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ENDOGENOUS STRESS: A STUDY OF THE NORMAL CELLULAR
RESPONSE TO OXIDATIVE DAMAGE

A Thesis in
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by
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ABSTRACT

Lifetime exposure to estrogen is implicated as a risk factor for breast cancer and increasing evidence points to estrogen as both initiator and promoter of tumorigenesis. However, the effects of estrogen placed upon the background of BRCA1 heterozygosity in nontumorigenic, human breast cells have not been elucidated. As a key player in the DNA damage response, we hypothesize that impaired BRCA1 function results in the accumulation of DNA damage secondary to the high level of estrogen metabolites and the generation of reactive oxygen species in breast tissue. Simultaneously, in the face of DNA damage, which might cause cell cycle arrest and apoptosis in wild type cells, BRCA1 heterozygotes are crippled in their ability to inhibit ER-associated growth stimulation, resulting in mutations. To this end we have treated two nontumorigenic, breast epithelial cell lines, one BRCA1 heterozygote (185del AG) and another wild type, with physiologically relevant doses of estradiol. Following treatment with 10 and 50 nm estradiol, dichlorfluorescein fluorescence increases similarly in both cell lines, corresponding to a 20-40% transient increase in oxidative stress. Estradiol and hydrogen peroxide (H$_2$O$_2$) increased the level of lipid peroxides and 8oxoG, an oxidative DNA adduct. The mutation frequency at the HPRT locus was determined following oxidative stress. Estradiol induced a 2 and 3.7-fold increase in HPRT mutations in wildtype and heterozygous cells. Severe oxidative stress induced by H$_2$O$_2$ did not alter the mutation frequency of wildtype cells but increased it 6-fold in heterozygous cells. Immunoprecipitation revealed that heterozygotes have less protein than wildtype cells both in the presence and absence of oxidative stress, but both cell lines increased levels of protein following oxidative stress. A proliferation assay demonstrated that while wildtype...
cells show a transient increase in cell number, growth of heterozygous cells is unaltered following exposure to estradiol. Cell cycle analyses of estradiol-treated wildtype cells revealed increased transit through G0/G1 into S phase in response to estradiol and a G2-M arrest following exposure to HP. Both responses were absent in heterozygotes. In multiple cellular and mutagenic assays, BRCA1 heterozygotes display an altered phenotype relative to wildtype cells, consistent with the definition of haploinsufficiency.
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LIST OF ABBREVIATIONS

8-oxoG  8-hydroxyguanine
6TG   6-thioguanine
ATM  Ataxia Telangiectasia mutated
ATR  Ataxia Telangiectasia Rad3-related
bcl-2  B cell leukaemia 2
BER  Base excision repair
BLM  Bloom’s helicase
BRCT  BRCA1-C-terminal
DCF  Dichlorofluorescein
DSB  Double strand breaks
ER  Estrogen Receptor
ERE  Estrogen responsive element
FasL  Fas Ligand
HNPCC  Hereditary nonpolyposis colorectal cancer
HP  Hydrogen Peroxide
HPRT  Hypoxanthine phosphoribosyl transferase
HR  Homologous recombination
MMR  Mismatch repair
MSI  Microsatellite instability
NHEJ  Nonhomologous end joining
RING  Really Interesting New Gene domain
ROS  Reactive oxygen species
<table>
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<tr>
<td>SSB</td>
<td>Single strand breaks</td>
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<td>TF</td>
<td>Transcription factor</td>
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INTRODUCTION

A. Endogenous Stress

In the past twenty five years, a plethora of data regarding the contribution of exogenous chemicals to tumorigenesis has flooded the scientific and lay communities. As sensitivity and specificity of testing methodologies and detection systems increased, the once held belief that DNA is maintained in a pristine condition and damaged only by exogenous sources began to fade. What is the contribution of our own cells to tumorigenesis?

A1. Oxidative Stress

There are several types and sources of endogenous stress that may initiate cellular changes that contribute to tumorigenesis. Oxidative damage is a major source of endogenous stress. Reactive oxygen species (ROS) are formed endogenously from a variety of sources including mitochondrial respiration, arachadonic acid metabolism, respiratory bursts in immune responses, and metabolism of both exogenous and endogenous carcinogens, such as estradiol [1]. Multiple studies have indicated that cells are continuously bombarded with high levels of ROS. Urinary excretion of byproducts of the removal of 8-hydroxyguanine (8-oxoG), an oxidatively damaged base, is approximately 2.5nmol/kg/day in normal human subjects, corresponding to 2,000 oxidative modifications to bases per cell each day [2]. When a cell’s damage recognition and repair pathways are comprised, ROS-induced damage may accumulate and contribute to tumorigenesis. In human lung cancers, as many as 200 in $10^5$ nucleotides have oxidative modifications [3]. Unrepaired oxidative damage can impair replication and transcription, alter cell kinetics, and affect signal transduction, and is implicated in many sporadic and hereditary types of cancer including BRCA1-mediated hereditary
breast cancer. Similarly, chronic inflammatory conditions such as Wilson’s Disease, ulcerative colitis, and *H. pylori*-induced gastritis are associated with an increased propensity for the subsequent development of cancer due to the high levels of oxidative stress produced by immune system and cancer cells [4-6].

Numerous ROS have been identified, but the most intensively studied ROS in the DNA damage field are the superoxide anion (O$_2^-$), the hydroxyl radical (OH$^•$), hydrogen peroxide (H$_2$O$_2$) (HP), and singlet oxygen. The hydroxyl radical is considered to be the most deleterious, but it is also the most short-lived [3]. ROS are able to exert cell-wide effects, modifying not only DNA but also RNA, lipids, and proteins. DNA damage includes base modifications, which are potentially mutagenic and cytotoxic, and single or double strand breaks (SSB, DSB). DSB are cytotoxic unless adequately repaired (Pfiefer Mutagenesis), and SSB may be converted to DSB during replication.

Several tiers of defense exist to protect a cell from the deleterious effects of ROS [7]. The first line of defense functions to remove ROS from the cellular environment. The enzymes superoxide dismutase and catalase neutralize the superoxide anion and HP to neutral or less reactive compounds. Antioxidant vitamins, amino acids, and other enzymes such as glutathione reductase work in a more general manner to scavenge for free radicals. When the levels of ROS exceed these defense mechanisms, the cell is under oxidative stress and damage to cellular structures may occur.

**A2. Oxidative base modifications and repair systems**

More than 20 different oxidative base modifications are known. The most prevalent purine and pyrimidine modifications are 7,8-dihydro-8-oxoguanine (8oxoG) and thymine glycol, formed following exposure to ROS [1]. The 8oxoG lesion is stable and is highly
mutagenic. 8oxoG in the DNA strand pairs with equal efficiency with cytosine or adenine, causing G:C to T:A transversions [8]. Incorporation of 8oxodGTP opposite A results in A:T to C:G transversions. The second line of defense against oxidative stress exists to prevent the incorporation of oxidatively damaged free nucleotides. 8oxoGTP and 8oxoATP are removed from free nucleotide pools by hMTF1 and degraded to their monophosphate forms. If 8oxoGTP is incorporated or if a guanine in the DNA strand is oxidized, this lesion is repaired by multiple repair systems including base excision repair and mismatch repair which represent the third line of defense [9, 10].

Base excision repair (BER) is the main pathway used to repair lesions which create a distortion in the double helix (Lindahl Nature 1993). In addition to the repair of oxidatively damage bases, it also repairs aklylated bases [7]. DNA glycosylases remove the damaged base to create an apurinic site. This action is followed by cleavage of the strand and the removal of 1-8 adjacent nucleotides creating a gapped substrate. The gap is filled by a DNA polymerase and the strands are again ligated. 8oxoG is removed from the DNA by the hOOG1 glycosylase, and mutations in this gene are associated with lung and kidney cancer [7]. In wild-type mouse embryonal fibroblast cell lines, 8oxoG is removed faster from transcribed than nontranscribed strands, indicating that transcription-coupled repair might also be involved in the repair of oxidative damage.

The mismatch repair system (MMR) also recognizes mispairs in the DNA arising from replication errors or that arise from oxidized or alkylated bases. Mouse embryonic fibroblasts with complete loss of the mismatch repair protein MSH2 showed increased levels of 8oxoG than their MSH-proficient counterparts following exposure to HP [11]. In humans at least six MMR proteins are required. Mismatches are recognized by the
MSH2 protein in conjunction with MSH6 or MSH3. Mismatch repair proteins MLH1 or MLH3 and PMS2 coordinate the interaction of other proteins that may be necessary for repair, such as DNA polymerases, helicase, and replication factors [12]. Studies of purified MMR complexes have demonstrated that the MMR complex recognizes mispairs, single strand breaks, abasic sites, and single nucleotide gaps [10]. This discovery places MMR as a key player in the response to oxidative damage to DNA and suggests interplay between MMR, BER, and strand break repair. MMR defects result in an increased spontaneous mutation rate, especially at microsatellites, which are repeating units of nucleotides found throughout the genome [13, 14]. Given that some growth regulatory genes such as Bax, MSH3 and 6, and insulin-like growth factor II receptor contain microsatellites, microsatellite instability (MSI) could have dramatic consequences for the cell [12].

A3. DNA strand breaks and repair

Single and double strand DNA breaks result from exposure to exogenous processes such as genotoxic agents and ionizing radiation, and endogenous sources such as oxidative damage to the DNA backbone, stalled replication forks, meiotic recombination, and immunoglobulin gene rearrangement [15]. DSB and SSB are cytotoxic if not repaired; thus, the cellular response to a DSB is complex and involves the interacting pathways of lesion recognition, cell cycle arrest, apoptosis, and repair. Non-homologous end-joining (NHEJ) and homologous recombination (HR) are the major two pathways of DSB repair. Both pathways are initiated by recognition of the DSB and subsequent phosphorylation of proteins, such as BRCA1, involved in signaling to repair pathways.
In HR, the Rad50/MRE11/NBS1 complex digests several nucleotides at the site of the break to generate single strand overhangs which are subsequently coated with Rad51. Formation of this complex allows for invasion of an identical, intact sister chromatid which is used as a template for repair. In NHEJ, the ends of the DSB are joined without the benefit of a template, which may result in the loss or gain of a few nucleotides. Proteins KU and DNA-PKcs bind to the single strands near the site of the breaks to promote ligation of the single DNA strands [15]. Although NHEJ is considered more error prone than HR, it appears to occur more frequently than HR and is the predominant method of DSB repair in G0/G1 [15]. NHEJ may be used most frequently simply because HR is only possible during S phase of the cell cycle when sister chromatids are near enough to be used as templates for repair [15].

A4. Alkylating Damage

Another source of endogenous damage in cells is alkylating damage, the sources of which are not as clearly elucidated as those generating ROS. Incubation of DNA with the reagent S-adenosylmethionine induces methylation of DNA bases. Nitrosated amino acids, polyamines, some peptides, and some components of bile are also implicated as potential alkylating agents. Ethylene gas, produced by microorganisms lining the gut, is converted to ethylene oxide which readily alkylates DNA and proteins [16]. Alkylating agents react most readily with ring nitrogens of purines and the most prevalent products of alkylating damage are N7-Me-dG and N3-Me-dA. Of these, N3-Me-dA is a potent replication blocker [16]. In terms of the ability to cause mutations, O6-Me-G is the most mutagenic alkylating lesion, and causes G:C to A:T transitions. Specialized repair
enzymes called alkyltransferases exist to demethylate the modified base [17]. In humans, O\textsuperscript{6}-MeG was the most common alkylating lesion in hepatic and leukocyte DNA.

A5. Summary of Endogenous Stress

The response to endogenous stress is complex and involves multiple pathways which must work in a coordinated fashion to prevent the potentially mutagenic and cytotoxic consequences of oxidative and alkylating damage. BRCA1 has been shown to interact with proteins from multiple repair pathways and may represent the “director” of repair, functioning to coordinate the cellular response to damage. This role will be discussed in detail, as well as the potential for estradiol to induce ROS, in future sections.

B. Breast Cancer

Interest in the role of endogenous stress and the interplay of cellular repair systems in the biology of breast cancer is growing rapidly. Oxidative stress appears to be a component of breast cancer, as the level of 8-oxoG was found to be ten times higher in human invasive ductal breast cancer cells relative to normal tissue taken from the same patient [18]. Elucidation of the role of ROS in the initiation of breast cancer may yield insight into potential protective measures, especially for those with an inherited predisposition resulting from mutations in DNA repair genes. These measures could have a huge impact on cancer treatment and prevention, especially since breast cancer is a very prevalent disease. 10% of all cases of breast cancer can be attributed to a hereditary gene mutation, half of which will be located within the BRCA1 gene [19]. Women carrying these mutations have an 85-95% lifetime risk of developing breast and/or ovarian cancer, which often arise decades earlier than their sporadic counterparts [20].
In addition to hereditary gene mutations, more common risk factors for the development of breast cancer include obesity, early onset of menses, late onset of menopause, and use of hormone replacement therapy. Cancer risk as the result of these conditions is attributed to the cumulative exposure to estrogen [21]. In obesity, for example, androgens are converted to estrogens by adipose cells. The focus of this study is to analyze the interplay of BRCA1 and estrogen-mediated cellular events.

B1. Estrogen

Epidemiological data from both sporadic and hereditary breast cancer implicate cumulative estrogen exposure as a modifier of lifetime breast cancer risk. Estrogen may function at several levels in the tumorigenesis pathway by impacting both growth rate and differentiation, and directly causing DNA damage and mutations.

Classically, estrogen is considered to act as a stimulus for cellular proliferation, mediated through interaction with the estrogen receptor (ER). In this model, the carcinogenic potential of estradiol is solely attributed to the accumulation of mutations as the result of increased DNA replication and associated spontaneous errors [22]. In the early 1970’s, mounting evidence suggested that the estrogen story was not so simple. Liehr and others asserted that the estrogen/ER model could not alone account for the carcinogenic potential of estrogen [23]. The following observations have helped to establish that estradiol induced carcinogenesis does not occur entirely through receptor-mediated activities: (1) In normal human mammary epithelium, proliferating cells do not express ER; (2) Hamster cells don’t express ER and estradiol is not mitogenic yet these cells demonstrate aneuploidy and morphological transformation in response to estrogen exposure; (3) Estradiol compounds such as 17-alpha ethylestradiol retain hormonal
competency yet are not carcinogenic; (4) The carcinogenic potential of estradiol can be attenuated by compounds which inhibit metabolic conversion of estradiol and/or scavenge free radicals. Today, we recognize three mechanisms responsible for estrogen-induced carcinogenicity: (1) the aforementioned receptor-mediated activity, (2) Metabolic activation of estrogen to genotoxic metabolites, and (3) ROS production resulting from redox cycling of estrogen metabolites.

Evidence exists to suggest that estradiol is preinitiator, initiator, and promoter of cancer. Hilakivi-Clarke has termed estradiol a preinitiator following the observation that in utero exposure to high levels of estradiol result in retention of less differentiated mammary structures which are much more likely to undergo neoplastic transformation [24]. Estradiol undergoes conversion to catecholestrogens by cytochrome P450 enzymes. These enzymes hydroxylate estradiol at both the two and four positions, with 2-hydroxyestradiol being the major metabolite. Polymorphisms in the CYP1A1 gene encoding proteins that increase the production of 4-hydroxyestradiol, which retains hormonal potency, increase the risk of breast cancer up to 2-fold in postmenopausal women [25]. Similarly, low activity alleles of the enzyme catechol-o-methyltransferase (COMT) which inactivates hydroxyestradiols, increase the risk of breast cancer 4-fold in postmenopausal women [25]. Catecholestrogens are capable of undergoing redox cycling between catechol and quinone forms, with a semiquinone formed as the intermediate step. This interconversion between semiquinone and quinone generates free radicals capable of damaging DNA and may account for the ability of estradiol to initiate tumorigenesis [26].
Estradiol-induced DNA damage has been studied *ex vivo* in cell culture systems and *in vivo* using hamster model systems. Among the types of damage caused by estradiol and its metabolites are single strand breaks, oxidative lesions such as 8-oxoG, and bulky DNA adducts following exposure to estradiol [27-30]. One of the bulky DNA adducts seen following exposure to estradiol are composed of oxidized lipid byproducts such as malondialdehyde. Mutations that arise from this damage include increased mutation frequency at the Na/K-ATPase and HPRT genes [31]. Also observed were gene amplifications, microsatellite instability, aneuploidy, and structural chromosomal alterations [32-35]. Animal models have demonstrated that these mutations are sufficient to increase tumor incidence, implicating estradiol as both mutagen and carcinogen [23]. Mammary cell lines exposed to physiologically relevant doses of estradiol undergo neoplastic transformation, characteristics of which include increased colony efficiency and loss of ductulogenesis [36-38]

2. BRCA1

2.1 BRCA1 Genetic background

The human *brca1* gene is located on chromosome 17q21 and spans about 100 kb of genomic DNA, 5.5 kb of which encodes 22 exons [39]. This gene is conserved in mammals but not in lower animals. In mice, complete loss of BRCA1 results in embryonic lethality [40]. Currently, there are over 200 reported mutations for humans which result in a phenotype. These mutations span the length of the gene and are primarily frameshifts that result in the production of truncated proteins. Common gene mutations include 5382insC, 4184del4 and the 185delAG (Figure 1) (data provided by Breast cancer Information Core 2004). The latter gene mutation is also known as the
Ashkenazi Jewish mutation, where 1% of the population carries this particular mutation (in contrast to 0.06% of the general population) [41]. This mutation results in the production of a truncated protein (38 amino acids) which is rapidly degraded within the cell [42]. Other mutations occurring in the 3’ region of the gene can result in proteins which are not degraded, but instead are dysfunctional or may even antagonize wildtype protein [43].

In addition to predicting the resulting protein product for a given mutation, it is also possible to make genotype-phenotype correlations (Figure 1). Mutations at the extreme ends of the gene result in the production of unstable proteins and are associated with tumors demonstrating a high proliferative index, mostly in the breast [44]. Mutations located in the central part of the gene are associated more with an increased risk of ovarian than breast cancer [44]. Thus, different mutations may have very varied consequences to the cell and to the individual depending upon where they are located.

Mutations in the brca1 gene are rarely seen in sporadic breast cancer [45]. However, low or undetectable levels of expression BRCA1 have been noted in sporadic breast and ovarian cancers [46]. Silencing of the gene may occur by several mechanisms. First, 40-80% of sporadic breast cancers have an allelic deletion at the BRCA1 locus [39]. Second, epigenetic silencing of the BRCA1 gene may occur by hypermethylation of its promoter. The majority of sporadic tumors with reduced BRCA1 levels will demonstrate both of these events [47].

2.2 Regulation of BRCA1

BRCA1 is regulated at the levels of both transcription and translation. Two promoters (alpha and beta) regulate transcription of the BRCA1 gene, one of which is bidirectional
and shared with the adjacent NBR1 gene [48]. Selective use of the two promoters results in the production of two transcripts with 5’UTR’s of varying size and content which impact their efficiency of translation. Controlled expression of the ratio of these two transcripts appears to represent refined control of BRCA1 transcription and translation [49]. These ratios are often altered in breast cancer tissue from their normal counterparts, suggesting a loss of this control [50].

BRCA1 mRNA and protein levels increase in response to both endogenous and exogenous factors. For example, estrogen has been shown to increase both BRCA1 mRNA and protein [51, 52]. While an estrogen responsive element has been seen in both promoters, these promoters respond more weakly to estrogen than classical estrogen responsive elements (ERE), suggesting alternate mechanisms may be involved [4, 53]. An indirect pathway may exist whereby estradiol stimulates the production of other transcription factors which then may influence BRCA1 promoters. Alternatively, estrogen may impact BRCA1 transcription independently of the ER by the induction of DNA damage [54].

On the translational level, one mechanism of regulation is phosphorylation of the BRCA1 protein. BRCA1 is hyperphosphorylated in G1 and S of the cell cycle, followed by de-phosphorylation at M [55]. Protein phosphorylation is proportional to the level of protein. Not only are levels of protein controlled by phosphorylation, the function of the protein also appears to be under similar control, as phosphorylation triggers BRCA1-mediated DNA damage repair [56, 57]. Thus, phosphorylation of BRCA1 may modulate binding of other proteins necessary to elicit the desired cellular response or may impact the localization of BRCA1 within the cells. Indirectly, phosphorylation of proteins which
associate with BRCA1 may also work to govern BRCA1 function [58]. Some evidence also exists to suggest that BRCA1 is sensitive to ubiquitination [59].

2.3 BRCA1 Functions

This gene encodes a 220 kDa protein consisting of 1863 amino acids and featuring two nuclear localization signals, an N-terminal RING (Really Interesting New Gene) domain, and two C-terminal BRCT (BRCA1-C-terminal). Both domains are thought to be involved in protein-protein interactions [20]. Though widely expressed, levels of the protein vary at different points within the cell cycle and peak in late G1/S [55]. The functions of BRCA1 are complex and varied. Its demonstrated ability to interact with a multitude of proteins suggests that BRCA1 may act as a scaffold for the assembly of protein complexes (Figure 2).

Major roles for BRCA1 will be reviewed here briefly, with special attention to those which may play a role in studies involving the interplay of estrogen and BRCA1.

A. Gene transcription

BRCA1 can both activate and repress gene transcription via a number of mechanisms. The C-terminus of BRCA1 is rich in acidic amino acid residues, a common characteristic of the activation domain of many transcription factors [20]. BRCA1 was co-purified with the RNA Polymerase complex and has been shown to interact with RNA helicase [60], [61]; thus, BRCA1 appears to be a component of the transcription machinery. BRCA1 may also play a role in the polyadenylation of mRNA through association with the BARD1 and CstF50 proteins [62].
In addition to acting directly as a transcription factor, BRCA1 can indirectly influence transcription. BRCA1 has been shown to bind to p53 and enhance its transcriptional activation of p21/WAF/CIP or Bax, eloquently reviewed by Wang and Greene [63]. Of particular interest to this project, BRCA1 binds to and inhibits the activity of c-Myc, the first downstream player in ER-mediated growth stimulation. By binding at the CDC25A promoter, BRCA1 can reverse the cellular transformation induced by c-Myc [64]. Also of importance is BRCA1’s ability to interact with hyperphosphorylated pRb, which may contribute to BRCA1’s suppression of proliferation [65].

BRCA1 can also impact gene transcription through chromatin remodeling. BRCA1 appears to function in a large protein complex that can either deacetylate or acetylate histones in order to appropriately limit or promote gene transcription [66].

B. DNA Repair

*Double Strand Break Repair* In 1997, Scully *et al* first observed that BRCA1 associates with RAD51, a necessary protein in the pathway of homologous recombination and error-free repair of DSB [67]. BRCA1 and RAD51 colocalized to nuclear foci during the S phase of the cell cycle and following DNA damage. DSB may occur as the result of DNA damage or may occur spontaneously. Vilenchik and Knudson have calculated that the rate of spontaneous DSB production is about 50 per cell per cell cycle [68]. In support of the role for BRCA1 in DSB repair via HR, BRCA1 deficient murine embryonic stem cells show defects in repair of DNA double strand breaks by HR but not NHEJ [69].
Cells deficient in BRCA1 showed increased sensitivity to radiation-induced DSB which is reversed with the restoration of BRCA1 (Scully et al 1999). The current model holds that ionizing and ultraviolet radiation activate ATM and ATR, respectively, which phosphorylate BRCA1 at serine residues, the site of which differs depending on the type of DNA damage. Following site specific phosphorylation, BRCA1 initiates the pathways of homologous recombination and/or cell cycle arrest or apoptosis [67].

**DNA Mismatch Repair** BRCA1 also interacts with MSH proteins which are essential for the repair of mismatched DNA lesions that arise spontaneously during DNA replication or in error prone repair of DSB [70]. The current model for BRCA1 interaction states that the MSH2-MSH6 complex binds to the site of a DNA mismatch and exchanges bound ADP for ATP, allowing BRCA1 to join the complex. Once onboard, BRCA1 may regulate mismatch repair and send downstream signals for cell cycle arrest and apoptosis [70].

**Transcription Coupled DNA Repair** Consistent with its role in gene transcription, BRCA1 also appears to play a role in transcription coupled repair. Gowen et al observed that BRCA1 deficient cells showed equal repair of both strands, transcribed and nontranscribed, in contrast to BRCA1 competent cells, which specifically repaired the transcribed strand. The introduction of BRCA1 back into the cells resulted in restoration of the ability to preferentially repair the transcribed strand [71]. Similarly, human cancer cells without BRCA1 were deficient in TCR of 8oxoG, suggesting that BRCA1 is essential for repair of oxidative damage [72].
B. Cell cycle/apoptosis

To function as a caretaker, BRCA1 stimulates repair following DNA damage, but also appears to initiate cell cycle arrest and/or apoptosis. BRCA1 is necessary for both G1/S and G2-M checkpoints [73] and overexpression of BRCA1 results in growth retardation in breast and ovarian cell lines [74]. Apoptosis may be mediated, in part, by BRCA1 induced downregulation of Bcl-2, upregulation of the Gadd45/JNK signaling pathway and upregulation of the FasL/Fas interaction [75, 76] but appear to be independent of p53.

BRCA1 functions may be mediated through BASC (BRCA1 associated Surveillance Complex) which is composed of several DNA repair proteins and tumor suppressors including MSH2, MSH6, MLH1, ATM, and BLM [77]. Site-specific phosphorylation of BRCA1 shuttles repair down the appropriate pathway. Thus, BRCA1 plays a role in maintaining genomic integrity [78]. “The spontaneous chromosome instability in BRCA-deficient cells underpins their propensity to undergo neoplastic transformation” [78].

2.4 Characteristics of BRCA1-related tumors

Breast cancers attributed to BRCA1 dysfunction have different histopathologic and genetic properties when compared to sporadic tumors. Often, these tumors have poor nuclear grade with a very high frequency of p53 mutations (85% as compared to 25% in sporadic tumors) [20]. Interestingly, the spectrum of p53 mutations is unique, suggesting that they may be acquired in a different manner [79]. In contrast to sporadic breast cancers, BRCA1-related tumors are frequently ER negative, perhaps partially explaining
why BRCA1 mutation carriers do not benefit from tamoxifen as a preventive agent [20, 80].

2.5 Heterozygosity and haploinsufficiency

Seidman and Seidman have elegantly elucidated the concept of haploinsufficiency in an article entitled “Transcription factor haploinsufficiency: when half a loaf is not enough.” [81]. Traditionally, reason dictates that loss of a single allele of a gene has negligible phenotypic effects provided another wildtype allele remains. In some cases, a single wildtype allele results in reduced production of protein. Half normal levels of some proteins, particularly transcription factors, are not sufficient to initiate transcription. More than 30 clinical syndromes have been described that result from a single mutated allele of a transcription factor gene. Seidman and Seidman propose a simple model to explain the phenotype seen in haploinsufficient individuals. Occupancy of promoter regions by transcription factors determines whether or not the target gene is transcribed; thus, decreased levels of transcription factors may alter the likelihood that a promoter is activated. In cases where transcription factors interact at multiple promoter sites, and gene transcription requires occupancy at each of these, then this gene will be even more sensitive to transcription factor concentration. In a simple example, assuming no redundancy, a promoter with three sites of TF binding would be eight times less active in an individual with only one allele in comparison to an individual with two functioning alleles [81].

As described earlier, BRCA1 alters gene transcription both directly and indirectly. In addition, given that certain mutant proteins arising from mutations near the 3’end of the gene have been shown to antagonize wildtype protein, it is conceivable that a single
mutated allele may have cellular consequences and may even result in less than half normal levels of BRCA1 [42]. If a single wildtype allele of BRCA1 is not enough to maintain normal functioning of BRCA1, then the cell risks additional cancer causing mutations and/or loss of the remaining BRCA1 allele. Puberty, for example, represents a time when this haploinsufficiency may have huge consequences. During this time, the epithelium of the breast is both rapidly proliferating and being exposed to elevated levels of mutagenic estradiol metabolites. If a BRCA1 mutation carrier is at increased risk during this time, it may send a cell down a tumorigenic pathway (also accounting for early onset of cancer.) This also may explain why BRCA1 gene mutations are relatively rare in sporadic cancers. By the time a BRCA1 gene or other tumor suppressor gene is lost in non mutation carriers, it is long after puberty and too late to have a significant impact [82].

Is there evidence to support BRCA1 haploinsufficiency? To date, there have been no published studies examining the role of BRCA1 haploinsufficiency in nontumorigenic breast epithelial cells, but there is evidence to suggest haploinsufficiency in other cell types. Three BRCA1 heterozygous lymphoblastoid cell lines demonstrated reduced fidelity of DNA end-joining when compared to control cell lines [83, 84]. Another study demonstrated increased radiosensitivity and impaired end-joining capabilities of fibroblasts and lymphocytes from eight BRCA1 mutation carriers relative to wildtype cells. Dermal fibroblasts from BRCA heterozygotes demonstrated reduced survival in an in vitro radiation clonogenic survival assay and lymphocytes showed an increased number of chromatid breaks after exposure to radiation [85].
Taken together, these studies present preliminary evidence of haploinsufficiency, but miss one key point: BRCA1-related cancers are tissue specific. To truly answer the question of BRCA1 haploinsufficiency, it is necessary to perform these studies in the tissue which clearly shows deficits on the clinical level. One potential explanation of tissue specificity is that either redundant activities compensate for lack of BRCA1 in the tissues that do not display increased tumorigenesis, or that unique exposure to mutagens exacerbates the BRCA1 deficiency. Additionally, growth patterns of the tissue itself may impact tissue specificity. Thus, it is key to examine the question of heterozygosity in the tissues that display tumorigenesis when BRCA1 is lost and to use genotoxic and mutagenic agents which are physiologically relevant.

C. Development of hypothesis one

Exposure to estradiol during the pregnancy of a young woman decreases the lifetime risk of breast cancer, presumably due to the ability of estradiol to induce the differentiation to mature breast structures which are less likely to undergo neoplastic transformation [86]. When the breast tissue of an older woman is exposed to increased levels of estradiol during pregnancy, the risk of breast cancer increases. Differentiation to more mature structures is only protective if the cells have not already accumulated genetic damage [87]. Interestingly, this age-related breast cancer risk pattern does not hold for BRCA1 mutation carriers. Young women carrying a BRCA1 mutation whose breast epithelium is induced to differentiate by estradiol have an increased risk rather than decreased. While the reasons for this are not completely elucidated, it is possible that the breast epithelium of mutation carriers has already accumulated genetic damage or is more susceptible to estradiol-induced mutagenesis. Other clinical observations help to
corroborate the role of estradiol in the onset of hereditary breast tumors. Sadly, the only current modifier of breast cancer risk for mutation carriers includes mastectomy and a decrease in circulating estrogen through removal of ovaries at an early age. Mutation carriers who are obese around the onset of puberty (increased levels of estradiol) have an increased lifetime breast cancer risk relative to their non-obese counterparts [87]. Male mutation carriers do not demonstrate an increased risk for breast cancer.

While the classical model of tumor suppressors states that cells of mutation carriers are undistinguishable from those of wildtype cells until the other allele is lost, the concept of haploinsufficiency raises the interesting possibility that the cellular environment in BRCA1 heterozygous cells is different than wildtype cells. This altered environment may result, in part, from decreased levels of BRCA1 protein in heterozygous cells. The majority of BRCA1 mutations result in reduced levels of wildtype protein within the cell. It is not yet known if the reduction of BRCA1 protein results in a phenotype, though this may be one explanation for the paradoxical increase in breast cancer risk following pregnancy in young women carrying a BRCA1 mutation.

Impaired or reduced levels of BRCA1 may alter the normal cellular response to estradiol on two levels. First, BRCA1 has been shown to attenuate the effects of estradiol mediated by the estrogen receptor, primarily transactivation of growth stimulatory genes [88, 89]. Growth stimulation and the subsequent accumulation of spontaneous DNA replication errors is one way in which estradiol can increase the tumorigenic potential of a cell. Second, metabolites of estradiol have been shown to be both genotoxic and mutagenic [23]. Thus, the BRCA1 heterozygous phenotype may result from two mechanisms which are not mutually exclusive and arise from the combined ability of
estradiol to act as both initiator and promoter of tumorigenesis. Reduced or altered 
BRCA1 function may not be sufficient to maintain genomic integrity and downregulate 
ER-induced growth stimulation in the presence of this unrepaired DNA damage. 

In this thesis, I examine the effects of estradiol on two populations of nontumorigenic 
breast cells, one wildtype for BRCA1 and the other carrying a single mutation 
(185delAG) of the BRCA1 gene to determine if BRCA1 haploinsufficiency confers an 
altered phenotype in response to estradiol. Chosen to represent the breast cells of 
mutation carriers before the onset cancer, both cell lines are nontumorigenic and are 
estrogen receptor negative.

The working hypothesis of this study is that BRCA1 heterozygosity will render the 
cells more susceptible to the cellular and mutagenic effects of estradiol. Specific Aim 1 
will test the hypothesis that BRCA1 heterozygosity will confer differential cellular 
changes indicative of an increased propensity for tumorigenesis following estrogen 
exposure. Specific Aim 2 will test the hypothesis that BRCA1 heterozygosity results in 
an increased rate of spontaneous and/or estrogen-induced mutations.

D. Development of Hypothesis Two

One potential target of ROS is microsatellite sequences, which are repetitive units of 
1-5 nucleotides found throughout the genome. Instability of these regions has been 
observed in many cancers and may serve as a marker for genome-wide instability. 
Alterations in microsatellite length are repaired by the mismatch repair system. Several 
researchers have investigated the role of endogenous oxidative damage in inducing MSI 
in human tumor cell lines. Reversion analyses of lung cancer cell lines demonstrated 
enhanced MSI in a (CA/GT)_{13} microsatellite following treatment with ROS-generating
chemicals [90]. Similarly, \( t \)-butyl hydrogen peroxide and N-methyl-N-nitro-N-nitrosoguanidine increased mutagenesis at microsatellite reporters in MMR-deficient colon cancer cell lines [91]. Growth of MMR-deficient human colon cancer cells in the presence of ascorbate decreased levels of spontaneous microsatellite instability in the genome [92]. While these data strongly support a role for ROS and alkylation in tumor cell MSI, it is not clear whether MSI is enhanced by endogenous DNA damaging agents in repair-competent, non-transformed cells. In particular, the MMR system is actively involved in the repair of endogenously generated DNA lesions and in the DNA damage signaling response as well as in correcting DNA synthesis errors [93]. In this thesis, I examine the ability of ROS to increase mutation frequency and induce microsatellite instability. The working hypothesis is that exposure to HP, an oxidative agent, will increase the mutation frequency and cause microsatellite instability at a \((TTCC/AAGG)_9\) microsatellite sequence located within the HSV-tk gene on a shuttle vector maintained in nontumorigenic, human lymphoblastoid cells.
Figure 1. Common mutations of the BRCA1 gene and genotypic-phenotypic correlations.
Figure 2. Protein-Protein Interactions of the BRCA1 protein. Abbreviations: BARD1, BRCA1-associated ring domain protein 1; BRCT, BRCA1 C-terminal repeat; ER-alpha, estrogen receptor alpha; HDAC1, 2, Histone deacetylase 1 and 2; Rb, Retinoblastoma Protein-1; TAD, Transcriptional Activation Domain
Chapter One: Development of experimental system to study BRCA1 heterozygosity and the effects of estradiol

A. Introduction/Rationale

Since the discovery of BRCA1 in the early 1990’s, many studies have examined cell function after complete loss of BRCA1, but few studies have addressed the state of BRCA1 heterozygosity. A handful of studies have aimed to examine potential haploinsufficiency, but have largely focused on fibroblasts or lymphocytes and stem from clinical concerns associated with hereditary breast cancers with irradiation. To our knowledge this is the first study using nontumorigenic human breast cells that are heterozygous for BRCA1. It is important that studies of BRCA1 heterozygosity utilize breast cells due to the fact that the tissue-specificity of the cancers suggests that an inherent property of the cell or their environment makes them uniquely susceptible. The use of human cells is necessary since BRCA1 heterozygous mice do not display an increased risk of breast cancer. The cell lines used in this study are also nontumorigenic, since we aim to examine the contribution of BRCA1 heterozygosity to the early initiation and promotion stages of tumorigenesis. Finally, though other chemical agents or radiation will elicit oxidative stress in breast cell lines, the tissue specificity of BRCA1-related tumors suggests a hormonal pathogenesis; thus, these studies utilize estradiol at doses which are physiologically relevant. Furthermore, redox cycling and the response of enzymes responsible for the neutralization of resulting oxidative species may respond very differently depending on the dose of estradiol. For example, high doses of estradiol (exceeding uM ranges) may initiate an apoptotic response while lower doses may result in cell cycle arrest and DNA damage repair. In order to mimic the hormonal environment in the breast cell, it is important that we strive for doses of estradiol that are
physiologically relevant. Similarly, estradiol levels fluctuate in a cyclical pattern in premenopausal women and this timing may be crucial to estradiols’ ability to induce tumorigenesis. We have tried to mimic the endogenous timing of hormonal exposure in premenopausal women using varying lengths of acute doses of estradiol.

B. Materials and Methods

B.1 Cell Lines

Two nontumorigenic human mammary epithelial cell lines, 90P and 184, were utilized in this study. Both were kindly provided by Cynthia Afshari [94]. Cell line 90P was derived from enzymatic digestion of normal tissue adjacent to a malignant breast tumor from a 35 year old female with a family history of early onset breast cancer. Cell line 184 was established from tissue removed during a reduction mammoplasty performed on a 21 year old female with no family history of breast cancer. DNA sequencing confirmed that 90P has a 185del AG in exon 2 of the BRCA1 gene, while 184 is wild type (see below). These cell lines were immortalized by infection with E6 and E7 proteins from Human Papillomavirus, type 16. Despite immortalization, both cell lines remain phenotypically normal and were judged non-tumorigenic following negative results in soft agar and nude mice studies [94]. All experiments are performed on cells lines less than 60 passage doublings. In some experiments, MCF-7 cells, provided by Maricarmen Planas-Silva (Penn State University College of Medicine), were used as controls.

B.2 Cell Culture

Cells are maintained in serum free media (Cambrex, MD or Cascade Biologics, OR) supplemented with SingleQuots of bovine pituitary extract (0.4%), human Epidermal
Growth Factor, human recombinant (3 ng/ml), hydrocortisone (0.5 ug/ml), insulin (5 ug/ml) (Cambrex, MD or Cascade Biologics, OR), transferrin (5 ug/mL) (Sigma, St. Louis), and isoproterenol (10^{-5} M). Cells were incubated at 37°C in a humidified environment with 2% CO\textsubscript{2} and were split at 70-80% confluence, neutralized with a trypsin neutralizing solution, and centrifuged prior to resuspension in media. Exposure of cells to estrogen was accomplished by the addition of estradiol (Sigma; St. Louis, MO), EtOH solvent, to the media. Solvent concentration was less than 0.1% in all experiments. For long term exposure, media and estradiol were refreshed every 2-3 days if cells were not confluent enough to be subcultured. Fresh dilutions of concentrated hydrogen peroxide (ICN Biochemicals, OH) solutions in sterile water were made for each experiment and added directly to culture media.

B.3 Confirmation of wildtype and mutant genotype

Genomic DNA was extracted from 90P and 184 cells (3-4x10^6) with a Qiagen Genomic DNA Extraction Kit, extracted with isopropanol, and washed with 70% EtOH. Concentration and purity of gDNA was determined by measurement of absorbance at 260 and 280 nm. PCR was performed on 2 and 200 ng gDNA utilizing a GeneAmp Gold PCR kit (PE Biosystems, CA) with the following primers for the BRCA1 gene: forward 5′ AAG GAC GTT GTC ATT AGT TC 3′ and reverse 5′ AAT CAG CAA TTA CAA TAG CC-3′. Cycling parameters were as follows: 95°C for 5 min, 35 cycles of 95°C for 45 s, 55C for 1 min, and 72°C for 1 minute. Product was purified with a Qiagen PCR Purification Kit and the concentration and purity was analyzed by spectrophotometry as described above. Sequencing PCR on 40 ug purified PCR product was performed with
the forward primer and reagents from the CEQ2000 Quick Start Kit (Beckman Coulter; Fullerton, CA) and the following cycling program: 30 cycles of 96°C for 20 sec, 50°C for 20 sec, and 60°C for 4 min. Samples were EtOH precipitated and resuspended in sample loading buffer prior to DNA sequence analysis on the CEQ 8000 Genetic Analysis System (Beckman Coulter).

B.4 Karyotyping of 90P and 184 cell lines

Cells were seeded onto coverslips and allowed to grow to approximately 60-70% confluence. Cells were mitotically arrested with colcemid and stained with BrDu for 24 hours. Cells were washed and incubated in prewarmed 0.8% prewarmed hypotonic solution for 35 minutes followed by fixation of cells in methanol/acetic acid (3:1). Initial fixation occurred for 25 minutes before the solution was removed and replaced with fresh fixative twice for an additional 60 minutes. Cells were transported to the Cytogenetics facility for further analyses. Chromosomal spreads were analyzed by Lee Gannutz (Penn State College of Medicine), certified cytogenetic technician. Two spreads were obtained for 184 cell line while eleven were obtained for the 90P cell line.

B.5 Western Blot Analysis of p53 and Rb

Cells (6x10^6) were collected and lysed overnight at 4°C followed by centrifugation at 14,000 rpm for 15 min at 4°C. Protein amounts ranging from 20-40 ug were heated to 85°C for 3 minutes and loaded onto an 8% acrylamide gel and electrophoresed at 150 V for 1-2 hrs. Protein was transferred to a PVDF membrane, blocked for 1 hour with 5% nonfat milk in TBS Tween, and then blotted with p53 (Oncogene) or Rb (Oncogene) antibody. Protein bands were visualized with an ECL Plus Kit and volume in each well was quantified with a phosphoimager and Image Quant software. Membranes were
stripped by incubation in Restore Western Blotting Stripping Buffer (Pierce) and reprobed with B-actin antibody.

B.5 Determination of Estrogen Receptor-alpha (ERα) status

B.5.1 Western blot analysis of ERα

Cell lysates was prepared as described above and used for Western blotting. Membranes were blotted with rabbit polyclonal IgG (HC-20) to ERα at a dilution of 1:1000. Detection of ERα was determined with an ECL Plus Kit (Amersham) and phosphoimager and quantitative comparisons were made with ImageQuant software.

B.5.2 Immunoprecipitation of ERα

Cells (6x10⁶) were collected and lysed overnight at 4°C followed by centrifugation at 14,000 rpm for 15 min at 4°C. Equal amounts of lysate from each cell line were precleared with 1 µg normal mouse IgG (Oncogene) and 20 µL Protein A/Protein G Agarose Conjugate (Santa Cruz, CA) for 1 hr at 4°C. Agarose beads were pelleted at 1500 rpm and protein concentration of supernatant was determined with a protein assay kit (Pierce, Illinois). Total protein (1 mg) was incubated with 1 µg rabbit polyclonal IgG (HC-20) to ERα (Santa Cruz, CA) and 15 µL packed agarose beads overnight at 4°C with gentle rocking. Agarose beads were collected by centrifugation at 2500 rpm for 15 minutes at 4°C. Pellet was washed four times with 1 ml PBS and spun as above. Following final wash, beads were resuspended in 35 µL electrophoresis buffer, heated at 85°C for 3 minutes and loaded onto an 6% acrylamide gel for Western Blot analyses. Samples ran at 150 V for approximately 1-2 hours. Following hybridization to membrane, blotting was performed with the same ERα antibody used for immunoprecipitation according to standard procedures. Detection of ERα
immunoprecipitates was determined with an ECL Plus Kit (Amersham) and phosphoimager and quantitative comparisons were made with ImageQuant software. Two independent experiments were performed.

B.6 BRCA1 Protein Levels

Cells were collected and used in immunoprecipitation and Western blotting as described above. Immunoprecipitation and Western blotting utilized BRCA1 Ab-1 and Ab-3 (Oncogene), respectively. BRCA1 protein levels were determined in two independent experiments and volume quantification was performed as described above to allow for comparisons between BRCA1 wildtype and heterozygous cell lines.

B.7 DCF Assay

90P and 184 cells were seeded (5x10^3 per well) into a 96 well plate in media and were divided into the following groups: ETOH and DMSO solvent control, estradiol (1x10^{-10}, 1x10^{-9}, 1x10^{-8}, 1x10^{-7}, and 1x10^{-6}) and 300 µM hydrogen peroxide. Cells were loaded with 5 uM 5’,6’-chloromethyl-2’,7’ dichlorodihydrogen-fluorescein diacetate (CM-H2DCFDA) (DMSO solvent) (Molecular Probes, OR) for 1 hour prior to initiation of treatment. Estradiol and HP were added to the media and treatment continued in the dark at 37°C for 2, 15, 30, 45, and 60 minutes. Fluorescence was quantified using a fluorescent plate reader at an emission and excitation wavelengths of 485±10 nm and 530±12.5 nm, respectively. For each treatment, a ratio of fluorescence in treated group relative to that seen in untreated control was calculated. Two separate populations of each cell line plated in triplicate were used in these experiments. Ratios of treated to
untreated were used in ANOVA and Tukey-Kramer Multiple Comparisons Test to analyze statistical significance of results.

B.8 Lipid Peroxidation Assay

Approximately $4 \times 10^8$ 90P and 184 cells were seeded into 4 T150 flasks containing media and designated as control, 10 nM estradiol, 50 nM estradiol, or 300 uM hydrogen peroxide. Estradiol (EtOH solvent) and HP were added directly to the media and incubated 24 hours. Cells were trypsinized and resuspended in 200 µL or 1mL of distilled H$_2$O, in cases where high cell number made single cell suspension difficult. 20 µL of cell solution were removed and counted using a hemacytometer prior to cell lysis by the freeze-thaw method. Cell lysates were transferred to glass tubes. A lipid peroxidation kit (Calbiochem) containing reagents which react with malondialdehydes and 4-NHE to yield a colorimetric product were utilized to determine levels of lipid peroxides in each group per kit protocol. Following incubation with reagents, the samples were centrifuged at 150,000 x g for 15 min at 4°C to remove cellular debris. Supernatant was analyzed by spectrophotometry at 586 nm and levels of MDA and 4-NHE were determined utilizing standard curves prepared according to kit instructions. Following 6 independent experiments, mean levels (±SEM) of MDA and 4-NHE in each group were normalized to cell number and compared statistically using ANOVA.

C. Results

C.1 Confirmation of wildtype and mutated BRCA1

Genomic DNA was used in PCR to amplify the region of chromosome 17 containing the BRCA1 gene and the DNA sequence of the resulting PCR product was determined.
Genomic DNA from cell line 184 was confirmed wildtype, as compared to the GenBank sequence, while 90P contained two overlapping sequences after the 184 position, signifying heterozygosity. A deletion of AG was found at position 185 along with a wildtype sequence. These data confirmed that reported by Annab and Afshari following single strand confirmation and direct sequencing analyses [94].

C.2 Karyotype analyses

Karyotypes were obtained to assess ploidy and the gross chromosomal conditions of cell lines 90P and 184. Representative spreads are shown in Figure 1.1 and 1.2. Analysis of multiple cells from each cell line indicate a largely diploid cell line with some consistent gross chromosomal alterations at a small number of chromosomes, as identified by the cytogenetic technician aided by a computer analysis program.

Two chromosomal spreads were obtained for the 184 cell line (Figure 1.1). Both spreads were diploid and the modal chromosome number was 46. One spread contained a chromosome which could not be identified, an abnormal chromosome 12, and a missing copy of chromosome 21. The second spread contained three unidentifiable chromosomes, missing copies of chromosomes 21 and 22, and an abnormal copy of chromosome 20.

Eleven 90P karyotypes were analyzed. This cell line is largely diploid, with only two spreads showing tetraploidy. The modal chromosome number is 46, with 7 of the 11 spreads showing a numerical alteration. There was an average of 1.5 structurally altered chromosomes per spread. Most abnormalities were seen at chromosomes 12, 15, 20, 21, and 22. The majority of the cells had an abnormal or missing copy of chromosome 21. In six of nine spreads, one copy of chromosome 12 had extra chromosomal material.
Similarly, chromosome 20 was abnormal or missing in five spreads and a single spread had an extra copy of chromosome 20. Three and four spreads demonstrated abnormal or missing copies of chromosomes 15 and 22, respectively. Though most spreads identified the loss of at least one chromosomal copy, each spread also had at least one unidentifiable chromosome, which may represent one of the missing chromosomes reported above with translocation material that precluded its proper identification. Importantly, no changes were observed at Chromosome 17 in the 90P cell lines, indicating that the cells are still heterozygous for BRCA1.

C.3 Protein Levels of p53, Rb, BRCA1, and ERα

Cell immortalization with HPV proteins E6 and E7 affects p53 and Rb protein levels, respectively. As important players in the process of tumorigenesis, absent or differential levels of these proteins in 90P and 184 cell lines may impact the interpretation of results. To address this, we compared levels of p53 and Rb in 40 µg of total protein from 90P and 184 using Western blots and ImageQuant software. In order to compare levels of these proteins with other commonly used cell lines, we also assessed levels of p53 and Rb in MCF7 and MCF10 cell lysates. Lysate (20 µg) from Molt-4, a tumorigenic lymphoblastoid cell line, served as positive control.

Both 90P and 184 cell lines show the presence of p53 protein (Figure 1.3 Panel A, Table 1). Immortalization of both cell lines also appears to have affected the p53 levels similarly as both cell lysates display roughly equivalent levels of p53. In contrast, absent or significantly reduced levels of p53 were found in MCF-7 and MCF-10 cell lysates. This analysis indicates that p53 protein is intact and present in roughly equivalent quantities in these 90P and 184 cell lines. In comparison to Molt-4 cells which
overexpress Rb, our cell lines showed significantly less protein (Figure 1.3, Table 1). Quantification revealed very low levels of protein in all cell lysates. Of these, 184 cells had the highest levels of protein, followed by 90P and MCF-10, with the least amount being seen in the MCF7 cell line. In contrast to p53, levels of Rb in our nontumorigenic cell lines appear to be roughly equivalent to those seen in MCF7 and MCF10 cell lines. 90P cells had Rb protein levels one half that seen in 184 cells. Though difficult to gauge the functional significance of these results, we are able to conclude that Rb levels are reduced relative to Molt-4.

The estrogen receptor is responsible for the initiation of transcription of genes containing estrogen responsive elements, many of which are involved in growth regulation. Thus, differential levels of ERα in 90P and 184 cell lines could potentially impact the cellular response to estradiol and complicate the comparison of 90P and 184 following exposure to estradiol. Using MCF-7 cells as a positive control, Western blot analyses revealed both 90P and 184 cells had reduced levels, 36 and 39 %, respectively, of ERα relative to MCF7 cells (Figure 1.3 Panel B, Table 1). These results suggest that these cell lines are ERα negative, and suggest that 90P and 184 have equivalent levels of ERα. To confirm that the levels of ERα in the 90P and 184 cell lines are indeed comparable, an immunoprecipitation of ERα was performed and these results are presented and quantified in Figure 2, Panel C and Table 1. Combined, these experiments suggest that the cell lines in use are ERα negative and also contain equivalent levels of ERα.

In order to determine if BRCA1 heterozygosity altered the level of BRCA1 protein, we performed immunoprecipitations for BRCA1 proteins. A representative experiment is
shown in Figure 1.3, Panel D and Table 1. In both experiments 184 wildtype cells had more BRCA1 protein than the 90P heterozygotes (2 and 4 fold, respectively).

C.4 DCF Assay

CM-H$_2$ DCFDA is the acetyoxymethyl ester of the fluorescent dye DCF. The addition of the acetate group confers a lipophilic state that allows for passive diffusion through the cell membrane. Once inside, intracellular esterases cleave the acetate groups and trap the dye inside the cells. The resulting nonfluorescent molecule, DCFH, can interact with reactive oxygen species to form the highly fluorescent DCF. This dye has been used as a very sensitive monitor of intracellular oxidative stress, as levels of fluorescence are directly proportional to levels of free radicals. In this experiment, DCFDA was employed to detect oxidative stress generated following exposure to five doses of estradiol ranging from $1 \times 10^{-10}$ M to $1 \times 10^{-6}$ M and 300 $\mu$M hydrogen peroxide as a positive control. This dose of HP was chosen based upon previous experiments (data not shown) in which this dose was found to be 70-80% cytotoxic to cells following a 24 hour exposure, consistent with the design of a mutagenesis experiment. The control group treated with only EtOH and DMSO was included to control for solvent effects. Measurements of DCF fluorescence were taken at 1,15, 30, 45, and 60 minutes following HP or estradiol addition. HP resulted in a large increase in ROS relative to control with maximal levels observed after 45-60 minutes of exposure (Figure 1.4, Panel A). In 90P cells, ROS rose nearly 30% higher than untreated control at 45 minutes, after which ROS steadily dropped to baseline levels. In 184 cells, the levels of ROS rose nearly 40% higher than untreated control at 60 minutes.
DCF fluorescence increased as a function of estradiol dose and time of exposure (Figure 1.4, Panels B and C). Though higher doses resulted in a measurable increase in ROS almost immediately, maximal levels of ROS were observed 30 minutes following treatment. By 60 minutes, ROS levels had returned to levels equivalent to or less than that seen in solvent control. At the estradiol dose of $1 \times 10^{-10}$ M, no statistically significant increase in ROS could be detected in either cell line at any timepoint. At the dose of $1 \times 10^{-9}$ M, an increase of 5-10% of ROS relative to the solvent control was seen in both cell lines, although this increase did not reach statistical significance. A statistically significant increase in ROS (approximately 13% in both cell lines) was observed following exposure to $1 \times 10^{-8}$ estradiol for 30 minutes ($90P \ p<0.01$, $184 \ p<0.001$) but by 45 minutes, ROS in this group had receded to baseline levels. Treatment with $1 \times 10^{-7}$ M estradiol resulted in a statistically significant increase in ROS in $90P$ cells at both 15 and 30 minutes ($90P \ p<0.05$ and 0.01 for 15 and 30 minutes, respectively) and in $184$ cells at 30 minutes ($p<0.01$). In $90P$ cells exposure to $1 \times 10^{-6}$ M estradiol increased levels of ROS around 7% relative to untreated control within the first minute of estradiol addition ($p<0.05$) and remained elevated 12% at 15 minutes ($p=0.001$) and 25% at 30 minutes ($p<0.001$ for both) before receding to baseline levels at 45 minutes. Similarly, in $184$ cells exposure to $1 \times 10^{-6}$ M estradiol increased levels of ROS significantly (10%) at 15 minutes ($p<0.05$). Maximal ROS from this dose was observed at 30 minutes (12%, $p<0.01$) and remained elevated 6.7% at 45 minutes ($p<0.05$) before receding to baseline levels at 60 minutes. To determine if $90P$ and $184$ experienced the same level of ROS following estradiol exposure, a Mann-Whitney test compared DCF fluorescence at 30 minutes when ROS levels are maximal for the two doses closest to those used in future
experiments (10 and 50 nm). The increase in ROS following exposure to estradiol is not statistically different in 90P and 184 cells (1x10⁻⁷ p=0.11 and 1x10⁻₈ p=0.89). These studies confirm that estradiol exposure induces ROS and that the levels of ROS are comparable in the two cell lines.

C.5 Lipid Peroxidation Assay

Following exposure to estradiol for 24 hours, lipid peroxide byproducts were measured with a kit that spectrophotometrically detects malondialdehyde and 4-hydroxynonenals. This experiment was repeated six times and data shown are mean values with standard error of the mean. ANOVA statistical analysis was performed.

Both 90P and 184 cells had 0.5 uM of lipid peroxides present in the untreated control. Hydrogen peroxide treatment doubled the level of lipid peroxides in 90P and increased the level by 30% in 184 treated cells (Figure 1.5). Following exposure to both doses of estradiol, lipid peroxides increased in both 90P and 184 cell lines. For 90P cells, the amount of lipid peroxides increased 2 and 3-fold following 10 and 50 nm estradiol, respectively. Estradiol-treated 184 cells had 2.8 and 2.2-fold higher levels of lipid peroxides following 10 and 50 nm estradiol treatment. The variability among experiments prevented these increases from reaching statistical significance (p=0.3 and 0.9 for 90P and 184, respectively). Thus, we conclude that estradiol and hydrogen peroxide treatment produced a qualitative increase in the level of lipid peroxide products. The degree of lipid peroxidation was similar among 90P and 184 cell lysates, reinforcing earlier observations that the oxidative treatment is equivalent in the two cell types despite potential differences in metabolic enzymes.
4. Discussion

At the onset of this project, the goal was to identify nontumorigenic, human, breast, epithelial cells that were either wildtype or heterozygous at the BRCA1 gene and shared the same estrogen receptor status. Since most hereditary breast tumors are ER negative, this status was preferable though not necessary [95]. An exhaustive search of cell repositories and published cell systems yielded only those chosen for this study. Though we would have preferred isogenic cell lines, those available are mostly heterozygous and null for BRCA1 or are derived from tumors. Construction of a heterozygote from null was also not desirable due to the technical difficulty associated with the restoration of BRCA1 to physiologically relevant levels.

The 90P and 184 cell lines have been used in a limited number of publications and little is reported about their genetic background. Our chromosomal analysis of these cell lines was very promising. Both lines are diploid and have few numerical alterations. Some chromosomes were consistently altered, but these did not include chromosome 17, where the BRCA1 gene is located. To place our findings into context with other cell lines used frequently in breast cancer studies, we compared karyotypes from our cell lines to the karyotypes of MDA-MD-231 and MCF7 breast cancer cell lines [96]. In contrast to the modal chromosome number of 46 in the 90P and 184 cell lines, MDA-MD-231 and MCF7 breast cancer cell lines have a modal chromosome number of 54 and 65, respectively. MCF7 cells were classified as triploid. Thus, these data suggest that our cell lines meet the initial requirement that they are as normal as possible in an
immortalized cell line. To facilitate this, we also used cells that had undergone fewer than 60 population doublings for our experiments.

Genetic heterozygosity has the potential to influence the levels of protein in a cell. In our system a single wildtype allele of BRCA1 appears to reduce the level of BRCA1 protein 2-4 fold. The functional significance of this reduction in protein levels and an analysis of the BRCA1 protein levels following exposure to estradiol will be examined in future experiments in this thesis.

Extensive effort was put forth to characterize levels of key proteins in the 90P and 184 cells used in this study since they are not from a single donor. To our advantage, both cells are immortalized via the same mechanism. In this case, HPV-16 will allow for immortalization of cells through alteration of p53 and Rb. Potentially different levels of p53 and Rb might impact the cellular response to DNA-damaging agent. However, these cell lines appear to have similar levels of p53 and both lack Rb almost completely. One disadvantage to this system is that BRCA1 may mediate some of its effects through interaction with p53 and/or Rb. BRCA1-mediated cell cycle arrest, for example, may occur by BRCA1 binding to p53. However, much evidence exists to suggest multiple pathways by which BRCA1 can exert a cellular influence, including a p53 independent cell cycle arrest [97]. Though p53 and Rb activity may be altered due to the immortalization, our analyses indicate that they will be altered similarly in both cell lines.

The same conclusion holds true for the estrogen receptor status of the cells. Previously reported studies of 184 have classified them as ER negative and have used them for studies where the ER was reconstituted using plasmid expression vectors. Most hereditary breast tumors are ER negative, thus the status of these cell lines as ER negative
is fortunate [80]. We are primarily concerned with the ability of estradiol to initiate ER-independent DNA damage; however, one major mechanism of estradiol action is mediated by the ER, making it essential that the cell lines used in this project share the same status. We were able to detect ER in lysates from these cells, but in a significantly lower quantity than that seen for the MCF7 cells, a strongly ER positive cell line.

The tissue specificity of BRCA1-related tumors hints at a role for hormones in tumorigenesis. Some studies have addressed the cellular response to acute doses of high levels of estradiol or its metabolites. In the case of a mutation carrier, however, breast tumors arise after years of BRCA1 heterozygosity and exposure to estradiol. Thus, we were interested in studying physiologically relevant doses of estradiol, yet these doses had to be high enough to detect a measurable increase in intracellular ROS. Across a broad range of estradiol doses from $1 \times 10^{-9}$ to $1 \times 10^{-6}$ M, an increase in ROS was detectable. Thus, we are able to conclude that physiologically relevant levels of estradiol can induce oxidative stress in breast cells.

These experiments also yielded another important comparison of 90P and 184 cells. While HP exposure can produce ROS without any conversion by cellular enzymes, estradiol must first undergo metabolism, during which free radicals are produced. There are a multitude of enzymes which modulate estradiol biosynthesis, metabolism, and the removal of metabolites. The CYP1A1 gene encodes enzymes which hydroxylate estradiol to form the hormonally inactive water soluble metabolites 2 and 4-hydroxyestradiol. Polymorphisms of this gene have been shown to influence circulating levels of estradiol and breast cancer risk [98]. Similarly, polymorphisms in the catechol-O-methyltransferase gene, which encodes the enzyme that deactivate catecholestrogens,
may also modify breast cancer risk [99]. Given that our cell lines are from two donors, there was some concern that different polymorphisms in these genes or varying levels of these proteins might influence the oxidative load resulting from a given dose of estradiol. Despite potential differences, these data do not validate that concern. Neither the level of ROS nor the lipid peroxides following exposure to estradiol were statistically different between the two cell lines. This suggests that 90P and 184 cell lines experience equivalent oxidative stress following each given dose of estradiol. Thus, we can conclude that estradiol treatments in the following experiments stress each cell line to similar degrees and that differential results are not due to different ROS loads.

These experiments serve to characterize and establish 90P and 184 cells as a valid model system to address BRCA1 haploinsufficiency. This concept is best probed through exposure of the cells to a compound which has been implicated in breast tumorigenesis and at levels that are physiologically relevant. Exposure to estradiol resulted in a measurable increase in ROS and lipid peroxides. Further experiments will address the cellular and mutagenic consequences of this increase in oxidative stress and the potential contribution of BRCA1 heterozygosity.
Figure 1.1. Representative spreads from karyotype analyses of 184 cell lines. (A) Chromosomal spread displaying single missing copies of chromosomes 8 and 22, two missing copies of chromosome 21 (red arrows), and three unidentified chromosomes (red box). (B) A second chromosomal spread of 184 displaying an abnormal chromosome 12, a missing copy of chromosome 21, and an abnormal chromosome which appears to be a third copy of chromosome 20 with additional chromosomal material, perhaps due to a translocation event.
Figure 1.2 Representative karytype analyses of 90P cells. (A) Chromosomal spread demonstrating missing copies of chromosomes 14 and 21, an abnormal chromosome 20 (red arrows), and one unidentifiable chromosome (red box). (B) A second spread showing similar alterations: abnormal chromosomes 12 and 20, a missing copy of 21, and an additional copy of chromosome 22.
Figure 1.3. Characterization of cell lines  (A) Western Blot analysis of p53 and Rb protein levels in 90P, 184, MCF7, MCF10A, and positive control lysate from MOLT-4. (B) Western blot analysis of estrogen receptor alpha (ERα) levels in 90P and 184 relative to ER+ MCF7 cell lysate. (C) Immunoprecipitation of ERα from 90P and 184. (D) BRCA1 protein levels, as detected by immunoprecipitation.
Figure 1.4 DCF fluorescence following exposure of 90P and 184 cells to estradiol or HP. (A) DCF fluorescence in cells treated with 300 µM HP for varying lengths of time. Blue diamonds, 184; Pink squares, 90P. (B) DCF fluorescence in 90P cells treated with varying doses of estradiol. (C) DCF fluorescence in 184 cells treated with varying doses of estradiol.
Figure 1.5 Lipid peroxides following exposure to estradiol. Bars correspond to solvent only control (gray bars), 10 nm estradiol (purple bars), 50 nm estradiol (white bars), or 300 uM HP (black bars). Data represent the mean values from six independent experiments ±SEM.
Table 1. Comparison of p53, Rb, ERα, and BRCA1 protein levels among cell lines

<table>
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<tr>
<th>Cell Line</th>
<th>Protein Relative to Wildtype (184 cells)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>p53</td>
</tr>
<tr>
<td>184</td>
<td>1.0</td>
</tr>
<tr>
<td>90P</td>
<td>1.28</td>
</tr>
<tr>
<td>MCF-7</td>
<td>0</td>
</tr>
<tr>
<td>MCF-10</td>
<td>0.068</td>
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<tr>
<td>Molt-4</td>
<td>0.91</td>
</tr>
</tbody>
</table>
Chapter Two: Genotoxicity and mutagenicity of estradiol

A. Introduction/Rationale

The earliest studies concerning the genotoxicity and mutagenicity of estradiol utilized a variety of tissue types, some of which are hormonally responsive, and a broad range of estradiol doses. As evidenced by numerous disparate reports in the literature, the effects of estradiol exposure are highly cell type and cell line specific. Similarly, dosage of estradiol is critical, as low doses of estradiol can have very different effects than high doses. The most recent studies of estradiol have utilized hormonally-responsive tissues and doses of estradiol that are physiologically relevant in order to accurately determine the role of estradiol in endogenous stress and its potential contribution to tumorigenesis. Russo et al published a recent report demonstrating that acute, low doses of estradiol are sufficient to induce neoplastic transformation of human mammary epithelial cells [37]. In Chapter 1, we have demonstrated that exposure to physiologically relevant levels of estradiol significantly increases intracellular ROS in breast epithelial cells. In this thesis chapter, I tested the hypothesis that estradiol-induced ROS results in DNA damage, as measured by an increase of 8-oxoG. As reviewed in the introduction, 8-oxoG is one of several potential ROS-modified bases. Due to the prevalence of 8-oxoG following ROS induction and its demonstrated mutagenic potential, an antibody to 8oxoG has recently been developed to facilitate more sensitive studies of both endogenous and ROS-induced 8oxoG. This antibody allows us to examine levels of 8oxoG using immunocytochemistry. This type of experiment is superior to earlier methods that involved extraction of DNA, a procedure that produces oxidative modifications and increases background.
The use of the hypoxanthine–guanine phosphoribosyltransferase (HPRT) gene to measure somatic mutations was developed after the discovery of this gene’s involvement in Lesch-Nyhan Syndrome, a neurological disorder characterized by severe mental retardation and tendencies toward self-mutilation. HPRT encodes an enzyme that salvages hypoxanthine and guanine for nucleic acid biosynthesis and phosphoribosylation. This enzyme also phosphoribosylates purine analogues, creating the basis for the HPRT mutational assay [100]. When exposed to 6-thioguanine (6TG) or 8-azaguanine, cells with a functional HPRT gene will convert these compounds to their cytotoxic forms while mutant cells will be resistant to these compounds. HPRT is a single copy gene located on the X chromosome in humans. Due to X inactivation, a single mutation in this gene will confer resistance to purine analogues in cells from both males and females [100]. One advantage to this system is that all types of mutations can be detected with this reporter gene and, if desirable, PCR and direct sequencing of the gene can be utilized to determine the mutational spectrum. This method of measuring mutagenesis has been utilized in a broad spectrum of tissue types to determine spontaneous and induced mutation frequencies following exposure to many different classes of mutagens [101-103]. The advantage of this system is that the HPRT gene is an endogenous locus and doesn’t necessitate establishing a stably-replicating plasmid based mutagenesis system. In plasmid based systems, however, multiple copies of plasmid can be maintained episomally, while only one mutational target (the HPRT gene) is present per cell in the HPRT mutation assay; thus, use of this assay necessitates plating large numbers of cells in order to view enough mutational events to make statistically significant conclusions.
B. Methods and Materials

B.1. 8-oxo-G immunocytochemistry

Immunocytochemistry protocols are somewhat altered when the target is DNA, due to the potential for preparation artifacts. This experiment followed the protocol suggested by the manufacturer of the 8-oxoG antibody. Briefly, cells were seeded onto treated glass slides and grown to a density of 60-70% and a hydrophobic pen was used to define three regions per slide. Cells were exposed to an EtOH control, 50 nM or 1 µM estradiol, or 300 µM HP for 30 minutes, 1 hour, or 3 hours. Control groups which utilized either primary or secondary antibody alone were treated with 1 µM estradiol. After exposure, cells were fixed with 70% EtOH for 10 minutes at RT. RNaseA (100 µg/mL in 10 mM Tris pH 7.5, 0.4 mM NaCl 1 mM EDTA) was added for 1 hour at 37º C. The DNA was denatured with 4N HCl for 7 minutes and neutralized with Tris-base (50 mM). To block nonspecific sites, slides were incubated in FBS (1% in 10 mM Tris-HCl, pH 7.5) for 1 hour at 37ºC. Antibody to 8-oxoG was added to the blocking media at a final concentration of 1:300 and was incubated ON at 4ºC. The slides were covered with biotin-conjugated universal secondary antibody and incubated for 30 minutes at 37ºC. To quench endogenous peroxidase, the slides were incubated in 3% HP in methanol for 30 minutes at RT, followed by incubation with streptavidin-HRP in PBS for 30 minutes at RT. Between each step, the slides were washed with excess PBS at least three times for greater than 2 minutes, with the exception of the final wash, which was supplemented with 1% Triton X-100. DAB solution (0.5 µg/mL with 0.001% HP in PBS) was added to the slides and incubated at RT for 10 minutes, followed by two washes with distilled water before mounting in water soluble mounting medium.
B.2 HPRT Mutagenesis

B.2.1 Determining sufficient levels of selection agent

Cells were seeded into 24 well plates and grown to 60-70% confluence. 6TG was added to the wells ranging from 0-100µM. After 5 days, wells were visually observed for cytotoxicity. Both cell lines showed equivalent cytotoxicity at all doses tested. The concentration which resulted in complete cell death within four days of treatment was chosen (5 µM) and future experiments used 4-times this level (20 µM), per standard procedure.

B.2.2 Determining mutation frequency

Cells (1-3x10^7) were treated with EtOH control, 50 nM estradiol, or 300 µM HP for 48 hours. Following treatment, cells were subcultured at a density of 1:3 and allowed to grow to 70% confluence. Once cells had reached confluence (approximately 3-4 population doublings), they were trypsinized and reseeded at a density of 6x10^5 cells into approximately 200 P100 dishes per treatment group. 6-TG was added to the media at a concentration of 20 µM 24 hours after plating. Exposure to 6-TG continued for 5 days, after which 6-TG-containing media was removed and replaced with fresh media. After 8-10 days dishes were stained with Giemsa and colonies greater than 5 cells were counted. Mutation frequency was determined by dividing the number of 6TG-resistant colonies by total number of cells plated, and then correcting for cloning efficiencies of cells. The cloning efficiency of each treatment group was determined during each experiment by plating 100 cells into P100 dishes in the absence of 6-TG. Colonies were stained with Giemsa and counted 7-10 days later. This experiment was performed twice for each cell line.
C. Results

C.1 8-oxoG levels following exposure to oxidative stress

Estradiol at 1 µM has been shown to result in the formation of 8oxoG, thus this concentration was used as a positive control. When either wildtype or heterozygous cells were treated with this concentration of estradiol, we detected a qualitative increase in bound antibody, as evidenced by intracellular brown staining, which was localized to the nucleus. This observation confirmed that the antibody was specific for DNA, as previous experiments in which the RNase treatment was not performed detected 8oxoG in the cytoplasm and nucleus. When primary or secondary antibody was excluded, we detected no signal (Figure 2.1, Panels A and B). Exposure to 50 nM estradiol and 300 µM HP resulted in a detectable levels of 8-oxoG at 30 min, 1 hour, and 3 hours, with staining being most intense at three hours. Representative examples of 30 minute and 3 hour timepoints are shown in Figures 2.2 and 2.3. We are unable to make quantitative comparisons between wildtype and heterozygous treated cells. Qualitatively, we are able to conclude that both wildtype and heterozygous cell lines demonstrate DNA damage in the form of 8oxoG following exposure to estradiol. This result is consistent with the lipid peroxidation assay (Figure 1.5), which demonstrated an increase in ROS-induced lipid peroxides in both cell lines following exposure to estradiol.

C.2 HPRT Mutation Frequency Analyses

Two independent experiments were performed for each cell line. Due to the large number of cells required to obtain enough mutant colonies for each analysis, we selected only the 50 nM estradiol treatment and used 300 µM HP as a positive control.
The average mutation frequency in the solvent only control wildtype cell line was determined to be $6.7 \times 10^{-7}$ for two independent experiments (Table 2). The average mutation frequency for the HP-treated cultures was similar to the control mutation frequency ($6.0 \times 10^{-7}$), despite high levels of ROS and the presence of 8-oxoG (Figures 2.2 and 2.3). The average estradiol-induced mutation frequency was $1.3 \times 10^{6}$, representing approximately a 2-fold increase in MF relative to the control mutation frequency. Given these results, we cannot conclude that estradiol significantly increased the mutation frequency. Further experiments are required to determine if these values reflect a true increase or are simply within the range of the background mutation frequency.

The average spontaneous mutation frequency in heterozygous cells was $3.0 \times 10^{-7}$ in two independent experiments (Table 2). Following exposure to HP, this value robustly increased to $1.8 \times 10^{6}$ (6-fold relative to control). The estradiol-induced mutation frequency was $1.1 \times 10^{6}$, representing a 3.7-fold induction of mutations relative to the average control mutation frequency.

**D. Discussion**

During the initial characterization of our model system, estradiol treatment resulted in increased DCF fluorescence corresponding to increased intracellular oxidative stress and a qualitative increase in lipid peroxidation products. We expanded these results in this chapter by demonstrating that estradiol treatment increased levels of nuclear adducts, as measured by 8-oxoG, indicating that the ROS induced were genotoxic. Exposure to estradiol or metabolites of estradiol has been shown previously to induce formation of 8-oxoG in hamsters [29]. In MCF-7 cells depleted of glutathione, 100 nM estradiol resulted in a 145% increase in 8-oxoG [104]. 8-oxoG is only one of several base
modifications resulting from oxidative damage. The availability of an antibody allowed
us to monitor levels of 8-oxoG as a marker of DNA damage following oxidative stress.
These results demonstrate that estradiol-induced ROS is genotoxic; furthermore, the
increased mutation frequency following exposure to estradiol suggests that under some
conditions estradiol also acts as a mutagen.

The average spontaneous mutation frequency of 184 and 90P cells was 6.2x10^{-7} and
3.0x10^{-7}, respectively. This is consistent with a report by Watanabe et al, which
identified a mutation frequency of 1.8x10^{-7} in a human, nontumorigenic, mammary
epithelial cell line [101]. An initial aim of this project was to compare the untreated
mutation frequency of each cell line to determine if BRCA1 heterozygosity resulted in an
increased spontaneous mutation frequency. We hypothesized that heterozygous cells
would have higher spontaneous mutation rates; however, we found that the mutation
frequency was somewhat higher in the wildtype population. Rather than being a
reflection of the repair status of the cells, this most likely reflects the manner in which
184 cells were immortalized. Cultures of mammary epithelial cells were exposed to
benzopyrene and those cells which continued to grow were selected and cultured for
longterm use [105]. These cell lines have also been in existence since the 1970’s and the
population doublings of the cells used by Afshari for HPV immortalization is unknown.

The ability of estradiol to induce mutations has been reported in the literature.
However, often the model systems used for these experiments are nonmammary or
tumorigenic. Much of the work done by Liehr and colleagues utilized renal tissue in
hamsters. Thus, we were interested in the mutagenic potential of physiologically relevant
levels of estradiol in mammary epithelial cells that are nontumorigenic. A recent report
by Russo et al demonstrated that estradiol exposure (0.007 and 70 nM) resulted in an increased colony efficiency and decreased ductalogenesis consistent with neoplastic transformation of immortalized but nontumorigenic human mammary epithelial cells, suggesting that estradiol may be acting as a mutagen in these cells [37]. In this chapter, we demonstrate that exposure to estradiol increased the mutation frequency at the HPRT locus two and 3.7-fold in wildtype and heterozygous cell lines, respectively. It is debatable whether or not the 2-fold increase seen in 184 cells is statistically significant, and more analyses would be necessary to determine if this increase is due specifically to estradiol exposure. However, the 3.7-fold increase in mutation frequency in heterozygous cells is more relevant. This increase is consistent with the reported increase in mutations at the HPRT gene in Chinese hamster V79 cells exposed to estradiol. At doses of 0.1 and 100 nM, the mutation frequency was increased 2.5-3.5-fold, respectively, from an untreated mutation frequency of 7.4x10^{-7} (Kong, Intl J. Oncology 2000). The fact that exposure to physiologically relevant levels of estradiol creates even a small increase in mutation frequency is noteworthy. An early criticism of the hypothesis that estradiol is a mutagen was the fact that the most mutagenic metabolites of estradiol are produced in the least quantities, and estradiol was not detected as a mutagen in the standard Ames test. Liehr and others asserted that if estradiol was a stronger mutagen, its presence would not be compatible with life [106]. In addition, redox cycling of estrogen metabolites creates ROS that are capable of causing mutations independently of the metabolites themselves [26]. Our results confirm those seen in other model systems that estradiol has the potential to be both genotoxic and mutagenic. Thus, the benefits of estradiol such as the induction of breast differentiation to mature structures less likely to undergo neoplastic
transformation must be balanced with the ability of estradiol to cause damage to DNA and induce mutations [87]. In a normal cell, protective measures are in place to ensure that this balance is maintained and the effect of estradiol remains favorable. BRCA1 may represent one protective protein key to the cells’ ability to limit the damaging effects of estradiol. When activity of this pathway is reduced or impaired, estradiol may contribute to tumorigenesis. The goal of the next analysis was to compare the mutagenic response to estradiol in BRCA1 wildtype and heterozygous cells and to determine if the reduced BRCA1 protein levels result in an increased susceptibility to estradiol and HP-induced mutations.

Despite the observations that both cell lines had comparable levels of ROS and 8-oxoG generated following exposure to HP, there was a striking difference in the magnitude of mutagenesis observed in HP-treated heterozygous and wildtype cell lines. While wildtype cells showed no increase in mutation frequency following this significant oxidative insult, the mutation frequency increased over 6-fold in heterozygous cells. This difference was again noted in estradiol-treated cells. While the wildtype cell line had a 2-fold increase in mutation frequency following exposure to estradiol, the mutation frequency in heterozygous cells increased 3.7-fold. This increase in mutation frequency observed in heterozygous cells occurred in the absence of estradiol-induced growth stimulation, and cannot be explained by random accumulations of mutations following growth stimulation (Chapter 3). Thus, we conclude that despite similar levels of ROS and resulting 8-oxoG lesions generated by estradiol, heterozygous cells demonstrated a more robust mutagenic response following exposure to both HP and estradiol. This is consistent with the hypothesis that reduced levels of BRCA1 present in baseline and
treated heterozygous populations relative to wildtype cells results in reduced repair of oxidative DNA lesions and a concomitant increase in mutations.
Figure 2.1. Control experiments for 8-oxoG Immunocytochemistry. (A) 90P cells treated with 1 uM estradiol for 30 minutes, no primary antibody, 200X (B) 184 cells treated with 1 uM estradiol for 30 minutes, no secondary antibody, 200X (C) 90P cells treated with EtOH, solvent only control for 30 minutes, 200X (D) 184 cells treated with EtOH, solvent only control for 30 minutes, 100X (E) 90P cells treated with 300 uM HP for 3 hours, 200X. Note intranuclear brown staining.
Figure 2.2. 8-oxoG Immunocytochemistry of 184 cells following exposure to estradiol. (A) 50 nM estradiol for 30 minutes, 100X (B) 50 nM estradiol for 30 minutes, 200X (C) 50 nM estradiol for 30 hours, 100X (D) 50 nM estradiol for 30 hours, 200X (E) 1 µM estradiol for 3 hours, 100X (F) 1 µM estradiol for 3 hours, 200X
Figure 2.3. 8-oxoG Immunocytochemistry of 90P cells following exposure to estradiol. (A) 50 nM estradiol for 30 minutes, 100X (B) 50 nM estradiol for 30 minutes, 200X (C) 50 nM estradiol for 30 hours, 100X (D) 50 nM estradiol for 30 hours, 200X (E) 1 µM estradiol for 3 hours, 200X (F) 1 µM estradiol for 3 hours, 400X
Table 2. Raw Data from HPRT mutation analyses of wildtype and heterozygous cells following exposure to estradiol and HP

<table>
<thead>
<tr>
<th></th>
<th>Wildtype cells (184)</th>
<th>Heterozygous cells (90P)</th>
</tr>
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<tbody>
<tr>
<td><strong>Trial 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cloning Efficiency</td>
<td># Mutants</td>
<td>#Cells Plated</td>
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<td>Control</td>
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<tr>
<td>Estradiol</td>
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<tr>
<td>HP</td>
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<tr>
<td><strong>Trial 2</strong></td>
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<td>#Cells Plated</td>
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<tr>
<td>HP</td>
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</table>

|              |                        |                          |
| **Trial 1**  | Heterozygous cells (90P) |                          |
| Cloning Efficiency | # Mutants | #Cells Plated | Corrected # Mutants | Mutation Frequency |
| Control      | 0.97          | 11                   | 5.20E+07            | 11                  | 2.18E-07           |
| Estradiol    | 0.64          | 23                   | 4.20E+07            | 36                  | 8.56E-07           |
| HP           | 0.30          | 39                   | 8.50E+07            | 130                 | 15.3E-07           |
| **Trial 2**  | Heterozygous cells (90P) |                          |
| Cloning Efficiency | # Mutants | #Cells Plated | Corrected # Mutants | Mutation Frequency |
| Control      | 0.99          | 17                   | 4.50E+07            | 17                  | 3.82E-07           |
| Estradiol    | 0.38          | 30                   | 6.00E+07            | 79                  | 13.2E-07           |
| HP           | 0.19          | 18                   | 4.50E+07            | 95                  | 21.1E-07           |
Chapter Three: Cellular Response to Estradiol

A. Introduction/Rationale

The BRCA1 protein is associated with the cellular response to DNA damage and BRCA1 protein levels rise following exposure to estradiol [54]. Animal models have demonstrated that BRCA1 protein levels are highest during periods of rapid mammary growth [107]. This increase in BRCA1 may serve to modulate the response to estradiol by inhibiting the transactivation of growth stimulatory genes and/or by inducing DNA damage repair, cell cycle arrest, or apoptosis [20]. In ex vivo studies, exposure to estradiol metabolites have resulted in cell cycle arrest and apoptosis, though these studies did not address the role of BRCA1. A recent report by Russo et al have demonstrated that acute exposure to physiologic doses of estradiol in a periodic fashion that mimics estradiol levels during the female menstrual cycle is capable of inducing transformation of normal breast cells in culture [37]. To investigate the role of BRCA1 in this scenario, we have examined the growth and cell cycle response of BRCA1 wildtype and heterozygous cells following exposure to estradiol both immediately following and 1 week after exposure.

In the past twenty-five years, some issues have been raised concerning the possible estrogenic ability of phenol-red containing media [108, 109]. Today’s standards of media production have drastically reduced the lipophilic contaminants of phenol red that have been recognized as the estrogenic component in the media. Though this issue is most important in estrogen receptor positive cell culture systems, we have examined this issue by comparing the growth response of our cells in phenol red-containing and phenol red-free media.
B. Methods and Materials

B.1 BRCA1 levels following exposure to estradiol or HP

Cells were plated as described in Chapter Two and treated with EtOH alone, 10 or 50 nM Estradiol, or 300 uM HP for 48 hours. In this and all following experiments the concentration of EtOH does not exceed 0.01%. Following treatment, cells were collected and immunoprecipitation was performed as described in Chapter Two. Levels of BRCA1 protein relative to untreated control were determined with volume quantitation and Image Quant software. Three independent experiments were performed and statistically analyzed with ANOVA and Tukey-Kramer multiple-comparison tests. Data shown is a representative experiment.

B.2 Cell proliferation following acute estradiol exposure

Cells were seeded into 96 well plates (1x10^3 cells/well) and incubated for 24 or 48 hours in the presence of EtOH only or 10 and 50 nM estradiol. CCK-8 reagent (10 uL) (Dojindo, Japan) which is reduced to a colored product by intracellular dehydrogenases, was added to the media of each well and incubation continued for 3 hours. Absorbance was measured at 450 nM with a reference wavelength of 630 nm. Absorbance of treated groups was analyzed relative to untreated control group. Data shown represents mean values (±SEM) from at least three independent experiments. Results were analyzed for statistical significance using ANOVA and Tukey-Kramer multiple comparisons test.

B.3 Analysis of the potential effects of phenol red on growth assays

Phenol red free media was obtained from Cambrex and two plates of cells were plated for the Cell proliferation assay described above. Though 10 and 50 nM estradiol were used in this thesis study, the range of estradiol was expanded for the analysis of phenol
red effects. If phenol red is estrogenic and masking the effect of the estradiol treatment, this effect should be maximal at lower doses of estradiol; thus, cells were exposed to 0, 0.25, 1.25, 10, and 50 nM estradiol. For each group median values for growth relative to untreated control were determined and statistical significance was determined with Kruskal-Wallis nonparametric ANOVA and Dunn’s Multiple Comparisons tests.

B.4 Cell cycle analyses following acute estradiol exposure

Approximately $1 \times 10^5$ cells were seeded into T25 flasks containing EtOH only or media with 300 uM HP or estradiol (10 nM or 50 nM). Cells were collected and centrifuged following a 12, 24, or 48 hour exposure and slowly resuspended in 1 ml of -20°C 70% EtOH with gentle vortexing. Following ethanol fixation, cells were stored at -20°C for 1-2 days before flow cytometry analyses. Prior to analyses, cells were centrifuged at 250xg for 5 min and washed in PBS to remove residual ethanol prior to pipette resuspension in 1 ml of Propidium Iodide solution (0.1 mg/mL). Cells were stained approximately 1 hour before analysis. Flow cytometry was performed on a Becton Dickson FACScan. Raw data was used to determine the percentage of cells in each phase of the cell cycle and was also analyzed with the ModFit software. Three independent experiments were performed. At each timepoint, the percentage of cells in each phase were compared to the solvent-treated control population and were expressed as a ratio. Data presented are mean values (± SEM) from three or four experiments. ANOVA and Tukey-Kramer multiple comparisons test were used to determine statistical significance of the results.
B.5 Long-term effects on growth following acute and chronic doses of estradiol

90P and 184 cells (5 x 10^4 /well) were seeded into 6-well plates and the following treatments were initiated: ETOH control, 10, or 50 nm estradiol for 24 hours, 48 hours, or 8 days. Growth was assessed by a direct count of viable cell number at Days 1, 3, 5, and 8. Total cell number or calculated cell population doublings were plotted for each treatment group. Two independent experiments were performed. One experiment examining the growth response to continuous estradiol was performed in T25 flasks and counted over a period of 15 days.

C. Results
C.1 BRCA1 Protein Levels following estradiol exposure

It has been reported that BRCA1 protein levels rise in response to DNA damage [110]. We examined baseline BRCA1 protein levels in the BRCA1 wildtype and heterozygous cells and following exposure to estradiol. In Chapter two, we determined that wildtype cells had an average of 3.7 fold higher levels of protein than heterozygous cells (Figure 1.3, Panel D). Further experiments were conducted in which cells were exposed to 10 or 50 nM estradiol or 300 µM HP for 48 hours prior to immunoprecipitation analyses. A representative example of three independent experiments is shown in Figure 3.1. Levels of BRCA1 protein rose in both cell lines following all three treatments. The ratio of protein in wildtype cells relative to heterozygous cells was increased from 3.7 in the control populations to 1.1 for 10 nM, 1.77 for 50 nM, and 2.75 for HP. These results demonstrate that heterozygous cells can increase protein in the face of oxidative stress, indicating that a single mutant allele doesn’t completely hinder this response. However, the heterozygotes are never able to reach the levels seen in wildtype cells under given
growth conditions. Further experiments will determine the functional significance of this difference in protein levels and will help to elucidate whether the presence of a single mutated allele cripples the cell’s response to oxidative stress.

C.2 Cell proliferation following acute estradiol exposure

Estradiol has the ability both to induce cellular growth and to cause DNA damage. In a normal cell, this potentially dangerous situation is mediated by caretaker proteins such as BRCA1 that function to recognize DNA damage, induce cell cycle arrest, and initiate repair. To determine if BRCA1 heterozygosity alters the cellular response to estradiol, BRCA1 wildtype and heterozygous cells were treated with 10 and 50 nM estradiol for 24 and 48 hours and their growth response was analyzed utilizing a growth assay based upon the conversion of a substrate to a measurable colored product following reduction by dehydrogenases from living cells.

In wildtype cells exposed to estradiol for 24 hours, both doses resulted in an approximately 30% increase in growth relative to the solvent control group (Mann-Whitney p for both doses <0.001, Figure 3.2). This increase was not sustained, as evidenced by a return to control levels at 48 hours. Heterozygous cells had a distinctly different growth response to estradiol exposure. Following a 24 hour exposure to estradiol, both doses increase growth 6% relative to control (Figure 3.2); however, this increase was not statistically significant (p>0.05). At 48 hours, 90P cells which had been exposed to 10 and 50 nM estradiol showed a 12% and 8% increase relative to control, respectively, but alterations are not statistically significant.
In summary, wildtype cells demonstrated a transient increase in cell growth following 24 but not 48 hours of estradiol exposure, while growth of heterozygous cells was not statistically altered in response to estradiol.

**C.3 Phenol Red Effects**

To investigate the potential influence of phenol red in growth analyses of these cell lines, heterozygous and wildtype cells populations were treated with a broad range of estradiol doses in the presence and absence of phenol red. At least five independent data points were generated for each cell line and its corresponding treatment (Figure 3.3). At the lowest dose, no statistically significant difference in growth was detected following exposure of either heterozygotes or wildtype cells to estradiol in either media (p=0.06). This observation was repeated following exposure to 1.25 nM estradiol (p=.15). At 10 and 50 nM estradiol concentrations, we again observed no statistically different results in the absence of phenol red (p>0.05 for both cell lines and doses). These data suggest that phenol red, a potential estrogen agonist at the ER, has little effect on the growth of our cell lines, and the presence of phenol red is not masking our ability to observe estradiol-induced growth increases. These observations are in agreement with Moreno-Cuevas et al who also did not observe any estrogenic effects of phenol red [109].

**C.4 Cell cycle analyses following acute estradiol exposure**

It has been reported that BRCA1 heterozygous fiobroblasts treated with UVA irradiation display faulty checkpoint mechanisms relative to wildtype cells [111]. BRCA1 has been implicated in checkpoints at both the G0/G1 boundary and G2-M, and a reduction of BRCA1 protein may prevent or shorten cell cycle arrest. Mouse embryonic fibroblasts with mutant BRCA1 protein demonstrate defective G2-M arrest resulting in
abnormal chromosome segregation, aneuploidy, and abnormal nuclear division [112]. To determine if breast epithelial heterozygotes demonstrate altered cell cycling relative to wildtype cells, 90P and 184 cells were treated with estradiol or HP for 24 or 48 hours before cell cycle analysis using flow cytometry.

Populations of solvent treated wildtype cells are predominantly in the G0/G1 and S phases at 12 hours post-plating (Figure 3.4, Panel D). In a representative experiment at 24 hours, the percentage in the G0/G1 phase drops by 12% relative to 12 hours, while the percentage of cells in G2-M increases 22%, as those cells in S phase at 12 hours move forward toward mitosis. By 48 hours, the percentage of cells in S is markedly reduced, and cells are being redistributed in the G2-M and G0/G1 phases, as the cells begin another round of the cell cycle (Figure 3.4, Panel C). Data from this control population indicates that the cells are actively moving through the cell cycle.

The control population of heterozygous cells behave similarly to wildtype (Figure 3.5, Panels A and B). At 12 hours, these cells are largely distributed in the G0/G1 and S phases. By 24 hours, the percentage of cells in G0/G1 is reduced while the number of cells in the G2-M phase is increased, indicating that the population has progressed through the first two phases of the cell cycle and is nearing completion of one complete cycle of DNA replication and division by 24 hours. This is confirmed by the data at 48 hours, which demonstrates that the cells have moved out of the G2-M phase and once again have entered the G0/G1 phase to begin another cell cycle. This data is consistent with cells that are actively replicating and also suggests that the two cell lines grow at similar rates.
Cell cycle analyses following exposure to HP provide the first indication that BRCA1 wildtype and heterozygous cells may respond differently to DNA damage (Table 3). In the wildtype cells, the ratio of cells in the G2-M phase is increased in the HP-treated groups relative to the solvent treated population at 12 hours. This increase is sustained at 24 hours, consistent with a G2-M arrest. By 48 hours, this increased percentage of cells in G2-M is no longer observed, but an increase in the percentage of cells in the S phase is observed. This suggests that the cells were released from arrest and are entering the cell cycle.

When exposed to HP, heterozygous cells do not respond in the same fashion as wildtype cell populations (Table 3). We observed no accumulation of cells in G2-M seen at 12 hours. These analyses demonstrate that HP-treated cells are not altered in cell cycle distribution relative to untreated controls. Specifically, the G2-M arrest seen in HP-treated wildtype cells is not reflected in the heterozygous population.

When treated with 10 or 50 nM estradiol, wildtype cells cycle differently than control populations (Table 4). At 12 and 24 hours, a greater percentage of the cells are in S phase and less remain in G0/G1, as though the estradiol treatment has pushed them forward more quickly into S than the control cell populations. This data suggests that within this time frame exposure to estradiol is able to influence the kinetics of wildtype cell replication and division. This data is consistent with the transient increase in cell growth seen in response to estradiol in the Cell proliferation assay (Figure 3.2).

In contrast to this alteration, heterozygous cells show no evidence of an estradiol-induced “push” into S phase at 12 or 24 hours. Rather, the percentage of cells in the S phase is reduced relative to the control population at all timepoints. Exposure to 10 nM
estradiol causes an accumulation of cells in G2-M, as evidenced by a 25 and 40% increase relative to the untreated control population. This data suggests that heterozygous cells may enter a transient G2-M arrest following exposure to estradiol.

C.5 Long-term growth analyses of estradiol-treated 90P and 184 cells

Cell population doublings (CPD) were determined for control and estradiol treated cells over a period of seven days (Figures 3.6 and 3.8). CPD are chosen as the units of growth rather than a direct cell number due to the tendency to overestimate growth differences when cell number alone is used. Two independent experiments were performed and the data that are presented are representative experiments. CPD were monitored for 8 days following either a 24 and 48 hour exposure to estradiol. Both wildtype and heterozygous cells underwent approximately 5 CPD during this time period and neither cell population showed an alteration in CPD when exposed to estradiol (Figures 3.6 and 3.8). In addition, wildtype cells were exposed to continuous estradiol throughout the seven day period. This population showed a decrease in cell growth of approximately 1 CPD (5.3 CPD in the control population vs 4.3 in both estradiol treated groups) (Figure 3.7, Panel A). To validate this observation, chronic treatment was repeated for a period of 15 days. At the conclusion of this time period, a decrease in growth of approximately 1 CPD was observed (Figure 3.7, Panel B). These experiments suggest that wildtype cells are inhibited by the continuous exposure to estradiol but not acute exposure. In the heterozygous cell populations, no alteration in growth was seen in the cells exposed to 24 or 48 hours of estradiol (Figure 3.7, Panels A and B). In the presence of continuous estradiol for 7 days, growth is inhibited by 1 CPD (Figure 3.9,
Panel A). When observed over 15 days, the CPD in the control group decreased from approximately 7 CPD at Day 10 to less than 6 at Day 15, while cells treated with estradiol maintain their current growth states (Figure 3.9, Panel B). Observation over a 15 day period allows for observation of cell populations that have reached confluence (approximately 7-8 CPD for heterozygous cells and 8-9 for wildtype cells). These experiments suggest that both treated and untreated wildtype cells die when they reach confluence, while heterozygous cells treated with estradiol are able to sustain growth.

D. Discussion

In previous chapters we described the mutagenic response to oxidative stress in BRCA1 wildtype and heterozygous cell lines. The goal of this chapter was to examine the cellular response of these cell lines to oxidative stress. In this specific aim, the goal was to: (1) determine the level of BRCA1 in each cell line following exposure to HP or estradiol; (2) analyze the short and long term growth characteristics of each cell line following oxidative stress; and (3) obtain cell cycle analyses to augment observations from Cell proliferation assays.

These experiments were performed in media containing phenol red. In the 1970’s, some concern was generated over the potential ability of lipophilic contaminants of phenol red to act as estrogen agonists at the ER. Moreno-Cuevas could not repeat these conclusions with media produced by today’s standards, and eight other reports in the literature spanning 25 years also did not validate these concerns, reviewed in [109]. Not only have we demonstrated that growth of estradiol-treated cells is not different in phenol
red-free media, this issue is also of less importance to this study since our cell lines do not display high levels of the ER, thus minimizing any effect of phenol red contaminants.

BRCA1 immunoprecipitation of the two cell lines confirmed the conclusion from previous experiments that heterozygotes have significantly lower levels of BRCA1 protein than wildtype cells. To our knowledge this is the first report of this discrepancy of protein levels in a mammary epithelial model system, though similar findings have been reported by Baldeyron et al in lymphoblastoid cells lines [84]. This suggests that the presence of a mutated allele results in the production of less than normal levels of protein. Several reasons for this reduction have been proposed but it is still not clear if this phenomenon is due to effects at the transcriptional, translational, or posttranslational level. BRCA1 has been shown to form multimers and it has been proposed that association with mutant protein may enhance degradation of wildtype protein [113]. In the case of the 185delAG, where protein appears to be rapidly degraded, reduction of protein level may reflect the inability of one allele to produce normal levels of protein.

We observed an increase in BRCA1 protein levels following exposure to ROS, with the most robust increase seen in the HP-treated cells. Many reports in the literature describe alterations to BRCA1 protein following oxidative damage, to our knowledge, it has not been previously reported that BRCA1 protein levels rise following oxidative stress in mammary epithelial cells. Since BRCA1 is essential for DNA damage repair, its upregulation may represent one of the cell’s responses to ROS-mediated DNA damage.

In this experiment, BRCA1 protein levels were also increased in both cell lines following exposure to estradiol. In the literature, it has been reported that estradiol increases BRCA1 mRNA and protein levels. In a study utilizing ER-positive cell lines,
estradiol treatment increased BRCA1 mRNA and protein levels 2.5-5 and 3 to 10-fold, respectively [52]. In this report, BRCA1 upregulation was coupled in each case with stimulation of growth and was inhibited by antiestrogen ICI-182780. While exposure to estradiol in our experiments increased BRCA1 protein levels to a similar degree as reported by this group, in our system this increase was not dependent upon cell proliferation. Heterozygous cells demonstrated an increase in BRCA1 protein without a concomitant increase in growth. We suggest that upregulation of BRCA1 protein is not dependent upon growth stimulation, and is therefore independent of the ER receptor and more likely coupled to induction of DNA damage.

The coupling of estradiol exposure and upregulation of BRCA1 protein may function as an elegant mechanism that senses and repairs DNA damage associated with estradiol exposure. Furthermore, through inhibition of the downstream player in the growth stimulatory pathway, BRCA1 can prevent the estradiol induced growth stimulation until DNA damage repair is completed. This protective mechanism may be especially important at times when the breast is rapidly proliferating, such as periods of high estradiol levels during puberty and pregnancy. Heterozygous cells may be most vulnerable during these times, as our results are consistent with the hypothesis that BRCA1 heterozygosity results in less BRCA1 protein than wildtype cells both at baseline and following exposure to oxidative stress. During puberty and pregnancy high estradiol levels contribute to rapid breast development. Inadequate BRCA1 function may allow for both unrepaired DNA damage and unopposed growth stimulation. Taken together, this scenario allows for not only loss of the other allele of BRCA1 but also the loss of other important growth regulatory genes as well and may account for the early onset of breast
cancer in BRCA1 mutation carriers. During times of high estradiol levels, mutation carriers may undergo periods of accelerated mutagenesis. Accumulation of enough mutations to initiate tumorigenesis may take a non-mutation carrier 50-60 years.

Given these differences in BRCA1 protein levels, we investigated the growth response of wildtype and heterozygous cell lines following exposure to HP and estradiol and examined the percentage of cells in each phase of the cell cycle following exposure to HP. The contrasting response of heterozygous and wildtype cells is most obvious in this analysis. Wildtype cells demonstrated an arrest in the G2-M phase while heterozygous cells were unaffected. This dose of HP is lethal to both populations several days following exposure. The response of wildtype cells is consistent with numerous reports in the literature, which demonstrate that high doses of HP result in a G2-M arrest [114, 115]. The heterozygous cell line does not demonstrate a permanent arrest in any phase of the cell cycle, which may be due to the fact that the cell line is unable to increase BRCA1 protein levels to a sufficient level to induce a cell cycle arrest.

To determine the growth responses of both cell lines following estradiol-induced ROS, a cell proliferation assay was used to determine relative cell growth following 24 and 48 hours of estradiol exposure. Despite similar ER status and levels of ROS, as seen following exposure to HP, our cell lines again did not respond to estradiol in the same fashion. While the wildtype cells demonstrated a small increase in cell number following exposure to estradiol, heterozygous cell number is unchanged. This is supported by cell cycle analyses which demonstrate that wildtype cells have an increased percentage of cells in the S phase at 12 hours post exposure. Heterozygous cells do not demonstrate this increase in S phase, but rather showed an accumulation of cells in the G2-M phase. By 48
hours the cell cycle distributions of both cell lines is indistinguishable from the solvent-treated control population.

The classical view of estradiol holds that estradiol stimulates cell growth. However, while this is the predominant action of estradiol in some cell lines, the effects of estradiol are more complex and vary depending on cell type and dosage. Estradiol can act as both mitogen and inhibitor of cell growth and these activities can be divided into ER-dependent and independent pathways. Estradiol induced growth stimulation is ER-dependent and occurs following ligand binding to the ER, dimerization, and translocation to the nucleus, where transactivation of genes involved in cell cycle regulation occurs [116]. Within 1-3 hours of exposure to estradiol, MYC mRNA and protein increase. This is followed by increases in other key players in cell cycle control including cyclin D1, cdk2, and cdk4, resulting in stimulation of the cells to cross the G1/S boundary [117]. The increased percentage of wildtype cells in S phase following estradiol stimulation may be a reflection of estradiols’ influence on the cell cycle. The fact that this response was not overwhelming is likely due to the fact that these cells are not considered ER-positive. While some ER was seen following immunoprecipitation, these levels are significantly lower than the ER-positive MCF7 cell line. The response of wildtype cells in the first 12 hours following exposure is consistent with ER-mediated events [118]. However, we feel that the contribution of ER-mediated events represents a small part of the response to oxidative stress, particularly since the level of ER is low in these cell lines. Additionally, analysis of growth following exposure to estradiol in the long-term demonstrates that stimulation of growth is not sustained. These transient alterations in cell growth were not seen in long term growth assays, where both cell lines were exposed
to both acute and chronic doses of estradiol and monitored for 5-6 passage doublings. No alterations in cell number were observed in this timeframe given these doses of estradiol.

ER-independent effects of estradiol are more likely to cause inhibition of growth and reflect the potential for estradiol to induce DNA damage. Treatment of cells with high doses of metabolites of estradiol have been shown to induce apoptosis of cells [119-121]. The initial accumulation of heterozygous cells in G2-M is likely a reflection of the ER-independent effects of estradiol associated with DNA damage and is analogous to the G2-M arrest seen in wildtype cells following exposure to HP.

Both cell lines are exposed to equivalent levels of ROS, yet this oxidative load results in different degrees of mutagenicity in the two cell lines. Since no significant increase in mutations is seen in wildtype cells, one possibility to explain the growth response of these cells is that DNA damage is effectively detected and repaired, freeing the cells to respond to estradiol. Heterozygous cells, with less BRCA1 protein, may be compromised in their response to DNA damage and may accumulate in the G2-M phase. The mutational analyses demonstrate that this response is not adequate to prevent mutations. A recent report by Shorrocks et al demonstrated that following UVA treatment both wildtype and heterozygous fibroblasts arrest in G0/G1; however, the BRCA1 heterozygotes were released into S much earlier than the wildtype cells, suggesting a defective G1/S checkpoint [111]. If heterozygous cells were initially unable to repair estradiol-induced damage and responded with a G2-M arrest, during which repair was inadequate, premature release of cells into S could account for the mutations induced by estradiol in this cell line.
The experiments in this chapter reinforce the conclusions in the previous chapter that wildtype and heterozygous cells do not respond to estradiol in a similar manner. The cellular response to both HP and estradiol are very different and may reflect dissimilar levels of BRCA1 protein. Similarly, the long-term growth analyses following exposure to both acute and chronic estradiol suggest only a minimal role for ER-mediated events in the response. Therefore, the responses of these cells likely represents those associated with DNA damage following oxidative stress.
Figure 3.1. BRCA1 proteins levels following exposure to estradiol. (A) Western blot analysis of immunoprecipitated BRCA1 protein. (B) Proteins in Panel A were quantified and compared to the solvent only control for 90P(open bars) and 184 (solid bars). Data shown is representative of three independent experiments.
Figure 3.2. Cell proliferation Assay following exposure to estradiol. BRCA1 heterozygous and wildtype cells were exposed to 10 nM (black bars) or 50 nM (open bars) estradiol for 24 or 48 hours. Data are presented as growth relative to control and represent mean values from at least six independent experiments ± SEM.
**Figure 3.3. Cell growth following 24 hour exposure to estradiol in phenol red containing and phenol red free media.** Black bars, 90P (+/-) cells with phenol red; Open bars, 90P (+/-) cells without phenol red; Hatched bars, 184 (+/+ ) cells with phenol red; Gray bars, 184 (+/+ ) cells without phenol red. Data bars represent median values obtained from at least 5 experiments. Data from 184 cells treated with 10 nM estradiol in the presence of phenol red represents 12 experiments with a range of 0.86-1.42. Its phenol red free counterpart represents 12 experiments with a range of 0.85-2.16. Similarly, data for 184 cells exposed to 50 nM estradiol in the presence of phenol red represents the median value from 12 data points with a range of 0.65-1.53. Data from these cells in the absence of phenol red represents 5 experiments with a
Figure 3.4. Cell cycle analyses of wildtype cells (184) treated with solvent only. (A) Cell cycle distribution as a function of time. Data represents three or four independent experiments and is shown as the percentage of cells in each phase relative to control population at 12 hours. (B) Representative example of cell distribution of cells in G0/G1, S, and G2-M. Violet, Go-G1; purple, S; Yellow, G2-M.
Figure 3.5. Cell cycle analyses of heterozygous cells (90P) treated with solvent only. (A) Cell cycle distribution as a function of time. Data represents three or four independent experiments and is shown as the percentage of cells in each phase relative to control population at 12 hours. (B) Representative example of cell distribution of cells in G0/G1, S, and G2-M. Violet, Go-G1; purple, S; Yellow, G2-M.
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<td>HP</td>
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**Data shown are mean values from 3-4 experiments and represent ratio of cells in treated groups relative to solvent control groups at each time point (SEM)**
Figure 3.6. Cell population doublings (CPD) in 184 (wildtype) cells treated with acute doses of estradiol. Blue diamond, solvent only control; Red Square, 10 nM estradiol; GreenTriangle, 50 nM estradiol. Data shown is representative of two experiments. Cells were exposed to estradiol and viable cell number was determined by trypan blue exclusion at Days 1, 2, 4, and 8 (A) Cells were exposed to 10 or 50 nM estradiol for 24 hours. (B) Cells were exposed to estradiol for 48 hours.
Figure 3.7 Cell population doublings of wildtype cells treated with solvent only or chronic doses of estradiol. Blue diamond, solvent only control; Red Square, 10 nM estradiol; Green Triangle, 50 nM estradiol. Cells were exposed to estradiol and viable cell number was determined by trypan blue exclusion at Days 1, 2, 4, and 8 or Days 5, 10, and 15. Cells were exposed to continuous 10 or 50 nM estradiol and growth was analyzed over eight days (A) or 15 days (B).
Figure 3.8. Cell population doublings (CPD) in heterozygous (90P) cells treated with acute doses of estradiol. Blue diamond, solvent only control; Red Square, 10 nM estradiol; Green Triangle, 50 nM estradiol. Data shown is representative of two experiments. Cells were exposed to estradiol and viable cell number was determined by trypan blue exclusion at Days 1, 2, 4, and 8. Cells were exposed to 10 or 50 nM estradiol for 24 hours (A) or 48 hours (B).
Figure 3.9 Cell population doublings of heterozygous (90P) cells treated with solvent only or chronic doses of estradiol. Blue diamond, solvent only control; Red Square, 10 nM estradiol; Green Triangle, 50 nM estradiol. Cells were exposed to estradiol and viable cell number was determined by trypan blue exclusion at Days 1, 2, 4, and 8 or Days 5, 10, and 15. Cells were exposed to continuous 10 or 50 nM estradiol and growth was analyzed over eight days (A) or 15 days (B).
Chapter Four: Feasibility study for the partial knockdown of BRCA1 using siRNA technology

A. Introduction/Rationale

This thesis projects uses only two cell lines, one of which is a BRCA1 heterozygote. One difficulty in the interpretation of our experiments is the absence of genetically identically BRCA1 heterozygous and wildtype cell lines. siRNA technology represents one approach to creating BRCA1 heterozygote from a wildtype cell. The availability of commercial siRNA complexes specific for BRCA1 facilitates this aim. Interaction of siRNA with target mRNA very effectively silences gene expression. While numerous reports demonstrate the ability of siRNA to completely knockdown target protein levels, this aim of my project was not to eliminate BRCA1 completely [122, 123]. Rather, this part of the thesis was designed to examine the feasibility of siRNA technology to reduce, but not eliminate, BRCA1 protein levels to mimic the levels in heterozygotes.

In this part of the thesis, I have shown that siRNA technology can be used to knockdown BRCA1 to at least 2-fold without completely losing BRCA1 gene expression. The knockdowns have been utilized in cell proliferation assays following exposure to estradiol for 24 hours. Due to the transient nature of siRNA experiments, we are unfortunately not able to use these knockdowns in long-term estradiol exposure assays or HPRT mutagenesis experiments. However, the cell proliferation assay will provide preliminary evidence concerning growth of estradiol-induced growth when the level of BRCA1 is reduced.

B. Methods and Materials

B.1 Construction of a heterozygote using siRNA technology
B.1.1 Determining Appropriate Transfection Method

BRCA1 duplex siRNA (5’-UCACAGUGUCCUUUAUGUA-dTdTdTdT-AGUGUCACAGGAAAUACAU-5’), non-Specific control siRNA, and Cy3 labeled Luciferase GL2 Duplex were obtained from Dharmacon RNA Technologies/Upstate cell signaling solutions (Lake Placid, NY). Initial studies addressed the transfection efficiency of both electroporation and liposomal reagents. Briefly, 6x10^5 cells were resuspended in 800 ul CytoPulse Media (CytoPulse Sciences, Inc., Columbia, MD) with 0-7.5 nM Cy3 labeled siRNA and were electroporated in three groups. Cells were resususpended in 10 mls media and incubated 24 hours before viewing with a fluorescent microscope equipped with a TRITC filter. To test liposomal transfection reagents, cells were plated in a 24 well plate and grown to 70% confluence. To determine the optimal amount of reagent and to assess cytotoxicity, 1-4 ul TransIT-TKO Transfection Reagent (Mirus, Madison, WI) was added to corresponding wells along with 0, 1,3,7,10, or 25 nM Cy3 labeled siRNA. Cells were viewed as described above and conditions resulting in most efficient delivery of siRNA to cells were chosen for future experiments.

B.4.2 Determining conditions for BRCA1 siRNA experiments

Wildtype cells were seeded into 6 well dishes (1x 10^5 cells/well) and grown overnight. Transfection reagent (4 uL/well) and media were prepared as described above with BRCA1 siRNA (0, 75, 100, 130, 160, or 200 nM) and added to wells. Incubation continued for 48 hours before harvesting of cells for immunoprecipitation as described previously in chapter two. Three independent experiments were performed and mean inhibition relative to control was calculated (±SEM). To determine if nonspecific inhibition of BRCA1 was occurring, two experiments utilized a nonspecific control
siRNA at the following concentrations: 0, 100, and 200 nM. These cells were harvested and BRCA1 protein levels were determined.

B.1.2 Duration of protein inhibition

Two final experiments were performed to determine the length of inhibition. Cells were transfected with 10 nM Cy3-labelled nonspecific siRNA. Fluorescent images were obtained at 2, 4, 6, and 12 days following transfection to determine if cells were shedding siRNA. Similarly, cells were transfected with 200 nM BRCA1 siRNA for 48 hours. Following this incubation, media containing siRNA was removed and cells were harvested at 2, 4, and 6 days following transfection. BRCA1 protein levels were analyzed as described above to determine the length of inhibition.

B.2 The cellular response of siRNA-constructed heterozygotes following exposure to estradiol

Heterozygotes were created according to the conditions determined in the previous experiments. Following 48 hours of incubation with siRNA complexes, cells were harvested and used in a short term cell proliferation as described above. Some experiments dealing with the continuous exposure to estradiol were performed in T25 flasks and counted over a period of 15 days.

C. Results

C.1 Delivery of siRNA

Two methods of siRNA delivery were analyzed. Electroporation resulted in no fluorescence following Cy-3 siRNA electroporated under various conditions (data not shown). Mirus transfection reagent, formulated specially for delivery of siRNA, demonstrated high transfection efficiency at each dose tested (Figure 4.1). Reagent (1-4
µL/well) did not result in cytotoxicity, as measured by relative confluence of cells. No floating cells were noted and attached cells divided normally. Though some cells appeared to have more siRNA than others, as measured by an increased intensity of fluorescence, nearly 100% of cells demonstrated some level of fluorescence. This experiment also demonstrated that 48 hours is an adequate incubation time for the siRNA-reagent complex to be internalized.

Cells were transfected with 10 nM Cy3-labelled nonspecific control in order to monitor the length of time that the siRNA is maintained within the cells (Figure 4.3). Cy3 fluorescence was detected until 12 days post-transfection, during which time the cells became confluent and began to die. Intracellular fluorescence was most intense at 48 hours and the media within the well also appeared hazy, suggesting that some Cy3-labelled siRNA was still located extracellularly. By Day 4, this haziness had decreased and intracellular fluorescence was detected. Less intense fluorescence was seen at Day 6 but the cells were still fluorescent and have maintained the siRNA. By Day 12, fluorescence was still observed within the cell but the media again appeared hazy, suggesting that the cells were losing the siRNA in some manner.

C.2 Conditions to generate heterozygote

Previous experiments have demonstrated that exposure to estradiol increases levels of BRCA1 protein. Thus, when constructing a heterozygote to be used in growth and cell cycle analyses following estradiol exposure, it may be necessary to use different concentrations of siRNA. In both the presence and absence of estradiol, transfection with BRCA1 siRNA resulted in a dose dependent decrease in BRCA1 protein relative to the control group with 0 nM siRNA (Figure 4.2). In the absence of estradiol, 75-160
nM BRCA1 siRNA did not significantly decrease the levels of BRCA1 protein relative to the 0 nM siRNA control within 48 hours of transfection. However, transfection with 200 nM BRCA1 siRNA reduced the level of BRCA1 protein 53% (T-test p=0.02). In the estradiol treated group, the decrease in BRCA1 protein levels began with 75 nM siRNA (approximately 20% decrease,) and continued to decrease in a dose-dependent fashion. Transfection with 100-160 nM siRNA dropped BRCA1 levels to approximately 50% (p_{100}=0.06, p_{130}=0.17, p_{160}=0.013), while 200 nM siRNA was decreased to approximately 34% (p=0.045) in the presence of estradiol. Previous experiments demonstrated that heterozygotes have 2-4 fold less BRCA1 protein than wildtype counterparts (Figure 1.3, Panel D and Figure 2.1). Thus, 200 nM siRNA appears to generate this condition in both the presence and absence of estradiol within 48 hours of transfection.

In order to determine the optimal time to use transfected cells in growth analyses, we analyzed BRCA1 protein levels at several timepoints post-transfection. Cells were transfected with 200 nM siRNA for 48 hours followed by washing to remove uninternalized siRNA. Immunoprecipitation for BRCA1 was performed at 2, 4, and 6 days post-transfection. Data values represent the mean value from two experiments. By 48 hours the level of BRCA1 protein was 75% of that seen in the 0nM siRNA control. BRCA1 levels continued to drop to approximately half the amount of BRCA1 protein relative to the control by Day 4 post-transfection. At Day 6, the levels of BRCA1 began to rise to near that seen at Day 2 (75%). These data indicate that the optimal time to perform 24 and 48 hour exposure analyses appears to be between days 2 and 4, when BRCA1 levels are lowest.
A nonspecific siRNA was used to determine if the alteration in BRCA1 protein levels was due to nonspecific cellular response to the presence of siRNA. The concentration of control siRNA was analogous to that used in the BRCA1 siRNA experiments. At the doses of 100 and 200 nM nonspecific control siRNA, no decrease in BRCA1 was seen relative to a 1 nM control and an untransfected control (Figure 4.2, Panel C). Image Quant analysis also revealed no statistical difference (ANOVA p=0.9). Thus, BRCA1 is not altered due to exposure to the transfection reagent or a foreign siRNA.

C.3 Cell proliferation following acute estradiol exposure in constructed heterozygotes

Following 24 hours of estradiol exposure, growth of populations of cells transfected with either BRCA1 or a nonspecific siRNA was determined. In previous chapters, growth of wildtype cells was increased approximately 30% when exposed to estradiol (Figure 3.2). This was the anticipated response in the nonspecific control group, as these cells should be identical to the untransfected wildtype group. In two independent experiments, this response was not seen in the cells transfected with nonspecific siRNA (Figure 4.5, Panel A), despite the fact that BRCA1 levels were not altered (Figure 4.5, Panel B). This observation was the first indication that siRNA treatment may alter cell growth independent of BRCA1 levels. BRCA1 knockdowns also showed no statistically significant alteration in growth when exposed to estradiol relative to the solvent only treatment group, which is similar to the response of the 90P cell line. In summary, neither the nonspecific nor BRCA1 siRNA transfected group showed any alteration in cell growth following estradiol. Cells from each transfection were collected and used for immunoprecipitation of BRCA1. Relative to the nonspecific siRNA group, BRCA1
siRNA reduced protein levels only 20 and 28% in the two independent experiments (Figure 4.5). This reduction is not consistent with previous experiments that demonstrated a reduction in BRCA1 by at least 50% (Figure 1.3). In previous experiments, cell lysates were collected from transfected cells following 2, 4, or 6 days without subculturing during this timeframe, unlike these cells which were subcultured at Day 48 and plated at subconfluence for growth analyses.

D. Discussion

The goal of this section of the project was to explore the feasibility of the use of siRNA technology to create a cell line with reduced levels of protein relative to wildtype. The purpose of the experiment was twofold. First, the majority of reports in the literature use siRNA to completely knockdown protein levels. Our first goal was to determine whether siRNA could be used to reduce levels of protein, consistent with heterozygosity. Second, we wished to verify that the cellular and mutational phenotypes of the 90P heterozygous cell line were due to heterozygosity and not due to other genetic variations between the 90P and 184 cell lines. While we were able to reduce BRCA1 protein to a level consistent with heterozygosity, the nonspecific siRNA control populations were altered in their cellular response to estradiol relative to untreated wildtype cells. Furthermore, BRCA1 protein levels do not appear to remain reduced once cells have been harvested and subcultured. These experiments demonstrate the siRNA technology can generate a transient knockdown in BRCA1 levels in cells. However, we can not be confident that these levels remain reduced following subculturing of these cells for experimentation.
While siRNA technology holds much promise, several questions remain. First, our experiments suggest that the process of siRNA transfection itself alters the cellular response to estradiol, without an apparent alteration in the level of the protein of interest. Second, when knocking down a protein which acts as a transcription factor, such as BRCA1, we cannot be certain that the transient reduction is sufficient for the complete phenotypic effects of haploinsufficiency to be demonstrated. For example, a reduction in BRCA1 protein for 5-6 days may not allow enough time for mRNA of downstream proteins, such as Rad51, to be depleted. Thus, the effects of haploinsufficiency may not be evident.

Third, is it also debatable whether the state of genetic heterozygosity is equivalent to that of protein level reduction. In the case of the 185delAG in BRCA1, where mutant protein is rapidly degraded, this is potentially quite analogous. However, in the case of other genetic mutations which produce dysfunctional protein that is not degraded, simply reducing protein levels in half may overlook the impact of the presence of mutation protein.

This technology holds great promise but has limitations. We feel that these results demonstrate that this technology can be used to partially reduce protein levels, but stable transfection of a plasmid expressing the siRNA sequence might be more appropriate. This concept is detailed further in the future directions section of this thesis.
Figure 4.1 Transfection Efficiency 48 hours following transfection of 25 nM Cy3-labelled siRNA into 184 cells. Varying concentration of transfection reagent were tested: 1 µL, (A); 2 µL (B); 3 µL (C); 4 µL (D) for their ability to deliver siRNA and potential cytotoxicity.
Figure 4.2. Immunoprecipitation of BRCA1 protein levels following transfection with BRCA1 siRNA for 48 hours. (A) BRCA1 protein levels following transfection with increasing amounts of siRNA in the absence of estradiol and (B) in the presence of 50 nM estradiol. (C) BRCA1 protein levels following transfection with 0, 100, or 200 nM nonspecific siRNA (D) Quantification of BRCA1 protein following siRNA transfection. (Blue bars, no estradiol; gray bars, with estradiol). Data represents mean values from three independent experiments ± SEM.
Figure 4.3. Cy3-labelled nonspecific siRNA was transfected with Mirus Transfection Reagent to monitor retention of siRNA over timecourse of experiments; brightfield (left) and rhodamine-filtered (right) (A) 48 hours post transfection (B) 4 days post transfection (C) 6 days post transfection (D) 12 days post transfection
Figure 4.4 BRCA1 protein levels in the days following transfection with 200 nM BRCA1 siRNA. (A) Representative experiment to demonstrate that BRCA1 protein levels decrease within 48 hours, are lowest at day 4, and begin to rise again at 6 days post-transfection. (B) Levels of BRCA1 protein relative to Day 0 control in the days following transfection. Data shown is representative of two independent experiments. Transfection of siRNA under these conditions generates a heterozygote between 48 and 96 hours after transfection.
Figure 4.5: Analyses of cells transfected with nonspecific or BRCA1 siRNA. (A) Cell proliferation assay of siRNA-transfected cells following exposure to estradiol for 24 hours. Data presented as growth relative to untreated control. (B) BRCA1 immunoprecipitation of cells used in experiments shown in A. Numerical values indication BRCA1 protein levels relative to nonspecific (NS) control.
Chapter Five: The effects of oxidative and alkylating damage on nontumorigenic, human lymphoblastoid cells

A. Introduction/Rationale

Microsatellite instability is a phenomenon that is well characterized in mismatch repair-deficient tumor cell lines, including the potential etiological role of endogenous DNA damage. However, our understanding of microsatellite mutational mechanisms in repair-proficient, nontumorigenic cells is limited. Microsatellites are repetitive sequences found throughout the human genome. Their involvement in cancer has been intensively investigated following the discovery that microsatellite instability (MSI) is found in both sporadic and hereditary cancers. The mismatch repair (MMR) system functions to correct base substitutions and frameshift errors as well as to correct expansions and contractions within microsatellite sequences [93], and hereditary nonpolyposis colorectal cancer (HNPCC) can be attributed to mutations in MMR genes [14]. MSI is also found in sporadic tumors including, but not limited to, cancers of the bladder, reproductive organs, lung, head and neck, and pancreas [124]. These alterations, which are not present in normal tissue, suggest that the tumor cell has acquired a “mutator phenotype” that allows a cell to accumulate mutations as it progresses [125]. In addition to serving as a marker for genome-wide instability, microsatellites have been postulated to function in gene expression [126]. These functional roles may be closely related to the formation of DNA secondary structure [127], the potential for which will differ depending on the sequence composition and length of the microsatellite. The ability to form non-B DNA structures may also contribute to many other processes such as chromatin folding, chromosomal organization, mutagenesis and repair [128].
Several researchers have investigated the role of endogenous DNA damage in inducing MSI in human tumor cell lines. Reversion analyses of lung cancer cell lines demonstrated enhanced MSI in a (CA/GT)$_{13}$ microsatellite following treatment by reactive oxygen species (ROS)-generating chemicals [90]. Similarly, $t$-butyl hydrogen peroxide and N-methyl-N-nitro-N-nitrosoguanidine increased mutagenesis at microsatellite reporters in MMR-deficient colon cancer cell lines [91]. Growth of MMR-deficient human colon cancer cells in the presence of ascorbate decreased levels of spontaneous microsatellite instability in the genome [92]. While these data strongly support a role for ROS and alkylation in tumor cell MSI, it is not clear whether MSI is enhanced by endogenous DNA damaging agents in repair-competent, non-transformed cells. In particular, the MMR system is actively involved in the repair of endogenously generated DNA lesions and in the DNA damage signaling response as well as in correcting DNA synthesis errors [93].

In this study, we have chosen to examine the susceptibility of the (TTCC/AAGG)$_9$ microsatellite to endogenous mutagens. Tetranucleotide repeats occur frequently in the human genome and the (TTCC/AAGG) microsatellite is one of the most common tetranucleotide sequences, occurring with a frequency of approximately 0.3 alleles/MB [129], [130]. Germline mutation frequencies for this microsatellite are high ($1-3 \times 10^{-3}$) [131], and studies of this repeat on the human Y chromosome have indicated that it is quite polymorphic in the population, with a gene diversity of 0.72, as calculated from allele frequencies [132].
B. Materials and Methods

B.1 Cells and Reagents

The LCL721 lymphoblastoid cell line was derived from a clinically normal donor [133]. Construction and characteristics of the oriP-\(tk\) episomal shuttle vectors used for mutational analyses have been described previously [134]. DMSO, hydrogen peroxide (HP), N-ethyl-N-nitrosourea (ENU) and catalase were obtained from Sigma. Antibodies used in the western blot analysis for mismatch repair protein MSH2 were obtained from PharMingen. Restriction enzymes and associated buffers were from Gibco BRL.

B.2 Hydrogen Peroxide and ENU Treatment

Cells were cultured in RPMI media supplemented with 10% FBS, 50 μg/mL gentamycin, and 150 μg/ml hygromycin. To determine if the presence of iron enhanced the damaging effects of hydrogen peroxide, cells were cultured with or without 100μM FeSO\(_4\)\(\cdot\)7H\(_2\)O (ICN Biomedicals Inc.) for 15-18 hours prior to treatment. Cells (1 x 10\(^8\)) in each group were resuspended in 20 mls of PBS and concentrated HP (PBS solvent) was added in varying concentrations (0-550 μM). Treatment continued for 30 minutes at 37°C in the dark with gentle rocking motion. The concentration of hydrogen peroxide that resulted in a 10-20% survival was chosen for mutagenesis studies.

For mutational analyses, three treatment groups were created: PBS control (no hydrogen peroxide), catalase (hydrogen peroxide and catalase), and treatment (hydrogen peroxide only). Cells (1x10\(^6\)/ml in PBS) were pretreated with 100 μM FeSO\(_4\)\(\cdot\)7H\(_2\)O for 15-18 hours, followed by treatment with 200 μM HP. Human erythrocyte catalase (5KU) was added to the catalase group just prior to the addition of HP. Cells were treated as
described above. When cell viability had increased to at least 65%, plasmid DNA was extracted as previously described [134]. Between treatment and plasmid extraction, cells underwent approximately 2-3 population doublings. Treatment of LCL721 cells with 1 mM ENU (DMSO solvent) was performed as previously described [135].

The mismatch repair status of these cell lines was determined by Western blotting with MSH2 antibody, as described in previous chapters.

B.3 Analyses of ROS Production and Oxidative Adducts

To determine the levels of ROS induced by HP, iron-loaded LCL721 cells were incubated in PBS with 10 µM 5’,6’-chloromethyl-2’,7’ dichlorodihydrogen-fluorescein diacetate (CM-H$_2$DCFDA) (Molecular Probes, Eugene, OR), DMSO solvent, for 40 min at room temperature. Following a PBS wash, groups of cells (4x10$^5$) were resuspended in PBS and treated with increasing levels of HP in the presence and absence of 5 kU of catalase for 30 min in the dark. Following treatment, cells were seeded into a 24 well plate and fluorescence was detected at excitation and emission wavelengths of 485 and 535 nm, respectively. Fluorescence from wells of each dose was summed and background fluorescence from the plate alone was subtracted. Statistical significance was determined using an ANOVA test.

To analyze the degree of oxidative damage immediately following treatment with HP and during the time of observed cell growth arrest, a lipid peroxidation assay (Calbiochem, San Diego, CA) was performed. Cells were iron loaded and treated as described above for HP mutagenesis experiments. Following the final wash, 2 x 10$^7$ cells were resuspended in PBS, lysed by repetitive freeze/thaw cycles, and used to assay for
levels of malondialdehyde and 4-hydroxyalkenals per manufacturer’s instructions. Remaining cells were placed in fresh media for five days, followed by adduct analyses. To control for cytotoxicity and proliferation differences between the treatment and control groups, the total lipid peroxidation levels were normalized to viable cell number.

B.4 HSV-tk Mutation Frequency and Specificity analyses

Plasmid DNA isolated from LCL721 cells was used to electroporate *Escherichia coli* strain FT334 (*tdk, upp, thi1, hsd20, supE44, lacY1, proA2, ara14, galK2, xyl5, mtl1, leuB6, rpsL20, recA13), as described [134]. To determine the HSV-tk mutation frequency, aliquots of *E.coli* were plated on VBA media containing 50 mg/ml chloramphenicol (Chlor) in the presence or absence of 40 µM FUdR [134]. The HSV-tk mutation frequency is defined as the number of FUdR and Chlor resistant colonies divided by the number of Chlor resistant colonies. A total of 7.9x 10^3 to 4.5x10^5 Chlor^R transformants were analyzed for each treatment group. Mutation frequencies among multiple groups were compared statistically using analysis of variance (ANOVA) and Fisher’s Least Significant Difference test (FLSD). A two-sided T-test was used when variability among groups precluded the use of ANOVA or when only two variables were being compared. The mutation frequency at the microsatellite was calculated as the proportion of mutations occurring in the microsatellite multiplied by the overall HSV-tk mutation frequency.

Plasmid DNA from the pJY5A.1 vector treatments was used to generate independent mutants in FT334 *E.coli* as previously described [134]. To detect large deletions within the HSV-tk gene, DNA derived from these independent clones was
digested with restriction enzymes *Ava*I and *Bgl*II at 37°C for 2 hours. DNA sequence changes were determined by dideoxy sequence analyses.

**C. Results**

**C.1 Selection of clones for analysis**

The magnitude of MSI following treatment with various DNA damaging agents was tested using HSV-*tk* shuttle vectors. The pND123 vector carries the HSV-*tk* control gene, while the pJY5A.1 vector carries a (TTCC/AAGG)$_9$ microsatellite inserted in-frame in the HSV-*tk* gene. Nontumorigenic, LCL721 cells express the hMSH2 proteins (Figure 5.1) and do not display the high microsatellite mutation rate associated with MMR-deficiency [136], [137]; thus, we can conclude that these cells are MMR-proficient. We first selected LCL721 clones carrying each vector that displayed similar mutation frequencies and mutational spectra. The clone bearing the pND123 plasmid had an initial mutation frequency of $1.1 \times 10^{-4}$, and molecular analysis of mutants revealed 8 gross rearrangements, 2 (+1) G/C frameshifts, and one GC→TA transversion. Similarly, the pJY5A.1-bearing clone had an initial mutation frequency of $2.1 \times 10^{-4}$ and mutant analysis showed 2 gross chromosomal rearrangements, 8 one unit expansions and 2 one unit deletions at the microsatellite, and 3 GC→TA transversions.

**C.2 HP mediated oxidative damage and LCL721 cytotoxicity**

A CM-H$_2$DCFDA assay demonstrated that treatment of LCL721 cells with HP resulted in a dose-dependent increase in fluorescence which was effectively reversed at every dose by catalase (Figure 5.2, Panel A). Catalase and PBS groups showed no statistical difference in fluorescence in any sample. These results demonstrate that HP
treatment increased intracellular levels of ROS and that the addition of catalase efficiently eliminated this oxidative stress.

Survival following HP treatment of the two LCL721 cell populations under study, pND123 and pJY5A.1, was determined over a 5 day period. At all concentrations tested, the presence of iron resulted in enhanced HP cytotoxicity (data not shown). Figure 5.3, Panel A presents data for the second day following treatment, when the effect of HP is maximal. No difference in cell viability was observed in response to the HP between the two groups of cells. Following treatment with 50 µM HP, a dramatic drop in viability was observed for both groups of cells; viability after 100 µM and 200 µM HP treatment decreased to 20% and 15%, respectively. No further decrease in viability was observed at higher doses.

Using 200 µM HP, treated LCL721 cells entered an apparent arrest, as evidenced by no increase in cell number for 7-10 days (Figure 5.3, Panel A). During this time period, a lipid peroxidation assay demonstrated greatly elevated levels of malondialdehyde (MDA) and 4-hydroxyalkenal, byproducts of lipid peroxides (~50µM total products/10^6 viable cells) immediately following treatment (Figure 5.3, Panel B). The inclusion of catalase in the treatment limited the production of lipid peroxide byproducts to less than 5 µM/10^6 viable cells, while none were detectable in the PBS control group. Five days following treatment, HP treated cells showed no increase in cell number; however, the level of lipid peroxides detected decreased 60-fold to 0.76 µM/10^6 viable cells (Figure 5.3, Panels A and B).
C.3 Mutagenic effect of HP treatment

HSV-tk mutation frequencies were determined for pND123 and pJY5A.1 clonal populations in each of the three groups (Figure 5.4, Panel A). For cells bearing the pND123 vector, HP treatment of cells increased the mutation frequency to $8 \times 10^{-4}$, 3.6-fold greater than the PBS control ($2.2 \times 10^{-4}$). The presence of catalase decreased the mutagenic effect of HP ($4.4 \times 10^{-4}$). Mutation frequencies for the PBS and catalase control groups are not statistically different from each other (T-test, $p=0.41$); however, the treatment group is elevated relative to both the control and the catalase (T-test, $p=0.07$).

To determine if HP enhances MSI at a [TTCC/AAGG]$_9$ allele, clonal populations bearing the pJY5A.1 vector were likewise analyzed and similar results were observed. The mutation frequency following treatment with HP ($6.6 \times 10^{-4}$) increased 4.5-fold relative to control ($1.5 \times 10^{-4}$), while the HP effect was again abrogated by catalase (Figure 5.4, Panel A). Statistical analysis resulted in the same set of conclusions as described for pND123 (ANOVA, FLSD, $p=.01$). To test whether HP treatment preferentially targets the microsatellite, we compared HP-induced mutagenesis between LCL721 clones bearing the pJY5A.1 and pND123 shuttle vectors and found no difference (T-test, $p=0.6$). This result suggests that while HP did increase the overall HSV-tk mutation frequency, it did not preferentially induce MSI at this target sequence.

In order to test this conclusion directly, plasmid DNA was extracted from independent mutants of each pJY5A.1 treatment group and the DNA sequence changes determined (Table 1). HSV-tk mutation frequencies are the summation of all types of mutational events that inactivate the HSV-tk gene, including point mutations and large rearrangements within the coding region in addition to microsatellite mutations. We
expect that each LCL721 cell will carry a mixture of different types of mutant plasmids, because we exert no selective pressure on the human cells for inactivating HSV-tk mutations. Mutational spectra were used to calculate the region-specific mutation frequency (Table 2). Molecular analyses of the PBS control and catalase treatment groups revealed that 62% and 70% of inactivating mutations occurred within the microsatellite, respectively. These results are consistent with our previous observations for spontaneous mutations recovered from the pJY5A.1 vector [138]. The observed microsatellite mutation frequency was \( \sim 1 \times 10^{-4} \) in the untreated, PBS and catalase-treated groups (Table 2). In our reporter cassette, the HSV-tk coding region serves as an internal control. We observed that the HSV-tk coding region mutation frequencies were constant among the untreated, PBS, and catalase groups (Table 2).

For the HP treatment group, 60% of the mutations occurred within the microsatellite sequence (Table 1). Within the HSV-tk coding region, HP-induced mutations consisted of large deletions (60%) and both GC → CG and AT → TA transversions (40%). A broader spectrum of point mutations were observed in the PBS control group, including GC → AT transitions and one base frameshifts. For the catalase group, mutations within the HSV-tk coding region resembled those of the HP group, with deletions again being the most frequent (Table 1).

The absolute frequency of microsatellite and HSV-tk coding region mutations in the HP-treated group was five and six-fold higher, respectively, than that of the PBS and catalase controls (Table 2). Thus, both the microsatellite and the downstream HSV-tk gene were equally susceptible to oxidative mutagenesis. However, the overall (combined) HP-induced mutation frequency for the [TTCC/AAGG]₉ vector was not
greater than that of the HSV-tk gene only-containing vector (Figure 5.4, Panel A), as should have been observed if mutagenesis at the HSV-tk gene and microsatellite targets were additive. Unexpectedly, DNA sequence analyses of the [TTCC/AAGG]_9 vector mutations revealed that the inherent HSV-tk mutation frequency was not constant between the two vectors over the course of the experiment. As noted above, 721 cell clones bearing each vector were chosen initially based upon the similar HSV-tk mutation frequency (Table 2). However, the accumulation of spontaneous mutations during the independent growth of the cultures resulted in a greater level of genetic drift within the pND123-bearing cell population than the pJY5A.1 population (Table 2, untreated versus PBS groups, HSV-tk mutation frequencies). Moreover, HP treatment resulted in only a four-fold increased HSV-tk mutation frequency within the pND123 vector, and treatment with catalase prevented only ~50% of this mutagenesis (Table II). In contrast, HP treatment of microsatellite vector-bearing cells (an independent population) resulted in a six-fold increased HSV-tk frequency, an increase that was completely inhibited by catalase.

C.4 Mutagenic effect of ENU treatment

To determine the effects of alkylating damage on microsatellite stability, clonal populations of cells bearing both vectors were treated with 1 mM ENU (DMSO solvent). The mutation frequency following treatment with only DMSO was 1.1x10^{-4} and 4.4x10^{-4} in the pND123 and pJY5A.1 groups, respectively (Figure 5.4, Panel B). In both groups, ENU treatment resulted in an increase in mutation frequency relative to the DMSO control (7.6 and 3.6-fold increases for pND123 and pJY5A.1, respectively), a statistically
significant increase for both groups (T-test, p=.02 and p=.04, respectively). As described previously for HP, the increase in mutation frequency within the pJY5A.1 group relative to its control does not exceed that observed for the pND123 group.

DNA from pJY5A.1 plasmid-bearing cells treated with ENU had a drastically different mutational spectrum, relative to solvent controls (Table 1). Of 25 ENU-induced mutants sequenced, only 4 were found to have changes in the microsatellite (16%) as compared to 53% microsatellite changes in the solvent-control and 56% in untreated cells[138]. All microsatellite mutations in the ENU-treated group were single unit expansions, which is consistent with the most common type of microsatellite alteration seen in the other groups and spontaneously [138]. Ten additional ENU mutant clones were generated and sequenced at the microsatellite, but none of them displayed alterations in microsatellite allele length. The majority of ENU-induced mutations were found within the HSV-tk coding sequence (84%), and consisted largely of GC→AT transitions. Mutation frequency calculations based on direct DNA sequence analyses confirm that ENU-treatment did not increase microsatellite mutagenesis, but resulted specifically in HSV-tk coding region mutagenesis (Table 2).

C.5 Mutagenic effect of DMSO treatment

We observed a differential effect of DMSO treatment between the pND123 and pJY5A.1 populations (Figure 5.4, Panel B). Exposure to DMSO did not elevate the HSV-tk mutation frequency of the pND123-bearing cells above the untreated control; however, the mutational frequency of the pJY5A.1 group was elevated 2.2-fold following exposure to DMSO, with deletions constituting the majority of mutations within the HSV-tk coding
region (Table 1). Over 50% of the DMSO-induced deletions removed the entire HSV-\textit{tk} gene, whereas such large deletions constitute only \~8\% of mutations in the PBS control group. The HSV-tk mutation frequency of the microsatellite-containing vector was increased three-fold after DMSO exposure, relative to the untreated control; however, no such increase was observed for DMSO treatment of the HSV-tk gene only control vector (Table 2).

D. Discussion

The goal of this study was to examine the effects of reactive oxygen and alkylating species on microsatellite mutagenesis in nontumorigenic, MMR-proficient human cells. Direct DNA sequence analyses revealed that HP treatment resulted in an increased mutation frequency at both the microsatellite and the HSV-tk coding regions that was abrogated by catalase (Table 2). Cellular treatment with ENU also resulted in a statistically significant increase in the HSV-tk mutation frequency for both vectors, relative to the DMSO control (Figure 5.4, Panel B). In contrast to HP, however, ENU treatment did not increase the frequency of misalignment mutations at the \([\text{TTCC/AAGG}]_9\) microsatellite (Table 2). We have demonstrated previously that ENU damage to DNA can induce eukaryotic DNA polymerase-mediated frameshift mutations \textit{in vitro} [139]. The ENU-induced mutational spectrum in this study is primarily base substitution mutations within the HSV-\textit{tk} coding region (Table 1), consistent with our previous \textit{ex vivo} results [135]. The production of such base substitution mutations within the \([\text{TTCC/AAGG}]\) microsatellite allele would not have been detected in our mutational assay.
We observed that treatment of MMR-competent human cells with HP did not affect the proportion of inactivating mutations that occur within the microsatellite sequence (Table 1). This result is in contrast to previous results of oxidative damage-induced microsatellite mutations in E.coli in which oxidative damage to DNA induced mutations exclusively within a [GT/CA] microsatellite [140]. Our observations that the [TTCC/AAGG]_9 microsatellite is not acting as an HP mutational “hotspot” suggest that the additional oxidative stress induced by HP does not alter the distribution of oxidative damage and repair, relative to the endogenous cellular environment. Thus, one interpretation of our results of increased mutation frequency with an unaltered specificity is that spontaneous mutations within the [TTCC/AAGG]_9 microsatellite arise from DNA damage by endogenous ROS arising in human cells.

This study of MSI in response to oxidative or alkylating damage is the first to use nontumorigenic human cells. Our MSI mutational findings are somewhat less pronounced than previous studies of ROS and alkylation-induced MSI at [CA/GT]_11, or tetranucleotide alleles in human lung and colon tumor cell lines [90, 91]. Several experimental differences exist between our design and these studies, including microsatellite sequence and length, chemical treatment, and detection methods. The most significant factor, however, may be the MMR-proficient status of our lymphoblastoid cell line. MMR-proficient tumor cells are more susceptible to HP induced cytotoxicity than their MMR-deficient counterparts [92],[141], probably due to the direct role of MMR in recognizing DNA damage and/or the indirect role in cell signaling, recruitment of the BER pathway, and apoptosis [142]. Thus, the HSV-tk mutational spectrum we observed after HP treatment likely reflects efficient repair of premutational oxidative lesions.
during growth arrest. Consistent with this hypothesis, the HSV-tk coding region spectrum of the HP treatment group is deficient in GC→TA transversions associated with mispairing by the 8-oxoguanine lesion [143]. The capacity for repair of oxidative damage also may be cell-type specific. Studies of lymphocytes have shown that ROS are produced and used for intracellular signaling and cell toxicity and that oxidative damage is cleared very efficiently [144], [145]. The clearance of lipid peroxide byproducts from the cells in this study suggests efficient clearance of ROS. These observations suggest that the capacity and time for repair of ROS-induced DNA damage is a major factor limiting the degree of induced MSI in our experiments, relative to previous reports in tumor cell lines.

Intriguingly, we observed that the HSV-tk mutation frequency of only cells bearing the microsatellite-containing vector was increased following exposure to DMSO (Figure 5.4, Panel B) and corresponded to a unique mutational spectrum, comprised of a high proportion of deletions within the HSV-tk coding region, particularly large deletions involving the entire HSV-tk gene (Table 1). While DMSO has not been found to be mutagenic in the traditional Ames test, other bacterial strains have shown that DMSO is mutagenic in a dose-dependent manner and is capable of increasing frequency of mutation at the HPRT locus [146], [92]. The ability of DMSO to form a methyl radical following exposure to oxidative radicals and to dehydrate DNA resulting in misreplication and stimulation of excision repair are two mechanisms that may contribute to the observed mutagenic effect [92], [146]. Consistent with misreplication, we have reported previously that DMSO treatment of DNA increases the frequency of polymerase α-primase and polymerase β errors using an HSV-tk in vitro assay [139].
Primary DNA sequence is a known modifier of ROS-induced DNA damage, due to intrinsic structural differences along the DNA molecule. Jackson et al have proposed that oxidative damage-induced MSI is due to the production of single and double strand breaks [140]. Therefore, a potential reason for why the [TTCC/AAGG] microsatellite was not a hotspot for ROS-induced MSI is that this allele sustained less damage than other DNA sequences (e.g., [GT/CA]_n). The reactivity of individual hydrogen atoms of ribose moieties with hydroxyl radicals parallels their accessibility to solvent [147]. Lower probabilities of irradiation-induced DNA strand breakage at certain DNA sequences can be explained by reduced minor groove width that reduces accessibility to the hydroxyl radical [148], and the sequence of our microsatellite [TTCC/AAGG] is similar to the low strand breakage sequences identified in this study. The potential for DNA secondary structure is also an important factor to consider regarding DNA damage-induced microsatellite mutagenesis. Previous studies in our laboratory demonstrated that the [TTCC/AAGG]_9-containing vector is sensitive to S1 nuclease in the microsatellite region, consistent with the formation of triplex structure *in vitro* [138], although we have no direct evidence that non-B DNA structures are formed in human cells. A triplex structure has been shown to be recognized by the nucleotide excision repair pathway[149, 150]. This stimulation of repair in the presence of significant DNA damage such as that seen following an oxidative or alkylation attack, may effectively reduce the mutagenicity of these treatments in specific areas.

In conclusion, our treatment of nontumorigenic human lymphoblastoid cells with HP and ENU proved mutagenic as evidenced by increases in HSV-\textit{tk} coding region mutation frequencies. However, HP resulted in a low level of increased microsatellite
mutation frequency while ENU treatment did not enhance MSI at a [TTCC/AAGG]₉ allele. The repair capacity of the cells examined is likely to be a major factor that limited the damage induced MSI. The lymphoblastoid cells used in our analyses might have efficient repair mechanisms in place to recognize and correct DNA. Finally, the DNA sequence composition of the microsatellite may affect the susceptibility of the allele to DNA damage.
Figure 5.1. Immunoblotting of MMR proteins MSH2 in 721 cell lysates. The presence MSH2 protein suggests that the cell lines are MMR proficient. This is consistent with the phenotype of the cells, which is absent of the characteristic increase in mutation frequency and microsatellite instability.
Figure 5.2. HP mediated ROS induction and cytotoxicity. (A). LCL 721 cells were treated with 100 µM FeSO$_4$ for 15-18 hours, followed by treatment with HP for 30 min. ROS induction as a function of [HP] in the absence (solid bars) or presence (hatched bars) of catalase was measured using DCFH fluorescence. Values are reported as DCFH fluorescence of treatment groups relative to untreated control and represent the mean of two independent experiments. (B). Viability measurements of LCL721 cell populations containing the pND123 control vector (☐) or the pJY5A.1 microsatellite vector (●) after treatment with the indicated concentrations of HP. Data shown are a representative experiment two days after treatment.
Figure 5.3. HP mediated growth arrest and damage. (A). LCL 721 cells were treated with 200 µM HP as described in legend to Figure 1. Viable cell number was determined by vital dye staining and hemacytometer counting at various times past treatment. Data shown for control vector (□) or the pJY5A.1 microsatellite vector (●) are representative of 2-3 independent experiments. Shuttle vector DNA was isolated for mutational analyses on Day 14. (B). Levels of MDA and 4-NHE lipid peroxides were assayed in each group immediately after or 5 days post treatment with 200 µM HP in the absence (open bars) or presence (solid bars) of catalase. Values for the untreated cell population are represented by hatched bar. Values were normalized for viable cell number, and are representative of two independent experiments.
Figure 5.4. Treatment-induced mutagenesis of HSV-tk control (pND123) or microsatellite-containing (pJY5A.1) vector bearing cell population. Data represent the average of three independent treatments ± standard deviations. Those columns with no error bars represent standard deviations too small to be represented on this scale. (A) Cells were exposed to a PBS control, 200 µM HP, or 200 µM HP in the presence of 5KU catalase for 30 min at 37°C. The mutation frequencies for the treatment groups are significantly elevated relative to untreated and catalase controls for both populations (p=0.07, pND123, T-test; p=0.01, pJY5A.1, ANOVA). The mutation frequencies measured for the two HP treated populations did not differ significantly (p=0.6, T-test). (B) Cells were exposed to a DMSO solvent control or 1mM ENU for 1 hr at 37°C. U, untreated cells. The mutation frequencies for the ENU treated groups are significantly different from the DMSO controls for both cell populations (p=0.02, pND123, T-test; p=0.04, pJY5A.1, T-test).
Table 4. HSV-tk mutational specificity after treatment of pJY5A.1 shuttle vector bearing LCL-721 cells

<table>
<thead>
<tr>
<th>Type of Mutation</th>
<th>PBS</th>
<th>HP</th>
<th>HP + Catalase</th>
<th>DMSO</th>
<th>ENU</th>
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<td>Number of Mutants Sequenced (Proportion of total)</td>
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<td>Microsatellite</td>
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*Deletion of entire HSV-tk gene refers to deletion of bp 1-1181
DISCUSSION AND FUTURE DIRECTIONS

Induction of oxidative stress in a cell has global consequences. While many reports in the literature have examined the cell cycle response to oxidative stress and others have examined DNA damage and mutagenesis following ROS formation, few papers have attempted to examine the interplay of these two processes in the same cell line following oxidative insult. Since the response to oxidative stress is complex, we have examined the cellular and mutagenic response to ROS induction using a multi assay approach. Furthermore, this project aims to examine the consequence of reduced levels of the BRCA1 protein, a key player in the global response to stress. To this end, we have utilized two nontumorigenic human mammary epithelial cell lines, one which is heterozygous and another which is wildtype for BRCA1.

In these experiments, we use oxidative stress generated by two different agents, estradiol and HP. While estradiol treatment is designed to represent physiologic levels of hormone, HP is being used as a positive control in a concentration which kills the vast majority of cells within three days of the treatment. These treatments serve to demonstrate the cellular and mutagenic response to oxidative stress in two situations: mild estradiol-induced oxidative stress at a level which is not cytotoxic following exposure to estradiol and severe, highly cytotoxic level of oxidative stress following exposure to HP. Consistent with these assumptions, DCF data demonstrates that estradiol induces ROS approximately 20-30%, while HP induces ROS 6-fold. If BRCA1 heterozygosity results in an altered response to oxidative stress, severe HP-induced oxidative stress will likely magnify this difference. Similarly, the difference in wildtype and heterozygote cells may be less pronounced following the milder estradiol treatment.
Using an HPRT mutation assay, we examined the hypothesis that decreased levels of BRCA1 protein will increase the mutagenic impact of ROS. BRCA1 is a protein involved in the response to and repair of DNA damage. In its role as sensor for DNA damage, it acts to initiate the appropriate response to limit the consequences of DNA damage. One way it does this is to induce DNA repair via multiple pathways which include DSB repair, mismatch repair, and transcription coupled repair. Initiation of repair limits the mutagenic potential of DNA damage. We hypothesized that BRCA1 heterozygotes, which have reduced levels of BRCA1 protein would have an impaired DNA damage response that would render them more susceptible to ROS-induced mutagenesis.

BRCA1 heterozygotes exhibit an increased mutation frequency following exposure to estradiol and HP. The difference is most striking following HP-induced oxidative stress, where the mutation frequency is near normal in wildtype cells but has increased 6-fold in heterozygotes. Similarly, following estradiol exposure the mutation frequency in wildtype cells was increased 2-fold, while heterozygotes demonstrated a nearly 4-fold increase in mutation frequency relative to that of the untreated controls. This is the first set of evidence that BRCA1 heterozygous and wildtype cells have different phenotypes (Table 2). To further investigate this possibility, the next experiment examined the cellular response of the cells to ROS. In the role of sensor for DNA damage, BRCA1 has been shown to induce cell cycle arrest or apoptosis. We hypothesized that reduced levels of BRCA1 protein might alter the cellular response to oxidative stress, perhaps by allowing inappropriate proliferation.
Cell cycle analyses following exposure to HP reveal a dramatic difference in the cellular response to HP-induced oxidative stress. A G2-M arrest is noted in wildtype cells but not heterozygous cells. Following exposure to estradiol, 184 cells demonstrate increased transit through G0/G1, which corresponds to a slight increase in cell number in growth experiments. This estradiol-induced alteration is not observed in BRCA1 heterozygotes. In this population, no increase in cell number is seen at either timepoint in the growth assay and no significant alterations are observed in cell cycle analyses. Thus, the cellular responses of BRCA1 heterozygotes and wildtype cells are also different.

Does BRCA1 heterozygosity result in haploinsufficiency? Before answering this question, it is first necessary to define the concept of haploinsufficiency. In this thesis, we will adopt the definition that haploinsufficiency is the state in which reduced levels of protein result in an altered phenotype. The data generated from this thesis project is consistent with the hypothesis that heterozygotes have both reduced protein levels and a different phenotype relative to wildtype cells, suggesting that these heterozygous cells are haploinsufficient.

While this definition of haploinsufficiency does not state that the altered phenotype in heterozygotes guarantees an increased propensity for tumorigenesis, BRCA1 mutation carriers clearly have an increased risk of cancer. Two models have been proposed to account for this increased risk of cancer. The first model centers on Knudson’s two-hit model. A cell that already has a mutated allele only needs one additional hit to lose BRCA1 completely and become initiated. Once lost, mutations in other genes are likely and the cell is capable of becoming tumorigenic. The second scenario proposes that heterozygous cells have an altered phenotype relative to their wildtype counterparts, such
that the state of heterozygosity alone is sufficient for initiation--making the loss of the remaining BRCA1 allele and other important regulatory genes more likely.

Evidence for this second model is most developed for p53, the most mutated gene in human cancer. p53 is, like BRCA1, very central to many processes which are key in the prevention of tumorigenesis. Heterozygous mice develop cancer around nine months and the incidence of cancer increases with age. In order to determine when the second allele of p53 was lost, Venkatachalam et al analyzed tumors from 217 heterozygous mice. In tumors from mice under the age of 18 months, only half of the tumors have lost the second allele of p53, indicating that loss of the second allele of p53 was not necessary for tumorigenesis. In tumors from older mice, loss of heterozygosity for p53 increased to nearly 85%. The results of this study suggest that the presence of one mutated allele is sufficient to confer an altered phenotype in the cell. In the case of heterozygosity of a tumor suppressor gene, this state may be sufficient for initiation of the cell.

Given the altered cellular and mutational phenotype of heterozygotes, we propose a model that centers on the reduced level of BRCA1 protein in heterozygotes (Figure D1). BRCA1 heterozygous cells have reduced levels of BRCA1 protein both at baseline and following the induction of DNA damage. Despite a demonstrated increase in intracellular ROS and the induction of 8oxoG, wildtype cells appear to recognize and repair DNA damage, allowing for growth without the accumulation of HPRT mutants. Given a similar oxidative insult, heterozygous cells are not able to recognize and repair DNA damage as efficiently as wildtype cells. This results in the lack of growth stimulation and transient pausing in the G2-M phase. These events are apparently not sufficient to limit
DNA damage, as evidenced by the 3.7 and 6-fold induction of mutations following exposure to estradiol and HP, respectively.

The potential for haploinsufficiency in BRCA1 mutation carriers was initially of concern since radiation is a part of the cancer treatment regimen. At least two reports in the literature have demonstrated that fibroblasts with a single mutant allele of BRCA1 demonstrate reduced survival following exposure to irradiation, increased DNA damage and reduced repair as evidenced by the formation of micronuclei, and resistance to apoptosis relative to wildtype fibroblasts [85, 151]. Studies in lymphoblastoid cells have yielded similar conclusions [84, 152]. While these studies provide preliminary evidence of haploinsufficiency, none of these addresses the question of haploinsufficiency in hormone responsive tissue using treatments representative of endogenous stressors.

We have demonstrated that BRCA1 heterozygous mammary epithelial cells have an altered phenotype relative to wildtype cells, consistent with the hypothesis that BRCA1 heterozygotes are haploinsufficient. BRCA1 is involved in cell cycle control and DNA damage recognition and repair; thus, the state of haploinsufficiency may contribute to tumorigenesis.

Why is the study of BRCA1 heterozygosity so important? Despite intensive study of BRCA1 in the ten years since its discovery, the approach to cancer prevention and treatment of BRCA1 mutation carriers has made little advancement. Currently, the major option available to women carrying this gene mutation is bilateral mastectomy and ovariectomy. While epidemiological studies are beginning to sort out the factors which increase the already highly elevated cancer risk in mutation carriers, such as pregnancy, very little of this information is translated to the clinical level. Besides radical, life-
altering surgeries, clinicians have very little to offer mutation carriers. This clinical
deficiency may stem from the commonly held belief that mutation carriers are
indistinguishable from non mutation carriers until they have lost the wildtype allele of
BRCA1. We have presented evidence to the contrary; heterozygotes display an altered
phenotype relative to wildtype. This and other studies have presented data to suggest that
heterozygotes are impaired in the response to DNA damage; hence, the presence of one
mutation may result in an initiated cell which is more likely to accumulate further
mutations that fuel neoplastic progression. Further studies of heterozygosity may help to
elucidate risk factors for breast cancer in mutation carriers. Evidence already exists to
suggest that the set of risk factors for mutation carriers may be different than that which
is implicated in non mutation carriers. Similarly, standard therapy regimen may not be
appropriate for mutation carriers. Studies addressing BRCA1 haploinsufficiency are
necessary to improve and develop prevention treatments and cancer therapeutic regimens.

**Future Directions**

This thesis project used one heterozygous, ER-negative cell line with one known
mutation of BRCA1 in comparison with an unrelated wildtype cell line. Future
experiments might expand these areas to address other BRCA1 mutations, ER-positive
cell lines, and further measure the repair capacity of heterozygotes. These are explored
briefly below.

Experiments with transient siRNA transfections are limited by the variability of the
BRCA1 levels once cells have been subcultured, as well as the short time frame during
which these knockdowns can be used for experiments. Stable transfection with an
inducible vector expressing the siRNA sequence would allow for knockdown of BRCA1
for extended periods of time. Similarly, this would allow for comparison between a genetically identical heterozygote and wildtype cell line.

The HPRT assay is laborious and expensive. Establishment of a reporter plasmid, such as that utilized in the lymphoblastoid experiments in chapter five would allow for more rapid analysis of induced mutations, as well as allow for characterization of the mutational spectra. Preliminary experiments to this end have been initiated and tested the efficacy of various plasmids and transfection methods. Attempts to transfec two plasmids, one bearing the HSV-tk gene and another bearing the EBNA-1 gene, as well as a single plasmid with both these genes, have been unsuccessful. Restriction digests with DpnI and Southern blotting analyses have shown no replication of the plasmid up to seven days following transfection. These experiments have demonstrated that in order for the maintenance of an episomal HSV-tk reporter plasmid, these cell lines need to first have the EBNA gene stably integrated and expressed at a level compatible with plasmid maintenance and replication. We have already evaluated the use of the HSV-tk mutational scheme in Chapter 5 of this thesis. Establishment of this system in the mammary cell lines would allow us to examine microsatellite instability in heterozygous cells. The presence of microsatellite instability may further elucidate the potential mutator phenotype of heterozygous cells.

Our model suggests that heterozygous cells demonstrate an altered cellular and mutagenic response following exposure to oxidative stress due to their impaired ability to repair DNA damage. While various other reports have demonstrated this deficiency in fibroblasts or lymphoblastoid cell lines, it has not yet been reported in mammary epithelial cell lines. Thus, a direct measurement of the repair capabilities of wildtype and
heterozygous mammary epithelial cell lines would be a novel observation. This could be measured by a variety of methods, but the most direct and most often cited method involves transfection of cell lines with a plasmid which has been linearized with restriction enzymes that make a blunt-ended double strand break. Following transfection into cells, the plasmid is extracted after a short time period. Plasmid that has been repaired is capable of being replicated. Thus, DpnI restriction enzyme analyses will demonstrate if the cell lines have repaired different amounts of plasmid. Reports in the literature have demonstrated that wildtype fibroblasts will repair approximately 40% of the ss-plasmid, while heterozygotes repair less than 10%. These analyses are dependent upon determination of appropriate conditions to allow plasmid replication in these cell lines.

The second approach to the question of haploinsufficiency might involve cell lines which are ER-positive. While DNA repair capabilities should be independent of the ER, it is possible that ER-mediated cell proliferation might magnify the effect of repair deficiency. Perhaps both of these processes are required for neoplastic transformation of heterozygous mammary cells following exposure to estradiol.

The 185delAG mutation in this study is one of the most common BRCA1 gene mutations. This mutation results in the production of a very small peptide which is rapidly degraded. While most other common mutations are also truncating mutations, most produce a protein which is large enough to escape degradation. These proteins may be dysfunctional and/or may antagonize the wildtype protein. Each of these scenarios may result in a potentially different phenotype. Inclusion of other mutations in the study
of BRCA1 haploinsufficiency is necessary to make larger conclusions about the treatments to prevent and treat cancer in mutation carriers.
Figure D1. Proposed model to account for altered phenotype of heterozygous cells. BRCA1 heterozygous cells have reduced levels of BRCA1 protein both at baseline and following the induction of DNA damage. Despite a demonstrated increase in intracellular ROS and the induction of 8oxoG, wildtype cells appear to recognize and repair DNA damage, allowing for cell proliferation without the accumulation of HPRT mutants. Given a similar oxidative insult, heterozygous cells are not able to recognize and repair DNA damage as efficiently as wildtype cells. This results in the lack of growth stimulation and transient pausing in the G2-M phase. These events are apparently not sufficient to limit DNA damage, as evidenced by the 3.7 and 6-fold induction of mutations following exposure to estradiol and HP, respectively.
REFERENCES


111. Shorrocks, J., et al., *Primary fibroblasts from BRCA1 heterozygotes display abnormal G1/S cell cycle checkpoint following UV irradiation but show normal levels of micronuclei following oxidative stress or mitomycin C treatment.* Int J Radiat Oncol Biol Phys, 2004. **58:** p. 470-478.


VITA

Mandy L. Maneval

A. Academic Experience
1999-present  Penn State College of Medicine, Cell and Molecular Biology Program and MD/PhD Training Program

B. Research Experience
1. Development of Protocol for the Genetic Transformation of Double Mutant Chlamydomonas reinhardii, Lock Haven University
2. Characterization of Bacterial Populations from Soil Enrichment Cultures Collected from the Drake Superfund Site, Lock Haven University
3. DNA Sequencing of the Lux Operon of Unknown Bioluminescent Bacteria Using Modified Sanger Dideoxy Silver Stain Method, Lock Haven University
4. Biodegradative Rate Analysis and Characterization Indigenous Soil Bacterial Isolates Collected from the Drake Chemical EPA Superfund Site, Lock Haven University
5. Searching for Novel Genes Responsive to Iron Using Subtractive Suppression Hybridization and Differential Screening, Penn State College of Medicine; Advisor: James Connor, PhD

C. Honors, Awards, and Activities
2003  Susan G. Komen Breast Cancer Foundation Dissertation Research Award
2000,2001  Associate Editor Wild Onions Literary Journal
2000-2001  Co-President American Medical Women’s Association (AMWA), Penn State chapter
1999-present  Phi Kappa Phi National Predoctoral Fellow
1999  Biology Honors Award
LHU Honors Program Scholarship
Phi Kappa Phi Chapter Scholarship
1997, 1998  Paul F. Klens Biology Scholarship
Pearl Basom Memorial Scholarship
Leah and Freida Fromm Memorial Scholarship
1997  Croda, Inc. Scholarship

D. Publications

E. Poster Presentations