FUNCTIONAL STUDIES OF ACCESSORY FACTORS ASSOCIATED WITH BASE EXCISION REPAIR

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ABSTRACT

BRIAN PACHKOWSKI: Functional Studies of Accessory Factors Associated with Base Excision Repair
(Under the direction of James A. Swenberg, D.V.M., Ph.D.)

Exposure to environmental and cellular mutagens is ubiquitous and, as a consequence, DNA is constantly faced with the possibility of becoming damaged. Base excision repair (BER) removes some of this damage to limit the impact of these exposures on cell physiology and ultimately human health. The function of core BER enzymes may be enhanced by other protein accessory factors, namely poly(ADP-ribose) polymerase-1 (PARP-1) and x-ray repair cross complementing gene 1 (XRCC1). The main hypothesis of this research was that genetic approaches using cellular knockout and complementation models can evaluate whether the accessory proteins PARP-1 and XRCC1 are determinants of BER efficiency.

While numerous biochemical studies have implicated PARP-1 in BER, the role of this protein in BER is somewhat uncertain. The first aim of this research was to evaluate the role of PARP-1 in BER in vertebrate cells. Chicken cells lacking PARP-1 were treated with an alkylating agent under different scenarios with subsequent endpoint measurements. PARP-1 was necessary as a survival factor during chronic exposure but did not appear relevant in acute exposures until the late stages of BER. In the absence of exposure, the DNA lesions measured were equal between PARP-1 proficient and deficient cells.

XRCC1 acts as a scaffold for numerous protein interactions necessary for proficient BER. However, the presence of polymorphic forms of XRCC1 in the human population may

influence DNA repair and disease susceptibility. The second aim of this research was to demonstrate the applicability of using transgenic cells in a combined study design for determining the biological significance of XRCC1 polymorphisms. Isogenic, mammalian cells lacking XRCC1 were transfected with various forms of the human *XRCC1* gene, exposed to different genotoxicants, and assessed for single strand break repair capacity. Only cells expressing the 280His variant showed a repair defect. Subsequently, evaluation of data from the Carolina Breast Cancer Study demonstrated associations between XRCC1 280His, smoking, and breast cancer.

Together these studies demonstrate that accessory factors can influence BER efficiency and illustrate the importance of a multi-disciplinary approach for investigating the link between genes, the environment, and disease risk.

DEDICATION

To my parents, John and Lorraine, and grandparents, Frank, Caroline, Michael, and Catherine, who have always inspired me.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

3-AB	3-aminobenzamide
5′-dRP	5′-deoxyribose phosphate
AA8	wild-type Chinese hamster ovary cell line, parental line of EM9
AP site	apurinic/apyrimidinic site
APE	apurinic/apyrimidinic site endonuclease
Arg	arginine
ARP	aldehyde reactive probe
ASB	aldehyde reactive probe slot blot
BER	base excision repair
BRCT	BRCA1 C-terminal
CBCS	Carolina Breast Cancer Study
CHO	Chinese hamster ovary
CI	confidence interval
CPT	camptothecin
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DSB(s)	double strand break(s)
DT40	wild-type chicken B-lymphocyte cell line
ECL	enhanced chemiluminescence reagents
EH	estimating haplotype frequencies
EM9	mutant Chinese hamster ovary cell line, XRCC1 deficient

ETS	environmental tobacco smoke
FBS	fetal bovine serum
FEN-1	flap endonuclease-1
GGE	glyoxal gel electrophoresis
Gln	glutamine
h	hour
H_2O_2	hydrogen peroxide
His	histidine
ICR	interaction contrast ratio
kDa	kilodalton
LIGIIIα	DNA ligase 3 alpha
LP-BER	long-patch base excision repair
LRT	likelihood ratio test
MEMα	minimum essential medium alpha
mL	milliliter
M	molar
mM	millimolar
min	minute(s)
MMS	methyl methanesulfonate
N7-meG	N7-methylguanine
N3-meA	N3-methyladenine
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced form of nicotinamide adenine dinucleotide

NAD(P)H	reduced form of nicotinamide adenine dinucleotide phosphate
NLS	nuclear localization signal
OGG	8-oxoguanine glycosylase
OR	odds ratio
PAR	poly(ADP-ribose)
PARP-1	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
PNKP	polynucleotide kinase phosphatase
POLβ	polymerase beta
roN7-meG	2,6-diamino-4-hydroxy-5-*N*-methyl-formamidopyrimidine
ROS	reactive oxygen species
S.D.	standard deviation
SNP	single nucleotide polymorphism
SSB(s)	single strand break(s)
SSBR	single strand break repair
SP-BER	short patch-base excision repair
TEMPO	2,2,6,6-tetramethylpiperidinoxyl
Trp	tryptophan
V	volt(s)
wt	wild-type
XRCC1	x-ray cross complementing gene 1 protein

LIST OF SYMBOLS

°C	degree Celsius
α	alpha
β	beta
δ	delta
ε	epsilon
μ	micro
′	prime

CHAPTER 1. INTRODUCTION

DNA damage represents an inevitable and ubiquitous event because of exposure to endogenous and environmental agents. Various repair pathways correct modifications to DNA in order to ensure genomic integrity, which can ultimately prevent adverse health effects. Of the many types of genetic lesions, base modifications and DNA single strand breaks (SSBs) occur as some of the most frequent insults to DNA. The base excision repair (BER) pathways act to limit such damage. Because of the frequency and potential lethality of BER intermediates, understanding the basic mechanisms of their repair and potential impact on human disease has attracted much attention. While not core pathway members, the proteins poly(ADP-ribose) polymerase-1 (PARP-1) and x-ray repair cross complementing gene 1 (XRCC1) have accessory roles that may affect BER efficiency. PARP-1 acts as a sensor of DNA breaks and generates signals that, in part, help to initiate DNA repair. However, the exact role of PARP-1 in BER within cells remains somewhat obscure. XRCC1 functions as a scaffolding factor and modulator of certain aspects of DNA repair. The existence of polymorphic forms of XRCC1 may compromise DNA repair proficiency and ultimately have implications on human disease risk. The present investigation explores the functional significance of PARP-1 and XRCC1 within BER to better ascertain their potential impacts at the cellular and population levels.

1.1 DNA Damage and BER

Various physical and chemical agents present in the environment can deleteriously modify DNA structure (Pitot and Dragan, 1996). Additionally, the generation of reactive by-products from cellular processes, including the immune response and xenobiotic metabolism (Marnett, 2000), leads to constitutive levels of DNA damage (Beckman and Ames, 1997; Nakamura and Swenberg, 1999). Chemical bonds within DNA, including those that attach DNA bases to the sugar-phosphate backbone or those within DNA bases, are inherently unstable and subject to spontaneous hydrolysis (Duncan and Miller, 1980; Lindahl, 1993). Together, the above phenomena lead to an array of damage products including: base adducts, abasic sites, deoxyribose fragments, strand breaks, as well as DNA-DNA and DNA-protein crosslinks (Pitot and Dragan, 1996).

Cells encumbered with DNA lesions must possess an adequate damage response to assure genomic integrity for continued viability, the accurate transmission of genetic material, and prevention of tumorigenesis. A number of DNA repair pathways, each consisting of a cadre of proteins responsible for the removal of classes of lesions, are charged with the abatement of DNA damage (Hoeijmakers, 2001). For example, nucleotide excision repair (NER) removes major distortions to the DNA helix (Mitchell et al., 2003) while BER repairs small, non-bulky modifications to DNA bases and discontinuities in the DNA strand (Barnes and Lindahl, 2004; Sweasy et al., 2006). Base mismatches formed by erroneous replication or by spontaneous events are corrected by mismatch repair (MMR) (Kunkel and Erie, 2005) and, through a single step mechanism, direct reversal removes methyl groups from O^6-methylguanine (Mishina et al., 2006). When double strand breaks (DSBs) form, the homologous recombination (HR) and non-homologous end joining (NHEJ) pathways repair

such damage (Helleday et al., 2007). While the individual pathways do show preference toward certain types of DNA damage, there is evidence that pathways can overlap or compensate for another to ensure the integrity of genetic material (Swanson et al., 1999; Pascucci et al., 2005).

Since these pathways are fallible, DNA lesions can escape repair and ultimately have deleterious effects. Damage to DNA can be converted to mutations, which if they were to occur in critical genes can lead to tumorigenesis. Unprocessed DNA damage can also promote cell death, a continuation of this phenomena over time can lead to degenerative diseases and affect longevity (Hasty, 2005). Inheritable defects occur in some repair pathways thereby causing cancer, premature aging, or neurological defects (Bohr, 2002). In contrast to these extreme conditions, much subtler alterations, as will be discussed below, exist in repair proteins that can affect an individual's response to a chemical exposure.

Of the different repair mechanisms, BER pathway acts as the sentinel against the hydrolytic, oxidative, and alkylation damage to DNA commonly arising from endogenous processes and some environmental exposures (Barnes and Lindahl, 2004; Sweasy et al., 2006). Intact BER appears to be a requisite biochemical process since complete abrogation of some BER genes, such *Ape*, *Xrcc1*, and *Polβ* confers embryonic lethal phenotypes in mice (reviewed in Larsen et al., 2007). The classical BER pathway has been reconstituted *in vitro* with 4 enzymes (Kubota et al., 1996) and could be summarized by five core steps involving: (i) removal of a damaged base via spontaneous hydrolysis or glycosylase activity with apurinic/apyrimidinic (AP) site formation; (ii) incision of the DNA strand on the 5′ side of the AP site by AP endonuclease (APE) to form a 5′-deoxyribose phosphate (5′-dRP) moiety; (iii) replacement of the appropriate nucleotide by polymerase β (POLβ); (iv) removal of the

nicked AP site by the dRP lyase activity of POLβ; and (v) DNA strand ligation by DNA ligase IIIα (LIGIIIα) (Srivastava et al., 1998) (Figure 1.1). These steps are believed to occur via a series of DNA-protein and protein-protein interactions and exchanges that occur as a series of repair complexes. In essence, BER intermediates are handed off from one protein to the next in a "pass the baton" fashion, which limits the exposure of the repair site to the surrounding environment (Wilson and Kunkel, 2000). In reality, BER is a much more complex process involving accessory factors, such as PARP-1 and XRCC1. Such factors do not cut, synthesize, or ligate DNA as do the core BER enzymes, rather PARP-1 and XRCC1 are believed to enhance pathway efficiency through posttranslational modifications or protein-protein interactions, respectively (Fan and Wilson, 2005).

The number of sub-pathways and the spectrum of lesions processed by BER increase the complexity of this pathway. Recently, a model has been proposed in an attempt to unify the different BER sub-pathways (Almeida and Sobol, 2007). This model is based on dividing BER into three general processes, each consisting of unique repair complexes or chemical events contingent on the BER substrate present. Accordingly, BER can be described as consisting of: (i) lesion recognition and/or strand scission as the ingress into BER; (ii) DNA gap tailoring to allow for nucleotide replacement; and (iii) DNA synthesis and/or ligation for repair completion. Of the different known BER sub-pathways short-patch (SP), long-patch (LP), and SSB repair (SSBR) will be discussed.

Entry into SP BER generally commences with the formation of alkylated bases such as N3-methyladenine (N3-meA) or N7-methylguanine (N7-meG), which are cleaved by a mono-functional glycosylase such as N-methylpurine DNA glycosylase (MPG). Alternatively, the glycosidic bond attaching the adducted base to the DNA backbone can

spontaneously hydrolyze to form an AP site. This lesion is cleaved on the 5′ side by APE to form a SSB with 5′-dRP and 3′-hydroxyl termini prior to gap tailoring initiated by POLβ. The dRP lyase activity of POLβ removes the 5′ terminal lesion allowing for the one nucleotide synthesis by POLβ with final strand ligation by DNA LIGIIIα. An additional entry into SP is with bifunctional glycosylases, which can recognize oxidative base damage and remove the damaged base while cutting the resulting AP site on the 3′ side for subsequent gap tailoring by APE (Wilson and Bohr, 2007). Completion of repair through DNA synthesis and ligation reactions can proceed with proteins associated with SP or the LP sub-pathway.

Similar to SP, entry into LP BER proceeds with the removal of damaged bases by glycosylase activity. However, during gap tailoring some BER intermediates may be refractory to β-elimination by the dRP lyase domain of POLβ. The presence of the oxidized abasic site 2-deoxyribonolactone (Sung and Demple, 2006), reduced AP sites formed by bifunctional glycosylases, or a massive accumulation of 5′-dRP lesions may cause a switch away from the complement of DNA synthesis and ligation enzymes used in SP. Accordingly, LP BER proceeds with the incorporation of 2 to 15 nucleotides at the damage site by POLβ or the replicative polymerases δ or ε. This synthesis occurs in conjunction with proliferating cell nuclear antigen (PCNA) and flap endonuclease-1 (FEN-1), which cleaves the oligonucleotides displaced by polymerase activity. Strand closure then precedes with DNA ligase I (LIGI) activity.

For SSBR, entry into this BER sub-pathway starts with the formation of frank DNA SSBs resulting from the oxidation of deoxyribose through hydrogen abstraction by reactive oxygen species (ROS). In addition to sugar fragmentation and base loss, deoxyribose

oxidation typically causes strand scission with the formation of characteristic DNA strand termini (Pogozelski and Tullius, 1998). These terminal lesions can lack the conventional 3′-hydroxyl and 5′-phosphate groups necessary for the ligation of a broken DNA strand (Caldecott, 2001). Gap tailoring events by APE and polynucleotide kinase phosphatase (PNKP) remove damaged termini subsequent to SSB detection by PARP-1 and XRCC1 recruitment. The remainder of SSBR consists of DNA synthesis and ligation by the activities of POLβ and LIGIIIα, respectively.

The proposition of removing base adducts via BER comes at the expense of generating intermediates, namely AP sites and SSBs, that have the potential of being more deleterious to the cell than the initial base lesion (Rinne et al., 2005). The spontaneous generation of AP sites is believed to be high in a cell, with 9000 AP sites generated each day (Nakamura et al., 1998), such a level is only exacerbated by the removal of non-bulky base damage formed by genotoxic agents. Without the presence of a nucleobase, AP sites have the potential to cause point mutations with a general rule that an adenine is inserted opposite these non-informative lesions during DNA replication (Simonelli et al., 2005). AP sites also elicit cytotoxicity by blocking DNA synthesis (Guillet and Boiteux, 2002).

The processing of AP sites to restore the DNA sequence involves the formation of SSBs. In addition to this indirect route of formation, SSBs form through a direct mechanism involving oxidative strand scission (Pogozelski and Tullius, 1998). Discontinuities in the DNA strand threaten cell function and overall survival. As suggested by a prokaryotic model, RNA polymerases may skip over SSBs in a template strand leading to the formation of mRNA transcripts with deletions, in effect causing a transcriptional mutagenesis (Saxowsky and Doetsch, 2006). SSBs may also block RNA polymerase II (Kathe et al.,

2004), thereby affecting gene expression levels. The accumulation of stalled RNA polymerases at SSBs has also been speculated to induce apoptotic cell death, particularly in non-dividing cells (Wilson and Mattson, 2007). In cells that divide, SSBs can lead to replication fork collapse and the formation of cytotoxic DSBs (Kuzminov, 2001).

To limit the potential mutagenic, cytotoxic, or clastinogenic effects from AP sites and SSBs, proficient BER is needed for the quick resolution of these repair intermediates. A departure from proficient repair, as demonstrated by the knocking out of certain BER genes, illustrates the profound effect of unrepaired BER intermediates. Gene deletions for BER proteins that are active post base removal, where AP sites and SSBs exist, show the most extreme phenotypes. For example, cells lacking POLβ are hypersensitive to methylating and ethylating agents that cause BER substrates (Horton et al., 2003). The etiology of such a phenotype is hypothesized to be due to a lack of dRP lyase activity, which allows for the persistence of SSBs formed during the normal course of BER (Sobol et al., 2000).

Similarly, the deletion of other downstream BER genes, such as *XRCC1* and *PARP-1*, lead to phenotypes consistent with a decrease in DNA repair capacity. While mice lacking *XRCC1* die during development (Tebbs et al., 1999), such a phenotype is observed in *PARP-1* mice only in conjunction with a deletion of *PARP-2* (Menissier de Murcia et al., 2003). Regardless of this discrepancy in embryonic viability, when models deficient in either XRCC1 or PARP-1 are treated with alkylating agents they exhibit a similar phenotype consisting of hypersensitivity, an impaired capacity to repair SSBs, and increases in deletion mutations (Thompson et al., 1982; Trucco et al., 1998; Op het Veld et al., 1998; Shibata et al., 2005). These observations imply that XRCC1 and PARP-1 work toward the repair of a similar repair intermediate. As will be discussed below, the functions of PARP-1 and

XRCC1 also appear to promote BER activities. Considering that the repair of mutagenic and cytotoxic lesions, which are constantly being formed, is potentially contingent on PARP-1 and XRCC1, understanding the functions of these proteins in living cells may provide a better understanding of the mechanisms involved in maintaining DNA integrity.

1.2 PARP-1 in BER

PARP-1 is considered to be the prototypical member of a family of enzymes capable of using NAD^+ as a substrate for the synthesis of poly(ADP-ribose) (PAR) for the posttranslational modification of certain nuclear proteins (Ame et al., 2004). Of the 18 separate genes associated with the PARP family, PARP-1 accounts for a majority of PAR synthesis (Shieh et al., 1998). PARPs and the ribosylation reactions they catalyze are linked to a number of cellular processes related to DNA metabolism including repair, transcription, and cell division (Schreiber et al., 2006). Such reactions may also have repercussions at the organismal level through their reported involvement in cancer, aging, and inflammation (Kim et al., 2005).

The human *ADPRT* gene resides on chromosome 1q41-q42 and encodes a 113 kDa PARP-1 peptide (Herzog et al., 1989). PARP-1 consists of four structural elements with known function: a N-terminal DNA binding domain (DBD), a bipartite nuclear localization signal (NLS), a central interaction/automodification domain, and a C-terminal catalytic domain (D'Amours et al., 1999). Two zinc finger motifs within the DBD allow for PARP-1 binding to primarily SSBs and DSBs but also undamaged and supercoiled DNA (Petrucco, 2003). As inherent to these sequences, the bipartite NLS directs nascent PARP-1 to the nucleus (Schreiber et al., 1992). Another notable amino acid sequence, a caspase cleavage

site, resides amid the NLS (Lazebnik et al., 1994). Within the interaction/automodification domain exist leucine zipper and BRCA1 C-terminal (BRCT) motifs that act as interfaces for protein-protein interactions (Alber, 1992; Bork et al., 1997). Additionally, this domain consists of glutamic acid residues that serve as PAR acceptors (Duriez et al., 1997). The catalytic domain, which possesses a NAD^+ binding site, initiates the synthesis of PAR as well as elongation and PAR branching reactions (Figure 1.2).

To become active in PAR synthesis, PARP-1 must bind a SSB. A "PARP shuttling" model can then describe the molecular events associated with PARP-1 activity (D'Amours et al., 1999). As a homodimer, PARP-1 binding to a SSB increases polymerase activity up to 500-fold (Simonin et al., 1993). With activation, PARP-1 mainly targets itself for the attachment of PAR polymers, which can reach up to 200 ADP-ribose units long (Ogata et al., 1981). The accumulation of PAR polymers during automodification builds a negative charge around PARP-1 causing dissociation from the anionic DNA polymer. With PARP-1 dissociation, ribosylation reactions cease until PARP-1 binds to additional SSBs.

PARP-1 also ribosylates over 30 protein substrates that participate in nucleic acid metabolism (D'Amours et al., 1999). The covalent addition of PAR polymers to glutamate or aspartate residues on acceptor proteins can lead to structural and functional alterations. Two prominent acceptors of PAR polymers are histones H1 and H2B (Ogata et al., 1980a; Ogata et al., 1980b). Such histone modification relaxes chromatin structure (Poirier et al., 1982), potentially increasing access to SSBs. In addition, certain proteins with PAR binding motifs can non-covalently interact with PAR polymers (Pleschke et al., 2000); such interactions may regulate protein interactions, localization, and degradation. The existence of PAR polymers, whether attached to an acceptor protein or PARP-1, is transient. The

enzyme poly(ADP-ribose) glycohydrolase hydrolyzes PAR polymers with such rapidity that the polymers have a half-life less than 1 minute when generated in response to DNA damage (Wielcken et al., 1983).

Due to its proclivity for binding to DNA SSBs, PARP-1 has garnered the reputation of being a molecular nick sensor and an active member in maintaining genomic stability (de Murcia and Menissier de Murcia, 1994). Since SSBs are formed during and repaired by BER, investigations have attempted to determine whether PARP-1 participates in this pathway. Initial approaches for assessing PARP-1 in DNA repair relied on a variety of methods including the use of chemical PARP inhibitors, antisense strategies, and the over-expression of a dominant-negative version of PARP (Burkle et al., 2000). However, the generation of viable *Parp-1* knockout mice provided an animal model for assessing the role of PARP-1 in BER (Shall and de Murcia, 2000). These animals exhibit a hypersensitive phenotype towards ionizing radiation and alkylating agents (de Murcia et al., 1997; Wang et al., 1997). Though PARP-1 deficient mice experience increased cancer susceptibility to agents that alkylate DNA (Tsutsumi et al., 2001; Nozaki et al., 2003), they resist tumor formation after exposure to chemicals that induce bulky DNA adducts (Gunji et al., 2006; Ogawa et al., 2006). Cells derived from PARP-1 knockout animals display similar hypersensitivities to ionizing radiation and alklyating agents, and show evidence of chromosomal damage (de Murcia et al., 1997; Trucco et al., 1998; Masutani et al., 1999). However, conflicting reports exist for whether PARP-1 is in fact required for proficient repair *in vivo* after exposure to alkylating agents (Trucco et al., 1998; Vodenicharov et al., 2000).

SSB formation and PARP-1 activation has been shown to recruit XRCC1 and other PARP-1 molecules to damage sites in living cells (El-Khamisy et al., 2003; Mortusewicz et al., 2007). Additional investigations using cell extracts or purified proteins have also established putative mechanisms by which PARP-1 could participate in BER. PARP-1 can bind to a structural analog of the 5′-dRP terminus formed by APE incision, and such a structure may be a source of competition between the two enzymes (Lavrik et al., 2001; Cistulli et al., 2004). PARP-1 interacts with POLβ (Dantzer et al., 2000) and, along with FEN-1, can also stimulate POLβ-dependent strand displacement synthesis necessary for LP BER (Prasad et al., 2001). LIGIIIα also participates in a functional and physical interaction with PAR and poly(ADP-ribosyl)ated PARP-1, which leads to increased strand ligation (Leppard et al., 2003).

When compiled, data regarding PARP-1 in BER suggest that the protein has the ability to: decondense chromatin; signal and recruit repair apparatus; protect repair intermediates; and enhance enzyme activity. Defining the role of PARP-1 in BER is further complicated since PARP-1 overactivation from extremely high levels of DNA damage causes massive depletions in NAD^+ and ATP levels leading to cell necrosis (Ha and Snyder, 1999).

1.3 XRCC1 and Polymorphisms

XRCC1 was first fully identified during gene complementation studies where the human *XRCC1* gene corrected the SSBR defective phenotype and high sister chromatid exchange levels observed in EM9 cells (Thompson et al., 1990). The EM9 model, a mutant of AA8 Chinese hamster ovary (CHO) cells was initially characterized by an enhanced

sensitivity to and mutagenesis by ethyl methanesulfonate (Thompson et al., 1982). Additionally, EM9 cells are sensitive to cell killing by a number of SSB inducing chemicals including: methylating agents, hydrogen peroxide (H_2O_2), camptothecin, and ionizing radiation (Thompson and West, 2000). Molecular analysis later established that the reason for the EM9 phenotype was due to a frameshift mutation at codon 220 in the *XRCC1* gene, which produces a truncated peptide about one-third the size of the wild-type protein (Shen et al., 1998a). Collectively, these data initially established XRCC1 as a central component for the efficient repair of SSBs.

The human *XRCC1* gene resides on chromosome 19q13.2 and encodes a 69.5 kDa peptide (Lamerdin et al., 1995). XRCC1 consists of an N-terminal domain and two BRCT domains, where one is located centrally (BRCTI) and the other at the C-terminal domain (BRCTII). These BRCT domains, which are found among a number of DNA repair and cell cycle checkpoint proteins, serve as modules for protein interactions (Huyton et al., 2000). Interspersed between these two BRCT domains are two linker regions and a NLS. Specific protein and DNA interactions have been assigned throughout the different XRCC1 domains (Caldecott, 2003b) (Figure 1.3).

While no known enzymatic activity has been described for XRCC1, this accessory factor is believed to function as a scaffold for the numerous protein-protein and protein-DNA interactions necessary for BER (Caldecott, 2003b). Through its various interaction domains, XRCC1 either stimulates, recruits, or stabilizes every core member of the BER pathway. *In vitro* experiments demonstrated that XRCC1 physically interacts with and stimulates a number of DNA glycosylases including 8-oxoguanine glycosylase (Marsin et al., 2003; Campalans et al., 2005). XRCC1 has been implicated in modulating the incision of AP sites

through a physical interaction with APE (Vidal et al., 2001). With a stabilizing effect, XRCC1 appears to be necessary for maintaining normal levels of LIGIIIα (Caldecott et al., 1994; Caldecott et al., 1995). An interaction between XRCC1 and POLβ has been described to be necessary for efficient SP BER (Dianova et al., 2004). However, such an interaction may also hinder POLβ strand displacement needed for LP BER (Kubota et al., 1996). In addition to the core members of BER, XRCC1 has been demonstrated to interact with a number of other BER related proteins including PNKP (Whitehouse et al., 2001), PCNA (Fan et al., 2004), aprataxin (APTX; Clements et al., 2004), tyrosyl DNA phosphodiesterase (TDP; Plo et al., 2003), and PARP-2 (Schreiber et al., 2002). An interaction between PARP-1 and XRCC1 has been described where activated PARP-1 recruits XRCC1 to damage sites in an effort that is believed to initiate or enhance repair (Masson et al., 1998; El-Khamisy et al., 2003). The ability of XRCC1 to interact with multiple partners allows for a model in which preformed repair complexes exist within the cell. While there is evidence for these XRCC1 complexes, a more likely scenario involves the conditional recruitment of BER proteins depending on the origin (i.e. direct versus indirect SSB formation) of the site to be repaired (Caldecott, 2003a; Luo et al., 2004)

The *in vivo* requirement for XRCC1 is quite evident. Animal studies have demonstrated that loss of the *Xrcc1* gene in mice leads to an embryonic lethal phenotype (Tebbs et al., 1999), which is rescued by transgene-complementation (Tebbs et al., 2003). In two separate studies the use of RNA interference established a need for XRCC1 in human cells. The resulting decrease in XRCC1 levels caused hypersensitivity, a decrease in SSBR capacity, and increased formation of micronuclei following chemical exposure (Brem and Hall, 2005; Fan et al., 2007). While the above manipulations of XRCC1 do not occur

naturally, more subtle human variations exist for this protein. These genetic variants may alter the influence XRCC1 has on BER and ultimately human health.

During the course of human evolution, mutations at single base pairs have occurred and been maintained in the population (Brookes, 1999; Sunyaev et al., 2000). These single nucleotide polymorphisms (SNPs), which by convention have an allelic frequency of 1% or greater, occur at a rate of 1 in 1000 base pairs and account for over 90% of genetic variation in the human genome (Brookes, 1999). While a number of SNPs exist within the *XRCC1* gene, three major SNPs have been identified within exons 6, 9, and 10, which correspond to amino acid positions 194, 280, and 399 of the XRCC1 protein, respectively (Shen et al., 1998b; Ladiges, 2006). Molecular analysis suggests that the allele frequencies for the SNPs within exons 6, 9, and 10 may be 0.25, 0.08, and 0.38, respectively (Shen et al., 1998b; Wang et al., 2003).

Within the coding region of the *XRCC1* gene, the presence of SNPs that change the amino acid sequence of the resulting gene product are of particular concern. In general, these nonsynonymous SNP (nsSNPs) may be functional by altering the stability, substrate specificity, catalytic ability, and interaction sites of the resulting variant protein (Sunyaev et al., 2001; Kelada et al., 2003). The three major XRCC1 polymorphisms are all nonsynonymous, where the wild-type amino acid arginine (Arg) is replaced with a tryptophan (Trp), histidine (His), or glutamine (Gln) at codons 194, 280, and 399, respectively. Each nsSNP may interfere with a specific XRCC1-protein interaction, which could then modulate DNA repair capacity and lead to the formation of mutations and possibly cancer (Figure 1.3). Polymorphisms, such as *XRCC1* variants, are generally considered low penetrance because they likely have a mild affect on disease risk, but occur in

an appreciable proportion of the population. Since certain gene variants can modify the effect of a chemical exposure this gives rise to the notion of a gene-environment interaction. Accordingly, individuals who possess certain genetic variants may be considered susceptible to certain diseases from environmental exposures.

To demonstrate the significance of XRCC1 polymorphisms, population based studies have attempted to show a link between the different *XRCC1* SNPs and cancer. Data are mixed for the codon 399 polymorphism with observed increases for breast and stomach cancers and decreases in esophageal and bladder cancers (reviewed in Goode et al., 2002 and Hung et al., 2005). The codon 194 variant may be slightly protective with observed decreases in breast, lung, and bladder cancers (reviewed in Goode et al., 2002 and Hung et al., 2005). The relatively few studies regarding the codon 280 polymorphism, which were hampered by small sample sizes, did not suggest any associations with cancer (reviewed in Goode et al., 2002 and Hung et al., 2005). These epidemiology data are not definitive, and like association studies for other genes, may suffer from low statistical power; be confounded by polymorphisms in other DNA repair genes; represent a false positive result; or fail to consider environmental exposures (Hung et al., 2005).

Mechanistic data generated through laboratory and other approaches may help clarify the biological importance of *XRCC1* polymorphisms. While *in silico* approaches have been developed to assess the significance of variant gene products, laboratory based functional assays have played a major role in attempting to confirm or refute epidemiologic findings regarding polymorphic genes and cancer risk (Au et al., 2003; Ng and Henikoff, 2006). These experimental approaches challenge genotyped human tissues with a mutagen and then compare biomarkers of DNA damage to determine relative repair proficiency. Since DNA

repair pathways consist of multiple proteins, each with potentially a number of variants, complex genotypes arise that may confound observations for a single variant of interest. To circumvent some of the issues inherent with measuring human samples (Berwick and Vineis, 2000; Mohrenweiser et al., 2003), a simpler model involving isogenic cells may be able to discern the functionality of a variant protein.

1.4 Rationale and Specific Aims

Efficient BER is necessary for maintaining cell viability and genomic integrity. Numerous studies report that PARP-1 and XRCC1 acts as accessory factors in BER. Accordingly, the activities of these proteins appear to enhance the efficiency of BER. The exact role of PARP-1 in BER remains under some debate. Conversely, the role of XRCC1 in BER is more established; however, the presence of polymorphic forms of XRCC1 may influence DNA repair. Some approaches that employ purified proteins or use human samples to assess these issues may not provide a precise representation of actual nuclear events or protein functionality. *We hypothesize that genetic approaches using cellular knockout and complementation models can evaluate whether the accessory proteins PARP-1 and XRCC1 are determinants of BER efficiency.* This hypothesis will be tested with the specific aims below.

To evaluate the role of PARP-1 in BER in vertebrate cells

The role of PARP-1 in BER has generated much controversy. To clarify this debate, cell free models have been employed to gain insight regarding the biochemical events associated with PARP-1 activity in BER. Such approaches, while informative, may not be

relevant to PARP-1 activity within living cells. Issues such as the interaction of PARP-1 with non-chromatin repair substrates such as plasmids and oligonucleotides; enzyme and DNA stoichiometry; and exogenous supplementation of NAD^+ may diminish the relevance of observations gleaned from *in vitro* approaches (D'Amours et al., 1999; Sukhanova et al., 2005). We hypothesize that PARP-1 is necessary for efficient BER during methyl methanesulfonate (MMS) exposure in vertebrate cells. To address this hypothesis, DT40 chicken cells and their isogenic PARP-1 null counterparts will be treated with MMS to generate BER substrates. Subsequent endpoint analyses for markers of DNA damage will determine whether PARP-1 influences cell survival and whether certain BER intermediates accumulate in the absence of PARP-1. Such an approach is expected to identify processes within BER that may be directly or indirectly affected by PARP-1 activity in response to chemical exposure.

To demonstrate the applicability of using transgenic cells in a combined study design for determining the biological significance of XRCC1 polymorphisms

The presence of XRCC1 polymorphisms in the human population represents a potential concern to public health. At the cellular level, the functional significance of XRCC1 variant proteins remains poorly understood, in part because of the use of experimental models with heterogeneous genetic backgrounds. Determining whether genetic polymorphisms enhance disease risk ultimately requires a multi-disciplinary approach linking biological plausibility with relevance to the human population (Costa and Easton, 2006). However, conventional study designs have been limited to either laboratory or population based approaches. We hypothesize that coupling laboratory and epidemiologic

data will provide a robust study design for identifying detrimental repair protein variants and for determining gene-environment interactions. Testing this hypothesis will initially be accomplished with the use of isogenic, XRCC1 deficient EM9 cells transfected with different polymorphic forms of the human *XRCC1* gene. These cells will then be evaluated based on their ability to repair SSBs caused by exposure to different genotoxicants. These functional analyses are expected to allow for the generation of a hypothesis that certain *XRCC1* genotypes are associated with disease risk from a relevant environmental exposure. This hypothesis will then be evaluated using an epidemiologic dataset, the Carolina Breast Cancer Study (Newman et al., 1995), to determine whether there are associations between any purported repair deficient genotypes, tobacco smoke exposure, and breast cancer risk.

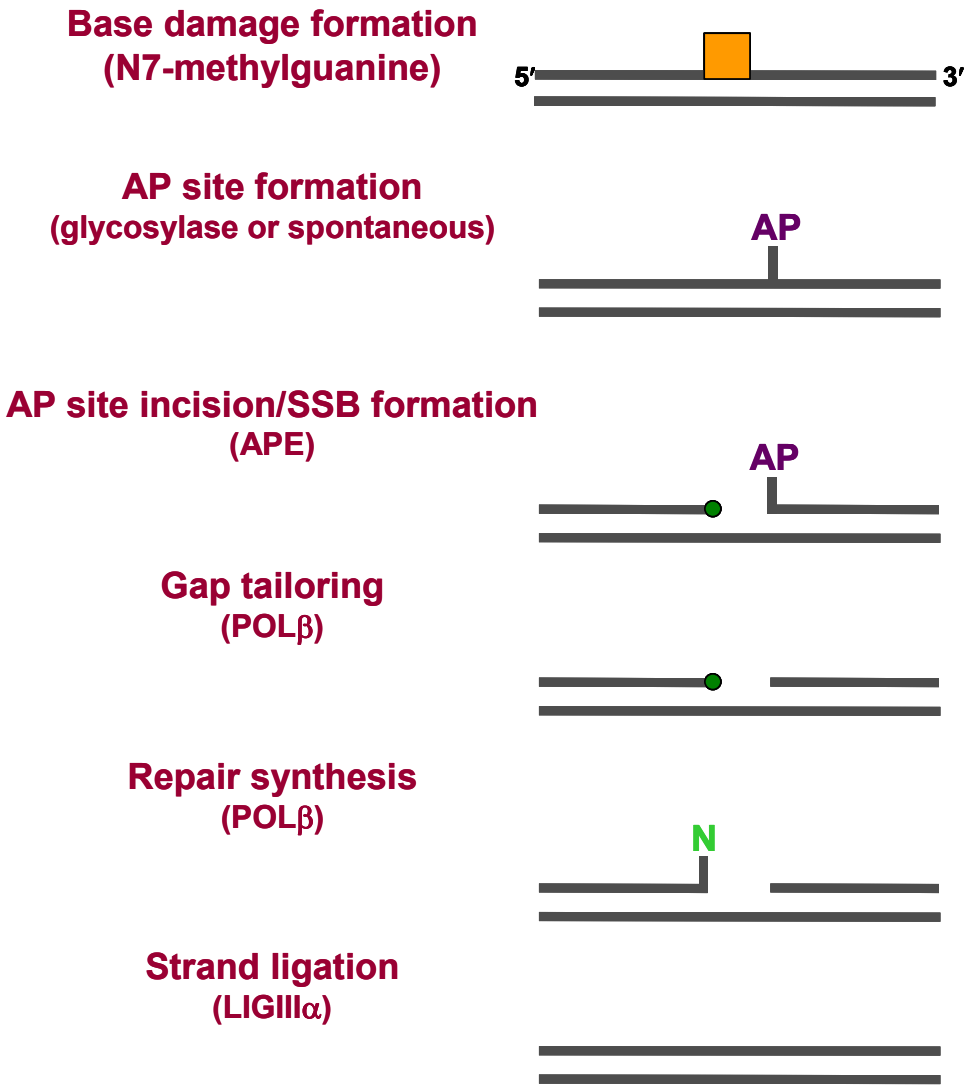


Figure 1.1. Classical BER pathway. Pathway event denoted on left with corresponding lesion, process, or protein in parenthesis.

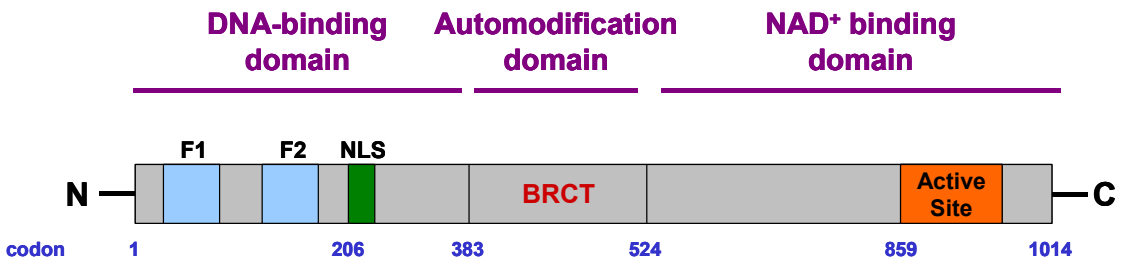


Figure 1.2. Schematic view of PARP-1.

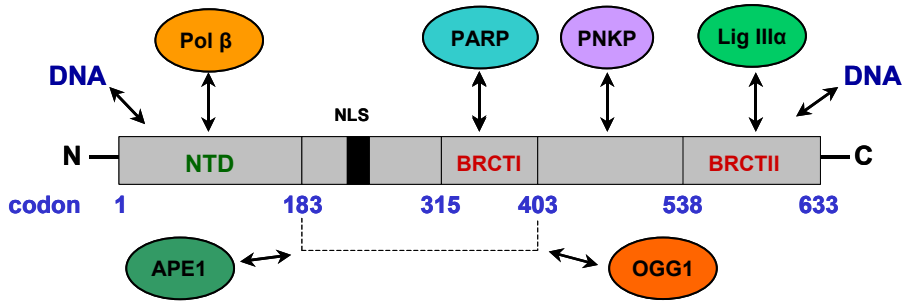


Figure 1.3. Schematic view of XRCC1 and protein interactions.

CHAPTER 2. ASSESSING A PARP-1 DEFICIENT PHENOTYPE

2.1 Abstract

Poly(ADP-ribose) polymerase-1 (PARP-1) is a BER protein that binds to DNA single strand breaks (SSBs) and subsequently synthesizes and transfers poly(ADP-ribose) polymers to various nuclear proteins. Numerous biochemical studies have implicated PARP-1 as a modulator of BER; however, the role of PARP-1 within BER in living cells remains unclear. To test the hypothesis that PARP-1 is necessary for efficient BER during methyl methanesulfonate (MMS) exposure in vertebrate cells, intact DT40 chicken cells and their isogenic PARP-1 null counterparts were challenged with different exposure scenarios for phenotypic characterization. With chronic treatment, PARP-1 null cells exhibited sensitivity to MMS but with an acute treatment did not accumulate base lesions or AP sites to a greater extent than wild-type cells. However, an increase in SSB content in PARP-1 null cell DNA, as indicated by glyoxal gel electrophoresis, suggested the presence of intermediates awaiting final strand ligation. These data suggest that during exposure, PARP-1 impacts the late stages of BER. We also propose that the function of PARP-1 in BER may be dependent on the relationship between the levels of DNA damage and intracellular NAD^+ concentration.

2.2 Introduction

Base excision repair (BER) limits DNA damage formed through spontaneous or oxidative processes associated with endogenous metabolism (Barnes and Lindahl, 2004). Additionally, BER removes non-bulky base damage, such as N7-methylguanine (N7-meG),

caused by exposure to mono-functional alkylating agents (Wyatt and Pittman, 2006). With formation of such alkylative damage, entry into BER can proceed with the removal of the adducted base from the DNA strand via spontaneous depurination or by the mono-functional methyl purine glycosylase. The resulting intact apurinic (AP) site is incised by AP endonuclease (APE), thereby generating a single strand break (SSB) with a 5′-deoxyribosephosphate (5′-dRP) terminus. Subsequently, polymerase β (POLβ) removes the 5′-dRP moiety and replaces the appropriate nucleotide to the DNA sequence. DNA ligase IIIα (LIG IIIα) finally seals the DNA strand to complete this sequence of events, which is commonly referred to as short-patch (SP) BER. Alternatively, the long-patch (LP) sub-pathway, which consists of a different complement of enzymes, can also operate to remove 5′-dRP residues and ligate DNA. Following the binding of proliferating cell nuclear antigen, POLβ or the replicative polymerases δ or ε participate in strand displacement synthesis creating a 2 to 8 nucleotide flap that is excised from DNA by flap endonuclease-1 (FEN-1). DNA ligase I subsequently closes the DNA strand (Fortini and Dogliotti, 2007).

A perturbation in BER enzyme activity can have profound cellular consequences. Cells deficient in the core BER enzyme, POLβ, are hypersensitive to chemicals such as methyl methanesulfonate (MMS), which induce BER substrates (Sobol et al., 2000). Such a phenotype is believed to be due to a lack of dRP lyase activity that causes an imbalance in BER, which then allows for the persistence of SSBs with a 5′-dRP margin formed during the normal course of BER. If not repaired prior to replication, SSBs can be converted to cytotoxic double strand breaks (DSBs) (Kuzminov, 2001). To increase pathway efficiency and limit the persistence of SSBs and other intermediates, a number of posttranslational modifications and accessory factors participate in BER (Fan and Wilson, 2005). One such

factor is the non-enzymatic protein x-ray repair cross complementing group 1 (XRCC1), which interacts with core BER enzymes and (Caldecott et al., 1995; Kubota et al., 1996; Vidal et al., 2001; Campalans et al., 2005) acts as a scaffold for the congregation of necessary repair enzymes around SSBs (Caldecott, 2003b). The influence of such accessory factors on DNA repair proficiency has been demonstrated by the hypersensitive and decreased repair phenotype of cells with mutant XRCC1 (Thompson et al., 1982).

A posttranslation modification believed to limit genotoxic stress is the synthesis and covalent addition of poly(ADP-ribose) (PAR) polymers to acceptor proteins associated with DNA metabolism (Schreiber et al., 2006). These ribosylation reactions are largely attributed to the nuclear protein poly(ADP-ribose) polymerase-1 (PARP-1), the archetypal member of a diverse family of a proteins capable of such reactions (Ame et al., 2004). PARP-1 surveys DNA for strand disruptions, binds to them, and synthesizes PAR polymers, through NAD^+ consumption, for attachment to itself and other proteins such as histones. While PAR polymers have a transient existence due to degradation by poly(ADP-ribose) glycohydrolase (PARG), ribosylation reactions influence chromatin structure and protein activity. Additionally, charge repulsion causes the dissociation of polyribosylated PARP-1 from DNA with the subsequent cessation of PAR synthesis.

The development of viable *Parp-1* knockout mice provided a model from which subsequent investigations could elucidate the necessity of PARP-1 in DNA repair. Cells from these animals are hypersensitive to alkylating agents and ionizing radiation, suggesting the participation of PARP-1 in BER (Shall and de Murcia, 2000). However, the requirement for PARP-1 in the processing of BER related damage remains tenuous due to the existence of conflicting observations (Trucco et al., 1998; Vodenicharov et al., 2000). To resolve this

disparity, biochemical studies have attempted to establish a role of PARP-1 in BER. PARP-1 can physically interact with and recruit XRCC1 to SSBs (Masson et al., 1998; El-Khamisy et al., 2003). Since interactions of XRCC1 with POLβ (Dantzer et al., 2000) and LIGIIIα (Leppard et al., 2003) have also been demonstrated, a model has emerged where PARP-1 activity could lead to the formation of a repair complex at SSBs, which consists of XRCC1, POLβ, and LIGIIIα (Leppard et al., 2003). PARP-1 also heterodimerizes with PARP-2, a functional homolog that possesses similar interaction capabilities, but lacks the affinity for SSBs and the capacity for PAR synthesis (Schreiber et al., 2002; Schreiber et al., 2006).

In an attempt to further solidify a requirement for PARP-1 in BER, we assessed the PARP-1 null phenotype in intact cells. We hypothesized that PARP-1 is necessary for efficient BER during MMS exposure in vertebrate cells. DT40 chicken cells and isogenic PARP-1 null cells were employed for this study. The DT40 based cell model employed here naturally lacks PARP-2 (Hochegger et al., 2006), allowing for an investigation without the contribution of this PARP-1 homolog to the genotoxic response. Cell lines were challenged under different MMS exposure scenarios for subsequent evaluation of endpoints, including survival and the accumulation of BER substrates throughout this pathway. We observed an influence of PARP-1 on BER at the later stages of this pathway, but PARP-1 did not appear necessary for constitutive BER.

2.3 Materials and Methods

Culture conditions and dish exposures

The generation of and culture conditions for DT40 and PARP-1 null cells were as described previously (Hochegger et al., 2006; Tano et al., 2007). For chemical exposure,

wild-type (PARP-1 proficient) and mutant DT40 (PARP-1 deficient) cells were seeded into 10 cm dishes with complete medium and allowed to incubate overnight to obtain the desired cell density (1×10^6/mL). Without changing medium, MMS (Aldrich) treatment solution (100×) was added to the cultures and cells were incubated at 39.5 °C for appropriate time points. After exposure, cells were harvested, washed with cold 1× PBS, pelleted, and then stored at -80°C until DNA isolation.

Cytotoxicity assay

Colony formation was determined in medium containing methylcellulose as described previously (Yamamoto et al., 2003).

DNA extraction

DNA isolation was performed with modification to the PureGene DNA extraction kit (Gentra Systems Inc., Minneapolis, MN, USA) as described previously (Nakamura et al., 2000).

Immuno-slot blot for ring opened N7-meG

Levels of N7-meG were measured based on the alkaline conversion of the adduct to 2,6-diamino-4-hydroxy-5-*N*-methyl-formamidopyrimidine (roN7-meG) with subsequent immuno-slot blot analysis (Elder et al., 1998; Rinne et al., 2005).

AP site assay

AP sites were measured as previously described (Nakamura et al., 1998) by aldehyde reactive probe (ARP, Dojindo Molecular Technology, Gaithersburg, MD, USA) labeling and slot blot analysis.

NAD(P)H depletion assay

During continuous MMS treatment, an imbalance in BER for DT40 cell lines was assessed in real-time by a colorimetric assay monitoring intracellular NAD(P)H (Tano et al., 2007). NAD(P)H depletion served as a proxy for NAD^+ consumption, an indicator of PARP-1 activation from SSB accumulation (Nakamura et al., 2003). To confirm the activation of PARP-1 during continuous MMS exposure, cells were also co-exposed in the presence of the PARP inhibitor 3-aminobenzamide (3-AB, 10 mM, Sigma).

Glyoxal gel electrophoresis assay

To qualitatively assay the extent of SSB formation in genomic DNA from treated cells, single stranded DNA was fractionated by neutral electrophoresis as previously described with modification (Drouin et al., 1996). Briefly, equal amounts of DNA (3 - 10 μg) samples to be compared were first denatured in 1.5 M glyoxal (Fluka), DMSO (50% (v/v); Sigma), and 10 mM sodium phosphate (pH 7) for 1 h at 50°C. Loading buffer, which consisted of 3.5% low melting agarose (Cambrex, Rockland, ME, USA), 0.01% bromophenol blue (Sigma), 0.01% xylene cyanol (Sigma), and 10 mM sodium phosphate (pH 7), was added to each sample prior to loading and separation of the DNA fragments on 0.7% agarose gels (Fisher) in 10 mM sodium phosphate (pH 7) for 16 h (30 V) at 4°C. Gels

were stained with acridine orange (5 μg/mL; Fisher) for 1 h and then destained in deionized water for subsequent visualization.

Integrating endpoint measurements for determining an imbalance in BER

To determine the number of N7-meG adducts associated with an imbalance in BER in DT40 cells exposed to MMS, NAD(P)H depletion values were log transformed (base 10) and plotted against their corresponding cumulative dose, defined here as the product of the MMS concentration and exposure time. The start of an imbalance in BER was defined as the point of departure from proficient BER, which was graphically depicted as the intersection of the linear regression line for the log NAD(P)H values with y = 2 (i.e. log 100% NAD(P)H relative to controls). The corresponding value along the x-axis was then designated as the cumulative dose that initiated an imbalance in BER. This cumulative dose was then applied to the response curve of N7-meG formed in DT40 cells during MMS exposure to determine the number of lesions present during the imbalance in BER.

Statistical analyses

Adduct and AP site data were log transformed to approximate linearity. Analysis of covariance (ANCOVA) was then performed to test for differences in the mean intercept and in the slopes of the linear dose-response curves between DT40 and PARP-1 null cells.

2.4 Results

Influence of PARP-1 on cell survival during MMS exposure

In this study, DT40 cells and their isogenic PARP-1 null counterparts served as an experimental model to investigate the *in vivo* role of PARP-1 in various aspects of BER. Since they lack PARP-2, DT40 cells allow for the investigation of the PARP-1 null phenotype without confounding by PARP-2 (Hochegger et al., 2006). When challenged with MMS for 10 days, PARP-1 null cells exhibited extreme hypersensitivity to cell killing (Figure 2.1). The consistency between this observation with previous analyses in vertebrate and mammalian cell models (Trucco et al., 1998; Hochegger et al., 2006) reaffirmed the role of PARP-1 as a survival factor after alkylative stress, presumably by limiting the accumulation of cytotoxic BER intermediates.

roN7-meG as an exposure marker

Subsequent experiments aimed to identify any BER defects in PARP-1 null cells, which may allow for the accumulation of repair intermediates that may ultimately elicit alkylation sensitivity. To rule out dissimilar MMS treatments between cell lines, N7-meG, the predominant lesion formed by this methylating agent (Wyatt and Pittman, 2006), served as a marker of exposure. Treating genomic DNA from MMS exposed cells with alkaline conditions causes imidazole ring opening (Tudek, 2003) of N7-methylpurines thereby allowing roN7-meG quantitation by an immuno-slot blot technique (Rinne et al., 2005). Over the exposure period, both cells lines showed similar formation of N7-meG with increasing exposure time (Figure 2.2). These adduct data show that the presence or absence of PARP-1 does not greatly influence the accumulation of base damage, particularly with increased exposure duration. These data confirmed the generation of N7-meG adducts with

MMS exposure and provide confidence for the interpretation of subsequent results that PARP-1 status, rather than inconsistent exposure conditions, would be the cause of any phenotypic differences between wild-type and mutant cells. Additionally, the proportional increase in adduct number with exposure time suggests that MMS was stable over this exposure time.

AP site measurement

AP sites were directly measured to determine whether a PARP-1 deficiency affected the accumulation of these lesions. The number of endogenous AP sites present in DT40 and PARP-1 null cells were similar (Figure 2.3). Both DT40 and PARP-1 null cells showed equivalent increases in AP site number with MMS exposure (Figure 2.3). Together, these data suggest that PARP-1 status does not influence AP site accumulation during continuous MMS exposure.

Determining SSB formation from MMS exposure

With MMS exposure, the accumulation of SSBs as intermediates of BER can lead to PARP-1 overactivation and NAD^+ consumption with depletion in intracellular NAD(P)H (Nakamura et al., 2003). In DT40 cells, as exposure time increased, levels of intracellular NAD(P)H decreased in a dose dependent manner (Figure 2.4A). Simultaneous treatment to MMS and the PARP inhibitor, 3-AB, protected against major depletions in NAD(P)H, confirming an active PARP-1 response to continuous MMS treatments (Figure 2.4B). These data suggest the synthesis of PAR by wild-type DT40 cells as an indicator of an imbalanced BER response to DNA alkylation.

PARP-1 null cells exposed to MMS in the presence or absence of 3-AB resisted a decrease in NAD(P)H of similar magnitude as wild-type DT40 cells treated under similar conditions (Figures 2.4C and D). This observation was expected due to the lack of PARP-1 and -2 activities in the null cells and was consistent with the response previously reported for PARP-1 null mouse embryonic fibroblasts (Nakamura et al., 2003). The relatively mild NAD(P)H depletion in PARP-1 null cells was unlikely to be attributable to a reduction in cell number, since no enhanced cell toxicity was observed by trypan blue exclusion when cells were exposed to 1 mM MMS for 4 h (data not shown). NAD(P)H depletion may have reflected the activity of other NAD^+ consumers, such as sirtuins, in response to genotoxic stress or mitochondrial dysfunction (Zhang, 2003). Regardless of the lack of a massive NAD(P)H depletion with MMS exposure in PARP-1 null cells, the observation from DT40 cells challenged under similar conditions suggests that mutant cells also experienced SSB accumulation during imbalanced BER.

While the NAD(P)H depletion assay provided an indication of SSB accumulation, we employed an electrophoretic method to visualize strand disruptions in the DNA of PARP-1 proficient and deficient cells exposed to MMS. When exposed to 1 mM MMS for up to 4 h, the migration of DNA from wild-type DT40 cells did not appear to increase with time (Figure 2.5A). Conversely, DNA from PARP-1 null cells did show greater migration with 3 to 4 h of MMS exposure. When cells were exposed to a range of MMS concentrations for 4 h, DNA from PARP-1 null cells migrated to a greater extent than that from wild-type cells, starting at 0.25 mM MMS and as a function of dose (Figure 2.5B). These gel data, particularly at long and high dose MMS treatments, provided evidence for SSB formation in PARP-1 null cells, which failed to show a major decrease in NAD(P)H due to a lack of

inherent PARP-1 activity. These data suggest greater formation of SSBs in PARP-1 null cells compared to DT40 cells, as demonstrated by enhanced DNA migration.

Determining an imbalance in BER

The cumulative dose of MMS that caused an imbalance in BER was determined to be 0.55 mM×hr (Figure 2.6). In calculating this cumulative dose, only data for 0.5, 0.7, 1 mM MMS were used and extreme exposure levels (1.4 and 2 mM) were excluded (Figure 2.4A). When applied to the regression equation ($y = 82.85x$) generated from the measurement of N7-meG adducts in DT40 cells (Figure 2.2), it was determined that such a cumulative dose had formed 46 N7-meG adducts per 10^6 nucleotides. At this adduct level, 6.1 N3-meA adducts per 10^6 nucleotides could be expected to occur when considering an approximate ratio of one N3-meA to eight N7-meG adducts formed during MMS exposure (Beranek, 1990).

2.5 Discussion

BER is tasked with the enormous burden of repairing DNA damage caused by the constant generation of endogenous genotoxicants and exposures to environmental agents. The basic requirement for active BER is evident since the singular disruption of most BER genes in mice leads to embryonic lethality, while a *Parp-1 Parp-2* double knockout is needed to produce such a lethality (Menissier de Murcia et al., 2003; Larsen et al., 2007). In its simplest form, BER can be reconstituted with four core enzymes *in vitro*: uracil-DNA glycosylase, APE, POLβ, and LIGIIIα (Kubota et al., 1996); however, the presence of accessory factors, such as PARP-1, are believed to modulate repair efficiency within cells

(Fan and Wilson, 2005). Much debate has centered on the significance of PARP-1 in BER, with proponents arguing that PARP-1 causes a positive or negative effect on BER capacity. Early cell free studies suggested that PARP-1 binding to SSBs inhibits repair by denying repair proteins access to damage sites (Satoh and Lindahl, 1992; Satoh et al., 1993). Conversely, the generation of mice deficient in PARP-1 and their exposure to alkylating agents and ionizing radiation established a need for PARP-1 in BER (Wang et al., 1995; de Murcia et al., 1997; Masutani et al., 1999). The use of intact cells or cell extracts from such animals produced mixed results, with some studies indicating a requirement for PARP-1 in BER (Trucco et al., 1998; Dantzer et al., 2000; Le Page et al., 2003; Parsons et al., 2005), while others showed no need for PARP-1 (Vodenicharov et al., 2000; Allison et al., 2003). Other studies, which have employed biochemical or *in vivo* models, have discovered possible roles for PARP-1 within BER (Lavrik et al., 2001; El-Khamisy et al., 2003; Leppard et al., 2003; Sukhanova et al., 2005; Mortusewicz et al., 2007).

We hypothesized that PARP-1 is necessary for efficient BER during MMS exposure in vertebrate cells. We chronically treated PARP-1 proficient and deficient DT40 cells to MMS for 10 days as our initial characterization of the PARP-1 null phenotype in this model. With acute MMS treatments we systematically evaluated aspects of BER to help clarify the significance of PARP-1 within this pathway. Since DT40 cells inherently lack PARP-2 (Hochegger et al., 2006), a functional homolog of PARP-1, this report is the first characterization of BER in cells lacking both PARP-1 and PARP-2, as well as, the first systematic evaluation covering the entirety of BER function (from base adduction to the presence of SSBs immediately before ligation) within living cells. PARP-1 null cells exhibited a hypersensitive phenotype when chronically treated with MMS. During an acute

treatment, both PARP-1 proficient and deficient cells had a similar accumulation of N7-meG and AP sites. However, cells lacking PARP-1 appeared to have a greater extent of SSB formation demonstrated by enhanced DNA migration during electrophoresis. Yet in the absence of MMS treatment, endogenous levels of AP sites were similar between cell lines.

The culmination of previous investigations supports an active role of PARP-1 in BER. During the repair of N7-meG or its depurinated sites, APE incision of AP sites forms SSBs with 5′-dRP termini. With low levels of damage, processing of 5′-dRP could proceed via a PARP-1 independent manner through a highly coordinated series of enzymatic steps involving XRCC1, POLβ, and LIGIIIα in which BER intermediates are handed off for subsequent processing to complete repair (Wilson and Kunkel, 2000). When DNA damage levels are high, 5′-dRP lesions may saturate the dRP lyase activity of POLβ and become uncoupled from the repair apparatus leading to an accumulation of SSBs, which may ultimately lead to cytotoxicity (Sobol et al., 2000). PARP-1 appears to have the ability to bind 5′-dRP moieties based on *in vitro* studies with a lesion of analogous structure (Lavrik et al., 2001). The events subsequent to PARP-1 binding and the initiation of ribosylation reactions could be an attempt to compensate for an imbalance in BER, by switching to LP or stimulating SP. Within the context of SP BER, PAR foci around the SSBs could serve as a recruitment signal for XRCC1 (El-Khamisy et al., 2003). With its arrival at the SSB, XRCC1 acts as a scaffold for the formation of a repair complex containing POLβ and LIGIIIα for the completion of BER (Caldecott et al., 1996). XRCC1 recruitment represents an apparently critical event since mutation of the XRCC1 BRCT1 domain, which interacts with PARP-1 and PAR, decreases SSB repair capacity and increases sensitivity to MMS (Taylor et al., 2002). Such observations were recapitulated in XRCC1 deficient cells treated

with MMS in the presence of a PARP inhibitor (Horton et al., 2008). Previously, PARP-1 was implicated in the LP sub-pathway since PARP-1 null cell extracts had inefficient LP capacity (Dantzer et al., 2000). Subsequent *in vitro* data suggest that PARP-1 binding to 5′-dRP is an active mediator in sub-pathway selection, allowing for the switch from the predominant SP to LP (Prasad et al., 2001). That study also demonstrated that a functional interaction between PARP-1 and FEN-1 stimulates POLβ strand displacement during LP.

In relation to our study, the resistance of PARP-1 proficient DT40 cells to chronic MMS exposure could reflect the ability of PARP-1 to enhance SP and eventually cause a switch to LP in an effort to limit toxic BER intermediates. At low levels of DNA damage, wild-type cells may solely rely on PARP-1 independent BER in which substrates are efficiently handed to sequential repair enzymes (APE, POLβ, and LIGIIIα) for complete repair without disruption. From our acute exposure, we detected a slight increase in AP sites at 1 mM MMS for 30 min and NA(D)PH depletion analysis showed that 1 mM MMS exposure theoretically starts NAD(P)H depletion at 33 min of exposure (calculated from 0.55 mM MMS). These results suggest that at such damage levels, 5′-dRP lesions may saturate dRP lyase capacity, become uncoupled from the repair apparatus, and then serve as substrates for PARP-1. With PARP-1 binding and NAD^+ consumption, the ribosylation of histones opens up the local DNA environment and automodification causes PARP-1 to dissociate from DNA, collectively facilitating repair enzyme access to damage sites. The generation of PAR within the vicinity of the SSB could further enhance SP by recruiting the XRCC1, POLβ, and LIGIIIα repair complex and stimulate LIGIIIα (Leppard et al., 2003) by acting as a source of ATP for strand ligation (Oei and Ziegler, 2000). With high levels of damage and continued PARP-1 binding, intracellular NAD^+ levels may not support the

efficient PAR synthesis needed for PARP-1 dissociation from DNA. This scenario could serve as the molecular switch to initiate LP BER, allowing for the functional interaction between PARP-1 and FEN-1 that stimulates strand displacement synthesis. Previously, the stimulatory effect of PARP-1 on LP was ablated when NAD^+ was added to an *in vitro* system, suggesting that the abortive dissociation of PARP-1 from DNA is critical for LP (Prasad et al., 2001). Under massive levels of DNA damage, the resulting depletion in NAD^+ would result in necrotic cell death. In contrast, the hypersensitivity of PARP-1 null cells could be explained by the fact that such cells are strictly limited to PARP-1 independent BER, which upon saturation would lead to an accumulation of uncoupled SSBs that are eventually converted to toxic DSBs.

We also employed a strategy of an acute, continuous MMS treatment of intact DT40 cells to determine when during the course of BER a repair defect could occur due to a lack of PARP-1. The direct analysis of DNA base damage and AP sites resulting from MMS exposure, the first such analysis to our knowledge, showed similar levels of each lesion regardless of PARP-1 status. The influence of a PARP-1 deficiency only became evident when analyzing markers of DNA damage occurring after SSB formation by APE activity. We assessed NAD(P)H depletion as a marker of NAD^+ consumption and PAR synthesis. Relative to PARP-1 proficient DT40 cells, PARP-1 null cells did not exhibit a similar extent of NAD(P)H depletion during MMS treatment, as expected due to the reliance of this assay on PARP activity. However, DNA from PARP-1 null cells appeared to have a greater SSB content than that from wild-type cells, as determined by an electrophoretic approach. Such an occurrence is supported by previous observations for increased SSBs in MMS treated cells with inhibited PARP activity, as demonstrated by the single cell electrophoresis assay

(Horton et al., 2008). Interestingly, in our study a difference in the extent of SSB formation was not reflected in AP site numbers, where both cells lines had similar levels. This observation suggests that in both cell lines, AP sites are processed with similar efficiency up to and including their removal by the dRP lyase activity of POLβ. Subsequently, the resulting intermediates awaiting ligation may be sealed with greater efficiency in wild-type cells than in PARP-1 null cells. A lack of both PAR synthesis and the eventual production of PAR degradation products associated with ATP synthesis could explain the SSB repair defect in PARP-1 null cells. Additionally, while demonstrated after hydrogen peroxide treatment, perturbation of the relationship between PARP-1 and PARG decreases SSB repair (Fisher et al., 2007), further suggesting a need for PAR anabolism and break down reactions for the complete repair of SSBs.

With the measurement of multiple DNA damage endpoints corresponding to individual stages of BER, we attempted to calculate the number of base adducts present during an imbalance in BER. By determining the start of a BER imbalance, as indicated by the initiation of NAD(P)H depletion in PARP-1 proficient DT40 cells, we correlated that MMS exposure level to the N7-meG response curve. Accordingly, our data suggest that 46 N7-meG adduct per 10^6 nucleotides, which corresponds to a level of 6.1 N3-meA per 10^6 nucleotides, were present during an imbalance in BER in DT40 cells when continuously exposed to MMS. To our knowledge, this calculation from empirical data represents the first association between adduct number and an imbalance in BER. Such an approach could prove informative when comparing different repair phenotypes and genotoxicants.

In summary, using an isogenic cell system we attempted to link the phenotype of PARP-1 deficient DT40 cells with previous biochemical studies to better define the role of

PARP-1 in BER. We conclude that PARP-1 enhances BER *in vivo*, particularly at the late stages during MMS treatment; however, PARP-1 may be dispensable during the processing of certain endogenous BER substrates. We also propose a model in which there is an ordered selection of BER sub-pathways that is predicated on the inverse relationship between intracellular NAD^+ levels and BER substrates. When substrate levels are low, PARP-1 independent SP predominates in lesion processing. As damage levels increase, PARP-1 becomes active in BER to enhance SP. In situations where levels of BER substrates continue to increase, the resulting decrease in NAD^+ levels from PARP-1 overactivation prohibits PARP-1 dissociation from DNA allowing for a switch to LP repair. Together the observations strengthen the positive role of PARP-1 in BER for preventing the accumulation of genotoxic lesions during chemical exposure.

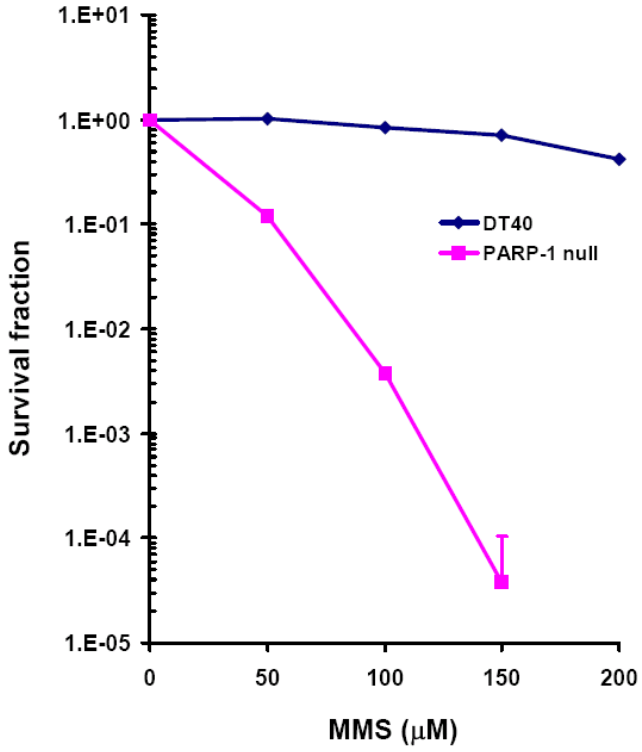


Figure 2.1. Sensitivity of DT40 and PARP-1 null cells to MMS. Survival curves of DT40 (PARP-1 proficient) and DT40-derived PARP-1 null cell exposed to MMS for 10 days. Each point represents the mean and S.D. (bars) from three independent experiments.

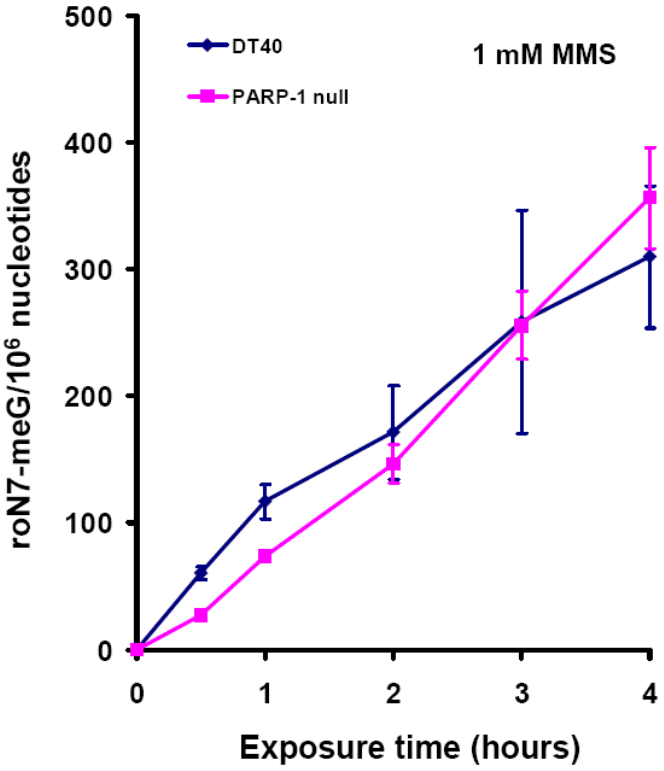


Figure 2.2. Measurement of ring-opened N7-meG as a marker of MMS exposure. Genomic DNA from DT40 and PARP-1 null cells exposed to 1 mM for up to 4 h was subjected to alkaline conditions to induce a ring-opened form of N7-meG for subsequent immuno-slot blot analysis. Each point represents the mean of four independent measurements. Bars indicate S.D.

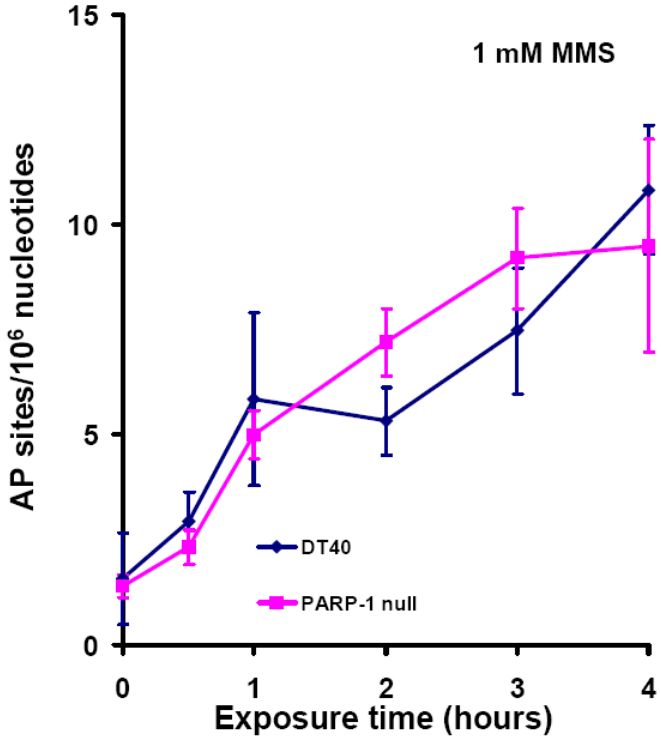
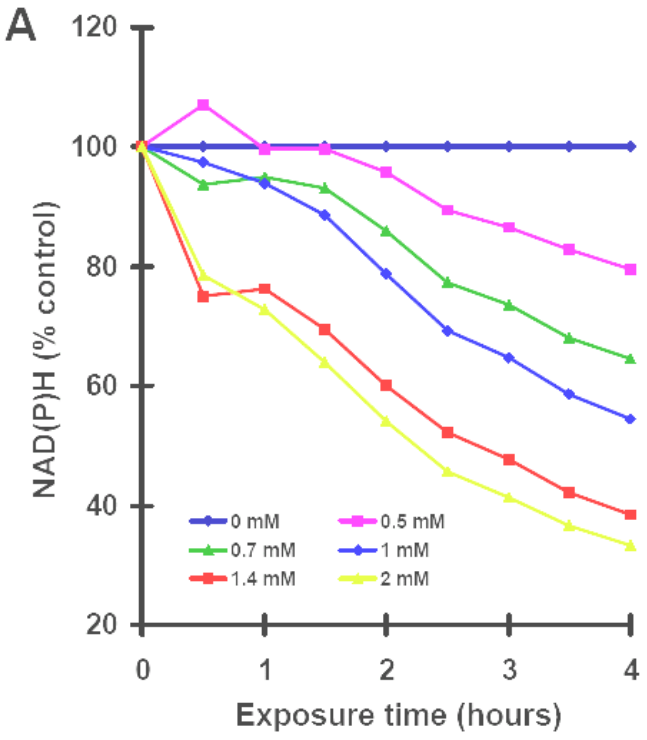
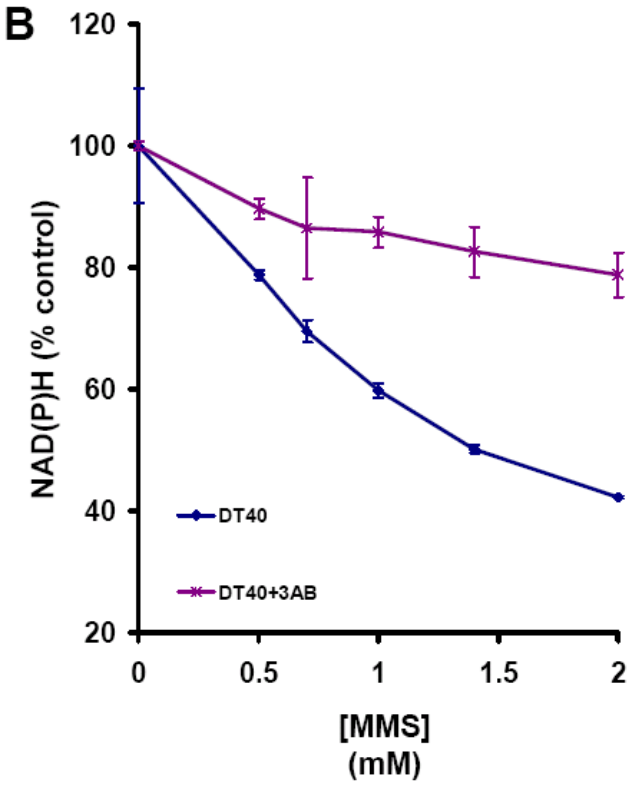
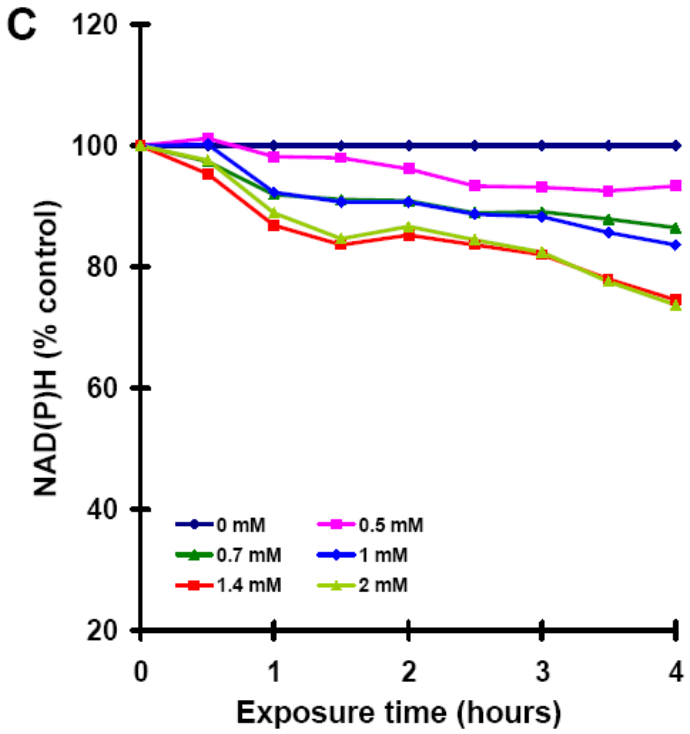


Figure 2.3. Measurement of AP sites in DT40 and PARP-1 null cells exposed to MMS. Genomic DNA from DT40 and PARP-1 null cells exposed to 1 mM for up to 4 h was reacted with ARP for slot blot analysis of AP sites. Each point represents the mean of four independent measurements. Bars indicate S.D.


A
120
100
80
60
40
20
NAD(P)H (% control)
0 mM
0.5 mM
0.7 mM
1 mM
1.4 mM
2 mM
0
1
2
3
4
Exposure time (hours)


B
120
100
80
60
40
20
NAD(P)H (% control)
DT40
DT40+3AB
0
0.5
1
1.5
2
[MMS]
(mM)


C
120
100
80
60
40
20
NAD(P)H (% control)
0 mM
0.5 mM
0.7 mM
1 mM
1.4 mM
2 mM
0
1
2
3
4
Exposure time (hours)

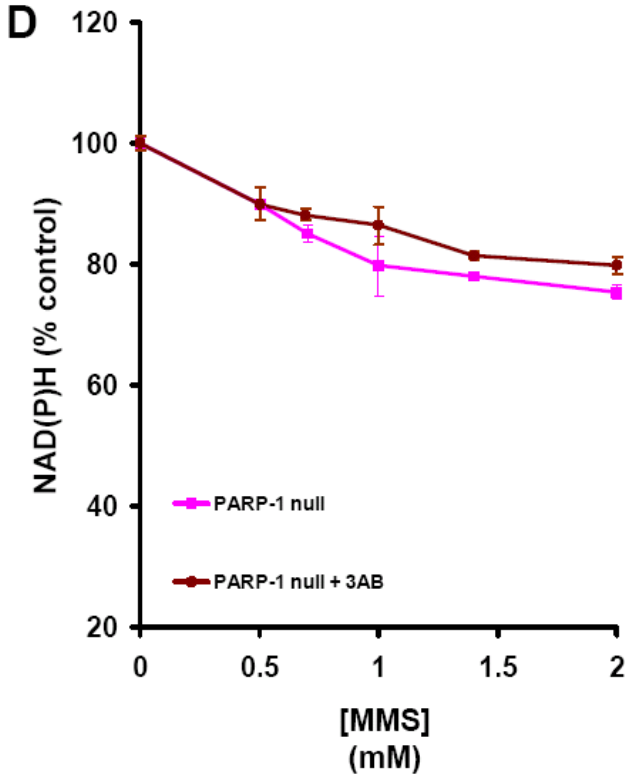

D
120
100
80
60
40
20
NAD(P)H (% control)
PARP-1 null
PARP-1 null + 3AB
0
0.5
1
1.5
2
[MMS]
(mM)

Figure 2.4. Depletion of intracellular NAD(P)H in DT40 and PARP-1 null cells. NAD(P)H levels in (**A**) DT40 and (**C**) PARP-1 null cells continuously exposed to various concentrations of MMS for up to 4 h. NAD(P)H depletion in (**B**) DT40 and (**D**) PARP-1 null cells exposed to various MMS concentrations for 4 h in the presence or absence of 3-AB (10 mM).

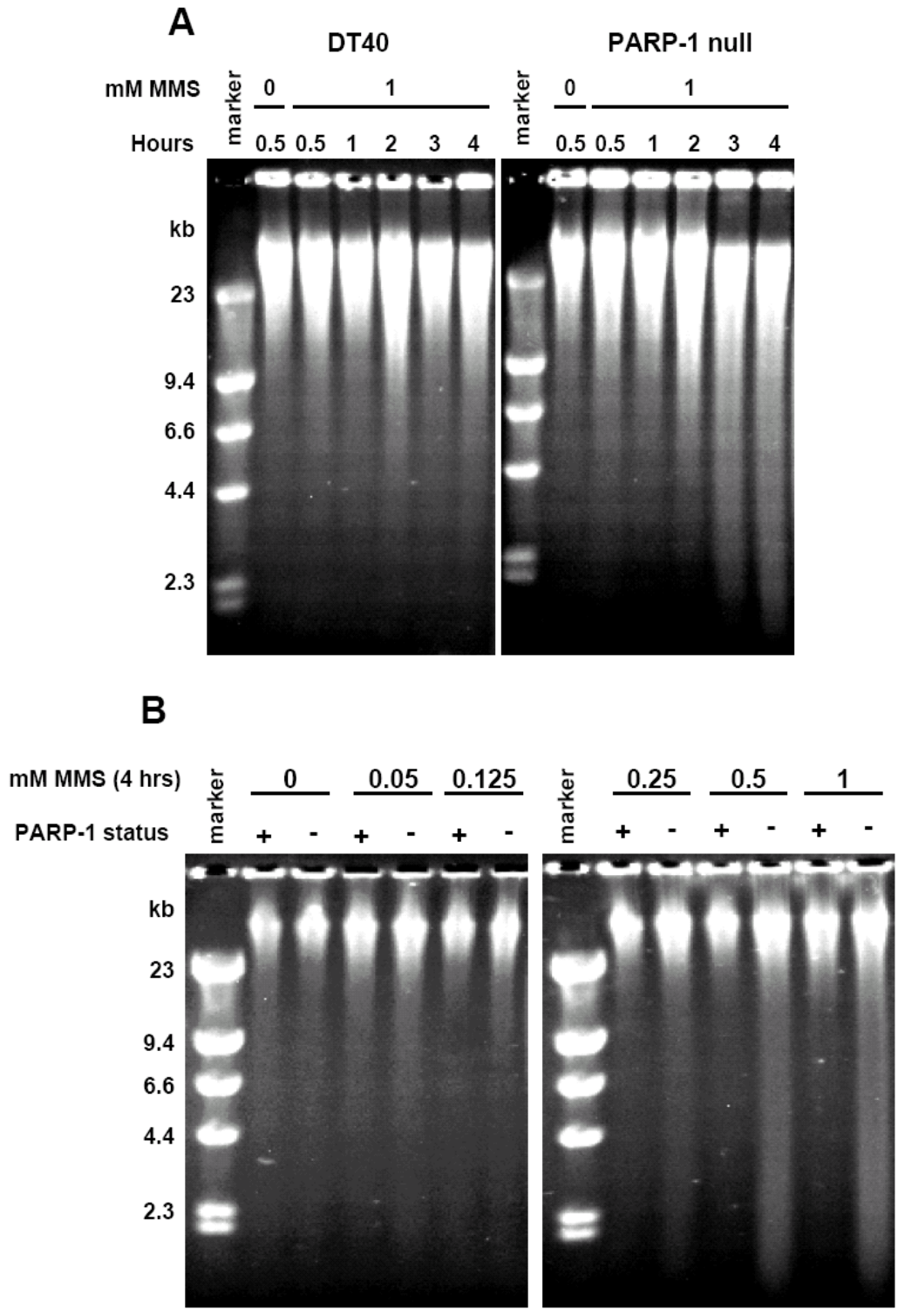

A
DT40
PARP-1 null
mM MMS
0
1
marker
Hours
0.5 0.5 1 2 3 4
kb
23
9.4
6.6
4.4
2.3
B
mM MMS (4 hrs)
0
0.05
0.125
0.25
0.5
1
PARP-1 status
+ - + - + - + - + - + -

Figure 2.5. Gel electrophoresis analysis of glyoxal denatured DNA from DT40 and PARP-1 null cells exposed to MMS. (**A**) The migration of genomic DNA from wild-type DT40 (left) and PARP-1 null (right) cells exposed to 1 mM MMS for up to 4 h. (**B**) The migration of genomic DNA from wild-type DT40 (+) and PARP-1 null (-) cells exposed to various MMS concentrations for 4 h. Gels are representative of two independent experiments.

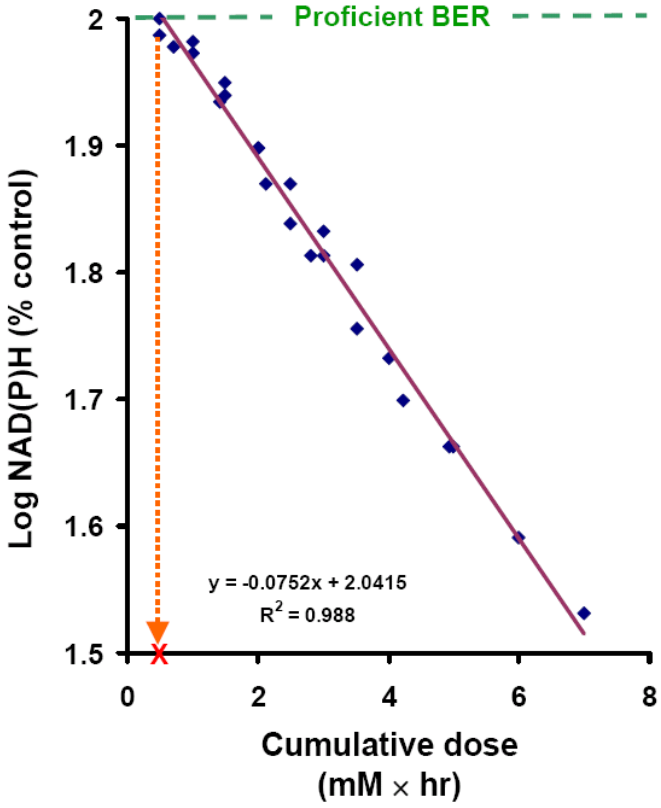


Figure 2.6. Determining the number of N7-meG adducts present at an imbalance in BER. Log transformed NAD(P)H values (from Figure 2.4A) for DT40 cells were plotted against the corresponding cumulative dose (the product of mM MMS and exposure time). The x value corresponding to the intersection of the resulting linear regression line and y = 2 (i.e. log 100% NAD(P)H relative to controls) was determined to be the start of an imbalance in BER. This cumulative dose was then applied to the response curve of N7-meG formed in wild-type DT40 cells during MMS exposure (Figure 2.2) to determine the number of lesions present at the beginning of an imbalance in BER.

CHAPTER 3. FUNCTIONAL STUDIES AND EPIDEMIOLOGIC DATA FOR XRCC1 POLYMORPHISMS

3.1 Abstract

Tobacco smoke produces oxidative and alkylative DNA damage that necessitates repair by base excision repair (BER) coordinated by X-ray cross-complementing gene 1 (XRCC1). We investigated whether polymorphisms in *XRCC1* alter DNA repair capacity and modify breast cancer risk associated with smoking. To demonstrate the functionality of the 280His variant, we evaluated single strand break repair (SSBR) capacity of isogenic Chinese hamster ovary (CHO) cells expressing human forms of XRCC1 after exposure to hydrogen peroxide (H_2O_2), methyl methanesulfonate (MMS), or camptothecin by monitoring NAD(P)H. We used data from the Carolina Breast Cancer Study (CBCS) a population-based, case-control study, which included 2077 cases (786 African Americans and 1281 whites) and 1818 controls (681 African Americans and 1137 whites) to examine associations among *XRCC1* codon 194, 280, and 399 genotypes, breast cancer, and smoking. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by unconditional logistic regression. Only cells expressing the 280His protein accumulated SSBs, indicated by NAD(P)H depletion, from both H_2O_2 and MMS exposures. In the CBCS, positive associations were observed between breast cancer and smoking dose for participants with *XRCC1* codon 194 Arg/Arg (P_{trend} = 0.046), 399 Arg/Arg (P_{trend} = 0.012) and 280 His/His or His/Arg (P_{trend} = 0.047) genotypes.

The 280His allele was in strong linkage disequilibrium with 194Arg (Lewontin's D′ = 1.0) and 399Arg (D′ = 1.0). These data suggest that less common, functional polymorphisms may lie within common haplotypes and drive gene-environment interactions.

3.2 Introduction

Polymorphisms in genes responsible for maintaining genomic integrity appear to be potential modifiers of disease risk (Berwick and Vineis, 2000; Vodicka et al., 2004). Consequently, a number of laboratory (Berwick and Vineis, 2000) and epidemiologic (Goode et al., 2002) investigations have attempted to show a link between polymorphic DNA repair genes and a variety of malignancies. With breast cancer being the most frequently diagnosed malignancy in women, there is enormous interest in demonstrating whether chemical exposures, genetics, or a combination of both are among the risk factors for this disease (Duell et al., 2001; Moullan et al., 2003; Kadouri et al., 2004; Kennedy et al., 2005). The role of cigarette smoking and breast cancer risk is controversial, with some epidemiologic studies showing positive associations, while others showed inverse associations or no association (Palmer and Rosenberg, 1993). A recent literature review concluded that breast cancer risk may be increased by smoking of long duration and by exposure to passive smoking, also referred to as environmental tobacco smoke (ETS) (Terry and Rohan, 2002). Additionally, functional and observational approaches have focused on interactions between polymorphisms of DNA repair genes and smoking (Duell et al., 2001; Shi et al., 2004; Kennedy et al., 2005), as an example of gene-environment interactions involved in the etiology of breast cancer.

X-ray cross-complementing gene 1 (XRCC1) acts as a scaffolding protein for base excision repair (BER) and single strand break repair (SSBR) (Caldecott, 2003a; Caldecott, 2003b). These overlapping pathways participate in the constitutive response to endogenous mutagens and exogenous exposures including tobacco smoke. Specifically, XRCC1-mediated pathways repair damage to DNA bases, from oxidation or covalent binding of non-bulky electrophiles, and to the deoxyribose-phosphate backbone. Quick resolution of this genetic damage is imperative since repair intermediates, such as abasic sites and single strand breaks (SSBs), are generally more genotoxic and cytotoxic than the initial lesion (Sobol et al., 2003). Three common polymorphisms within the *XRCC1* gene have been identified at codons 194, 280, and 399 (Arg194Trp, Arg280His, and Arg399Gln) (Shen et al., 1998b). These nonconservative amino acid changes may alter XRCC1 function. This change in protein biochemistry leads to the supposition that variant alleles may diminish repair kinetics thereby influencing susceptibility to adverse health effects including cancer (Rebbeck et al., 2004).

Laboratory experiments and epidemiologic studies have failed to reach a consensus regarding the functional effects of XRCC1 polymorphisms (Hung et al., 2005). Some laboratory investigations of *XRCC1* codon 399Gln functionality in human cells suggested that this polymorphism is associated with increased levels of DNA damage after exposure to various mutagens (Lunn et al., 1999; Au et al., 2003; Wang et al., 2003). Other reports offered conflicting evidence suggesting that the 399Gln polymorphism has no adverse effect on DNA repair (Pastorelli et al., 2002; Tuimala et al., 2002; Kiuru et al., 2005). The 194Trp variant protein does not appear to negatively alter the DNA repair capacity of human cells (Tuimala et al., 2002; Wang et al., 2003). Functional studies using lymphocytes suggested

that the 280His polymorphism diminishes genomic stability (Pastorelli et al., 2002; Tuimala et al., 2002).

In the present study we further characterized and confirmed the ability of isogenic mammalian cells transfected with human *XRCC1* cDNA to amend SSBs caused by genotoxic stress. We directly assessed the functionality of the 280His and 399Gln variant proteins through their expression within EM9 cells, a theoretical XRCC1 knockout model (Thompson and West, 2000), and comparison with repair proficient cells. The choice of chemicals for exposure, hydrogen peroxide (H_2O_2) and methyl methanesulfonate (MMS), qualitatively mimics some of the genotoxic events resulting from tobacco smoke exposure, namely DNA oxidation and purine alkylation by *N*-nitrosamines. As a result, we could infer how BER and SSBR capacity in humans would be affected by XRCC1 variants after exposure to tobacco smoke. Additionally, exposure to the topoisomerase 1 inhibitor camptothecin allowed for the novel functional evaluation of XRCC1 variants within tyrosyl-DNA phoshodiesterase1 (TDP1)-mediated pathways (Barrows et al., 1998; Park et al., 2002). We then applied our observations to a population-based, case-control study to evaluate the hypothesis that the *XRCC1* 280His allele increases the risk of breast cancer from exposure to tobacco smoke. We found that combining the use of transgenic cells and a novel screening assay for DNA repair capacity with a traditional epidemiologic approach proved to be an effective union for providing an increased understanding of gene-environment interactions.

3.3 Materials and Methods

Cell Line Preparation and Cell Culture

Preparation of EM9 cells expressing the human wild-type (EM9-WT), 280His (EM9-280His) or 399Gln (EM9-399Gln) variant proteins, or an empty pCMV vector (EM9-V) and culture conditions were described previously (Nakamura et al., 2003; Takanami et al., 2005). After reaching 90-100% confluency, cells were harvested by trypsin (Sigma) for subculturing or chemical exposure.

Chemicals

Unless noted, all chemicals used for cell exposures and the NAD(P)H assay were purchased from Sigma. MMS was obtained from Aldrich (Milwaukee, WI). Dosing and control solutions of chemicals were prepared with 1× phosphate-buffered saline (PBS, pH 7.4; Invitrogen).

Chemical Exposures and NAD(P)H Assay

Exposed cells were analyzed for an imbalance of SSBR by non-invasively monitoring intracellular NAD(P)H levels using a colorimetric assay (Nakamura et al., 2003) with modification. Briefly, prior to chemical exposures cells were seeded onto a 96-well plate (5×10^3 cells/50 μL/well) in Dulbecco's modified Eagle medium with nutrient mixture F-12 (DMEM/F-12, Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (Invitrogen) for an overnight incubation. For continuous exposures (ie. MMS and camptothecin), each well had been adjusted to a volume of 110 μL with complete medium, dye, and test chemical. For H_2O_2 exposures, cells were exposed to H_2O_2 for 30 minutes at 37 °C after replenishment with 50 μL of serumless DMEM/F-12

medium. To quench the oxidation reactions, DMEM/F-12 containing 20% FBS, catalase (18 units/mL) and dye were added to each well to give a final volume of 110 μL.

NAD(P)H levels were then monitored as previously described (Nakamura et al., 2003). Statistical evaluation of functional assay data was preformed using SAS 9.1 (SAS Institute, Cary, NC). Due to the approximate negative exponential decay with increasing dose, NAD(P)H values were log transformed for multiple linear regression. For each chemical exposure, two-sided *t* tests were performed comparing the regression coefficients for the wild-type response and the regression coefficient of each of the other cell lines to determine statistical significance with an alpha level of 0.05.

Carolina Breast Cancer Study

The Carolina Breast Cancer Study (CBCS) is a population-based, case-control study of invasive and *in situ* breast cancer conducted in 24 counties of central and eastern North Carolina (Newman et al., 1995). Incident cases were identified using a Rapid Case Ascertainment System in cooperation with the North Carolina Central Cancer Registry. Controls were selected from Division of Motor Vehicles (women younger than 65 years old) and United States Health Care Financing Administration lists (women 65 years old or greater). In-person interviews were conducted to obtain blood samples and information on potential breast cancer risk factors (Newman et al., 1995; Millikan et al., 1998).

Cases of invasive breast cancer were enrolled in two phases (Phase 1: 1993-1996, Phase 2: 1996-2001), with over-sampling of African American and younger women (Millikan et al., 2003). Controls were frequency matched to cases based upon age and race (± 5 years) using randomized recruitment (Weinberg and Sandler, 1991). Cases of *in situ*

breast cancer were enrolled between 1996 and 2001, and included women with ductal carcinoma *in situ* (DCIS) and DCIS with microinvasion to a depth of 2 mm. All cases of *in situ* breast cancer were eligible, with no over-sampling according to age or race. Controls were frequency matched to *in situ* cases based upon age (± 5 years) and race. Race was classified according to self-report. Less than 2% of participants reported Native American or other race, and were classified as white.

A total of 1803 cases (787 African Americans, 1016 whites) and 1564 controls (718 African Americans, 846 whites) were enrolled in the invasive study, and a total of 508 cases (107 African Americans, 401 whites) and 458 controls (70 African Americans, 388 whites) were enrolled in the *in situ* study. Contact and cooperation rates for the CBCS, and characteristics of cases and controls, have been published previously (Millikan et al., 2003). Response rates for blood draws and obtaining DNA were 90% for cases and 90% for controls. DNA samples were available for a total of 2077 cases (786 African Americans and 1281 whites) and 1818 controls (681 African Americans and 1137 whites). Odds ratios (ORs) for breast cancer risk factors did not differ significantly between persons who gave DNA and those who did not (data not shown). XRCC1 codon 194 and 399 results for part of Phase 1 of the CBCS were published previously (Duell et al., 2001). The present results combine genotypes from the entire CBCS (Phase 1 and 2 and *in situ* studies). Results did not differ for African Americans and whites, or for invasive and *in situ* disease, so results are combined to increase precision.

Genotype Analysis

DNA was extracted from peripheral blood lymphocytes by standard methods using an automated ABI-DNA extractor (Nuclei Acid Purification System, Applied Biosystems, Foster City, CA) in the UNC SPORE Tissue Procurement Facility. Genotyping was conducted using the ABI 7700 Sequence Detection System, or "Taqman"™ assay (Applied Biosystems, Foster City, CA). The following loci were genotyped: *XRCC1* codon 194 (rs 1799782), codon 280 (rs 25489) and codon 399 (rs 25487). Primer and probe sequences as well as annealing temperatures for each genotyping assay are listed in Appendix A. Probes were labeled on the 5′ end with either FAM or VIC (Applied Biosystems, Foster City, CA). Probes were labeled on the 3′ end with the quencher dye 6-carboxy-*N,N,N′,N′*-tetramethylrhodamine (TAMRA).

PCR reactions were performed in 15 μL reaction volumes. Reactions contained 0.7X Universal Master Mix (Applied Biosystems, Foster City, CA), 200 nM of each allele specific probe, 900 nM of each primer, and 15 ng of genomic DNA. After reactions tubes were set up, amplification was performed using a Perkin-Elmer GenAmp 9700 thermocycler. Reaction tubes were placed into the thermocycler after the temperature reached 50 °C. PCRs were carried out using the following conditions: 50 °C for 2 minutes (AmpErase UNG Activation), 95 °C for 10 minutes (AmpliTaq Gold Activation), and 40 cycles of 92 °C for 15 seconds (denature) and the temperature listed in Appendix A for 1 minute (anneal/extend). Samples that failed to amplify were repeated. Those samples that failed to amplify on the second run were scored as missing. Missing genotypes for each loci were as follows: *XRCC1* codon 194 (22 cases, 1 control), codon 280 (41 cases, 8 controls) and codon 399 (72 cases, 20 controls). A 10% random sample of genotypes were repeated for each locus, and results

were identical to the initial analysis. For each genotyping assay, DNA samples from the Coriell tissue repository (Coriell Institute for Medical Research, Camden, NJ) that had previously been sequenced at the National Cancer Institute (www.nci.snp500.gov) were used as positive controls.

Statistical Methods

Departures from Hardy-Weinberg equilibrium were evaluated by calculating expected genotype frequencies among controls based on observed allele frequencies and comparing the expected frequencies to observed genotype frequencies using χ^2 tests. Differences between allele or genotype frequencies in cases and controls were estimated using χ^2 tests or Fisher's Exact tests when expected counts were less that 5. Tests for statistical significance were two-sided with an alpha level of 0.05. SAS Genetics (version 8.2; SAS, Cary, NC) was used to estimate *XRCC1* codon 194 + 280 + 399 haplotype frequencies, and to compare haplotype frequencies in cases and controls. Haplotype estimates from SAS Genetics are based upon the EH algorithm (Zhao et al., 2000). Lewontin's D′ value, an estimate of the extent of linkage disequilibrium, was calculated using SAS Genetics for pair-wise comparisons of *XRCC1* codon 194 and 280, and 280 and 399.

Unconditional logistic regression was used to calculate ORs for breast cancer and 95% confidence intervals (CIs). PROC GENMOD in SAS (version 8.2; SAS Institute, Cary, NC) was used to incorporate offsets derived from sampling probabilities used to identify eligible participants (Weinberg and Sandler, 1991) and to adjust for race (African American, white) and age (as an 11-level ordinal variable that reflected 5-year age categories).

Analysis of smoking effects used a common referent group of women who were not exposed to active or passive smoking. Ever active smokers were defined as women who smoked at least 100 cigarettes in their lifetime. Exposure to passive smoking was defined as living with a smoker after the age of 18 (ETS after 18). Women who smoked on the reference date (date of diagnosis for cases or date of selection for controls) were classified as current smokers, while those women who no longer smoked on the reference date were designated former smokers. Women were asked about the amount of cigarettes smoked (packs per day) and the duration of smoking (the total number of years the participant smoked regularly). Information on duration of smoking was obtained by asking participants, "Keeping in mind that you may have stopped and started several times, overall how many years have you smoked regularly?" Dose of smoking was obtained by asking, "On average, how many cigarettes did you smoke per day?" Odds ratios for smoking dose and duration were calculated separately for current smokers and former smokers, and these groups were combined in the present analysis since positive associations were observed in both groups. Information regarding dose and duration of smoking was missing for 3 white cases. Multivariable logistic regression was used to adjust for potential confounding factors. Confounding was evaluated by determining whether adding a variable to a model resulted in a change in the beta coefficient of at least 10% for the exposure of interest. The following confounding variables were identified for the association of smoking and breast cancer: age at menarche (<12, ≥ 12 years), a composite term for age at first full-term pregnancy and parity (nulliparous, parity = 1 and age at first full-term pregnancy < 26, parity = 1 and age at first full-term pregnancy ≥ 26, parity ≥ 2 and age at first full-term pregnancy < 26, parity ≥ 2 and age at first full-term pregnancy ≥ 26), family history of breast cancer (yes/no for first-

degree relative), and alcohol consumption (never/ever). ORs for *XRCC1* genotypes and breast cancer were unchanged after adjusting for smoking and the other covariates listed, and thus are presented adjusted for offsets (sampling probabilities), age and race only. Participants with missing values for any of the variables in a regression model were omitted from the analysis.

Stratified analyses were used to investigate modification of ORs for smoking and breast cancer by *XRCC1* genotype. ORs for smoking were calculated according to each *XRCC1* genotype separately. In addition, we wished to estimate effects for *XRCC1* codon 194 and 399 separately, while ignoring codon 280 genotype, in order to compare our results with previous epidemiologic studies of *XRCC1*. Tests for trend for smoking dose and duration were conducted by calculating P-values for the beta coefficient in logistic regression models with smoking dose or duration coded as an ordinal variable. Results were similar for African American and white participants, therefore only combined results are shown. The term "Any genotype" refers to one or more copies of the less common allele, e.g. XRCC1 codon 194 "Any Trp" refers to "Arg/Trp or Trp/Trp genotype."

Interactions between XRCC1 genotypes and smoking on a multiplicative scale were evaluated using likelihood ratio tests (LRTs). An alpha value of 0.20 was used for statistical significance to account for the lower power of the test (Selvin, 1996).

Interactions on an additive scale were assessed by estimating independent and joint effects for XRCC1 genotypes and smoking using a single referent of never smokers and low risk XRCC1 genotype. Departures from additive effects were assessed using interaction contrast ratios (ICRs). ICRs greater than zero imply greater than additive effects, or synergy (Rothman and Greenland, 1998).

3.4 Results

Data from our functional evaluation of the 280His polymorphism support the hypothesis that some polymorphisms in DNA repair genes can alter the efficiency of repair pathways. To determine the phenotypic response to oxidative stress, the transfected EM9 cell lines were exposed to H_2O_2 (Figure 3.1). At the concentration range tested, the EM9-WT cells showed no depletion in NAD(P)H. However, the EM9-V cells exhibited a dose-dependent decrease in NAD(P)H to levels less than 40% of controls at the highest dose. Likewise, the EM9-280His cells showed a similar response with a 50% decrease in NAD(P)H after 4 hours of recovery. EM9-399Gln cells showed a slight reduction in NAD(P)H levels (83% of control level) at the highest dose.

During MMS exposures (Figure 3.1) we observed clear differences in terms of SSBR proficiency between the EM9 cell lines. After 4 hours of continuous exposure to MMS, EM9-280His cells showed greater NAD(P)H depletion than EM9-WT cells at a concentration as low as 62.5 μM. These data strongly suggest that the efficient removal of alkylated bases and other repair intermediates may be hindered by the expression of the *XRCC1* 280His genotype. EM9-399Gln cells appeared to have a similar depletion of intracellular NAD(P)H as EM9-WT cells in the cellular response to alkylative stress. EM9-V cells showed a massive reduction in NAD(P)H with only 30% of control levels at the highest dose.

To determine the influence of XRCC1 polymorphisms on interactions with proteins involved in a TDP1-mediated pathway, we exposed the transfected cell lines to the topoisomerase 1 inhibitor camptothecin (Figure 3.1). After 4 hours of continuous exposure

the EM9-WT, EM9-399Gln, and EM9-280His cell lines showed less than 10% decreases in NAD(P)H relative to controls, indicating no influences of XRCC1 genotypes on this repair pathway. The repair deficient EM9-V cells showed a 25% decrease in NAD(P)H at the highest dose level.

Because data indicated that the XRCC1 280His variant was a functionally detrimental polymorphism, we evaluated *XRCC1* genotype and smoking history data from the CBCS. Genotype frequencies, allelic frequencies and ORs for breast cancer for *XRCC1* codon 194, 280, and 399 genotypes are presented in Table 3.1. Allele and genotype frequencies were similar in African Americans and whites and between cases and controls within each racial group with respect to *XRCC1* codons 194 and 280. The frequency of the codon 399Gln variant was greater in white controls (q = 0.35) than African American controls (q = 0.14). Genotypes for each XRCC1 locus were observed to be in Hardy Weinberg equilibrium among African American cases, African American controls, white cases and white controls (data not shown). For each locus, comparisons between the Arg/Arg genotype and the variant genotypes did not yield any statistically significant increases in ORs for breast cancer. Haplotype frequencies for *XRCC1* in African American and whites are presented in Table 3.2. Haplotype frequencies did not differ between cases and controls for either racial group. The 194Arg + 280Arg + 399Arg haplotype was the most common in both African Americans and whites. The 280His allele was in strong linkage disequilibrium with 194Arg ($D' = 1.0$) and 399Arg ($D' = 1.0$) in both racial groups. Results for non-African Americans were not affected by exclusion of the 2% of participants who were non-white.

Odds ratios for breast cancer and smoking stratified by *XRCC1* codon 194 genotypes are presented in Table 3.3. Results are presented combining African Americans and whites,

and are adjusted for race. Former smokers with the Arg/Arg genotype showed an increased risk for breast cancer (OR = 1.4, 95% CI = 1.1 to 1.7). Participants with the Arg/Arg genotype showed statistically significant trends for increasing breast cancer risk with increased smoking dose (P = 0.046) and duration (P = 0.017). LRTs were significant for the interaction of smoking duration with codon 194 genotype (Table 3.3). ICRs for codon 194 Arg/Arg genotype and smoking status, dose, and duration were 0.35, 0.39, and 0.39, respectively. No associations were observed for participants with 194 Any Trp genotype.

ORs for smoking and breast cancer stratified by *XRCC1* codon 280 genotype are presented in Table 3.4. A statistically significant positive association between passive and former and breast cancer was observed for participants with the 280 Any His genotype. Although the test for trend was statistically significant only for smoking dose (P = 0.047), ORs were elevated for all levels of smoking dose and duration, as well as current and former smoking and passive smoking. LRTs were significant for the interaction of smoking status, dose and duration with XRCC1 codon 280 genotype (Table 3.4). ICRs for XRCC1 codon 280 Any His and smoking status, dose, and duration were 0.87, 0.65, and 0.80, respectively.

Results for smoking and *XRCC1* codon 399 genotype are presented in Table 3.5. ORs for former smoking and trend tests for smoking dose (P = 0.012) and duration (P = 0.001) were statistically significant among participants with codon 399 Arg/Arg genotype. LRTs were significant for the interaction of smoking duration with XRCC1 codon 399 genotype (Table 3.5). ICRs for XRCC1 codon 399 Arg/Arg genotype and smoking status, dose, and duration were 0.17, 0.32, and 0.39, respectively. There was no association among participants with codon 399 Any Gln genotype.

3.5 Discussion

In our functional evaluation of XRCC1 polymorphisms, we determined that relative to the wild-type protein the 280His variant decreased the DNA repair capacity of mammalian cells exposed to chemical stresses, such as oxidation and alkylation, associated with tobacco smoke. This observation that the 280His variant is functionally relevant guided the subsequent evaluation of data from the CBCS for a potential gene-environment interaction between *XRCC1* genotypes and exposure to tobacco smoke. Several epidemiologic studies have implicated the XRCC1 399Arg allele in the etiology of bladder, head and neck, and lung cancer (David-Beabes et al., 2001; Stern et al., 2001; Olshan et al., 2002). However, our functional assay demonstrated that the *XRCC1* 399Arg allele appears to be functionally competent like the 194Arg allele as demonstrated in previous studies (Taylor et al., 2002; Tuimala et al., 2002; Takanami et al., 2005), suggesting that another genetic modifier may be the causative factor that increases breast cancer risk. The less common 280His variant appears only within the 194Arg + 399Arg haplotype of *XRCC1* (Table 3.2). Because ORs for smoking and breast cancer were stronger for *XRCC1* codon 280 His-containing genotypes, compared to codon 194 Arg/Arg or codon 399 Arg/Arg, our results suggest that codon 280His is the relevant functional polymorphism in *XRCC1* with respect to smoking and breast cancer. Other complex phenotypes have been shown to be influenced by less common and rare alleles within common haplotypes (Cohen et al., 2004). These phenomena demonstrate the importance of investigating less common alleles that lie within common haplotypes in human populations.

Here we demonstrated that the *XRCC1* 280His variant attenuated the DNA repair capacity of transgenic cells after exposure to oxidative stress. Additionally, our functional

evaluation substantiated a previous observation (Takanami et al., 2005) that the 280His variant hinders the efficient repair of DNA damage from alkylative stress. These observations were evident from greater NAD(P)H depletions caused by PARP-1 overactivation in response to the accumulation of SSBs. Relative to the EM9-WT and EM9-399Gln cells, NAD(P)H depletions in EM9-280His cells were greater after exposure to H_2O_2 or MMS (Figure 3.1), suggesting an inability to efficiently amend DNA damage. Because NAD(P)H depletion in EM9-399Gln cells was similar to that in EM9-WT cells, it appears that the 399Gln variant protein does not negatively impact XRCC1-mediated repair. When exposed to the topoisomerase 1 inhibitor camptothecin all cell lines, excluding the repair deficient EM9-V line, had levels of NAD(P)H near 100% of control levels (Figure 3.1). These data suggest that the functionality of XRCC1 polymorphisms is relevant only to the removal of damaged bases or frank SSBs and not abortive topoisomerase 1 activity.

Prior functional studies in human and rodent cell models support our observations regarding the *XRCC1* 399Gln and 280His variants. Human cells with the 399Gln allele were not sensitive to bleomycin-induced DNA damage compared to lymphocytes with the codon 399 Arg/Arg genotype (Tuimala et al., 2002). Expression of the 399Gln variant protein in an EM9 background restored DNA repair capacity and cell survival to a level similar to that of EM9-WT cells after exposure to MMS (Taylor et al., 2002). Lymphocytes from individuals carrying the 280His allele showed increased genetic damage from bleomycin exposure relative to 280 Arg/Arg homozygotes (Tuimala et al., 2002). The 280His polymorphism was also associated with increased chromosomal aberrations in lymphocytes (Kiuru et al., 2005). While not assessed in this study, prior investigations of the Arg194Trp variant protein in human cells have demonstrated that this protein does not alter DNA repair capacity from

bleomycin exposure (Tuimala et al., 2002). Additionally, after MMS exposure EM9 cell lines expressing the 194Trp variant protein (EM9-194Trp) as well as EM9-WT and EM9-399Gln cells responded similar to repair proficient AA8 cells in terms of survival (Takanami et al., 2005).

A decrease in DNA repair capacity precipitated by the 280His variant appears to be biologically plausible. The 280 codon of the XRCC1 polypeptide lies within the AP endonuclease (APE) binding domain (Caldecott, 2003a). The nonsynonymous Arg280His polymorphism causes the replacement of arginine with histidine, which changes the amino acid sequence of XRCC1. This change in protein biochemistry could potentially alter XRCC1 structure and its ability to interact with APE. The 280His protein only appears to have a negative effect during the course of BER or SSBR induced by either base damage or DNA oxidation, processes which both involve APE. During the repair of SSBs formed by camptothecin exposure, a process independent of APE activity, EM9-280His cells show a phenotypic response similar to that of EM9-WT cells (Figure 3.1). Additionally, when expressed in EM9 cells the 280His variant protein failed to localize to DNA damage foci with the same efficiency as the wild-type protein (Takanami et al., 2005).

The association of *XRCC1* genotypes and breast cancer has been examined in thirteen epidemiologic studies (Kim et al., 2002; Han et al., 2003; Moullan et al., 2003; Shu et al., 2003; Smith et al., 2003a; Smith et al., 2003b; Deligezer et al., 2004; Figueiredo et al., 2004; Forsti et al., 2004; Sigurdson et al., 2004; Chacko et al., 2005; Metsola et al., 2005; Shen et al., 2005), in addition to a previous report from Phase 1 of the CBCS (Duell et al., 2001). Only the CBCS included significant numbers of African Americans. For codon 194, positive associations were observed for Trp-containing genotypes in four studies (Smith et al., 2003a;

Smith et al., 2003b; Sigurdson et al., 2004; Chacko et al., 2005), an inverse association in one study (Han et al., 2003), and no association in five studies (Kim et al., 2002; Moullan et al., 2003; Deligezer et al., 2004; Forsti et al., 2004; Shen et al., 2005;). Increased risk for codon 280 His-containing genotypes was observed in one study (Moullan et al., 2003) and no association in three studies (Sigurdson et al., 2004; Chacko et al., 2005; Metsola et al., 2005). For codon 399, positive associations were observed for Gln-containing genotypes in three studies (Kim et al., 2002; Sigurdson et al., 2004; Chacko et al., 2005), and no association in nine studies (Kim et al., 2002; Moullan et al., 2003; Shu et al., 2003; Smith et al., 2003a; Smith et al., 2003b; Deligezer et al., 2004; Figueiredo et al., 2004; Forsti et al., 2004; Metsola et al., 2005; Shen et al., 2005). A meta-analysis by Hung et al. (2005) combined results from ten breast cancer studies (Duell et al., 2001; Kim et al., 2002; Han et al., 2003; Moullan et al., 2003; Shu et al., 2003; Smith et al., 2003a; Smith et al., 2003b; Deligezer et al., 2004; Forsti et al., 2004; Chacko et al., 2005). Summary odds ratios were close to the null for codon 194 and 399 genotypes and breast cancer (Hung et al., 2005). Three epidemiologic studies of breast cancer analyzed *XRCC1* haplotypes (Han et al., 2003; Moullan et al., 2003; Chang-Claude et al., 2005), and results were consistent with the presence of the less common codon 280 His allele solely on the codon 194 Arg + codon 399 Arg chromosomal background.

Four breast cancer studies examined interactions between *XRCC1* genotypes and smoking (Han et al., 2003; Figueiredo et al., 2004; Metsola et al., 2005; Shen et al., 2005). Han et al. (Han et al., 2003) reported a trend of increasing breast cancer risk with increasing duration of smoking among study participants with the codon 194 Arg/Arg genotype, but not among codon 194 Trp-carriers, consistent with the results of our study. For codon 399,

Metsola et al. (2005) and Shen et al. (2005) reported interactions between Gln-containing genotypes and smoking, but no interactions were observed by Figueriedo et al. (2004) and Han et al. (2003). Metsola et al. (2005) reported a stronger association for codon 280 His-containing genotypes and breast cancer among heavy smokers. A meta-analysis by Hung et al. (2005) of tobacco-related cancers (lung, upper aerodigestive tract, bladder, stomach, liver, pancreas, and myeloid leukemia) found a protective effect for codon 194 Trp-containing genotypes among ever smokers. Codon 399 Gln-containing genotypes were associated with increased risk of tobacco-related cancers among light smokers, but a decreased risk among heavy smokers (Hung et al., 2005). These results are compatible with our observation of a stronger association between breast cancer and increased duration and dose of smoking among study participants with codon 194 Arg/Arg and codon 399 Arg/Arg genotypes. Hung et al. (Hung et al., 2005) reported a summary odds ratio close to the null for codon 280 His-containing genotypes and tobacco-related cancers, but the data was too sparse to stratify on smoking history. For results of additional epidemiologic studies of *XRCC1*, see Hung et al. (2005) and Goode et al. (2002).

Evaluating gene-environment interactions using a transgenic cell system as a screen for functional polymorphisms has advantages over human cell-based functional assays. The use of isogenic EM9 cells expressing human XRCC1 protein allowed for direct functional characterization of variant proteins without confounding by other genetic modifiers. Additionally we found this CHO model to be preferable over genetically matched lymphocyte cell lines from cases and controls carrying the 280His allele since human lines exhibit different rates of growth (data not shown), a potential source of confounding and a concern for assay variability. The use of a sensitive, real-time NAD(P)H assay to assess

BER/SSBR capacity afforded us the flexibility to reproducibly test a number of exposure scenarios in a relatively short amount of time. The stable transfection of plasmids harboring human cDNA of other polymorphic genes into isogenic knockout cells would extend the applicability of this approach. Our combined study design provides a robust examination of the biological significance for XRCC1 polymorphisms. The precise functional evaluation of XRCC1 polymorphisms through a laboratory study lends biologic plausibility to the findings from an epidemiologic study of breast cancer susceptibility. The strategy could prove useful for clarifying the biological significance of other genetic polymorphisms in DNA repair genes, particularly those with low allelic frequencies.

In summary, we further characterized the functionality of the XRCC1 280His polymorphism and used these observations to clarify the relationship between this allele, breast cancer, and smoking. The *XRCC1* codon 280His allele is in linkage disequilibrium with the more common variants for two other *XRCC1* polymorphisms at codon 194 and 399. Functional and epidemiologic data suggest that the XRCC1 codon 280His allele may be more important than codon 194 or 399 alleles with respect to smoking and breast cancer. Haplotype analyses, particularly using anonymous tagSNPs, may prove useful for identifying genetic heterogeneity when functional alleles are unknown. However, identification of functionally relevant alleles within defined haplotypes, as presented here, will also contribute important information for understanding gene-environment and gene-gene interactions.

Table 3.1. *XRCC1* genotype frequencies, allele frequencies and odds ratios for breast cancer from the Carolina Breast Cancer Study.

		African Americans			Whites	
Locus	Cases	Controls	OR[a] (95% CI)	Cases	Controls	OR[a] (95% CI)
XRCC1 Codon 194[b]						
Arg/Arg	671 (86.7%)	593 (87.0%)	Referent	1126 (87.9%)	987 (87.0%)	Referent
Arg/Trp	101 (13.0%)	86 (12.6%)	1.0 (0.7-1.3)	148 (11.5%)	141 (12.4%)	0.9 (0.7-1.2)
Trp/Trp	2 (0.3%)	3 (0.4%)	0.7 (0.1-4.4)	7 (0.6%)	7 (0.6%)	0.9 (0.3-2.8)
Any Trp	103	89	1.0 (0.7-1.3)	155	148	0.9 (0.7-1.2)
Fisher's exact test[c]		P = 0.83			P = 0.81	
XRCC1 Codon 194[d]						
Arg	0.93	0.93		0.94	0.93	
Trp	0.07	0.07		0.06	0.07	
Fisher's exact test[c]		P = 0.97			P = 0.48	
XRCC1 Codon 280[b]						
Arg/Arg	710 (92.8%)	642 (94.3%)	Referent	1146 (90.2%)	1030 (91.2%)	Referent
Arg/His	54 (7.1%)	38 (5.5%)	1.3 (0.8-2.0)	125 (9.8%)	97 (8.6%)	1.2 (0.9-1.6)
His/His	1 (0.1%)	1 (0.2%)	1.1 (0.1-18.0)[e]	0	2 (0.2%)	nd
Any His	55	39	1.3 (0.8-1.9)	125	99	1.2 (0.9-1.6)
Fisher's exact test[c]		P = 0.55			P = 0.17	

		African Americans			Whites	
Locus	Cases	Controls	OR[a] (95% CI)	Cases	Controls	OR[a] (95% CI)
XRCC1 Codon 280[d]						
Arg	0.96	0.97		0.95	0.96	
His	0.04	0.03		0.05	0.04	
Fisher's exact test[c]		P = 0.28			P = 0.47	
XRCC1 Codon 399[b]						
Arg/Arg	536 (70.4%)	493 (72.9%)	Referent	504 (40.5%)	480 (42.8%)	Referent
Arg/Gln	203 (26.7%)	172 (25.5%)	1.1 (0.9-1.5)	581 (46.7%)	494 (44.0%)	1.1 (0.9-1.3)
Gln/Gln	22 (2.9%)	11 (1.6%)	1.8 (0.8-3.8)	159 (12.8%)	148 (13.2%)	1.0 (0.8-1.3)
Any Gln	225	183	1.2 (0.8-1.5)	740	642	1.1 (0.9-1.3)
Chi square test[c]		P = 0.22			P = 0.42	
XRCC1 Codon 399[d]						
Arg	0.84	0.86		0.64	0.65	
Gln	0.16	0.14		0.36	0.35	
Chi square test[c]		P = 0.16			P = 0.51	

[a] Adjusted for offsets and age.
[b] Genotype frequencies N (%)
[c] Comparing cases and controls
[d] Allele frequencies (95% CI)
[e] Unstable estimate
nd = not determined

Table 3.2. XRCC1 haplotype frequencies in African Americans and whites.

Codon 194	Codon 280	Codon 399	Cases	Controls	Chi square test[b]
African Americans					
Arg (C)[a]	Arg (G)	Arg (A)	0.73	0.76	P = 0.13
Arg	Arg	Gln (G)	0.16	0.14	P = 0.15
Arg	His (A)	Arg	0.04	0.03	P = 0.28
Arg	His	Gln	<0.001	< 0.001	nd
Trp (T)	Arg	Arg	0.07	0.07	P = 0.85
Trp	Arg	Gln	< 0.001	<0.001	nd
Trp	His	Arg	< 0.001	<0.001	nd
Trp	His	Gln	< 0.001	<0.001	nd
Whites					
Arg (C)	Arg (G)	Arg (A)	0.53	0.54	0.48
Arg	Arg	Gln (G)	0.36	0.35	0.56
Arg	His (A)	Arg	0.05	0.05	0.50
Arg	His	Gln	< 0.0001	< 0.0001	nd
Trp (T)	Arg	Arg	0.07	0.07	0.78
Trp	Arg	Gln	<0.0001	< 0.0001	nd
Trp	His	Arg	< 0.0001	< 0.0001	nd
Trp	His	Gln	< 0.0001	< 0.0001	nd

[a] Nucleotide in parentheses.

[b] Comparing cases and controls.

African Americans and whites (cases and controls combined): D′ = 1.0 for Arg194 + His280, D′ = 1.0 for His280 + Arg399.

Table 3.3. Odds ratios for smoking and breast cancer according to *XRCC1* codon 194 genotypes from the Carolina Breast Cancer Study. Data are combined for African Americans and whites.

		XRCC1 codon 194		
	Arg/Arg		Any Trp	
Smoking status	Cases / Controls	OR (95% CI)[a]	Cases / Controls	OR (95% CI)[a]
Unexposed to active and passive smoking[b]	345 / 310	Referent	55 / 45	Referent
Passive smoking (ETS > 18)	598 / 546	1.1 (0.9-1.3)	96 / 76	1.2 (0.7-2.0)
Former active	559 / 424	1.4 (1.1-1.7)	72 / 66	1.0 (0.5-1.7)
Current active	292 / 300	1.0 (0.8-1.2)	35 / 50	0.6 (0.3-1.1)
LRT: P = 0.23				
Dose of active smoking (packs per day)				
1/2 or less	273 / 248	1.1 (0.9-1.4)	31 / 42	0.8 (0.4-1.5)
1/2 to 1	334 / 257	1.2 (1.0-1.6)	44 / 35	0.9 (0.5-1.7)
1 or more	239 / 214	1.3 (1.0-1.6)	31 / 39	0.8 (0.4-1.5)
Trend test		P = 0.046		P = 0.24
LRT: P = 0.27				
Duration of active smoking (years)				
<= 10	222 / 199	1.1 (0.8-1.4)	28 / 41	0.6 (0.3-1.2)
11 - 20	204 / 192	1.1 (0.8-1.4)	29 / 24	1.1 (0.5-2.2)
> 20	419 / 330	1.4 (1.1-1.7)	49 / 50	0.9 (0.5-1.7)
Trend test		P = 0.017		P = 0.53
LRT: P = 0.16				

[a] Adjusted for offsets, age, race, age at menarche, age at first full term pregnancy/parity composite, family history and alcohol.

[b] Referent group.

Table 3.4. Odds ratios for smoking and breast cancer according to *XRCC1* codon 280 genotypes from the Carolina Breast Cancer Study. Data are combined for African Americans and whites.

		XRCC1 codon 280		
	Arg / Arg		Any His	
Smoking status	Cases / Controls	OR (95% CI)[a]	Cases / Controls	OR (95% CI)[a]
Unexposed to active and passive smoking[b]	371 / 315	Referent	28 / 39	Referent
Passive smoking (ETS > 18)	621 / 583	1.0 (0.8-1.2)	67 / 37	2.8 (1.4-5.7)
Former active	563 / 450	1.2 (1.0-1.5)	58 / 38	3.0 (1.4-6.2)
Current active	299 / 324	0.9 (0.7-1.1)	26 / 24	2.0 (0.9-4.6)
LRT: P = 0.01				
Dose of active smoking (packs per day)				
1/2 or less	271 / 268	1.0 (0.8-1.2)	28 / 21	2.5 (1.1-5.8)
1/2 to 1	333 / 269	1.1 (0.8-1.4)	38 / 23	2.9 (1.3-6.7)
1 or more	252 / 233	1.1 (0.9-1.4)	18 / 17	2.7 (1.0-6.8)
Trend test		P = 0.34		P = 0.047
LRT: P = 0.02				
Duration of active smoking (years)				
<= 10	223 / 223	0.9 (0.7-1.1)	23 / 16	2.7 (1.1-6.7)
11 – 20	209 / 199	1.0 (0.7-1.3)	19 / 16	2.2 (0.9-5.5)
> 20	424 / 348	1.2 (1.0-1.6)	41 / 30	2.7 (1.2-6.1)
Trend test		P = 0.11		P = 0.08
LRT: P = 0.03				

[a] Adjusted for offsets, age, race, age at menarche, age at first full term pregnancy/parity composite, family history and alcohol.
[b] Referent group.

Table 3.5. Odds ratios for smoking and breast cancer according to *XRCC1* codon 399 genotypes from the Carolina Breast Cancer Study. Data are combined for African Americans and whites.

		XRCC1 codon 399		
	Arg / Arg		Any Gln	
Smoking status	Cases / Controls	OR (95% CI)[a]	Cases / Controls	OR (95% CI)[a]
Unexposed to active and passive smoking[b]	186 / 192	Referent	200 / 158	Referent
Passive smoking (ETS > 18)	373 / 353	1.2 (0.9-1.5)	304 / 264	1.2 (0.9-1.3)
Former active	309 / 254	1.5 (1.1-2.0)	313 / 230	1.2 (0.9-1.6)
Current active	172 / 174	1.1 (0.8-1.6)	145 / 173	0.7 (0.5-1.0)
LRT: P = 0.34				
Dose of active smoking (packs per day)				
1/2 or less	158 / 165	1.2 (0.8-1.6)	139 / 120	1.0 (0.7-1.4)
1/2 to 1	197 / 145	1.5 (1.1-2.1)	175 / 144	1.0 (0.7-1.4)
1 or more	123 / 116	1.4 (1.0-2.1)	141 / 136	1.0 (0.7-1.4)
Trend test		P = 0.012		P = 0.73
LRT: P = 0.26				
Duration of active smoking (years)				
<= 10	119 / 136	1.0 (0.9-1.6)	128 / 100	1.1 (0.7-1.5)
11 - 20	123 / 110	1.4 (1.0-2.0)	104 / 103	0.8 (0.5-1.2)
> 20	236 / 181	1.7 (1.2-2.3)	222 / 197	1.0 (0.7-1.4)
Trend test		P = 0.001		P = 0.79
LRT: P = 0.05				

[a] Adjusted for offsets, age, race, age at menarche, age at first full term pregnancy/parity composite, family history and alcohol.
[b] Referent group.

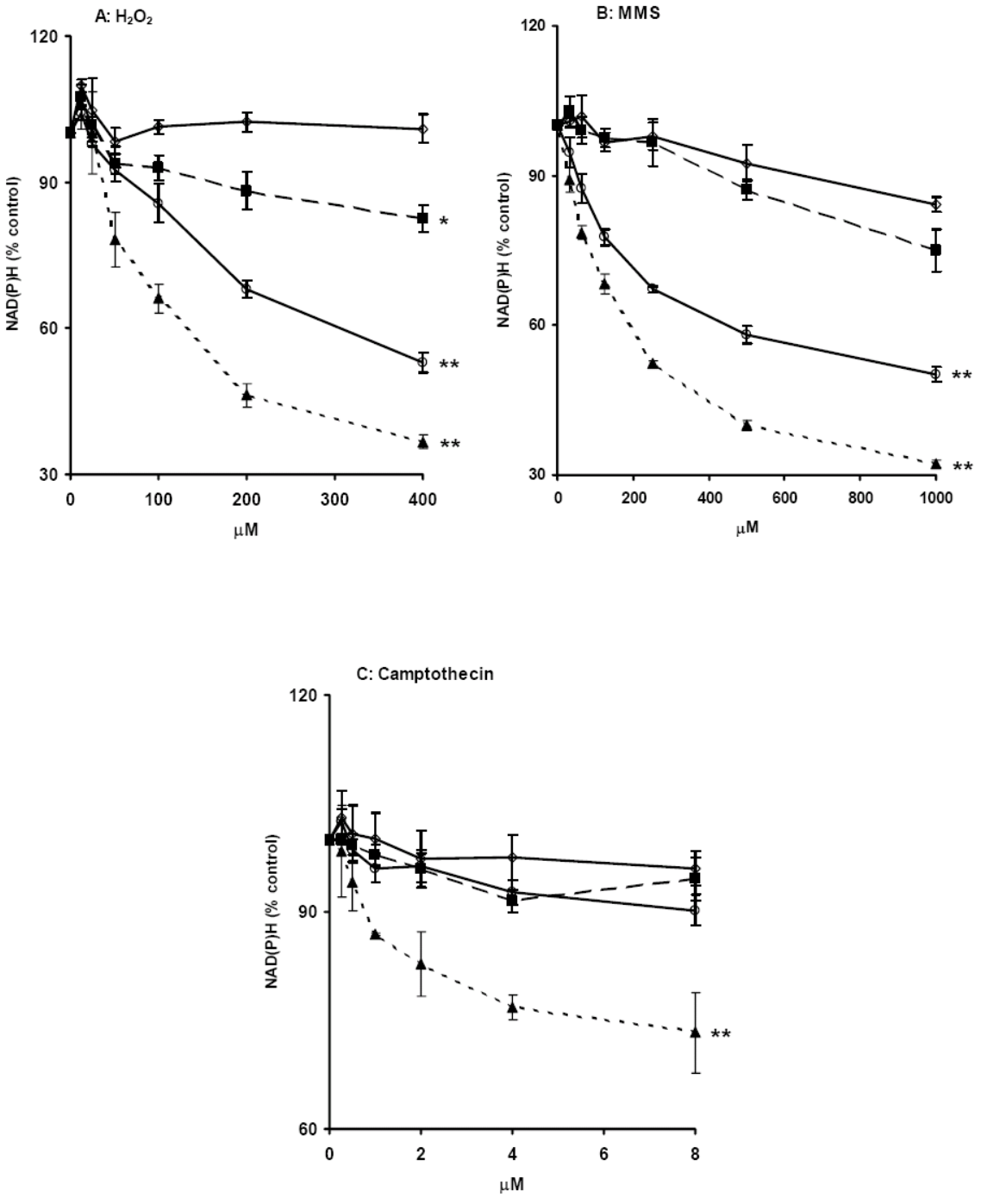

A: H2O2
B: MMS
C: Camptothecin
NAD(P)H (% control)
μM
*
**

Figure 3.1. Graphical representation of NAD(P)H data as an indirect indicator of SSB accumulation. EM9 cells expressing human forms of *XRCC1* including wild-type (EM9-WT, ◇), the 399Gln (EM9-399Gln, ■) or 280His (EM9-280His, ○) polymorphisms, or an empty vector (EM9-V, ▲) were exposed A) for 30 minutes to hydrogen peroxide (H_2O_2), B) continuously to methyl methanesulfonate (MMS), or C) continuously to camptothecin. NAD(P)H levels were monitored in real-time for 4 hours during (MMS and camptothecin) or after exposure (H_2O_2). NAD(P)H data for exposed wells were represented as a percentage relative to NAD(P)H levels (100%) in corresponding control wells of the same cell line dosed with PBS. Chemical exposures were conducted in triplicate and expressed as mean with standard deviation and were repeated on different days. Asterisks indicate significant difference from wild-type line; *, $P < 0.05$; **, $P < 0.01$

CHAPTER 4. GENERAL DISCUSSION

4.1 Summary and Conclusions

Previous research has shown that a deficiency in a BER enzyme can lead to a decrease in the ability of a cell to repair DNA damage. The goal of this study was to investigate the functionality of PARP-1 and XRCC1 as accessory factors in BER. For PARP-1, some reports have questioned the need for this enzyme in BER. With regards to XRCC1, previous investigations to assess whether polymorphic forms confer a defective repair phenotype have yielded mixed results. We demonstrate that both of these accessory factors can influence DNA repair efficiency, particularly in response to a genotoxic exposure.

Even with data from a number of different models suggesting a positive role of PARP-1 in BER, debate continues regarding its participation in this pathway. Therefore, the impetus for chapter 2 was to explore the necessity of PARP-1 in BER. When DT40 cells lacking PARP-1 were chronically exposed to MMS they showed extreme hypersensitivity suggesting a general need for PARP-1 in limiting the adverse effects of alkylative DNA damage. During an acute exposure both PARP-1 proficient and deficient cells showed an equal accumulation of N7-meG and AP sites thereby confirming that PARP-1 is relevant only to the late stages of BER in this avian system. Under similar exposure conditions, an imbalance in BER was determined to occur in cells with PARP-1, as determined by NAD(P)H depletion, an indirect indicator of PARP-1 activation. PARP-1 deficient cells exposed to MMS lacked the extreme NAD(P)H depletion observed in their PARP-1 proficient counterparts, but by virtue of analogous exposure conditions should have

experienced an imbalance in BER. For PARP-1 null cells, electrophoretic analysis qualitatively demonstrated enhanced DNA migration from MMS exposure suggesting SSB accumulation. Similar time and dose dependent increases were not observed in PARP-1 proficient cells. These observations suggest that the repair defect in PARP-1 deficient cells is associated with events downstream of SSB formation, particularly the ligation of SSBs. This study demonstrated that PARP-1 does influence the efficiency of BER in vertebrate cells during exposure to an alkylating agent. Since DT40 cells inherently lack PARP-2, we provide an assessment of BER efficiency without the contribution of this functional homolog of PARP-1. The data generated from this chicken cell model also support results derived from mammalian cell systems, confirming a requirement for PARP-1 in BER across taxa.

In chapter 3, through a multi-disciplinary approach, we provide evidence that XRCC1 polymorphisms influence DNA repair at the cellular level and modify cancer risk with an environmental exposure within a human population. CHO cells that inherently lack XRCC1 were transfected with human polymorphic forms of XRCC1 and exposed to genotoxicants known to induce SSBs. Cells expressing the 280His variant showed a decrease in repair capacity toward damage caused by MMS and H_2O_2. Alone these data provide basic information regarding the significance of XRCC1 polymorphisms at the cellular level. However, these cellular data also allowed for the generation of a hypothesis assessing whether a variant allele had relevance to human cancer risk. Through a collaborative effort, analysis of data from the CBCS showed an association between the 280His polymorphism and breast cancer when there was exposure to tobacco smoke. Because of its low frequency, not many studies have been able to assess the risk associated with the 280His polymorphism. These data lend further evidence for a genetic and environmental link in the etiology of

breast cancer. This study also provided a "proof of principle" for a unique study design, which employed both a transgenic model and human data, that may further help to determine the significance of genetic variation on disease risk.

Overall, these studies demonstrate that the accessory factors PARP-1 and XRCC1 can play a significant role in determining BER efficiency in response to chemical exposures.

4.2 Significance of Study

The findings from this study may help improve the scientific basis for the assessment of risk from environmental exposures. Some environmental carcinogens act through a genotoxic mode of action that involves DNA alkylation or oxidation, which can be processed by BER. The induction of BER genes has been demonstrated to be a potential biomarker for some chemical exposures (Rusyn et al., 2004), thereby further illustrating the importance of this pathway. Basic research regarding BER members, such as PARP-1, provides greater insight about the underlying molecular events responsible for the proficiency of this defense mechanism. Additionally, understanding the basis for interindividual responses and susceptibility to environmental exposures is a challenging endeavor in public health research. However, identifying functional genes variants and their relevance at the cellular and population levels, such as in the case of the XRCC1 280His polymorphism, may improve upon linking genetic variation and different environmental exposures to the etiology of certain diseases.

4.3 Future Studies

Quantitation of SSBs in DT40 cells

Our analysis of PARP-1 proficient and deficient cells determined that both cell lines accumulated an equal amount of AP sites during MMS exposure. However, DNA from PARP-1 deficient cells appeared to have a higher SSB content based on increased DNA migration during GGE analysis. The qualitative analysis suggests that the BER step after AP site removal, namely strand ligation, may be hindered in the absence of PARP-1. In follow up research, a more quantitative analysis of SSB formation via single cell gel electrophoresis (Comet assay) should provide more robust evidence in support of our assertion that PARP-1 positively impacts DNA ligation at the end of BER.

A criticism of the Comet assay has been the inadvertent conversion of alkali labile sites, such as AP sites, into SSBs, which would lead to an overestimation of SSB formation (Nakamura et al., 2003). In the case of PARP-1 proficient and deficient cells, this occurrence would be irrelevant since AP site levels were equal as determined by slot blot analysis and therefore would not confound SSB analysis by the Comet assay. However, in instances where the number of AP sites may be different between cell lines or unknown, analysis by the Comet assay may require careful interpretation. A strategy to circumvent this issue would be to protect AP sites from alkaline cleavage, thereby preventing their contribution to SSB measurements. Hydroxylamines including methoxyamine can bind AP sites and prevent β-elimination reactions and the resulting strand scission caused under alkaline conditions. Ultimately, extension of the Comet assay protocol to include the use of hydroxylamines administered to cells prior to their lysis may lead to the analysis of SSBs without the contribution of alkali labile sites.

Applying BER imbalance data

In assessing the phenotypic difference between PARP-1 proficient and deficient cells, endpoint measurements were made for the different steps within BER, including the number of N7-meG adducts formed during chemical exposure. NAD(P)H depletion was also assessed to indicate an imbalance in BER during exposure. We were able to determine what cumulative dose (0.55 mM × hr) had initiated an imbalance in BER and the corresponding number of N7-meG adducts formed at this level of exposure. The future application of this approach may involve comparing cell lines with different DNA repair mutations for assessing the relative significance of certain repair defects. For example, preliminary results suggest that DT40 cells deficient in POLβ experience an imbalance in BER at a cumulative dose of 15.5 μM × hr, which is about 35 times lower than that in wild-type DT40 cells.

Further characterization of XRCC1 variant functionality

Our study in conjunction with data from a previous report demonstrated that with a chemical exposure the 280His variant of XRCC1 increased sensitivity to cell killing and decreased SSB repair within an EM9 background (Takanami et al., 2005). With the use of additional isogenic models, examination of the 280His variant in other cell types would provide additional information regarding the significance of this polymorphism. Humanized mouse models expressing XRCC1 variants may provide such a platform for assessing the significance of these polymorphisms at the cellular, tissue, and whole animal levels (Ladiges, 2006). Mathematical models have also been devised to understand the effect of BER polymorphisms (Sokhansanj and Wilson, 2006). Ultimately, data from the above cellular

and animal systems may further refine these models to increase the understanding of the biological significance of XRCC1 polymorphisms.

Appendix A. Assay conditions for genotyping *XRCC1* polymorphisms.

Locus	SNP	dbSNP	Forward primer Reverse primer	Probe sequences[a] VIC probe FAM probe	Annealing temperature (°C)
XRCC1 codon 194	Exon 6-22 C -> T Arg 194 Trp	rs 1799782	AGGATGAGAGCGCCAACTC CCCACGAGTCTAGGTCTCAAC	Arg 194 (C) VIC TTGTTGATCCgGCTGAA Trp 194 (T) FAM TTGTTGATCCaGCTGAA	62.0
XRCC1 codon 280	Exon 9+16 G -> A Arg 280 His	rs 25489	CCCCAGTGGTGCTAACCTAATCTA GCTCGGGCAGGGACTG	His 280 (A) VIC TCCAACTCaTACCCC Arg 280 (G) FAM TCCAACTCgTACCCC	60.0
XRCC1 codon 399	Exon 10-4 A -> G Arg 399 Gln	rs 25487	GAGTGGGTGCTGGACTGTCA TGCCCAGCACAGGATAAGG	Gln 399 (G) VIC CTGCCCTCCCgGAGGTAAGGC Arg 399 (A) FAM CTGCCCTCCCaGAGGTAAGGCC	62.0

[a]Lower case indicates sequence variant. Probes for XRCC1 codon 280 and 399 were designed for the anti-sense strand. XRCC1 codon 194 and 280 probes are minor-groove binding. All DNA sequences are listed 5′-3′.

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