of the oncogenic c-myc (Prochownik, 2008) in mammary glands of ACI rats exposed to E<sub>2</sub> and IR (Figure 3.5) in the presence of elevated levels of p53 protein corresponds to this mechanism. Furthermore, recent evidence has convincingly established that, in addition to Mdm2 p53-dependent effects on genomic stability, Mdm2 may, independently of its p53-function, influence genomic stability and mediate neoplastic cell transformation (Bouska and Eischen, 2009A; Bouska Eischen 2009B).

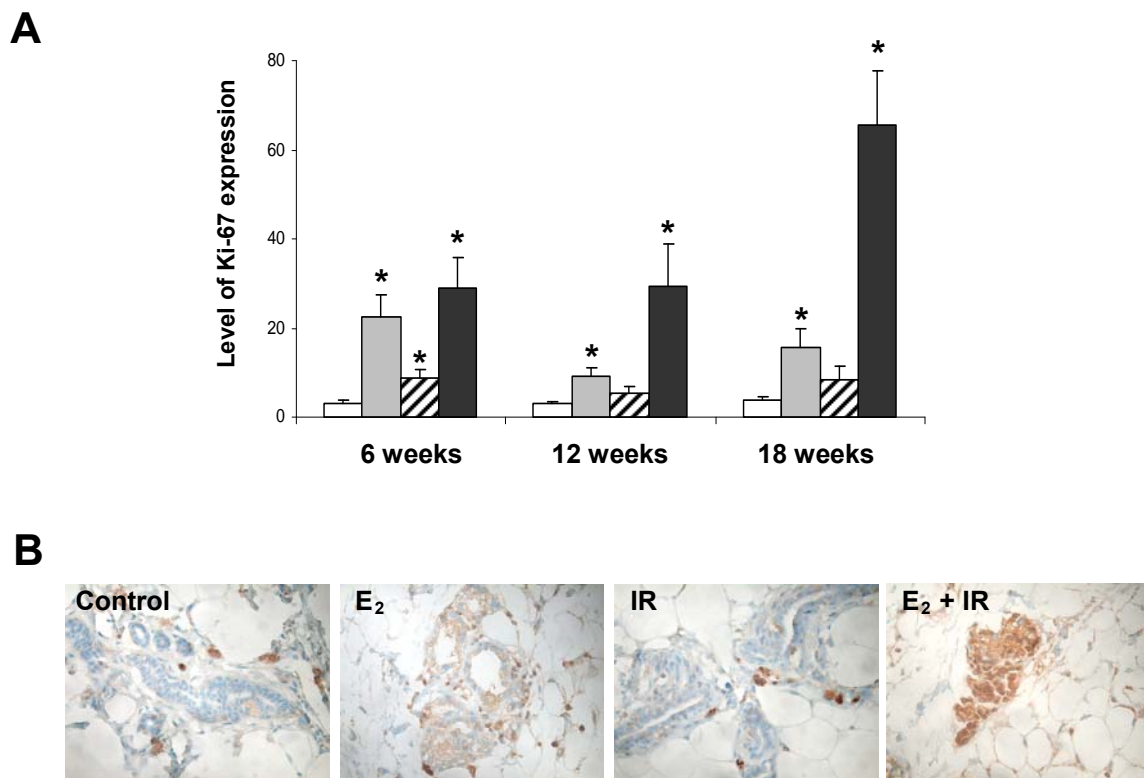
In conclusion, the results of our study demonstrated that alterations in cell proliferation and apoptosis occur in the early stages of mammary carcinogenesis in ACI rats induced by continuous exposure to E<sub>2</sub> or E<sub>2</sub> plus IR. We also found that combined exposure to E<sub>2</sub> and IR has a synergistic effect on cell proliferation and apoptosis resulting in an imbalance of these processes, a pro-tumorigenic event that may trigger neoplastic cell transformation. These results indicate that the evaluation of the extent of cell proliferation and apoptosis before the onset of preneoplastic lesions may be a potential biomarker of breast cancer risk.

### 3.6. FIGURES AND TABLES

**Table 3.1. Summary of pathomorphological changes in the mammary gland of control ACI rats and ACI rats exposed to estrogen (E<sub>2</sub>), radiation (IR), or estrogen plus radiation (E<sub>2</sub>+IR) for 18 weeks (mean ± S.E.M.; n=6).**

\*Significantly different from the age-matched control. Scoring: 1 – normal; 2 – mild hyperplasia; 3 – moderate hyperplasia; 4 – moderate/severe hyperplasia; 5 – severe hyperplasia.

<b>Group</b>	<b>Histopathology score</b>		
	<b>6 weeks</b>	<b>12 weeks</b>	<b>18 weeks</b>
Control	1.0 ± 0.0	1.3 ± 0.5	1.0 ± 0.0
E <sub>2</sub>	3.5 ± 1.2*	3.7 ± 1.0*	2.8 ± 1.0*
IR	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
E <sub>2</sub> + IR	4.7 ± 0.5*	2.8 ± 0.8*	2.8 ± 1.0*

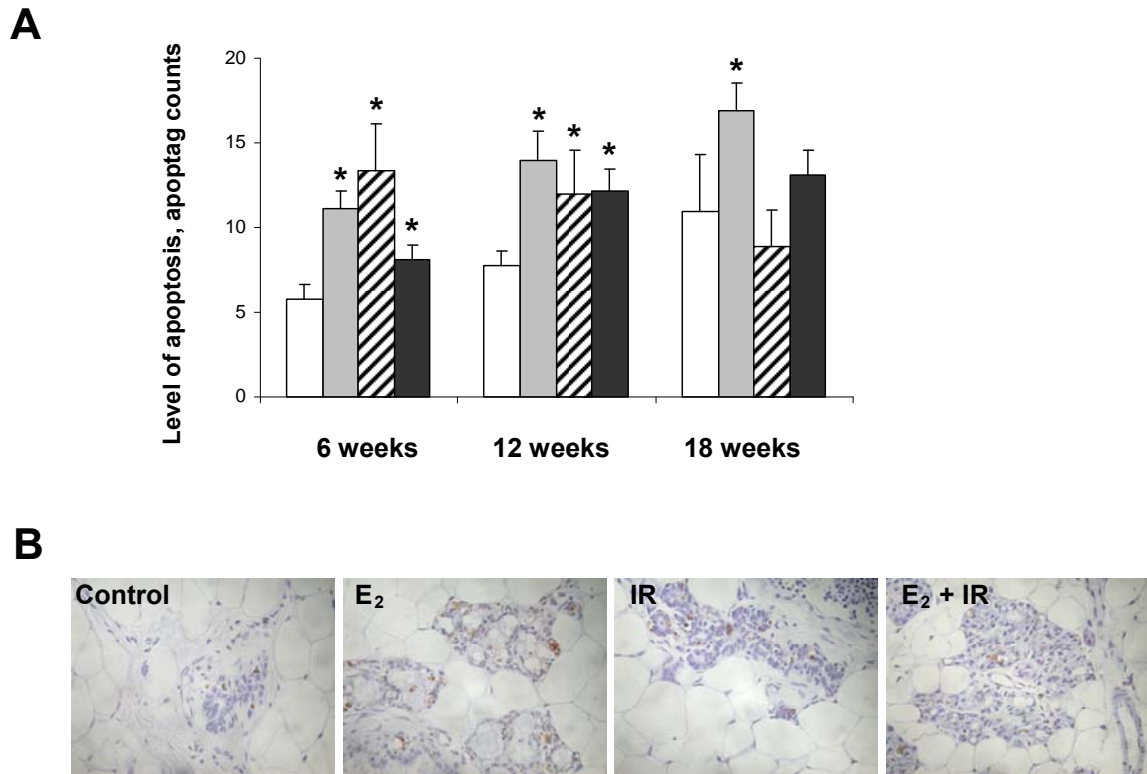


**Figure 3.1. Cell proliferation in the mammary glands of ACI rats exposed to E<sub>2</sub>, IR, or E<sub>2</sub>+IR.**

**(A)** Ki-67 labeling indices in mammary tissue sections of control rats and rats exposed to E<sub>2</sub>, IR, or E<sub>2</sub>+IR. Data are presented as means  $\pm$  S.E.M. (n=6) relative to control.

\*Significantly different from the age-matched control. White bars – controls, grey – estrogen-treatment, striped – radiation-treatment, and black – estrogen- plus radiation-treatment.

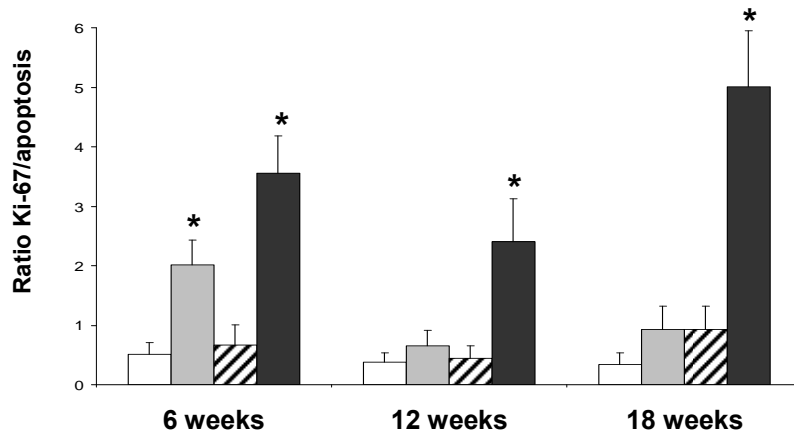
**(B)** Representative images of Ki-67-stained mammary epithelial cells (brown) counterstained with hematoxylin and eosin after 18 weeks (Original magnification x40).



**Figure 3.2. Apoptotic cell death in the mammary glands of ACI rats exposed to E<sub>2</sub>, IR, or E<sub>2</sub>+IR.**

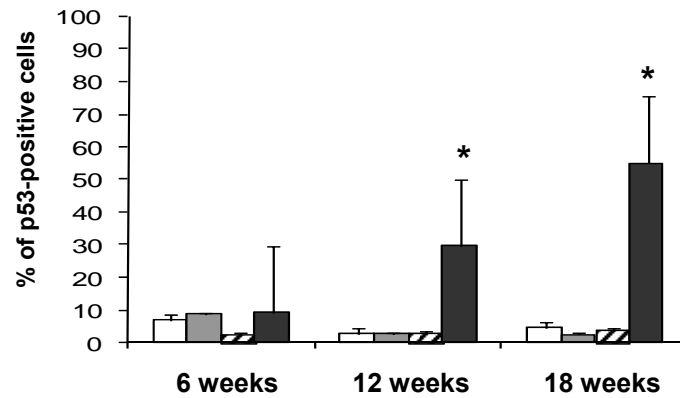
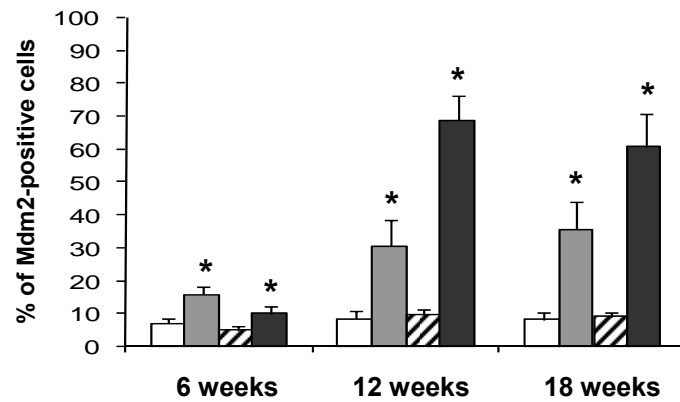
**(A)** TUNEL indices in mammary tissue sections of control rats and rats exposed to E<sub>2</sub>, IR, or E<sub>2</sub>+IR. Data are presented as means  $\pm$  S.E.M. (n=6) relative to control.

\*Significantly different from the age-matched control. White bars – controls, grey – estrogen-treatment, striped – radiation-treatment, and black – estrogen- plus radiation-treatment. **(B)** Representative images of TUNEL-stained mammary epithelial cells (brown) counterstained with hematoxylin and eosin after 18 weeks (Original magnification x40).



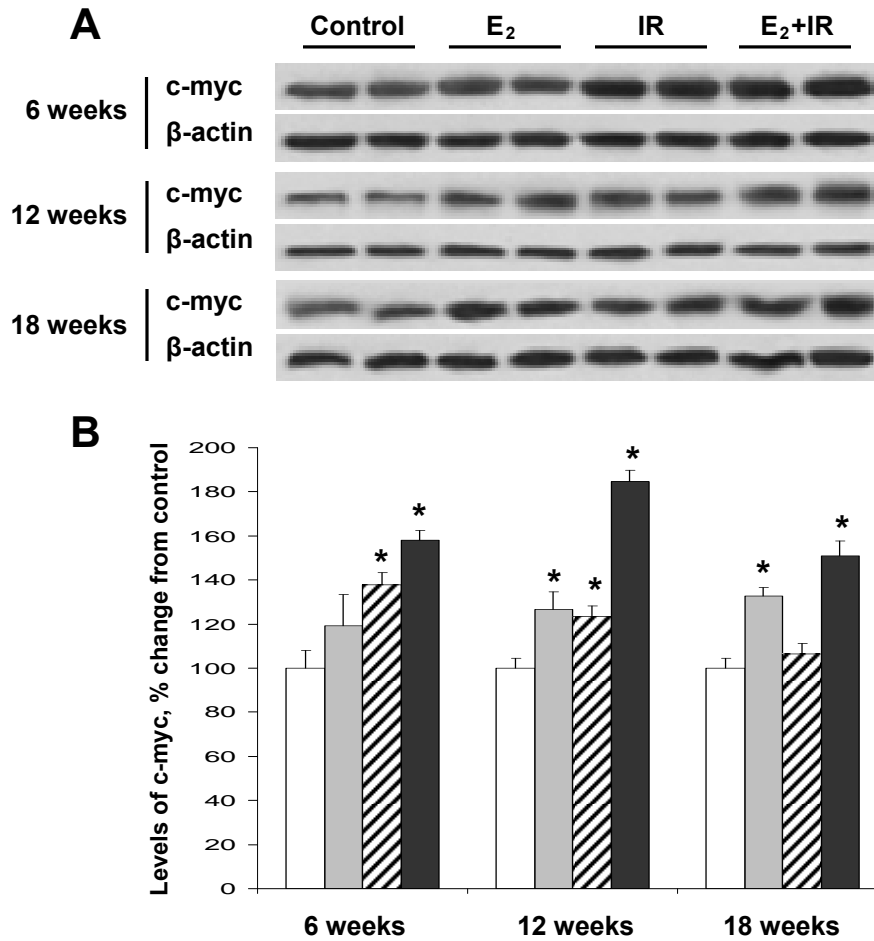
**Figure 3.3. Ratio Ki-67/apoptosis in the mammary glands of ACI rats exposed to E<sub>2</sub>, IR, or E<sub>2</sub>+IR.**

\*Significantly different from the age-matched control. White bars – controls, grey – estrogen-treatment, striped – radiation-treatment, and black – estrogen- plus radiation-treatment.

**A****B**

**Figure 3.4. Effect of E<sub>2</sub>, IR, or E<sub>2</sub>+IR exposure on the number of p53- and Mdm2-positive cells in the mammary glands of ACI rats.**

Data are presented as percent change of p53- or mdm2-positive cells relative to control  $\pm$  S.E.M. (n=6). \*Significantly different from the age-matched control. White bars – controls, grey – estrogen-treatment, striped – radiation-treatment, and black – estrogen-plus radiation-treatment.



**Figure 3.5. Western blot analysis of c-myc in the mammary glands of ACI rats exposed to E<sub>2</sub>, IR, or E<sub>2</sub>+IR.**

Western immunoblot analysis of c-myc protein levels normalized against β-actin.

**(A)** Representative western immunoblot images of c-myc and β-actin protein. **(B)** The quantitative analysis of c-myc protein levels. Data are presented as means ± S.E.M.

(n=6) relative to the age-matched control ACI rats. Control values at each time point were considered as 100%. \*Significantly different from the age-matched control. White bars – controls, grey – estrogen-treatment, striped – radiation-treatment, and black – estrogen- plus radiation-treatment.

**4. EXPOSURE TO ESTROGEN AND IONIZING RADIATION CAUSES  
EPIGENETIC DYSREGULATION AND ACTIVATION OF MITOGEN-  
ACTIVATED PROTEIN KINASE PATHWAYS IN THE MAMMARY GLANDS  
OF ACI RATS**

<sup>1</sup>Chapter 4 to be submitted in its entirety to EMM:  
Kutanzi K, Koturbash I, Pogribny I, Kovalchuk O. Exposure to estrogen and ionizing radiation causes epigenetic dysregulation and activation of mitogen-activated protein kinase pathways in the mammary glands of ACI rats.

#### 4.1. ABSTRACT

The impact of environmental mutagens and carcinogens on the mammary gland has recently received a lot of attention. Among the most generally accepted carcinogenic agents identified as factors that may increase breast cancer incidence are ionizing radiation and elevated estrogen levels. However, the molecular mechanisms of mammary gland aberrations associated with radiation and estrogen exposure still need to be further elucidated, especially, the interplay between elevated hormone levels and radiation. Therefore, in the present study, we investigated molecular changes induced in the rat mammary gland by estrogen exposure, ionizing radiation, and the combined action of these two carcinogens using a well-established ACI rat model. We found that continuous exposure of intact female ACI rats to elevated levels of estrogen or to both estrogen and radiation resulted in significant hyperproliferative alterations in the mammary gland. In contrast, radiation exposure alone did not induce hyperplasia. Interestingly, despite the obvious disparity in the mammary gland morphology, we did not detect significant differences in the levels of genomic methylation among animals exposed to estrogen, radiation, or both agents together. Specifically, we observed a significant global genomic hypomethylation at 6 weeks of exposure. However, by 12 and 18 weeks, the level of global DNA methylation returned to the level of age-matched controls. We also demonstrated that combined exposure to radiation and estrogen significantly altered the levels of histone H3 and H4 methylation and acetylation. Most importantly, we for the first time demonstrated that estrogen and radiation exposure caused a significant induction of MAPK p42-44 and p38 pathways, which was paralleled by changes in H3S10 phosphorylation, a well-established factor of genome and

chromosome instability. The precise role of MAPK pathways and their inter-relationship with H3S10 phosphorylation and genome instability in mammary gland tissue needs to be further delineated.

## 4.2. INTRODUCTION

Breast cancer is the most commonly diagnosed malignancy in women and the leading cause of death among women between the ages of 35 to 55 years (Schairer et al., 2004). It is estimated that only 5% of new breast cancer cases are attributed to abnormal function of susceptibility genes (Ronckers et al., 2005), while the etiology of the remaining 95% of cases still remains unclear. The emerging evidence suggests a crucial role of environmental mutagens and carcinogens in breast cancer etiology (Weyandt et al., 2008; Brody et al., 2007; Ronckers et al., 2005).

Among the most generally accepted environmental carcinogenic agents identified as the factors that may increase breast cancer risk are ionizing radiation (IR) and elevated estrogen levels (Cavalieri et al., 2006; MacMahon, 2006; Ronckers et al., 2005; Doisneau-Sixou et al., 2003; Land et al., 2003). Indeed, the results of numerous epidemiological studies have strongly established a link between increased breast cancer incidence and exposure to ionizing radiation in atomic bomb survivors and women exposed to various diagnostic and therapeutic irradiations (Ronckers et al., 2005; Land et al., 2003; Storm et al., 1992; Boice et al., 1991). For instance, elevated incidence of breast cancer has been reported in patients with scoliosis and tuberculosis (Boice et al., 1991), women treated for benign breast disease (Mattsson et al., 1993), and in cancer survivors who received radiation therapy (Ronckers et al., 2005). The average dose of IR-exposure linked to breast carcinogenesis ranges between 0.2 and 20 Gy (Ronckers et al., 2005). Additionally, experimental *in vitro* and *in vivo* studies have also established that IR can alter the functioning of normal mammary gland epithelial cells and trigger their neoplastic transformation (Calaf and Hei, 2000; Russo et al., 1996). However, the

exact nature of these radiation effects on the mammary glands need to be further explored.

Additionally, recent epidemiological studies have also convincingly established a causative role of estrogen in human breast cancer development, especially in young premenopausal women (Yager and Davidson, 2006). More importantly, women with elevated estrogen levels are considered to be a high-risk group for breast cancer development (Subramanian et al., 2008; Russo and Russo, 2006) and are likely to be exposed to diagnostic radiation procedures more frequently. Similarly, many patients with estrogen-induced breast cancer undergo radiation treatment and are exposed to relatively high X-ray doses to the healthy breast. *In vitro* application of both IR and estrogen leads to the malignant transformation of normal breast epithelial cells (Calaf and Hei, 2000). However, the underlying mechanisms behind estrogen-induced mammary gland genome instability and carcinogenesis, especially the effects of combined exposure to estrogen and IR on mammary glands, are not fully understood and remain to be elucidated.

Epigenetic changes, including DNA methylation and histone modifications, and variations in gene expression have been associated with changes in the molecular function of breast cells and the development of breast cancer (Dalvai and Bystricky, 2010; Veeck and Esteller, 2010; Hinshelwood and Clark, 2008; Widschwendter and Jones, 2002). Furthermore, it is believed that epigenetic alterations in breast cancer are more prominent than genetic changes (Martens et al., 2009) and that these epigenetic alterations may predispose cells to genomic instability and the acquisition of genetic changes during carcinogenesis (Sawan et al., 2008; Feinberg et al., 2006). Alterations in

genomic methylation patterns is a well-known epigenetic feature of cancer cells (Jones and Baylin, 2007) with regional hyper- and hypo-methylation of specific genes being very important in breast carcinogenesis (Veeck and Esteller, 2010; Hinshelwood and Clark, 2008 Widschwendter and Jones, 2002). Despite recent advances in uncovering breast cancer-related epigenetic abnormalities, the extent and timing of epigenetic dysregulation induced in the mammary gland in response to different carcinogenic agents, remains poorly understood.

In light of these considerations, the goal of the present study was to investigate association between morphological and molecular changes induced in the rat mammary glands by exposure to estrogen, radiation, and the combination of these two carcinogens.

## 4.3. MATERIALS AND METHODS

### 4.3.1. Animal Treatment and Tissue Preparations

Female ACI rats were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). The animals were housed 2 per cage in a temperature-controlled (24°C) room with a 12 hour light-dark cycle and given *ad libitum* access to water and NIH-31 diet. Handling and care of animals was in strict accordance with the recommendations of the Canadian Council for Animal Care and Use. The procedures have been approved by the University of Lethbridge Animal Welfare Committee.

At 8 weeks of age, the rats were randomly allocated into 4 groups of 18 rats each: i) sham-treated controls; ii) estrogen-treated (E<sub>2</sub>) groups; iii) IR-treated (IR) groups; and iv) estrogen- plus IR-treated (E<sub>2</sub>+IR) groups. The estrogen-treated groups were implanted with estrogen constant release mini-pellets (7.5 mg/90 days release, Innovative Research of America Inc., Sarasota, FL.) subcutaneously in the shoulder region under ketamine and xylazine-induced anesthesia (Pogribny et al., 2007; Harvell et al., 2000; Shull et al., 1997). Animals from the irradiated groups were exposed to a single, whole-body dose of 3 Gy of X-rays 1 week later (90 kVp, 5 mA). Six rats per group were humanely euthanized after 6, 12, and 18 weeks of treatment.

The paired caudal inguinal mammary glands (and fat pad) were excised from the overlying skin. One gland was frozen immediately in liquid nitrogen and stored at -80°C for subsequent analyses. The contralateral gland and fat pad were carefully spread onto a 5x8 cm glass slide and excess fat and other tissue were trimmed. The gland was then placed flat in a cassette *in toto*. This provided a histological specimen with frontal plane orientation, in which the gland profile is comparable to that of a mammary whole mount.

This orientation allows visualization of the arborizing pattern of the duct system and associated alveoli more clearly and completely than is possible using a transverse section of the gland. The specimens were then fixed in 10% neutral buffered formalin for 48 h, processed, embedded in paraffin, sectioned at 4 microns, and mounted on glass slides. The sections were stained with hematoxylin and eosin for histopathological examination.

#### **4.3.2. Histopathology**

Tissue sections were stained with hematoxylin and eosin using a standard protocol. The morphological changes observed during the development of mammary cancer were independently assessed by two pathologists in a blinded fashion. A semi-quantitative system was used to record the severity of epithelial hyperplasia of the mammary gland.

#### **4.3.3. Global DNA Methylation Analysis**

Genomic DNA was isolated from rat mammary tissue by using the Qiagen Dneasy™ Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The extent of global DNA methylation was evaluated with a well-established radiolabeled [<sup>3</sup>H]-dCTP extension assay (Pogribny et al., 1999). In brief, 1 µg of genomic DNA was digested with 20 U of methylation-sensitive HpaII restriction endonuclease (New England Biolabs, Beverly, MA) for 16-18 h at 37°C. A second DNA aliquot (1 µg) of undigested DNA served as a background control. A single nucleotide extension reaction was performed in a 25 µl reaction mixture containing 1.0 µg DNA, 1X PCR buffer II, 1.0 mM MgCl<sub>2</sub>, 0.25 U AmpliTaq DNA polymerase, and 0.1 µl of [<sup>3</sup>H]dCTP (57.4 Ci/mmol), and the mixture was incubated at 56°C for 1 h. Samples were applied to DE-81 ion-exchange filters and washed three times with

0.5 M Na-phosphate buffer (pH 7.0) at room temperature. The filters were dried and processed for scintillation counting. The [<sup>3</sup>H]dCTP incorporation into DNA was expressed as mean disintegrations per minute (dpm) per µg of DNA after subtraction of the dpm incorporation in undigested samples. Two technical repeats of each experiment were conducted to insure consistency of the data.

#### **4.3.4. Western Blot Analysis of Protein Expression**

Total protein was extracted from mammary tissue as previously described (Tryndyak et al., 2007), using 1% sodium dodecyl sulfate (SDS) and sonication to homogenize the tissue. Equal amounts of proteins (20 µg) were separated by SDS-polyacrylamide electrophoresis (PAGE) and transferred to PVDF membranes (Amersham, Baie d'Urfé, Québec). The membranes were probed with primary antibodies against p-p42-44, p-p38 (1:500, Cell Signaling Technology, Beverly, MA); APE1, DNA Polymerase β, NBS1, KU70, and β-Actin (1:1000, Abcam, Cambridge, MA). Horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and the ECL Plus immunoblotting detection system (Amersham, Baie d'Urfé, Québec) were used to reveal antibody binding. Chemiluminescence was detected with Biomax MR films (Eastman Kodak, New Haven, CT). All membranes were stained with Coomassie Blue (BioRad, Hercules, CA) to confirm equal protein loading. Signals were quantified using NIH ImageJ 1.63 Software and normalized to loading controls. Experiments were repeated twice to ensure reproducibility.

#### **4.3.5. Immunohistochemical Analysis**

Following pathological examination, the tissues were assembled into tissue microarrays

(TMAs) with 4.5mm cores. TMAs (Pantomics, Inc, Richmond, CA) offer great benefits to perform fast and efficient analysis of large amounts of data using less samples and reagents.

The tissues were fixed to the slides by baking at 60°C for 1 hour, deparaffinized, rehydrated and steamed in antigen retrieval citrate buffer (pH 6.0) (DAKO, Carpinteria, CA). Endogenous peroxidase activity was quenched in 3% H<sub>2</sub>O<sub>2</sub>, and the slides were blocked in 3% goat serum (Santa Cruz Biotechnology, Santa Cruz, CA). The slides were probed with primary antibodies against DNMT1, DNMT3a, (1:400, Santa Cruz Biotechnology, Santa Cruz, CA), H3K4me3, H3K9me3, H4K20me3 (1:500, Abcam, Cambridge, MA), AcH4K5, AcH4K8, AcH4K12, and pH3S10 (1:300, Cell Signaling Technology, Beverly, MA). Binding was detected using avidin-biotinylated horseradish peroxidase and visualized with DAB (ABC Staining System, Santa Cruz Biotechnology, Santa Cruz, CA). The tissues were counterstained with hematoxylin (Santa Cruz Biotechnology, Santa Cruz, CA). Staining for DNA methyltransferases and histone modifications was scored semi-quantitatively in a blinded fashion in at least 5 high power fields in each of 6 animals per group.

#### **4.3.6. Statistical Analysis**

Statistical analysis was conducted using the student's *t*-test. P-values <0.05 were considered significant.

## 4.4 RESULTS

### **4.4.1. Estrogen- and Radiation-Induced Morphological Changes in Rat Mammary Glands**

Exposure of ACI rats to constitutively elevated levels of  $E_2$  significantly altered mammary gland morphology characterized by extensive cell proliferation (Figure 4.1). The increased levels of cell proliferation were noted as early as 6 weeks of  $E_2$ -exposure and persisted further for 12 and 18 weeks of treatment. Interestingly, the severity of these hyperplastic changes decreased slightly at 18 weeks compared to the 6 and 12 week values, but still remained 2.3 times greater than the control values (Figure 4.1B). In contrast to  $E_2$ - and  $E_2$ - plus IR-treated animals, no hyperplastic changes were found in mammary glands of IR-exposed animals (Figure 4.1B). However, it was noted that the mammary glands of IR-exposed ACI rats exhibited fibrotic changes, characterized by a general loss of functional units at 12 and 18 weeks. The most pronounced morphological changes were found in animals that were exposed to both radiation and estrogen (Figure 4.1B).

### **4.4.2. Level of Global DNA Methylation in Estrogen- and Radiation-Exposed Rat Mammary Glands**

To dissect the role of epigenetic changes in rat mammary gland tissues in response to estrogen exposure, IR and the combined action of both carcinogens, we first analyzed changes in levels of global DNA methylation in rat mammary gland tissues of control and treated rats using a sensitive HpaII-based cytosine extension assay that measures the proportion of unmethylated CCGG sites in the genome. DNA methylation patterns exhibit a degree of plasticity, which can be reflective of the cellular response to environmental stimuli, including

chemical and physical agents (Szyf, 2003; Christman et al., 1993). We observed a significant increase in the [<sup>3</sup>H]dCTP incorporation ( $p < 0.05$ ), indicative of global genome hypomethylation at 6 weeks, a stage marked by extensive hyperplastic changes hyperplasia in E<sub>2</sub>- and E<sub>2</sub>- plus IR-treated groups (Figure 4.2). Interestingly, we also detected a significant 3.3-fold loss of global methylation as compared to controls ( $p < 0.05$ ) in response to IR treatment alone (Figure 4.2). However, by 12 and 18 weeks, the extent of global DNA methylation in all groups was not significantly different from those in age-matched controls.

#### **4.4.3. Expression of DNA Methyltransferases in Estrogen- and Radiation-Exposed Rat Mammary Glands**

DNA methyltransferases, DNMT1 and DNMT3a, are the key cellular enzymes responsible for maintaining proper DNA methylation patterns in mammalian cells. Any changes in cellular levels of these enzymes may lead to altered DNA methylation levels. Hence, we assessed whether or not the observed changes in DNA methylation were accompanied by DNMT1 and DNMT3a alterations. Figure 4.3A shows that estrogen exposure resulted in a significant increase in the number of DNMT1 positive cells at 6, 12, and 18 weeks after exposure. This increase was most pronounced after 18 weeks of estrogen treatment. At that time, the number of DNMT1-positive cells was over 4 times greater ( $p < 0.05$ ) than in control rats (Figure 4.3A). Similarly to E<sub>2</sub> exposure, combined E<sub>2</sub> and IR treatment led to significant increase in the levels of DNMT1 positive cells in the groups of animals at 6, 12 and 18 weeks. Contrarily, IR exposure alone did not affect the number of DNMT1 positive cells (Figure 4.3A).

E<sub>2</sub> exposure and combined exposure to E<sub>2</sub> and IR also led to a pronounced and statistically significant increase in the number of DNMT3a-positive cells after 6 and 12 weeks of treatment, while irradiation alone did not induce any significant changes in the number of DNMT3a-positive cells over the 18 week period (Figure 4.3B).

#### **4.4.4. Expression of DNA Repair Proteins in Estrogen- and Radiation-Exposed Rat**

##### **Mammary Glands**

Another main factor that may affect the status of DNA methylation is the integrity of the DNA (Valinluck and Sowers, 2007; Weitzman et al., 1994). It is well established that E<sub>2</sub> and IR are strong DNA damaging agents that cause a variety of DNA lesions, including DNA strand breakage, damage to bases, and oxidative DNA damage (Rajapakse et al., 2005; Jenner et al., 2001; Prise et al., 2001; Zhang et al., 2001). In light of these considerations, we investigated whether or not altered DNA methylation in the mammary glands of E<sub>2</sub>-, IR- and E<sub>2</sub>- plus IR-exposed ACI rats may be associated with compromised DNA integrity.

Currently, it is widely accepted that altered levels of proteins in the base excision repair (BER) pathway, a key pathway involved in repair of oxidative DNA damage, are sensitive *in vivo* markers of oxidative DNA damage (Powell et al., 2005; Rusyn et al., 2004). Figure 4.4A shows that exposure to E<sub>2</sub> for 6 weeks induced a significant ( $p < 0.05$ ) 1.4- and 1.2-fold increase in the levels of apurinic/apyrimidinic endonuclease 1 (APE1) and DNA polymerase  $\beta$  (Pol. $\beta$ ), respectively. Combined exposure to estrogen and IR also led to significantly elevated levels of APE1 and Pol. $\beta$  after 6 weeks of treatment. At later times (after 12 and 18 weeks of treatment), the changes in the levels of APE1 and Pol. $\beta$  that were seen in the 6 weeks E<sub>2</sub> and

E<sub>2</sub>- plus IR exposure groups diminished. Similar to the E<sub>2</sub>+IR group, exposure to IR alone resulted in a delayed decrease in the levels of these BER proteins at 18 weeks.

Estrogen and IR exposure may also result in increased levels of DNA strand breakage. The NBS1 protein is part of the nuclear multi-protein complex composed also of MRE11 and RAD50 (the MRN complex), which plays a crucial role in response to DNA double-strand breaks as well as in DNA strand break repair by homologous recombination (HR) and non-homologous end joining (NHEJ). Importantly, in mammals, most double-strand breaks are repaired by non-homologous end joining (Jackson et al., 2002; Sargent et al., 1997). Ku70 is essential for NHEJ and is induced in mammalian cells exposed to ionizing radiation (Kumaravel et al., 1998; Gu et al., 1997). Therefore, we measured the levels of NBS1 and Ku70 in the mammary gland of control rats and rats exposed to E<sub>2</sub>, IR, and both E<sub>2</sub> and IR. Figure 4.4B shows that estrogen exposure and combined exposure to estrogen and IR increased cellular levels of NBS1 and Ku70 after 6 weeks of treatments. At later times (after 12 and 18 weeks of treatment), the levels of NBS1 and Ku70 decreased as compared to the age-matched controls, similar to the observed reduction of APE1 and Pol.β.

#### **4.4.5. Histone Modifications in Estrogen- and Radiation-Exposed Rat Mammary Glands**

DNA methylation is closely connected with alterations in the other components of chromatin structure, primarily with histone modifications (Cedar and Begman, 2009; Fuks et al., 2003; Bird and Wolffe, 1999). Histone modifications including acetylation, methylation, and phosphorylation are important in the regulation of gene transcription and overall genome stability (Moss and Wallrath, 2007; Tryndyak et al., 2006; Pogribny et al., 2006; Jenuwein and Allis, 2001). Therefore, we investigated the effects of E<sub>2</sub>-, IR- and E<sub>2</sub>- plus IR-exposure on

the levels of histone H3 and H4 methylation and acetylation (Figure 4.5). We noted that E<sub>2</sub> exposure, either alone or in combination with radiation, led to significant increases in the levels of H3K4me<sub>3</sub> (a modification associated with chromatin relaxation and activation of gene expression) after 12 and 18 weeks of treatment as compared to controls. IR exposure alone affected the levels of H3K4me<sub>3</sub> only in the 12-week group.

Additionally, we have evaluated the status of histone H4 acetylation at lysines 5, 8 and 12. Exposure to E<sub>2</sub> resulted in significant increases in H4K5, H4K8, and H4K12 acetylation after 6, 12 and 18 weeks of estrogen treatment. Changes of similar magnitude were induced by the influence of combined exposure to E<sub>2</sub> and IR, whereas IR exposure alone had no effects on H4K12 acetylation levels (Figure 4.5).

In addition to these two well-studied histone modifications, histone phosphorylation has also been implicated in gene activation and, most importantly, in controlling genome stability (Grant, 2001; Thomson et al., 1999). Therefore, we studied the effects of E<sub>2</sub>-, IR-, and E<sub>2</sub> plus IR-exposure on the levels of H3S10 phosphorylation in rat mammary glands. Figure 4.6 shows that exposure to E<sub>2</sub> caused a small but statistically significant increase of H3S10 phosphorylation after 6 weeks. At later time (12 and 18 weeks), the levels of H3S10 phosphorylation in rat mammary glands further increased and were 2.9- and 3.0-fold, respectively, greater than in control rats. Contrarily, IR treatment resulted in a significant 4.3- and 1.4-fold decrease in the levels of H3S10 phosphorylation after 6 and 18 weeks of exposure, respectively. Interestingly, at 12 weeks after exposure, IR caused a 1.9-fold increase in the levels of H3S10 phosphorylation. Combined application of E<sub>2</sub> and IR led to a statistically significant 1.9-,

4.1-, and 3.2-fold increase in the levels of H3S10 phosphorylation in rat mammary gland tissues (Figure 4.6).

#### **4.4.6. Alterations in the Mitogen-activated Protein Kinase Pathways in Estrogen- and Radiation-Exposed Rat Mammary Glands**

The results of recent studies have linked exposure to genotoxic agents with the induction of MAPK pathways (Dyson et al., 2005; Chadee et al., 1999; Barratt et al., 1994). Indeed, Figure 4.7A shows that radiation alone and in combination with estrogen exposure caused a significant induction of p-p42-44 MAPK at 6, 12 and 18 weeks of treatment. Similarly, exposure to IR alone resulted in increased levels of phosphorylated p38 protein at each of the time points, whereas estrogen exposure alone led to increased p-p38 levels only after 12 and 18 weeks of treatment (Figure 4.7B). Likewise, E<sub>2</sub> exposure led to increased p-p38 levels after 12 and 18 weeks of treatment. Contrarily, combined exposure to both carcinogenic agents did not affect p-p38 levels. Interestingly, we did not observe any statistically significant changes in the levels of JNK phosphorylation after treatment of ACI rats with E<sub>2</sub>, IR, and E<sub>2</sub> plus IR (data not shown).

#### 4.5. DISCUSSION

Currently, breast cancer is the most common malignancy among North American women (Jermal et al., 2009; Schaierer et al., 2004). Most breast cancer patients undergo radiation diagnostics and are also treated with radiotherapy. In addition to being an important treatment modality, IR is a potent tumor-causing agent that has been linked to breast cancer development. However, the exact changes induced by IR exposure in mammary gland tissue remain largely unknown. In addition, the interplay between elevated estrogen levels and the magnitude of IR responses in the mammary gland has to be defined.

In the present study, we demonstrated that two very different carcinogens, estrogen and radiation, applied either separately, or in conjunction, exert numerous cellular and molecular epigenetic effects on the mammary gland of female ACI rats. This was evidenced by estrogen-driven morphological alterations, deregulation of cellular epigenetic processes, and altered cell signaling and DNA repair pathways. Importantly, we have identified several important differences in the extent and timing of these changes in response to two known carcinogens.

The results of the present study demonstrated that, despite the obvious disparity in morphology induced by E<sub>2</sub> and IR, we did not detect significant differences in the levels of genomic methylation among animals exposed to E<sub>2</sub>, IR, and E<sub>2</sub>- plus IR. Specifically, E<sub>2</sub>, IR, and both carcinogens together caused significant global DNA hypomethylation only at 6 weeks after treatment. Interestingly, the extent of DNA methylation returned to normal levels by 12 and 18 weeks after exposure.

Several possible explanations exist for a comparable loss of global DNA methylation at 6 weeks, including DNA damage and/or a DNA repair-based mechanism. It has been demonstrated previously that DNA damage can interfere with the methylating

ability of DNA methyltransferases by stalling DNA methyltransferase at the sites of lesions (Smith, 1998; Turk et al., 1995). Furthermore, during DNA repair synthesis, polymerases incorporate cytidine but not methylcytidine, thus the presence of DNA lesions and activation of DNA repair mechanisms may also contribute to DNA hypomethylation. The results of our study demonstrate a close association between the induction of DNA repair enzymes (Figure 4.4) and the loss of global DNA methylation (Figure 4.2) after 6 weeks of exposure, supporting these suggestions.

Alternatively, DNA hypomethylation may arise as a by-product of estrogen-driven cell hyperproliferation. This suggests that DNA methyltransferases may be overwhelmed by the rate of replication synthesis, such that they are unable to maintain methylation patterns on the nascent strands. As one might expect, we observed significant induction of DNMTs, especially DNMT3a, in the estrogen-treated groups, which may serve as a cellular compensatory mechanism. The observation that IR, in conjunction with exposure to elevated E<sub>2</sub>, did not contribute to a more pronounced loss of DNA methylation than exposure to either carcinogen alone, as well as the increase in DNA methylation levels in the mammary glands of exposed rats from a relatively hypomethylated state at 6 weeks to unchanged levels at 12 and 18 weeks, further affirm the potential for overburdened methylation machinery. However, the increased expression of DNMTs may initiate aberrant gene-specific *de novo* methylation events and result in gene silencing. Indeed, persistent up-regulation of DNMTs has been reported to play a significant role in transcriptional silencing of gene expression by hypermethylating promoter CpG islands during breast carcinogenesis (Starlard-Davenport et al., 2010; Roll et al., 2008; Agoston et al., 2005). These findings strongly correlate with our current

observations of the induction DNMT1 and DNMT3a expression in the E<sub>2</sub>- and E<sub>2</sub>- plus IR-treated groups.

In addition to DNA methylation changes, exposure of ACI rats to E<sub>2</sub> and IR, applied either separately, or in conjunction, resulted in noticeable histone modification changes, especially alterations of histone H3S10 phosphorylation levels. Generally, phosphorylation of H3S10 is crucial for proper chromosome condensation and segregation, with nearly all H3 molecules phosphorylated at this residue during entry into mitosis. Overall, phosphorylation of H3S10 is therefore regarded as a marker of mitosis (Fishle, 2008; Hendzel et al., 1997). The presence of a greater percentage of cells expressing this marker, especially in the highly proliferative estrogen group, supports this literary evidence. Therefore, increased levels of pH3S10 are in good agreement with the observed hyperplasia.

Recent data also suggest that H3 histone phosphorylation is a major contributing factor for genome and chromosome instability and thus may play a role in cellular transformation and carcinogenesis (Choi et al., 2005; Ota et al., 2002). Indeed, our results demonstrated a substantial increase in histone H3S10 phosphorylation that correspond to previous findings of over-expression of Aurora-A kinase and chromosomal instability during mammary carcinogenesis in ACI rats (Li et al., 2004).

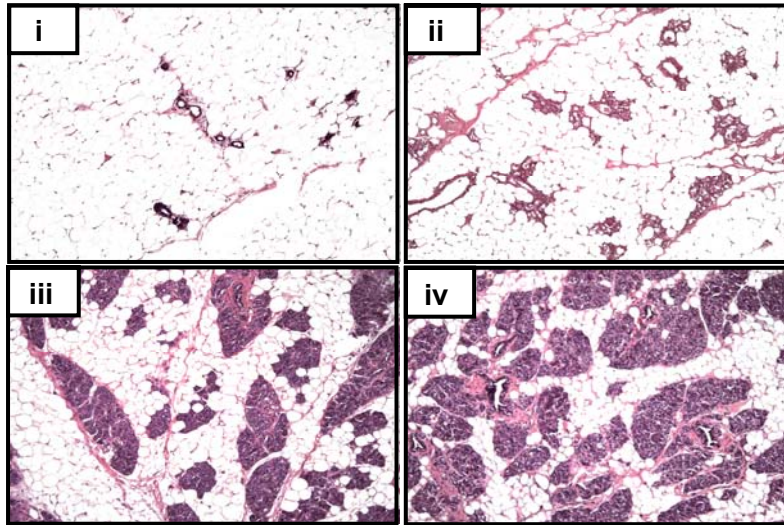
Furthermore, previous studies have demonstrated that genotoxic DNA-damaging agents can induce phosphorylation of H3S10 through the activation of the MAPK pathway (Dyson et al., 2005). There are four distinct MAPK cascades that include the extracellular signal-regulated kinase (p42-44 ERK)/MAPK 1 and 2 pathway; the p38 pathway; the c-jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK)

pathway, and the mitogen-activated protein kinase/ERK5 pathway. MAPK pathways transmit signals that partake in control of cell proliferation and cell death. Recent studies have reported that breast cancer frequently exhibits activation of MAPK pathways (Normanno et al., 2006; Torii et al., 2006; Sivaraman et al., 1997). The activation of p38 and p42-44/ ERKs was proven to induce cellular invasion and motility and contribute to the invasive breast cancer phenotype (Choi et al., 2005; Shin et al., 2005; Kim et al., 2003). Furthermore, MAPK pathways have been implicated in genotoxic stress responses. Indeed, exposure to breast carcinogens, such as radiation and estrogen, has been shown to activate MAPK pathways (Chou et al., 2009; Liao and Hung, 2003; Song et al., 2002). The activated MAPK pathways may in turn influence H3S10 phosphorylation. Here, we demonstrate a correlation between H3S10 phosphorylation and activation of p42-44 ERK1/2 and p38 MAPK pathways. This correlation may suggest that estrogen and radiation-induced activation of ERK1/2 and p38 may partake in H3S10 phosphorylation. The precise role of MAPK pathways and their inter-relationship with H3S10 phosphorylation, genome instability and genotoxic stress-induced changes in the mammary gland tissue to be further delineated.

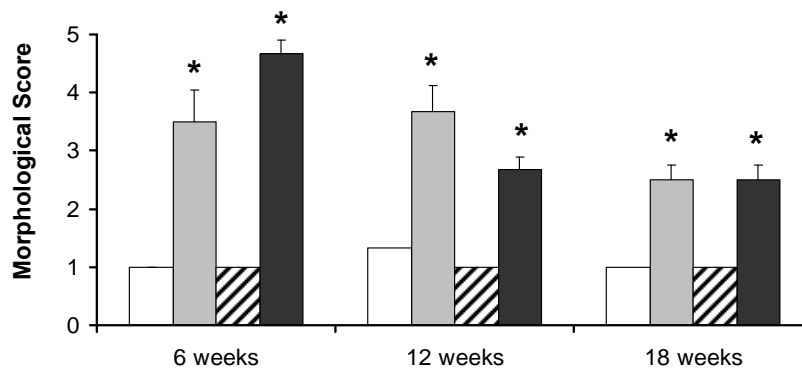
Importantly, these molecular changes were detected in morphologically normal mammary glands suggesting that these early molecular changes may be a critical event that drives tumorigenic process (Ellsworth et al., 2004). Therefore, future studies are needed to dissect the roles of the aforementioned epigenetic and signaling changes in mammary gland carcinogenesis.

#### 4.6. FIGURES

A

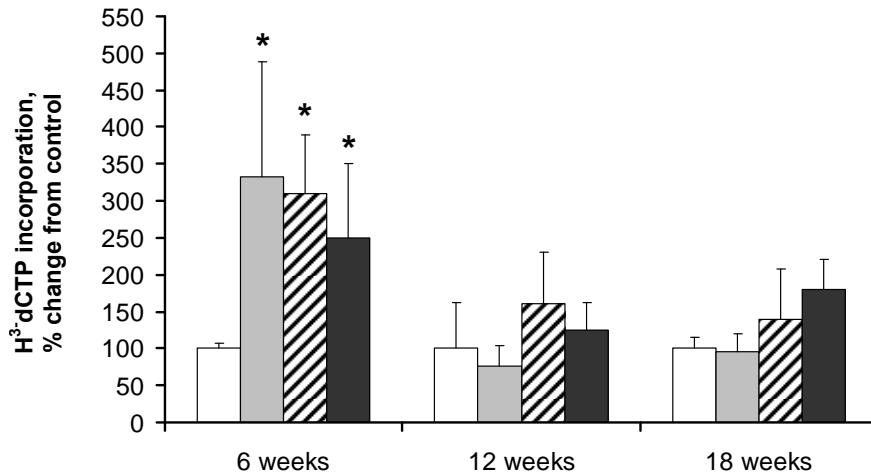


B



**Figure 4.1. The progression of morphological changes in the mammary gland of female ACI rats after exposure to constitutively elevated levels of estrogen.**

(A) Representative images of i) normal; ii) mild; iii) moderate; and iv) severe hyperplasia (Original magnification x10). (B) Average score of morphological changes in the mammary gland after exposure to sham treatment, estrogen, radiation, or estrogen and radiation at 6, 12, 18 weeks (mean  $\pm$  S.E.M, n=6). \*Significantly different from the age-matched control. Scoring: 1 – normal; 2 – mild hyperplasia; 3 – moderate hyperplasia; 4 – moderate/severe hyperplasia; 5 – severe hyperplasia.

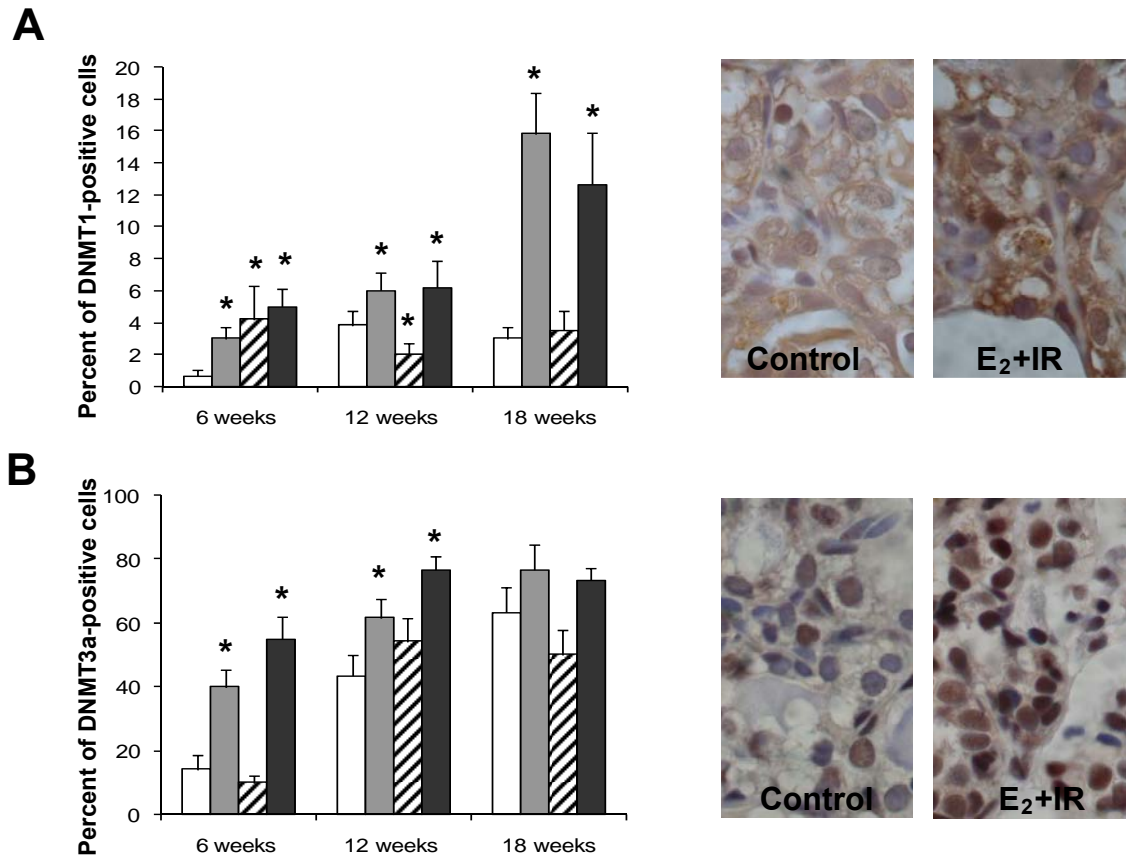


**Figure 4.2. Dysregulation of DNA methylation patterns in the mammary gland of estrogen-exposed, radiation-exposed, and combined estrogen- plus radiation-exposed rats.**

Levels of global DNA methylation as measured by the DNA cytosine extension assay.

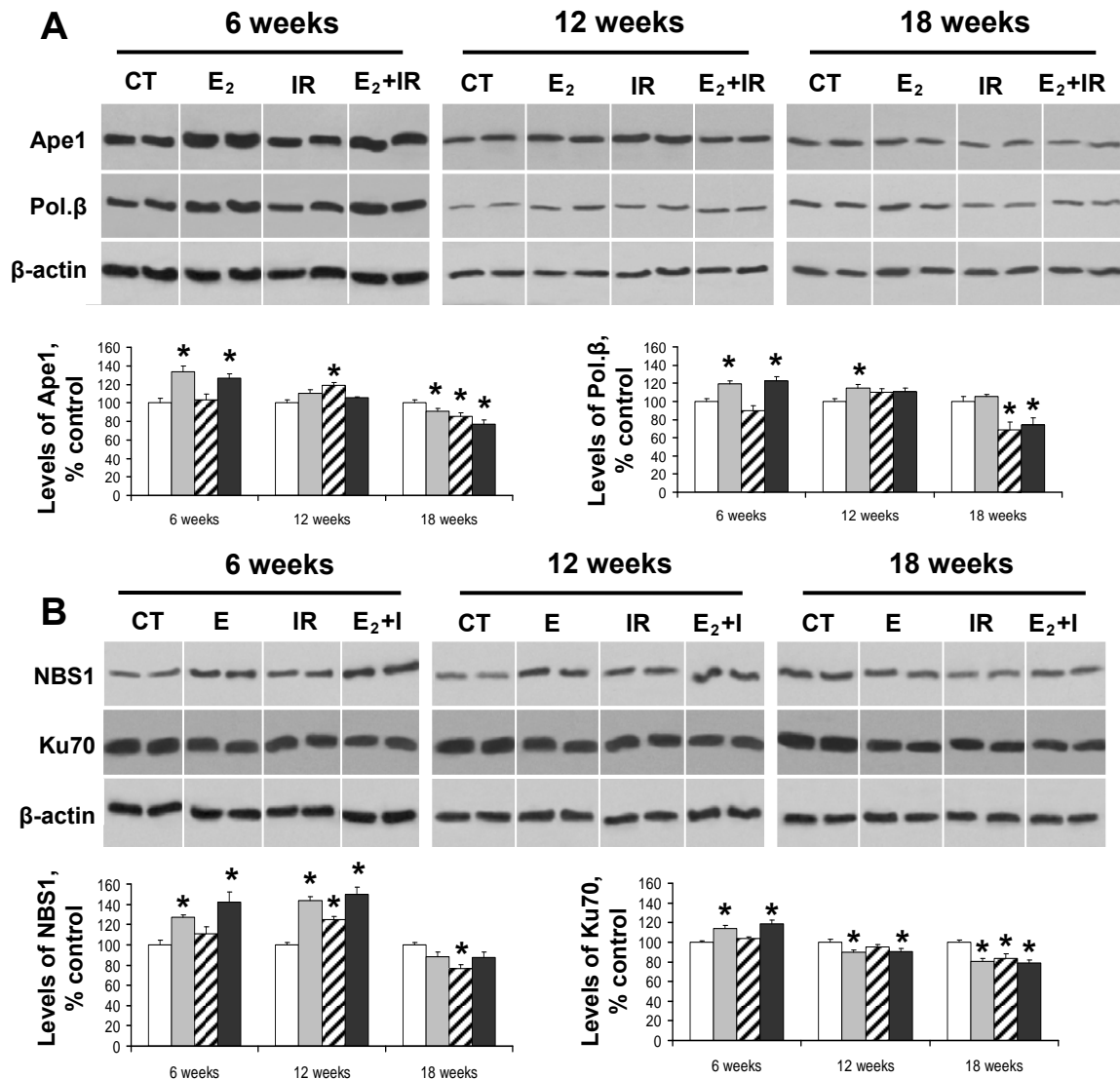
Data from two independent technical repeats is presented as means  $\pm$  S.E.M. (n=6).

\*Significantly different from the age-matched control. White bars – controls, grey – estrogen-treatment, striped – radiation-treatment, and black – estrogen- plus radiation-treatment.



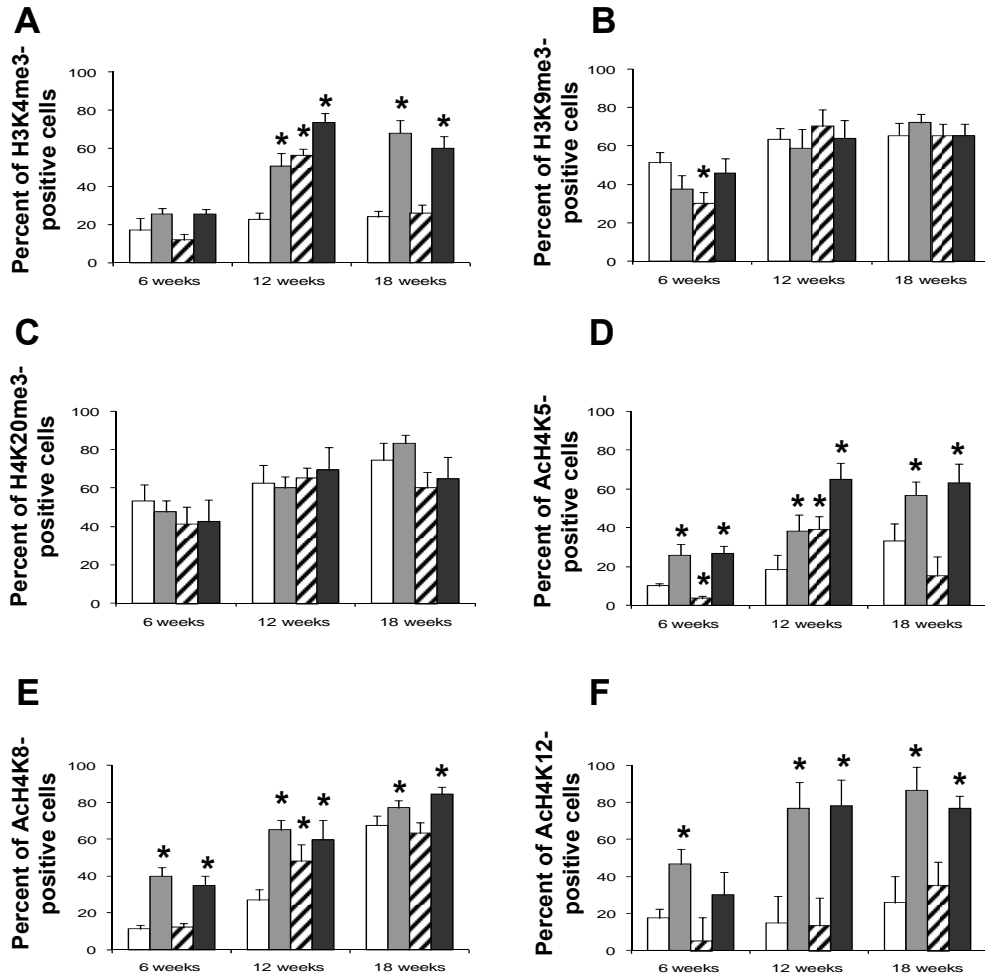
**Figure 4.3. Dysregulation of DNA methylation machinery in the mammary gland of estrogen-exposed, radiation-exposed, and combined estrogen- plus radiation-exposed ACI rats.**

Levels of DNA methyltransferases were determined by immunohistochemical analysis, evaluating the percentage of positive cells and staining intensity. Representative images of (A) DNMT1 and (B) DNMT3a in the mammary gland of sham-treated and estrogen-plus radiation-exposed ACI rats at 18 weeks are shown on the right panel (Original magnification x100). Data presented as means  $\pm$  S.E.M. (n=6). \*Significantly different from the age-matched control. White bars – controls, grey – estrogen-treatment, striped – radiation-treatment, and black – estrogen- plus radiation-treatment.



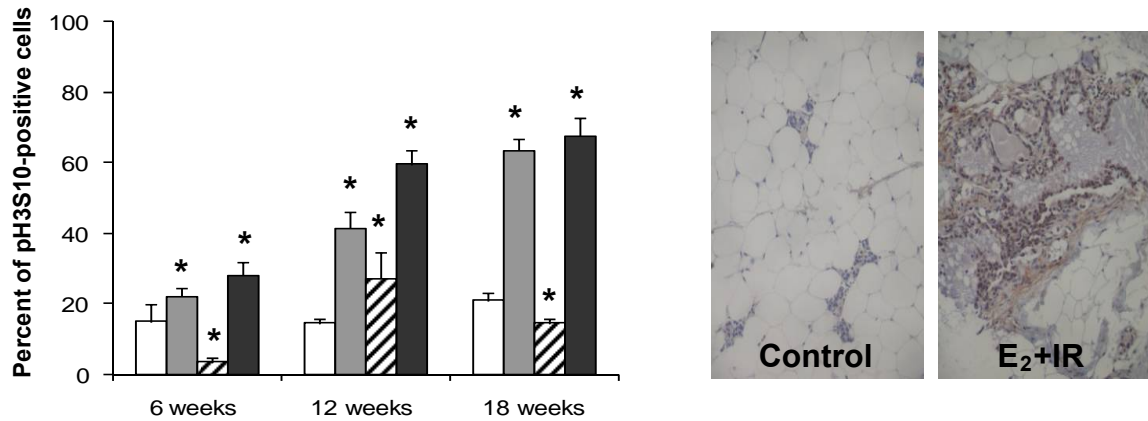
**Figure 4.4. Levels of DNA repair proteins in the mammary gland of female ACI rats exposed to estrogen, radiation, and estrogen plus radiation.**

Representative western immunoblots of **(A)** Ape1 and DNA Pol.  $\beta$  proteins; and **(B)** NBS1 and Ku70 proteins from two independent technical repeats. Protein levels were normalized to  $\beta$ -actin. Data presented as mean values  $\pm$  S.E.M. (n=6). \*Significantly different from the age-matched control. White bars – control-treatment (CT), grey – estrogen-treatment (E<sub>2</sub>), striped – radiation-treatment (IR), and black – estrogen- plus radiation-treatment (E<sub>2</sub>+IR).



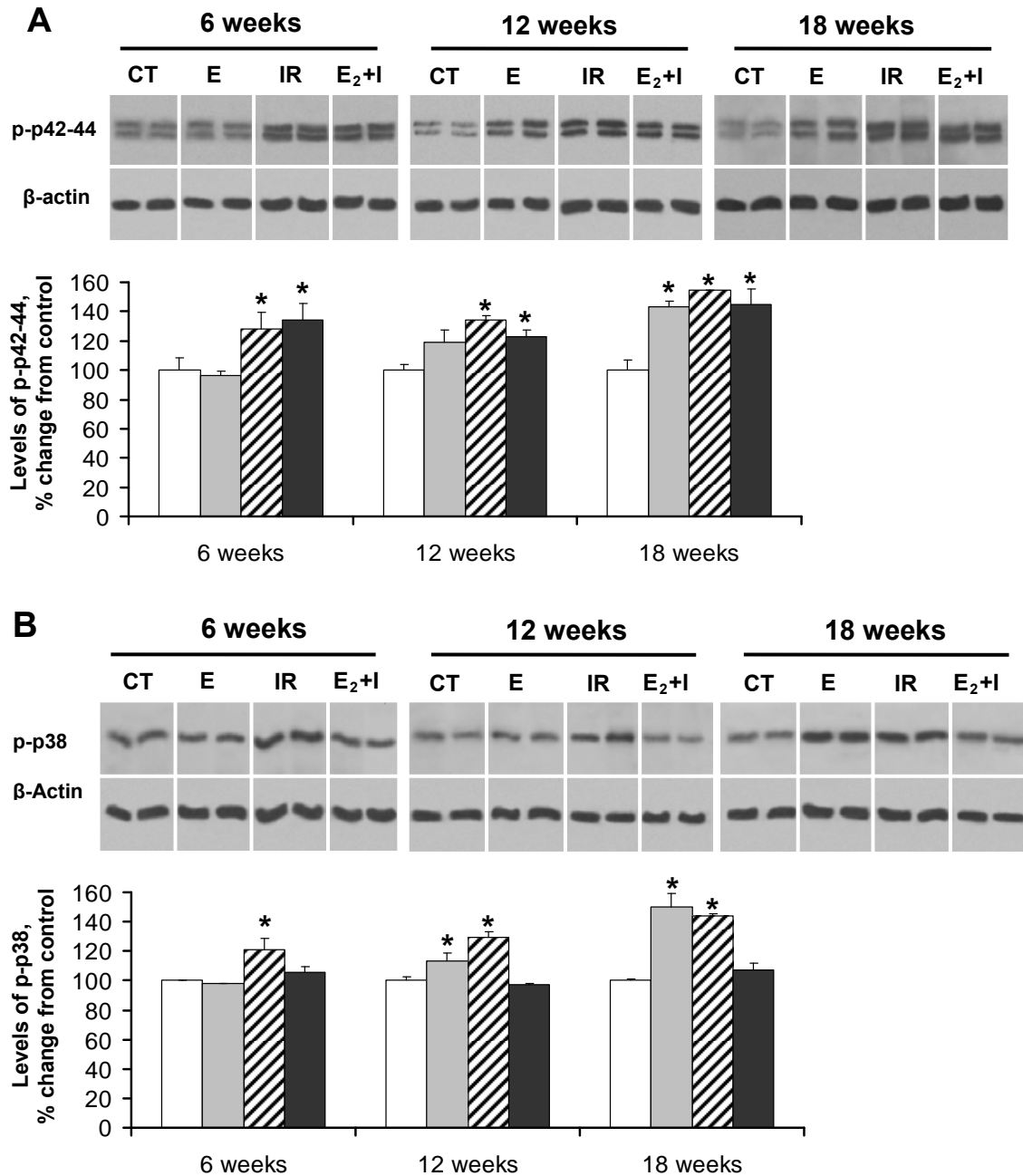
**Figure 4.5. Effects of estrogen and radiation exposure on key histone markers associated with either silenced or active regions of the genome.**

Immunohistochemical analysis of (A) H3K4me3, (B) H4K9me3, (C) H4K20me3 histone marks, (D) AcH4K5, (E) AcH4K8, and (F) AcH4K12. Data presented as means  $\pm$  S.E.M. (n=6). \*Significantly different from age-matched controls. White bars – controls, grey – estrogen-treatment, striped – radiation-treatment, and black – estrogen- plus radiation-treatment.



**Figure 4.6. Immunohistochemical staining for phosphorylated H3S10 (pH3S10) in the mammary gland of estrogen-exposed, radiation-exposed, and combined estrogen- plus radiation-exposed ACI rats.**

Data are presented as means  $\pm$  S.E.M. (n=6). \*Significantly different from the age-matched control. The right panel shows a representative stain of sham-treated and estrogen- plus irradiated- animals ( $E_2+IR$ ) at 18 weeks (Original magnification x40). White bars – control-treatment, grey – estrogen-treatment, striped – radiation-treatment, and black – estrogen- plus radiation-treatment.



**Figure 4.7. Alterations in the mitogen-activated protein kinase pathway.**

Western blot analysis of (A) p-p42-44 and (B) p-p38. Sample loading was normalized to  $\beta$ -actin. Data are presented as means  $\pm$  S.E.M. (n=6). \*Significantly different from the age-matched control. White bars – control-treatment (CT), grey – estrogen-treatment (E<sub>2</sub>), striped – radiation-treatment (IR), and black – estrogen- plus radiation-treatment (E<sub>2</sub>+IR).

## 5. FINAL DISCUSSION AND CONCLUSIONS

Breast cancer is the most common form of cancer in Canadian women, with over 22,700 women predicted to be diagnosed with the disease in 2009 – an average of 437 women diagnosed every week (Statistics Canada, 2009). Of the current cases, an estimated 5,400 women will die (Statistics Canada, 2009). Many of these cancers are estrogen-responsive and are exposed to radiation as both a diagnostic tool and as a treatment modality. Similarly, women with elevated estrogen levels are considered to be at a higher risk for breast cancer and would likely undergo diagnostic radiation exposures on a more frequent basis. Based on literary evidence, there is growing concern regarding the potential synergistic effects.

Furthermore, numerous studies have demonstrated inter-individual variation in breast cancer susceptibility. In the past, emphasis was placed on the value of genetic screening of high risk individuals to promote earlier detection of breast cancer. More recently studies have focused on the diagnostic and prognostic value of screening for epigenetic markers.

The value of early biomarkers is based on the characteristic delay (years or even decades) between the first exposure to a known or suspected carcinogen and the eventual occurrence of cancer after this latency period. During this period, intermediate biomarkers reflective of the biological changes along the causal pathway to malignancy can be used to monitor the risk of tumor formation, as well as the critical window of preventative intervention. These markers would prove even more valuable if they exhibit reversibility following chemopreventative intervention and that this modulation ultimately leads to a decrease in breast cancer.

Potentially reversible molecular markers for the use of breast cancer chemoprevention include epigenetic alterations, such as DNA methylation and histone modifications. Unlike genes inactivated by mutation, genes silenced by epigenetic mechanisms are intact and can be potentially reactivated by “epigenetic drugs”. Future studies may look towards restoring epigenetic balance as a means to prevent cancer.

## 5.1. EPIGENETIC CHANGES IN THE RAT MAMMARY GLAND

The objective of this thesis was to identify early epigenetic markers of carcinogenic exposure in the rat mammary gland, prior to tumor development. More specifically, the aim of this thesis was to examine the epigenetic response to two different carcinogens, estrogen and ionizing radiation, either alone or in conjunction. The cellular and molecular changes that accompanied the observed disturbances in the epigenome were also noted.

### **The main findings of this thesis are that:**

1. The epigenome is altered in response to exposure to elevated levels of estrogen, ionizing radiation, or a combination of the two carcinogens. Importantly, we have identified several important differences in the extent and timing of these changes in response to these two known carcinogens.
2. These changes were also reflected in the cellular and molecular biology of the mammary gland. Exposure to constitutively elevated levels of estrogen was sufficient to induce hyperplastic changes, while only fibrotic changes were detected in response to IR alone.
3. Exposure to estrogen alone and the combined exposure to estrogen and irradiation led to imbalances in apoptosis and cellular proliferation as early as 6 weeks after treatment.
4. Changes in apoptosis and proliferation were accompanied by alterations in the cellular levels of p53, an important regulator of the cell cycle and apoptosis, as well as Mdm2 and c-myc, which antagonize p53's nuclear functions.

5. The aforementioned molecular changes were paralleled by estrogen- and radiation-induced epigenetic dysregulation that could be detected prior to tumor development. This was evidenced by disturbances in DNA and histone methylation, hyperacetylation of H4 molecules, and increases in H3 serine 10 phosphorylation.
6. Epigenetic changes can be detected early after exposure to elevated levels of estrogen. These changes were evidenced by the profound dysregulation of DNA methyltransferases and histone modifications as early as 1 week after the implantation of the estrogen mini-pellet.
7. These epigenetic modifications were, to some extent, reversible upon removal of the exogenous source of estrogen. These changes were also reflected in the morphological state of the mammary glands, in which a regression of the estrogen-induced hyperproliferative changes was observed.
8. Interestingly, estrogen-induced hyperacetylation of H4 molecules and the upregulation of the *de novo* methyltransferase, DNMT3a, persisted for up to 8 weeks after the removal of the estrogen mini-pellet. These changes were significantly less severe than in animals exposed to continuously elevated levels of estrogen.

In summary, the results of the present study confirm our hypothesis that estrogen- and radiation-induced changes in the female mammary gland are mediated by perturbations in the epigenome. Exposure to these two carcinogens, either separately, or in conjunction, induces morphological changes that are mediated by global DNA hypomethylation and

aberrant expression of methyl-binding proteins that interact with chromatin-modifying enzymes. These changes are associated with altered gene expression and imbalances in proliferation and apoptosis. We propose that these epigenetic alterations may contribute to genomic instability and carcinogenesis. Further research is required to understand the impact of dose and administration on the carcinogenic potential of these agents, as well as to examine their effects when combined with other carcinogens.

### **5.1.1. Limitations and Future Considerations**

The first hurdle to overcome in the course of modeling human breast cancer is to identify an appropriate model. Clearly, breast cancer must be recognized as a heterogeneous disease, with different subtypes exhibiting different molecular markers and treatment response. Although rodent models exhibit similar histopathological features and hormone-responsiveness as do human breast cancers (Ruhlen et al., 2009; Li et al., 2002), these models, at best, could only be used to study certain human breast cancer subtypes or even specific pathways. The fact that some species and strains more closely resemble their human counterparts in specific features than others, making them a more suitable model to study a particular stage of carcinogenesis or subtype, should be taken into careful consideration when designing an experiment and interpreting the resulting data.

Our studies focused on the ACI rat as a model of estrogen-induced mammary carcinogenesis, which features biological markers reflecting the general characteristics of ductal carcinoma *in situ* (Weroha et al., 2006; Li et al., 2004; Li et al., 2002). The intent of these experiments was to identify epigenetic biomarkers that could be detected early

after carcinogenic exposure. This information may provide insights as to the diagnostic value and potential reversibility of these changes. In recognizing the limitations of the current knowledge of the genetic contributions of this particular strain, such as single nucleotide polymorphisms, future studies will need to address the interplay between genetic and epigenetic backgrounds and their effect on cancer susceptibility.

As with any modeling system, one must acknowledge difficulties in extrapolating data generated in a model organism to the heterogeneous nature of human populations, which portray a diverse array of genetic, environmental, and lifestyle factors. In the studies presented herein, we estimated the concentration of circulating estrogen to be similar to the levels shown in previous studies, which indicate serum estrogen levels, from implants ranging from 3 to 27.5 mg, plateau at approximately that observed during pregnancy (Weroha et al., 2005; Harvell et al., 2000; Shull et al., 1997), a period when breast cancer risk is elevated in humans (Shull, 2007). However, perhaps a more relevant point to consider would be the level of estrogen within the breast tissue itself. Studies have shown that high levels of aromatase activity within the breast tissue is the primary source of estrogen, and directly correlates with increased tumor growth, suggesting that local levels of estrogen in breast is a more important indicator of breast cancer risk than circulating estrogen (Santen et al., 2002; Santen et al., 1999).

Furthermore, given the nature of estrogen as a long distance endocrine signaling molecule, which targets a number of tissues throughout the body, one must also consider the impact of elevated estrogen levels in other organs. It is well-known that one of the primary targets for estrogen is the uterus, in which high levels of estrogen have been strongly associated with increased risk of endometrial cancer (Lépine et al., 2010).

Elevated levels of estrogen have also been shown to target the pituitary gland, resulting in endocrine disruption and prolactin-producing pituitary tumors (Spady et al., 1999). The complexities of the organismal response to elevated levels of estrogen have yet to be explored.

Similarly, the effects of radiation on other radiosensitive tissues such as spleen and thymus should be addressed. Previous studies have found that radiation-induced changes in radiation-target tissues can lead to significant and persistent epigenetic changes and genome instability in other tissues (Koturbash et al., 2006).

The observed decrease in body weights observed in our studies, particularly in the rats exposed to both estrogen and radiation, suggest that these carcinogens may be affecting the organism on a larger scale. Changes in the whole-organism environment would likely play an important role in breast cancer risk. Clearly, the extent to which carcinogens exert their effects needs to be considered at both the tissue and organismal level.

## 5.2. SIGNIFICANCE AND RELEVANCE TO HUMAN HEALTH

Cancer continues to be one of the most common diseases that affect humans worldwide. As the knowledge of the molecular basis of cancer grows, improved diagnostic methods and more effective strategies have been implemented, leading to earlier detection, increases in patient survival, and higher quality of life.

The recent acknowledgement of cancer as both a genetic and an epigenetic disease has resulted in several initiatives implementing epigenetics in cancer prevention and treatment. Unlike genetic alterations, which are difficult to reverse in clinical settings, the very nature of epigenetics makes it easier to reactivate epigenetically-silenced tumor suppressor genes by pharmacological means. Moreover, the identification of promoter hypermethylation and histone modifications associated with inactivation of tumor suppressors serve as early molecular biomarkers for which chemopreventative treatments can be targeted to block or reverse these epigenetics aberrations during the early stages of carcinogenesis. However, the role of epigenetics in the genesis of mammary cancer and the cause of individual susceptibilities are largely unknown.

### 5.3. FUTURE DIRECTIONS

#### 5.3.1. Modeling Epigenetics

Since carcinogen-induced cancer and the experimental application of novel “epigenetic” drugs are often considered unethical in human studies, rodent models, which share similar metabolic and histological features as humans, are utilized (Li et al., 2004; Russell, 2003; Li et al., 2002; Harvell et al., 2000; Shull et al., 1997; Tomatis, 1979).

There are a number of factors that one must take into consideration when choosing an appropriate model. Past studies have clearly outlined genetic difference, identifying alleles conferring resistant and susceptibility phenotypes (Shull, 2007; Schaffer et al., 2006). Differences in the epigenome are also becoming increasingly recognized for their role in cancer predisposition (Pogribny and Beland, 2009; Pogribny et al., 2009; Bagnyukova et al., 2008). These data suggest that possessing collective epigenetic features that reinforce long-term transcriptional status in an inappropriate manner may predispose individuals to genome instability and, therefore, susceptibility to diseases. Future studies may look to challenge different strains with the same carcinogen and evaluate whether differences in epigenetic backgrounds are responsible. Of course, one must take both the genetic and epigenetic contributions into consideration when evaluating their susceptibilities.

To more clearly define which specific epigenetic modifications contribute to increased susceptibility or resistance, one would need to examine the development of cancer in genetically identical organisms which possess different epigenomes. For example, rats on a methyl-deficient diet exhibited lower levels of DNA methylation and increased susceptibility to carcinogenic exposure than their genetically identical

counterparts fed a folate-sufficient diet (Pogribny et al., 2007). Further studies along a similar line could shed light on the epigenetic patterns that alter susceptibility to breast cancer, providing the underlying framework for identifying epigenetic signatures of women with increased risk for developing mammary carcinogenesis.

These studies need to address such questions as: Which epigenetic marks contribute to the formation of cancer? Which sequence of epigenetic events can be detected first and how do they contribute to the progression of cancer? What is the significance of alterations in the histone code and which specific patterns contribute to cancer predisposition? Can these epigenetic changes be stably reversed to prevent or halt tumor growth? Are there secondary consequences to the use of “epigenetic” drugs?

### **5.3.2. Identifying High Risk Individuals**

Clearly, the importance of identifying breast cancer risk factors is many-fold. Firstly, the assessment of susceptibility genotypes will identify women at higher risk for breast cancer. Secondly, genome-wide profiling of epigenetic modifications have identified early biomarkers of carcinogenesis (Figuroa et al., 2009; Goto et al., 2009; Martin-Subero et al., 2009; Chekhun et al., 2007; Esteller, 2006). Importantly, these epigenetic modifications can predispose cells to genetic changes (Baylin and Ohm, 2006; Gould et al., 1996). Clearly, the ability to maintain an appropriate epigenomic landscape for homeostasis through the maintenance of DNA methylation patterns and histone modifications are crucial for reducing the risk of cancer formation.

The development of “epigenetic” drugs is currently being investigated as an alternative approach to treat individual predispositions for their specific genetic and

epigenetic status, and may increase the effectiveness of radio- and chemotherapeutic treatment modalities (De Schutter and Nuyts, 2009; Beltran et al., 2008; Pray, 2008; Arce, 2006; Karpf, 2006). Epigenetic therapy may prove to be particularly useful in chemopreventative approaches, especially for those individuals who have been diagnosed with aberrant epigenetic alterations but have not yet acquired neoplastic lesions (Fay et al, 2005; Herman, 2005). Epimutations, or aberrant DNA methylation and histone-modification patterns, are observed in individuals with no history of malignancy (Arai et al, 2009; Holst et al., 2003) and can be used as an indicator of the likelihood of developing cancer (Arai et al., 2009; Laird, 2003). If these epimutations are corrected with DNA methylation and HDAC inhibitors, it can delay or completely prevent tumorigenesis in these individuals (De Schutter and Nuyts, 2009; Beltran et al., 2008; Pray, 2008; Arce, 2006; Karpf, 2006; Fay et al, 2005).

Having a detailed map of specific epigenetic patterns in each tissue type in their normal and in cancerous states would make detection of premalignant epimutations feasible, even from as little biological material as a drop of blood (Teschendorff et al., 2009; Esteller, 2006; Laird, 2003). In the future, one could expect to see a more comprehensive screening protocol of individuals classified by the latest literary evidence of being at “high risk” for developing disease. Furthermore, comprehensive knowledge of the epigenome would open up a new avenue for the development of various drugs designed to target a specific region of the genome in which an epimutation has occurred.

Understanding that cancer is a heterogeneous disease, the importance of treating individuals based on their specific genetic or epigenetic predisposition has prompted interest for individual pharmacokinetics. Clinicians are beginning to realize that a “one-

size-fits-all” is no longer an appropriate approach for treating cancer. With the advent of technology enabling the mapping of the human epigenome and epigenetic profiling of cancer, the tools to define each person’s unique genetic and epigenetic makeup are becoming available. This information will help them design individually tailored medicine based on their specific circumstances.

### **5.3.3. Epigenetic Profiling of Cancer**

Cancer is associated with hypermethylation of tumor suppressor genes and abnormal expression of DNMTs. These changes are often reinforced by repressive chromatin marks, which condense the chromatin, thereby blocking access of transcription factors to the DNA. Genome-wide scanning techniques have proven to be valuable in assessing the validity of targeting DNA methylation and histone modifications as a treatment for cancer (Figuroa et al., 2009; Goto et al., 2009; Martin-Subero et al., 2009; Chekhun et al., 2007; Esteller, 2006).

Diagnosis of cancer through the analysis of epigenetic patterns is a promising prospect, although the complex nature of DNA and histone modifications will likely make this approach difficult. Perhaps the biggest difficulty to overcome lies in that the epigenome is responsive to a number of environmental and intrinsic factors, dynamically modifying gene expression during development in both normal and malignant cells. Despite this, a number of epigenetic hallmarks of cancer cells have been identified, including DNA promoter hypermethylation of tumor suppressors and the global loss of DNA methylation (Fraga et al, 2005B; Macaluso et al., 2003). These marks have been utilized in numerous studies which clearly demonstrate the potential for epigenetic

profiling to distinguish cancerous tissue from their normal counterparts with high specificity (Kanai, 2009; Esteller, 2006; Laird, 2003). Certainly, although these features are considered hallmarks of genome instability and carcinogenesis, specific epigenetic patterns are not identical among different types of cancer or even at different stages of progression. However, this can be used advantageously, in which epigenetic profiling of cancer has not only been used to successfully detect specific epigenetic patterns associated with different types of cancer, but also can be used to differentiate sub-types (Goto et al., 2009; Martin-Subero et al., 2009; Stumpel et al., 2009; Teschendorff et al., 2009; Mi et al., 2008). Moreover, epigenetic profiling can be used to predict prognosis and response to treatment (Hesson et al., 2007; Seligson, 2005; Esteller et al., 2000). For example, the epigenetic profiling of multi-drug-resistant human breast adenocarcinoma cells revealed hyper- and hypomethylation of genes that may contribute to the acquired drug-resistant phenotype in cancer (Chekhun et al., 2007).

By comparing the epigenetic signatures of different cancers and their stages of progression, the underlying molecular mechanisms driving tissue- and cancer-specific alterations can be targeted for epigenetic reprogramming. It is also important to recognize that different carcinogens sculpt the epigenetic landscape in different ways, and even the epigenetic response to the same type of carcinogen can be quite different, depending on the dose and exposure regime (Koturbash et al., 2005; Pogribny et al., 2005; Woloschak and Chang-Liu, 1990). Moreover, studies indicating gene expression can be used to distinguish between spontaneous and carcinogen-induced mammary carcinogenesis (Imaoka et al., 2008), suggest similar findings may be observed in epigenetic patterns, which regulate gene expression.

#### **5.3.4. Exposure to Multiple Carcinogens**

Most studies focus on the effect of one carcinogen at a time. Although this may make it easier to trace the molecular repercussions of the carcinogen, this does not reflect real life scenarios, in which people are exposed to numerous chemical and physical agents throughout their lifetime (Charles. 2001). Furthermore, these studies rarely take into account differences in the genetic and epigenetics make-up of individuals.

A plethora of studies have been initiated to address the combined potential of chemical and physical carcinogens (Kantorowitz et al., 1995; Watanabe et al., 1993; Maisin et al, 1987; Borek et al., 1986). Studies of combined exposure have indicated that the carcinogens were reported to exert an additive, sub-additive, or supra-additive (synergistic) effect. In breast tissue, radiation and estrogen are known carcinogens, in which their combined exposure produces a response larger than the sum of either carcinogen alone (Broerse et al., 1987; Shellabarger et al, 1983; Holtzman et al., 1979).

Other factors were also shown to influence the extent to which carcinogens are able to exert their effects. Age, for example, was shown to be a major determinant of susceptibility, in which the mammary gland of younger populations tend to exhibit increased radiation sensitivity with the administration of estrogen (Bartstra et al, 1998).

These interactions must be taken into consideration in a clinical setting, in which many chemotherapy agents are also carcinogens. Although radiotherapy may be used either alone or in combination with surgery for the treatment of malignant tumors, other adjuvant treatments, such as chemotherapy and hormone therapy are also utilized. Secondary cancers, which are unrelated to the first cancer that was treated, may occur months or even years after initial treatment (Leone et al., 1999; Storm et al., 1992; Boice

et al., 1992). These cancers are more frequent in patients who receive both radiation therapy and chemotherapy compared to either treatment alone (Wong et al, 2003; Araujo et al., 1991). Clearly, further studies need to be conducted to elucidate the interacting mechanisms of combined modality treatments leading to secondary complications and malignancies.

### **5.3.5. Epigenetic Drugs**

A number of biotech companies have focused on technologies identifying DNA-methylation and histone modification biomarkers and diagnostics based on differences in DNA methylation between healthy and diseased tissue (Futscher and Domann, 2006; Taipale et al., 2005; Conacci-Sorrell et al., 2002; Futscher et al., 2002; Geiman and Robertson, 2002; Jawhari et al., 1999). It is anticipated that these markers can then be targeted by “epigenetic” drugs for reprogramming prior to tumor development and during the progression to malignancy.

One specific goal of epigenetic therapy is to restore normal epigenetic patterns and to prevent the cells from acquiring further epigenetic-mediated alterations that could lead to silencing of genes crucial for normal cell function. Epigenetic drugs include demethylating agents and HDAC inhibitors which target aberrantly heterochromatic regions, leading to reactivation of tumour-suppressor genes and/or other genes that are crucial for the normal functioning of cells (Egger et al., 2004; Yoo et al., 2004; Christman et al., 2002). Several drugs targeting DNA methylation and histone deacetylation enzymes have already been approved and others are in clinical trials (Wang and Dymock, 2009).

Inactivation of DNMTs is one of the most effective ways of inhibiting DNA methylation and re-establishing more normal patterns of gene expression. However, targeting the methyltransferase enzyme can lead to inadvertent consequences, including the loss of specificity and overall decrease in methylation levels across the genome (Szyf, 2008; Yoo et al., 2004). Nevertheless, decreased activity of DNMT resulting from the administration of DNMT inhibitor has been shown reactivate genes involved in controlling cell proliferation, differentiation, apoptosis and other key homeostatic mechanisms, and has also been shown to halt neoplastic changes (Figueroa et al., 2009, Laird, 2003; Laird et al., 1995). This positive result indicates that DNMT inhibitors warrant further study, although they should be used with caution (Gius et al., 2004).

HDAC inhibitors (HDAC-i) block the enzymes that remove acetyl groups from histone tails, which leads to the accumulation of acetylated histone residues, followed by changes in cellular processes that have become defective in cancerous cells. Interestingly, the resulting hyperacetylation of histones is thought to contribute to genomic instability, which ultimately triggers the cell-cycle checkpoint (Qui et al., 2000). Alternatively, HDAC inhibitors have been shown to activate genes involved in cell differentiation, apoptosis, and the inhibition of angiogenesis and metastasis (Nebbioso et al., 2005; Peart et al., 2005; Shetty et al., 2005; Michaelis et al., 2004).

The combination of demethylating agents with HDAC-i is of special interest, having been found to act synergistically to reactivate the expression of more than a thousand genes in primary tumors of breast cancer patients (Arce et al., 2006; Li et al., 2005; Cameron et al., 1999). In addition, these drugs were found to down-regulate genes implicated in multidrug resistance, suggesting that they may have the potential to

resensitize cells to chemotherapeutic agents (Arce, 2006; Pérez-Plasencia and Duenas-Gonzalez, 2006).

Indeed, demethylating drugs and HDAC-i have proven to be valuable in combinational with other therapeutic modalities (Arce et al., 2006). Concurrent administration with classical cytotoxic agents, such as doxorubicin and cyclophosphamide, which are commonly prescribed for the primary treatment of breast cancer, was shown to be more effective than their single application (Arce et al., 2006). Nowadays, the majority of patients with cancer are treated with radiotherapy. To optimize the results obtained with this treatment modality, strategies to target tumor-specific cells have shown that treatment with HDAC-i, as well as demethylating agents, can increase the killing effect of radiation on tumor cells *in vitro* and *in vivo* (Chen et al., 2007; Chinnaiyan et al., 2005; Arundel and Leith, 1987), while acting as a radiation protectant in normal tissue. The exact mechanism through which these drugs mediate anti-tumour activity has not been elucidated, and further studies focusing on the chemo- and radiosensitizing potential of these epigenetic drugs in a clinical setting may be promising avenues to explore.

#### 5.4. CONCLUSION

In conclusion, the field of epigenetics has evolved dramatically over the last few decades. However, there are still many gaps in our understanding of the epigenome; the list of DNA and histone modifying enzymes is far from complete. Future studies are required to identify and elucidate the exact role of the key players involved in the generation of epigenetic patterns and the complex interplay that exists between them. Only with the complete understanding of these epigenetic modifiers will the development of the most effective therapies be possible.

The potential of epigenetic alterations to act as biomarkers for cancer detection and treatment will be important to for developing preventative strategies that may one day make the currently unavoidable cancer deaths obsolete. Until that time, however, the reversible nature of epigenetic modifications has led to the development of several drugs aiming to restore a normal epigenetic balance. These drugs appear to be promising new therapeutic agents that can be implemented alone or as part of combinational therapies. Follow-up studies are required to monitor the efficacy of these epigenetic drugs and any long-term repercussions they may have should be taken into consideration.

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