

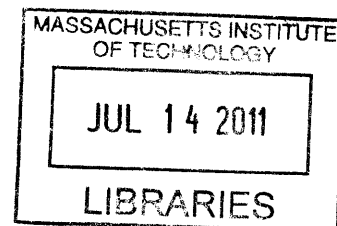
XRCC1 & DNA MTases: Direct and Indirect Modulation of Inflammation-Induced DNA Damage

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Inflammation and Cancer: The role of XRCC1 and DNA methyltransferases in mediating responses to inflammation-mediated DNA damage

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Thesis Abstract

Cancer causes 13% of all deaths worldwide. Inflammation-mediated cancer accounts for ~15% of all malignancies, strongly necessitating investigation of the molecular interactions at play. Inflammatory reactive oxygen and nitrogen species (RONS), including peroxynitrite and nitric oxide (NO[•]), may potentiate malignancy. We hypothesize that the base excision repair (BER) pathway modulates susceptibility to malignancy, by modulating the BER-intermediate levels, large scale genomic rearrangements and toxicity following exposure to RONS. We further hypothesize that DNA methyltransferases are responsible for the memory of genotoxic insult, and the epigenetic propagation of genomic instability, following exposure to genotoxins.

Here, we exploited cell lines engineered to carry deficiencies in BER to study repair of DNA damage induced by RONS. Toxicity and BER-intermediate levels were evaluated in XRCC1 proficient and deficient cells, following exposure to the peroxynitrite donor, SIN-1 and to NO[•]. Using the alkaline comet assay, we find that while XRCC1 proficient and deficient CHO cells incur equivalent levels of SIN-1 induced BER-intermediates, the XRCC1 null cells are more sensitive to killing by SIN-1, as assessed by clonogenic survival. Furthermore, using bioreactors to expose CHO cells to NO[•], we found that the BER-intermediate levels measured in XRCC1 null cells were lower than in WT cells. We found that while XRCC1 can facilitate AAG-mediated excision of the inflammation-associated base lesions ethenoadenine and hypoxanthine, *in vitro*; XRCC1 deficient human cells were no more susceptible to NO[•] than WT cells. However, in live glioblastoma cells, XRCC1 is acting predominantly downstream of AAG glycosylase. This work is some of the first to assess the functional role of XRCC1, in response to RONS and suggests complexities in the role of XRCC1.

We also demonstrate that the underlying basis for the memory of a genotoxic insult and the subsequent propagation of genomic instability is dependent on the DNA methyltransferases, Dnmt1 and Dnmt3a. We found that a single exposure led to long-term genome destabilizing effects that spread from cell to cell, and therefore provided a molecular mechanism for these persistent bystander effects.

Collectively, our findings impact current understanding of cancer risk and suggest mechanisms for suppressing genomic instability, following exposure to inflammatory genotoxins.

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Preface

The work contained herein represents the herculean efforts of numerous industrious and insightful individuals from different labs at MIT and beyond. To these people, and especially to members of the Engelward Lab—both past and present—I am extremely grateful.

At the time of writing, Chapter II had been drafted as a manuscript for submission. The project was initiated by the author. David Svilar, in the Sobol Lab at the University of Pittsburgh conducted the molecular beacon excision assay and the glioblastoma western blot. Xiao-Hong Wang and Ying-Chih Lin made the glioblastoma cells with modified BER capacity. Somsak Prasongtanakij performed the MMS repair kinetic using the 2nd generation comet chip (CoaCh). All other data were generated by the author with technical support from Laura Trudel.

Chapter III, while unpublished at the time of writing, served as a basis for the development of a second generation of the comet on a chip device. This subsequent work was published in *PNAS* in March of 2010. Work on the project was initiated by Sukant Mittal, in the Bhatia Lab (Laboratory for Multiscale Regenerative Technologies) and the author with support from Werner Olipitz. The project has since been advanced by David Wood, David Weingeist, Somsak Prasongtanakij and Jing Ge.

Chapter IV was published in *Oncogene* in November of 2010. The project was initiated by Rebecca Rugo and experiments were designed and conducted by Rebecca Rugo with support from Tiffany Yee and the author. KN Mohan and JR Chaillet, at the University of Pittsburgh engineered the doxycycline controllable Dnmt1 system and performed western analysis; Bevin Engelward prepared the manuscript with support from the author; Bevin Engelward and Joel Greenberger oversaw the study design. All authors discussed the results and commented on the manuscript.

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through the joys and the woes
be they mentor, family-member or friend
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the end.

Ad maiorem Dei gloriam

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List of Abbreviations

5'dRP	5'-deoxyribose-5-phosphate
8-oxoG	8-oxo-7, 8-dihydro-2'-deoxyguanosine
AAG	Alkyladenine DNA glycosylase
AMP	Adenosine monophosphate
AP	Apurinic/ apyrimidinic
APE	Apurinic/apyrimidinic endonuclease
ARTs	ADP-ribosyl transferases
BER	Base excision repair
BRCT	BRCA1 C-terminus
CoaCh	Comet on-a-chip
DNA	Deoxyribonucleic acid
DNMT	DNA methyl transferase
DSB	Double strand break
EMS	Ethyl methanesulfonate
FaPy-G	Formamidopyrimidine glycosylase
H ₂ O ₂	Hydrogen peroxide
IFN	interferon
IL	Interleukin
L-NMA	N ^G -methyl-L-Arginine monoacetate
LPS	Lipopolysaccheride
Me-Lex	MeOSO ₂ (CH ₂) ₂ -lexitropsin
MMS	Methyl methanesulfonate
PAR	Poly (ADP-ribose)
PARP	Poly (ADP-ribose) polymerase

PCNA	Proliferating cell nuclear antigen
PNKP	Polynucleotide kinase 3'-phosphatase
Pol	Polymerase
RFC	Replication factor-C
RONs	Reactive oxygen and nitrogen species
ROS	Reactive oxygen species
SCE	Sister chromatid exchange
SCGE	Single cell gel electrophoresis
SSB	Single- strand break
TDP1	Tyrosyl-DNA phosphodiesterase 1
TMZ	Temozolomide
UDG	Uracil DNA glycosylase
UDS	Unscheduled DNA synthesis
WHO	World Health Organization
XRCC1	X-ray cross complementing

Chapter I

Introduction

Cancer is the leading cause of death globally, accounting for 13% of all deaths worldwide (~8 million deaths) (Ferlay *et al.*, 2010). Additionally, cancer related deaths are projected to rise to ~12 million deaths by the year 2030, further stressing the impact of this illness to current and future generations. Most importantly, according to the World Health Organization more than 30% of cancer deaths can be prevented, perhaps providing an exploitable chink in the armor of this seemingly intractable malady, once the mechanisms at play have been established.

1.1 Inflammation and Cancer

Cancer can be defined as a collection of genetic disease states, arising from the multi-staged accumulation of heritable changes in gene expression and resulting in invasive tumors. These heritable changes can be mutations, permanently fixed transmissible changes in the coding sequence of genomic DNA (Friedberg, 2006), and/or epigenetic changes, heritable changes that do not involve changes in DNA sequence (Lund, 2004; Vogelstein, 2004). More specifically, these heritable changes are changes that allow cells to divide, independent of growth stimuli, evade cell cycle checkpoints, become pro-angiogenic, become immortal, evade apoptosis; metastasize, gain mutations, or indeed, possess any combination of these and additional attributes (Hanahan and Weinberg, 2000).

A recognized and useful, but appreciably simplified, model of carcinogenesis is the *two stage model of carcinogenesis* (Philip, 2004). This model is based on the idea that carcinogenesis is a multi-step process with sequential stages of initiation and promotion, followed by progression (Fig. 1-1). This model hypothesizes that somatic cell mutations occur as a consequence of endogenous or exogenous exposure to carcinogens. Cells that

have been 'initiated', by induction of tumor promoting mutations and/or changes in methylation patterns, subsequently accumulate and can persist indefinitely in otherwise normal tissues until promotion. Certain micro-environmental conditions act as 'promoters', giving initiated cells a selective advantage and leading to the accumulation of more tumor-promoting mutations. These micro-environmental conditions may be the result of chemical irritants (*e.g.* phorbol esters), endogenous factors released at the site of wounding (*e.g.* cytokines, complement components), or tissue damage (*e.g.* from organ resection). These conditions allow for the proliferation of the 'initiated' population, and or for the selective growth of this population by Darwinian-like selection. Inflammation is now increasingly appreciated as a critically important micro-environmental condition that both promotes tumorigenic mutations (potentially at both the initiation and the promotion stages); and that yields a selective advantage to pre-malignant cells, allowing for progression to malignancy.

In 1863 Rudolf Virchow, "the father of modern pathology", proposed that cancer originated from sites of chronic inflammation, based on the observation of leukocyte infiltration at neoplastic sites (Balkwill, 2001; Coussens, 2002). Today, almost a century and a half after Virchow's initial observations, the association between chronic inflammation and cancer is well established. Indeed, 15-25% of all malignancies are thought to be associated with a chronic inflammatory state (Hussain, 2003; Kuper, 2000; Ohshima *et al.*, 2003). Concordantly, many factors that cause chronic inflammatory conditions are now known to be associated with malignancy (Table 1-1) (Shacter, 2002). However, the precise molecular processes that drive this determinedly complex relationship remain elusive. This is not surprising as inflammation is itself, an intricately complex, multi-component response

to a plethora of stimuli including irritants, pathogens and autoimmunity. Indeed, chronic inflammation is characterized by prolonged, concurrent, active tissue repair and damage as a result of numerous chemically mediated vascular and cellular responses (Kumar, 2005; Philip, 2004). As such, a singular etiological relationship between chronic inflammation and cancer is improbable.

Initial models for inflammation mediated carcinogenesis focused on proliferation (Coussens, 2002; Preston-Martin, 1990; Tamir, 1996). However, while it is generally appreciated that enhanced cellular proliferation is a risk factor for cancer, this singular attribute is not sufficient to cause cancer. Instead, consistent with the two-stage model of carcinogenesis described above, chronic inflammation is now thought to provide a micro-environment conducive to initiation, promotion and indeed subsequent progression of precancerous lesions to malignant tumors (Philip, 2004). In this regard, the role of reactive oxygen and nitrogen species (RONS) is thought to be critically important. RONS have the ability to induce mutations by directly or indirectly damaging DNA (Hussain, 2003) and by epigenetic processes (Cerutti, 1985; Dickey, 2009). Additionally, RONS create conditions wherein initiated cells have selective growth and survival advantages (Shacter, 2002).

1.2 Reactive Oxygen and Nitrogen Species, DNA Damage and Genomic Instability

Of particular interest to our group in the etiology of inflammation-associated cancer, is the role played by reactive oxygen and nitrogen species (RONS) in the initiation and promotion of malignancy. The chronic inflammatory milieu is characterized by infiltration of dendritic cells, mast cells and phagocytes (neutrophils, eosinophils, monocytes,

macrophages) (Shacter, 2002). When exposed to any of a number of different inflammatory stimuli, for example bacterial lipopolysaccharide (LPS) or IFN γ , macrophages and neutrophils can become activated and generate large quantities of RONS (Fig. 1-2). These oxidants, including superoxide, hydrogen peroxide, nitric oxide (NO \cdot), hypochlorous acid and hydroxyl radical, serve to kill bacteria and invading parasites (Dedon, 2004; Lonkar and Dedon, 2011). However, collateral damage is often suffered by proximal somatic cells. The biomolecular targets of inflammatory RONS in these target cells are varied and include proteins, membranes and cellular and mitochondrial DNA. Importantly, RONS-driven reactions with these cellular targets lead to deleterious phenotypes including DNA double strand breaks DSBs (Clemons *et al.*, 2007), cytotoxicity (Szabo *et al.*, 1996; Zhang *et al.*, 1994) and genomic instability via point mutations (deRojas-Walker *et al.*, 1995; Gal and Wogan, 1996) and large scale genomic rearrangements (Kiziltepe, 2005; Li, 2006) (Fig. 1-2), that may all potentiate malignancy.

NO \cdot is an important reactive molecule, present in the inflammatory environment. At relatively low concentrations, this diatomic radical regulates processes in the nervous, immune and cardiovascular systems (Bredt and Snyder, 1994; Cary *et al.*, 2006; Thomas *et al.*, 2008). However, NO \cdot can be genotoxic at higher concentrations (Burney *et al.*, 1997; Wink *et al.*, 1991). Indeed, via its reactions with superoxide and oxygen to form peroxynitrite (ONOO \cdot) and nitrous anhydride (N $_2$ O $_3$) respectively, NO \cdot has been shown to make numerous DNA lesions both *in vitro* (Burney *et al.*, 1999b; Wink *et al.*, 1991) and *in vivo* (Gal and Wogan, 1996). For example, ONOO \cdot can oxidize guanine making 8-nitro-guanine (Yermilov *et al.*, 1995), which can spontaneously depurinate leaving an abasic site, or be further oxidized to 8-oxo-guanine (8-oxo-G) (Dedon, 2004; Epe *et al.*, 1996; Kennedy

et al., 1997; Uppu *et al.*, 1996), which can then also spontaneously depurinate (Dedon, 2004), also yielding an abasic site. 8-oxo-G is particularly mutagenic, causing G:C→T:A transversion mutations (Shibutani *et al.*, 1991). Furthermore, depending on the levels of CO₂, ONOO⁻ can make direct DNA single strand breaks (SSBs) via direct oxidation of the sugar backbone. Nitrosoperoxycarbonate, the product of the reaction of peroxyxynitrite and CO₂, skews the reactivity of peroxyxynitrite from reaction with the deoxyribose to form SSBs, to reaction with nucleobases, as described (Tretyakova, 2000; Yermilov, 1996). In contrast, N₂O₃ primarily creates base deamination products; converting guanine to xanthine, adenine to hypoxanthine and cytosine to uracil (Burney *et al.*, 1999a). Additionally, RONS can cause lipid peroxidation which creates secondary metabolites that can then alkylate DNA, creating alkylation lesions like 1, N⁶-etheno adenine (εA), for example (El Ghissassi *et al.*, 1995; Nair *et al.*, 1998). εA blocks replicative polymerases (Frick *et al.*, 2007), is mutagenic causing AT→GC transitions and A:T→T:A and A:T→T:A transversions (Basu *et al.*, 1993; Pandya and Moriya, 1996) and is associated with the signature p53 and Kras mutations observed in rat and human liver cancers, following exposure to vinyl chloride (Barbin, 1998; Barbin, 2000).

Many of the DNA lesions resulting from exposure to RONS can promote a number of deleterious phenotypes including toxicity (Szabo *et al.*, 1996; Zhang *et al.*, 1994) elevated DNA double strand breaks (DSBs) (Clemons *et al.*, 2007) and genomic instability, via point mutation (deRojas-Walker *et al.*, 1995; Gal and Wogan, 1996) and large-scale genomic rearrangements (Kiziltepe, 2005; Li, 2006). Consequently, the repair of RON-mediated DNA damage may be an important mechanism for mitigating the toxic and tumorigenic effects of these lesions. Indeed, an individual's capacity to repair inflammation-mediated DNA lesions may represent a hitherto poorly-defined risk factor for inflammation-mediated malignancy

(Fortini, 2003; Frosina, 2007; Meira LB, 2008; Sweasy, 2006; Tudek). For example, Meira et al. demonstrated that DNA damage induced by a chronic inflammatory stimulus (derived from dextran sulfate sodium in drinking water) contributes to the formation of pre-neoplastic lesions in AAG deficient mice (Meira LB, 2008).

1.3 Base Excision Repair

Overview

In mammalian cells, base excision repair (BER) is responsible for repairing many of the inflammation-mediated lesions. The base excision repair (BER) pathway consists of three sub-pathways: short patch—monofunctional glycosylase, short patch—bifunctional glycosylase and long patch BER (Fig. 1-3, for overview). These three sub-pathways can be further simplified to five enzymatically catalyzed steps: *lesion excision*, *strand scission*, *gap tailoring*, *DNA synthesis* and *ligation*, conducted by numerous proteins in a complex set of interactions (Table 1-2).

The nomenclature “short” vs. “long” patch refers to the length of excised/ inserted bases, in effect the repair gap size; short patch is a single nucleotide excision/insertion, whilst long patch is an insertion of 2-7 nucleotides (Fortini, 2003; Frosina, 1999). Additionally the nomenclature “monofunctional” vs. “bifunctional” glycosylase refers to the number of enzymatic steps (functions) conducted by the enzyme. Monofunctional glycosylases carry out the single enzymatic activity of *lesion excision*, whilst the bifunctional glycosylases carry out both *lesion excision* and *strand scission* steps via a connate AP lyase

activity. Interestingly, all glycosylases for oxidative DNA damage possess this bi-functionality (Hegde *et al.*, 2008).

The first step, *lesion excision*, involves the identification and hydrolytic cleavage of the N-glycosidic bond of an aberrant base to yield an apurinic/ apyrimidinic (AP) site by a lesion-specific monofunctional or bifunctional glycosylase. However, the AP site may also be generated spontaneously. Monofunctional glycosylases possess only N-glycosidic hydrolytic activity. In contrast, and as mentioned, bifunctional glycosylases possess N-glycosidic hydrolytic activity and AP lyase activity that carries out the *strand scission* step by cleaving the DNA backbone 3' to the abasic site (Fig. 1-4A).. However, in the case of short patch-monofunctional glycosylase BER, the strand scission step is conducted by APE1 (apurinic/apyrimidinic endonuclease 1), after base removal by the glycosylase. APE1 nicks the phosphodiester backbone 5' of the AP site, yielding a 5' ligation-blocking lesion, 5'-deoxyribose-5-phosphate (5'dRP) (Fig. 1-4B).

Both the 3' and the 5' blocking lesions must be restored to 3'-OH and 5' phosphate by *gap tailoring* processes to allow for DNA synthesis and ligation. The 5'dRP is removed by the dRP lyase activity of Pol β . The 3' blocking lesions are varied depending on the initiating glycosylase but are removed by PNKP (polynucleotide kinase 3'-phosphatase) and TDP1 (Tyrosyl-DNA phosphodiesterase 1) (Wilson, 2007).

For long patch BER, rather than rely upon a DNA lyase to remove the 5'dRP, cells have the option of incorporating several new nucleotides, thus creating a longer repair patch (Frosina 2001; Fortini 2003). In addition to the length of the gap size, long patch and short patch BER are differentiated by the enzymes involved. Short patch has a single nucleotide gap, with Pol β conducts the DNA *re-synthesis*. Long patch involves *re-synthesis* of

a longer gap, typically two to seven nucleotides long and involves either Pol β or the replicative polymerases δ/ϵ . In long patch BER, the length of the *re-synthesis* gap necessitates displacement of the lesion containing strand and this is conducted by proteins that will be discussed below. Long patch BER could potentially be initiated by either monofunctional or bifunctional glycosylases, however, it is thought that specific intermediate proteins in the monofunctional glycosylase pathway (*e.g.* 5'dRP) (Fortini *et al.*, 1999; Sung, 2006), as well as the local concentrations of specific BER proteins as a function of cell cycle (*e.g.* PCNA, Pol δ/ϵ) (Bravo and Macdonald-Bravo, 1987; Savio *et al.*, 1998), signal for the long patch pathway. Additionally, there is evidence that XRCC1 and ligase III α can regulate the switch between short and long patch BER (Petermann *et al.*, 2006). Following excision, the strand is then resealed or *ligated* by the action of ligase I or the XRCC1- ligase III α complex to restore undamaged DNA.

Additionally, there are other proteins that, although not formally considered part of the BER pathway, play a role in single strand break repair. These proteins may play a facilitative role (Table 1-2) in lesion detection and or protein co-ordination *e.g.* poly (ADP-ribose) polymerase (PARP) 1&2, XRCC1 and may thus influence the rate at which single strand breaks are repaired. A more detailed description of each of the BER steps follows.

BER Steps

Lesion excision

Lesion excision is carried out by an evolutionarily conserved group of hydrolytic enzymes known as glycosylases (Table 1-3). These enzymes are monomeric proteins, each with the ability to act on a limited number of damaged or inappropriate (Table 1-4).

A glycosylase's specificity for its cognate substrate(s) is variable. While UDG is highly specific for uracil (Hegde *et al.*, 2008) other glycosylases, such as the alkyladenine DNA glycosylase (AAG), recognize and excise many substrates including alkyl purines (*e.g.* 3-methyl adenine, ethenoadenine) and hypoxanthine (Friedberg, 2006)(See Table 1-4). The specific mechanisms by which glycosylases search for, interrogate and excise damaged bases are complex and the subject of intriguing speculation and intense inquiry (Friedman and Stivers, 2010). However, glycosylases are known to excise damaged or inappropriate bases by a "base-flipping" mechanism in which the damaged base is rotated from the DNA double helix into the glycosylase active site pocket, where hydrolysis of the N-glycosidic bond occurs (Geir *et al.*, 1996; Roberts and Cheng, 1998). Further, glycosylases can be categorized as being either monofunctional or bifunctional in their activity; a designation which refers to their ability to carry out the glycosyltic step and incision of the phosphodiester backbone. Monofunctional glycosylases can perform only the N-glycosidic hydrolysis, typically using an activated water molecule as a nucleophile to attack the sugar C1' of the damaged base. Whereas, the bifunctional glycosylases can perform both the N-glycosidic hydrolysis and incision of the back bone. The bifunctional glycosylases utilize the N-terminal proline or ϵ -NH₂ of lysine as the nucleophile for the glycosyltic reaction. Additionally, bifunctional glycosylases can carry out the base excision and lyase steps in a concerted reaction, but these steps can also be decoupled. For example, OGG1 has weak lyase activity and yields primarily AP sites, post-excision of 8-oxoG (Hill *et al.*, 2001).

Strand scission.

The N-glycosyltic action of monofunctional glycosylases results in the formation of an abasic site (a sugar devoid of a base). An endonuclease, primarily APE1 in human cells, then performs incision of the phosphodiester DNA backbone 5' to the AP site, generating a

3'-OH and a residual 5' dRP lesion. Alternatively, following bifunctional glycosylase excision of the damaged base, AP endonucleases demonstrate AP lyase activity that allows for incision of the DNA backbone 3' to the AP site (as described in the lesion excision section, above). This AP lyase activity can proceed via a β -elimination reaction or a β , δ -elimination reaction, generating a 3' phospho α , β -unsaturated aldehyde or a 3' phosphate, respectively (Baute, 2008).

Gap tailoring

The strand scission activities of APE1 and bifunctional glycosylases result in specific 5' and 3' blocking lesions. These termini are called blocking lesions as they block replication of DNA by polymerases. Other types of 5' and 3' blocking lesions can also be generated by different genotoxins. Indeed, most radiation and ROS induced DNA SSBs result in non-conventional termini (Tables 1-4A and B). Ultimately, these non-conventional strand break ends block replication and are toxic (Sobol, 2000; Sobol *et al.*, 2003), mutagenic (Takemoto, 1998) and recombinogenic (Sobol *et al.*, 2003). Thus, in order to restore conventional ends, *i.e.* a 3'-OH from which a polymerase can extend, and or a 5' phosphate for the subsequent ligation reaction, tailoring of the nonconventional DNA strand nick is required. APE1, in addition to the phosphodiesterase endonuclease activity discussed above, possesses weak 3' phosphatase and 3' exonuclease (Marenstein *et al.*, 2004; Wiederhold *et al.*, 2004) activity, allowing for restoration of a 3'OH terminus from a 3'-phospho α , β -unsaturated aldehyde (product of bifunctional glycosylase β -elimination). The 3' phosphate blocking lesion (product of NEIL β , δ -elimination) is restored to the conventional 3'OH by the 3' phosphatase activity of PNKP (Breslin and Caldecott, 2009) and TDP1 (Tyrosyl-DNA phosphodiesterase 1). In contrast, 5' blocking lesions are tailored by polymerase β and PNKP (Wilson, 2007). For example, the 5'dRP product of APE1 phosphodiesterase activity is

restored to a conventional 5' phosphate via the action of the 5'dRP lyase domain of polymerase β (Sobol *et al.*, 1996). This lyase activity has been determined to be the rate limiting step of at least the monofunctional glycosylase initiated BER pathway *in vitro* (Srivastava *et al.*, 1998) and arguably *in vivo* as well, since the majority of endogenous AP sites found in rats and human liver are cleaved 5' to the AP site (Nakamura and Swenberg, 1999).

DNA synthesis

Once a conventional 3'OH terminus has been restored, polymerase β or polymerase $\delta/\epsilon/\lambda$ can then synthesize DNA to fill the gap (Blank *et al.*, 1994; Braithwaite *et al.*, 2010; Wang *et al.*, 1993). Pol β , although similar to the replicative polymerases, is distributive and has no exonuclease activity. This 39 kDa polypeptide has a 31 kDa C-terminal polymerase domain and an 8 kDa N-terminal lyase domain for the removal of the 5'dRP blocking lesion (see *Gap tailoring* above), after the polymerase domain conducts its DNA synthesis activity. Pol β acts primarily, but not exclusively, in the context of short patch BER. Pol δ and Pol ϵ perform the gap-filling DNA synthesis step, typically in the context of long patch BER. Pol δ is made up of four subunits: p125—the polymerase and 3' to 5' exonuclease proof reading domain, p66, p50—which interacts with proliferating cell nuclear antigen (PCNA), and p12. Indeed, the long patch BER polymerases are associated with many other proteins including replication factor-C (RFC), which loads the processivity-augmenting clamp, PCNA. Importantly, Pol β and Pol δ/ϵ can all participate in long patch BER, since they all interact with the long patch machinery proteins like flap endonuclease, FEN1 (Liu *et al.*, 2004). FEN-1 is essential in long patch BER for the removal of the variable-length oligonucleotide displaced from DNA by the replicative machinery. Recently solved crystal structures of this protein with the substrate and product DNA indicate that this protein uses helices to carry out

recognition and threading of 5'-overhangs for subsequent hydrolysis (Tsutakawa *et al.*, 2011).

Ligation

In BER, ligation is carried out by one of two ATP-dependent ligases: ligase I or ligase III α . Although typically associated with ligation of Okazaki fragments in DNA replication, ligase I can act in both long and short patch BER, as this protein interacts with both Pol β (Prasad *et al.*, 1996) and PCNA (Levin *et al.*, 1997; Tom *et al.*, 2001). In contrast, Ligase III α acts primarily in an XRCC1-dependent complex (Mackey *et al.*, 1997; Nash *et al.*, 1997) in short patch BER (Fortini, 2003) and in an XRCC1-independent pathway in mitochondrial BER (Gao *et al.*, 2011). In fact, it has recently been suggested by Gao *et al.* that ligase III α is critical for mitochondrial genome integrity, but is dispensable for nuclear BER (Gao *et al.*, 2011). Nevertheless, obligate termini for the ligation reaction are a 3'OH and a 5' phosphate. Once in place, DNA ligases act in a three step reaction to seal DNA single strand nicks. DNA ligases first use a lysine side chain to form a covalent linkage to AMP (adenosine monophosphate), using ATP. The ligase-AMP intermediate then transfers AMP to the 5' phosphate, thereby activating 5' phosphate for nucleophilic attack by the terminal 3'OH. Then finally, as the 3' OH attacks the activated 5' phosphate, the nick is sealed and intact DNA is restored. AMP is released as a byproduct in this final step.

Facilitative functions

The functions and proteins discussed above represent the core BER pathway and the minimal components sufficient to reconstitute BER *in vitro*. However, there are additional proteins that are known to facilitate the role of the major BER proteins *in vivo* (See Table1-2). Among these, and yet to be fully discussed, are PARP1 and PARP2, and XRCC1.

PARP1 and PARP2 are members of the ADP-ribosyl transferases (ARTs) family of proteins (Yelamos, 2011) and are enzymes that use β -NAD⁺ as a substrate to poly(ADP-ribosyl)ate other proteins and auto- poly(ADP-ribosyl)ate themselves in response to DNA strand breaks and other stimuli. In this regard, PARP proteins play an important role in detecting DNA nicks via two CX₂CX₂₈, 30HX₂C zinc fingers (DNA-break-sensing motif) and then catalyzing formation of long and branched polymers of poly(ADP-ribose) (PAR) on proteins, including other BER proteins like XRCC1, or at sites of DNA damage, with hundreds of negatively charged subunits (Schreiber *et al.*, 2006). PARP also interacts with AP sites, presumably to protect the lesion until APE1 can perform the strand scission step (Khodyreva *et al.*). Additionally, PARP proteins also physically interact with XRCC1, conceivably to facilitate DNA repair via BER (Masson *et al.*, 1998) and homologous recombination (Ahmed *et al.*, 2010).

PARP proteins play roles in cell-survival and death and there is a growing appreciation of numerous other roles for PARP proteins, stemming from the ability of these proteins to post-translationally modify other proteins, *e.g.* in epigenetic regulation of transcription by modifying histones by damage-dependent and damage-independent processes (Poirier *et al.*, 1982); but also other functions independent of their poly(ADP) ribosylation activity, *e.g.* in interacting directly with transcription factors like NF- κ B and subsequently regulating NO[•] expression by immune cells (Oliver *et al.*, 1999).

PARP proteins therefore function as sensors and signal transducers of DNA damage, and while not canonically considered a component of BER, PARP plays a role in facilitating the repair of DNA single strand breaks (Godon *et al.*, 2008; Ström *et al.*, 2011). DNA single strand breaks, in as far as they represent a physical discontinuity in the DNA back bone, are

indistinguishable from intermediates of base excision repair (*i.e.* products of DNA strand scission through pre-ligated 3' and 5' termini). Thus, the repair of DNA SSBs is considered a sub-pathway of base excision repair, and as such PARP1 and PARP2 are thought to facilitate BER (Pachkowski *et al.*, 2009; Schreiber *et al.*, 2006; Yelamos, 2011).

1.4 X-ray Repair Cross Complementing Group 1 (XRCC1)

Like PARP1 and PARP2, XRCC1 plays a facilitative role in BER and is particularly protective against alkylation DNA damage. The X-ray repair cross-complementing group I (XRCC1) is a protein devoid of enzymatic activity, and is thought to function as a scaffold protein that facilitates BER by recruiting and interacting with other BER proteins (Thompson, 2000)(See Table 1-2). Additionally, The XRCC1 protein preferentially binds to single strand breaks and AP sites in DNA, forming a Schiff base covalent complex with the AP site (Mani *et al.*, 2004; Nazarkina *et al.*, 2007). XRCC1 is extremely gregarious, interacting in a variety of ways with many different BER proteins. XRCC1 contains two BRCT domains. The C-terminal BRCT domain of XRCC1 interacts with (Nash *et al.*, 1997) and stabilizes ligase III α (Caldecott *et al.*, 1994); the N-terminal BRCT domain interacts with PARP1 (Beernink *et al.*, 2005; Nazarkina *et al.*, 2007), inhibiting PARP's activity (Masson *et al.*, 1998). XRCC1 has also been demonstrated to interact either physically or functionally with the core pathway BER proteins. Indeed, XRCC1 is known to interact with glycosylases: AAG (Campalans *et al.*, 2005), OGG1 (Campalans *et al.*, 2005; Marsin *et al.*, 2003), NEIL1 and NEIL2 (Campalans *et al.*, 2005), NTHL1 (Campalans *et al.*, 2005); gap tailoring enzymes: APE1 (Vidal *et al.*, 2001) and PNKP (Mani *et al.*, 2007); Polymerase β (Dianova *et al.*, 2004; Wong and Wilson, 2005)

and ligase III α , in the aforementioned XRCC1-ligase III α complex (Caldecott *et al.*, 1994). XRCC1 also interacts with the facilitative polymerase processivity protein PCNA (Fan *et al.*, 2004; Mani *et al.*, 2007).

By interacting with these proteins, XRCC1 accelerates the rate at which DNA base lesions and SSBs are repaired (Campalans *et al.*, 2005; Doulias, 2001; Thompson, 1990; Thompson, 2000). These lesions could be induced by ionizing radiation, *e.g.* γ rays (Thompson, 1990; Vanankeren, 1988) and x-rays (Schwartz, 1987), or by hydrogen peroxide (Dianova *et al.*, 2004), or by alkylating agents like MMS, EMS (Thompson *et al.*, 1982; Zdzienicka *et al.*, 1992) and the cancer therapeutic Temozolomide (TMZ) (Horton, 2008). Indeed, XRCC1 deficient cells are deficient in the repair of DNA SSBs (Taylor, 2002; Wong and Wilson, 2005). Further, XRCC1 has also been implicated in the repair of DNA DSBs, since it co-localizes with the homologous recombination-associated protein Rad51 (Fan *et al.*, 2004; Taylor, 2000), and is highly expressed in testes and especially in pachytene (meiotic recombination stage) spermatocytes and round spermatids (Ahmed *et al.*, 2010; Zhou and Walter, 1998a). More directly, XRCC1 deficient hamster cells have been shown to have decreased rates of DSB repair (Schwartz, 1987).

XRCC1 deficient cells also show elevated baseline (Thompson, 1985; Zdzienicka *et al.*, 1992) and MMS-exposure-induced levels of sister chromatid exchanges (SCEs) (Zdzienicka *et al.*, 1992), a measure of homologous recombination and the result of large scale genomic rearrangements between sister chromatids. Whether the increased SCEs are due to increased DSBs that result from SSBs *e.g.* via replication fork break-down (Helleday, 2007), or more directly by way of Rad51-mediated homologous recombination (Fan *et al.*, 2004; Taylor, 2000), or by a recently discovered XRCC1-dependent DSB repair pathway (Ahmed *et*

al., 2010; Audebert *et al.*, 2004; Charbonnel *et al.*, 2010), is yet to be determined. Nevertheless, XRCC1 is thought to play pivotal role in base excision repair and the maintenance of genomic instability.

1.5 Base Excision Repair, Inflammation Mediated DNA Damage and Malignancy

Since inflammatory RONS can cause mutagenic and toxic DNA damage that is associated with malignancy, as described in section 1.2 above, and since base excision repair acts on many of the lesions known to be made by inflammatory RONS, it is logical to posit that BER could modulate cancer risk. Indeed, various deficiencies in BER are associated with increased genomic instability and cancer risk. A few pertinent exemplars follow, below.

Glycosylases

AAG deficient cells as are highly sensitive to killing by alkylation damage and have elevated levels of post-MMS-exposure SCEs (Engelward *et al.*, 1996a); but knockout mice are, surprisingly, only mildly sensitive and have no increased incidence of cancer (Engelward *et al.*, 1996a). In contrast, AAG deficient mice do have increased levels of base lesions ϵ A and 8-oxoG, and display increased pathology and colon tumorigenesis on exposure to inflammatory chemicals (Meira LB, 2008). This apparent discrepancy in the tumorigenic potential of AAG deficiency may be due to tissue-specific differences, the differential action of translesion polymerases and the levels of damage (spontaneous vs. induced).

Although MYH knockout mice are viable and display no overt phenotype, deficiencies in MYH result in increased frequency of mutation (Xie *et al.*, 2004). Additionally, biallelic inherited mutations in MYH are associated with colorectal cancers (Al-Tassan *et al.*,

2002; Sampson, 2005). These malignancies display G:C→T:A transversions in the APC gene (Al-Tassan *et al.*, 2002), which controls the proliferation of colon cells and the oncogene K-ras (Lipton *et al.*, 2003). The G:C→T:A transversion mutation is consistent with an inability to correct the 8-oxo-G:A mispair that MYH addresses (See Table 1-4).

The S326C polymorphic variant of OGG1, the bifunctional glycosylase responsible for the removal of oxidized and ring-opened purines, *e.g.* 8-oxoG and FaPy-G, is associated with increased risk of esophageal (Xing *et al.*, 2001), prostate (Xu *et al.*, 2002) and lung (Le Marchand *et al.*, 2002; Sugimura *et al.*, 1999; Wikman *et al.*, 2000) cancers (Goode, 2002). Additionally, a role for OGG1 in tumor suppression has been suggested, based on the fact that its location on chromosome 3, is a region that frequently experiences loss of heterozygosity in kidney and lung tumors (Chevallard and F., 1998).

Low levels of NEIL1 protein and inactivating mutations in this glycosylase are associated with gastric cancer (Shinmura *et al.*, 2004). However, there are redundancies in the functions of oxidative damage glycosylases, so much so that single-glycosylase knock-out mice display subtle or no phenotypes. It is only when two or more glycosylases are eliminated, that symptoms then become apparent (Hegde *et al.*, 2008; Xie *et al.*, 2004).

APE1

While the complete abrogation of APE1 is embryonically lethal, heterozygotic cells and animals are sensitive to oxidative stress and heterozygote mice have elevated levels of spontaneous mutations (Huamani, 2004).

Interestingly, BER can also modulate cancer disease prognosis, once diagnosed. For example, increased levels of XRCC1 and APE protein increase the survival rate in bladder cancer patients being treated by radiotherapy (Sak *et al.*, 2005).

XRCC1

As mentioned in section 1.4 above, XRCC1 deficient cells have elevated baseline and post-MMS-exposure SCE frequencies (Thompson, 1985; Zdzienicka *et al.*, 1992). Additionally, XRCC1 deficient cells display increased formation of MMS-induced micronuclei, another measurement of chromosomal instability, on exposure to MMS (Brem, 2005). In humans, various polymorphic variants have been associated with deficient SSB repair (Au, 2003; Takanami, 2005) and malignancy (Goode, 2002; Ladiges *et al.*, 2003). For example, the R399Q polymorphism is associated with increased risk of breast (Duell *et al.*, 2001) and stomach cancer, while the R194W polymorphism is associated with decreased risk of bladder, esophageal (Lee *et al.*, 2001) and lung (Ratnasinghe *et al.*, 2001; Stern *et al.*, 2001) cancers.

While relatively little has been published on the role of XRCC1 in response to NO, XRCC1 is known to protect against oxidative DNA damage (Kulkarni *et al.*, 2008). This is consistent with the ability of XRCC1 to associate with and stimulate oxidative DNA damage excision glycosylases *e.g.*, OGG1 and NEIL (Campalans *et al.*, 2005), and with gap tailoring enzymes specific to oxidative damage, *e.g.* PNKP (Mani *et al.*, 2007). Thus, one could posit that cells would be similarly dependent on XRCC1 for the repair of NO[•] oxidative (and alkylation) DNA damage.

Importantly, while the above-enumerated BER deficiencies are nominally associated with increased cancer risk, there is a growing appreciation that it is the intermediates of BER, that potentiate the deleterious phenotypes associated with BER deficiencies (Fortini, 2003; Sobol *et al.*, 2003; Sossou, 2005; Spek *et al.*, 2002; Wilson, 2007). Thus, it can be surmised that any perturbations to the pathway that might alter BER intermediate levels

would similarly alter cancer risk. Such perturbations would include imbalances in the levels of BER proteins, pathway polymorphic variants with differential functional efficiencies and exposure to environmental genotoxins that overwhelm the repair pathway's innate efficiency (repair capacity).

NO[•] and bystander effect-induced genomic instability

NO[•] has also recently been implicated in the communication of DNA DSBs and genomic instability via a transmissible epigenetic mechanism known as the bystander effect (Dickey, 2009; Shao *et al.*, 2002). This phenomenon, comprising of the propagation of increased toxicity, SCEs and genomic instability from genotoxin-exposed cells to naïve, neighboring cells, has traditionally been associated with ionizing radiation (IR) exposure (Huo, 2001; Lewis, 2001a; Little, 2003; Nagar, 2003; Nagasawa, 1992; Zhou *et al.*, 2000). However, our group has shown that a persistent hyper-recombination phenotype can be induced in both the distant progeny of cells exposed to the cross-linking agent, mitomycin C (MMC), and their naïve bystander cells (Rugo, 2005). Additionally, other groups have demonstrated that NO[•], possibly in the context of an inflammatory-like response, is capable of generating a bystander effect (Burr, 2010; Dickey, 2009; Shao *et al.*, 2002).

While, the specific molecular signals underlying this phenomenon are, as yet, unknown; epigenetic mechanisms have been implicated (Kovalchuk, 2008; Lorimore, 2003). Of particular interest, in this regard, is the potential role of DNA methyl transferases (DNMTs). It has been shown that DNMT1 protein levels increase in distal, bystander cells, but not in directly exposed cells; and conversely global methylation levels decrease in

directly exposed cells, but not in distal bystander cells (Koturbash, 2006). The bystander effect therefore represents an additional mechanism, albeit poorly understood, by which RONS can mediate genomic instability.

1.6 Goals and thesis design

Our overall objective is to determine how base excision repair proteins, in particular XRCC1, act and interact to modulate DNA damage and toxicity on exposure to inflammatory genotoxins. We also sought to define, how these phenotypes are epigenetically propagated. The potential applications of understanding the mechanistic and molecular underpinnings of these processes abound; from improved means of preventing inflammation-associated cancer, to enhanced methods for treatment of the disease (since many chemotherapeutics impinge on base excision repair). Our general hypotheses were two-fold:

First, we hypothesize that XRCC1 plays a role in modulating the toxicity and DNA damage levels associated with exposure to the reactive oxygen and nitrogen species derived from NO[•] (Fig. 1-5). Secondly we hypothesize that the epigenetic transmission of genomic instability via the bystander effect, known to be propagated by NO[•], is mediated by DNA methyltransferases.

The work described herein is therefore the culmination of efforts to synthesize and test these hypotheses.

1.7 References

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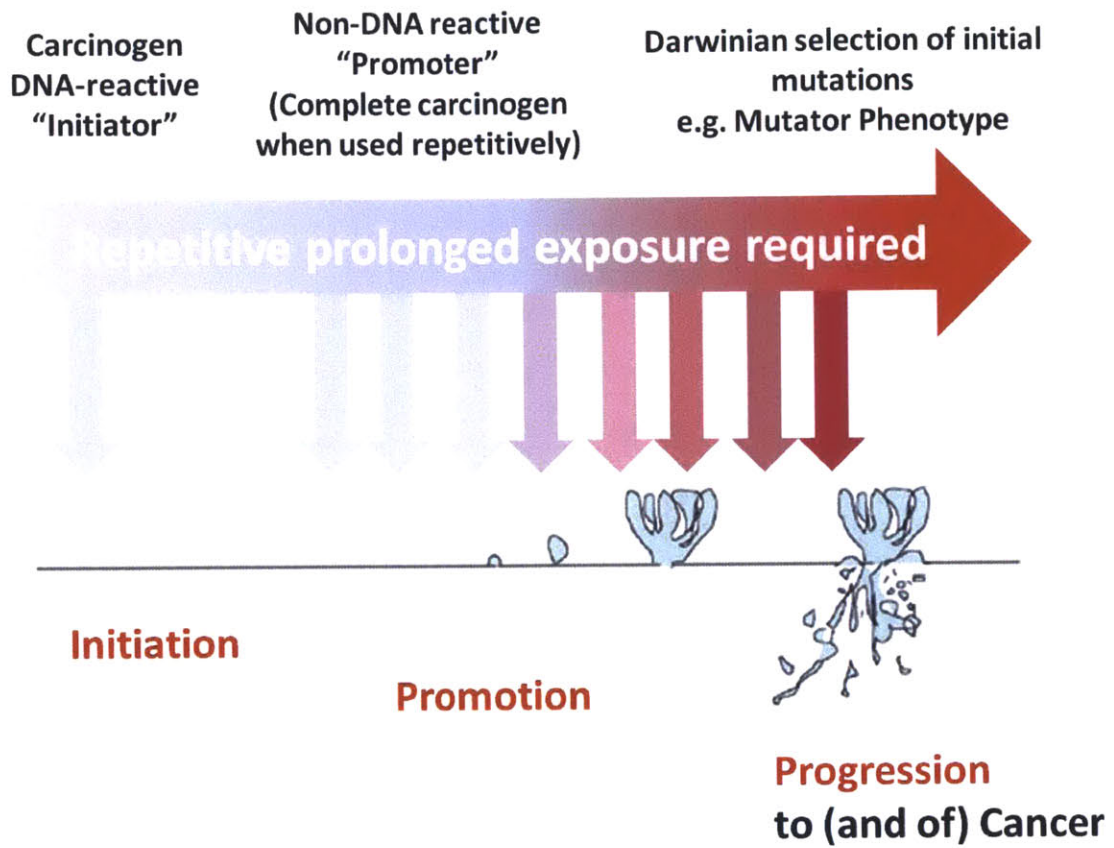


Figure 1-1. Two stage model of carcinogenesis. Promotion is followed by progression (modified from Philip, 2004). See text for details.

Malignancy	Inflammatory Stimulus/ Condition
Bladder	Schistosomiasis
Cervical	Papillomavirus
Ovarian	Pelvic inflammatory disease/tissue remodeling
Gastric	<i>H. pylori</i> induced gastritis
MALT lymphoma	<i>H. pylori</i>
Esophageal	Barrett's Metaplasia
Colorectal	Inflammatory bowel disease
Hepatocellular	Hepatitis virus B and C
Kaposi's Sarcoma	Human herpes virus type 8
Cholangiocarcinoma	Cholangitis
Oral squamous carcinoma	Gingivitis , lichen planus
Bronchial	Silica, asbestos, cigarette smoke
Mesothelioma	Asbestos

Infectious agent
 Non-infectious agent

Table 1-1. Chronic inflammatory conditions that are associated with malignancy (modified from Shacter, 2002).

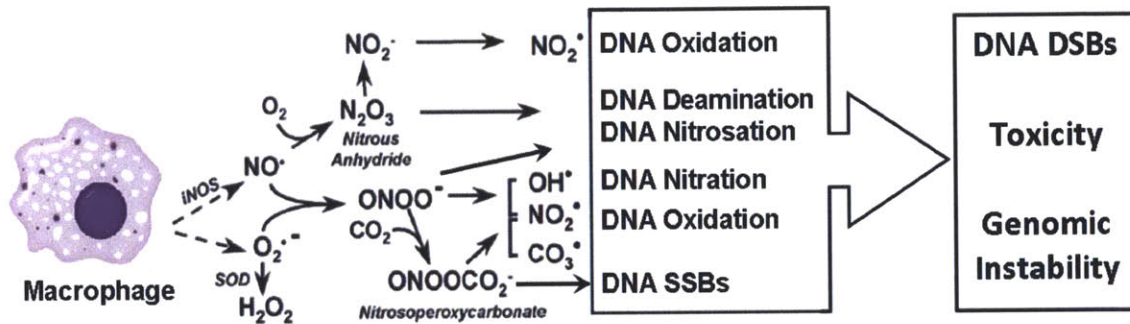


Figure 1-2. Model for RON-mediated DNA DSBs, Toxicity and genomic instability (modified from Dedon, 2004). See text for details.

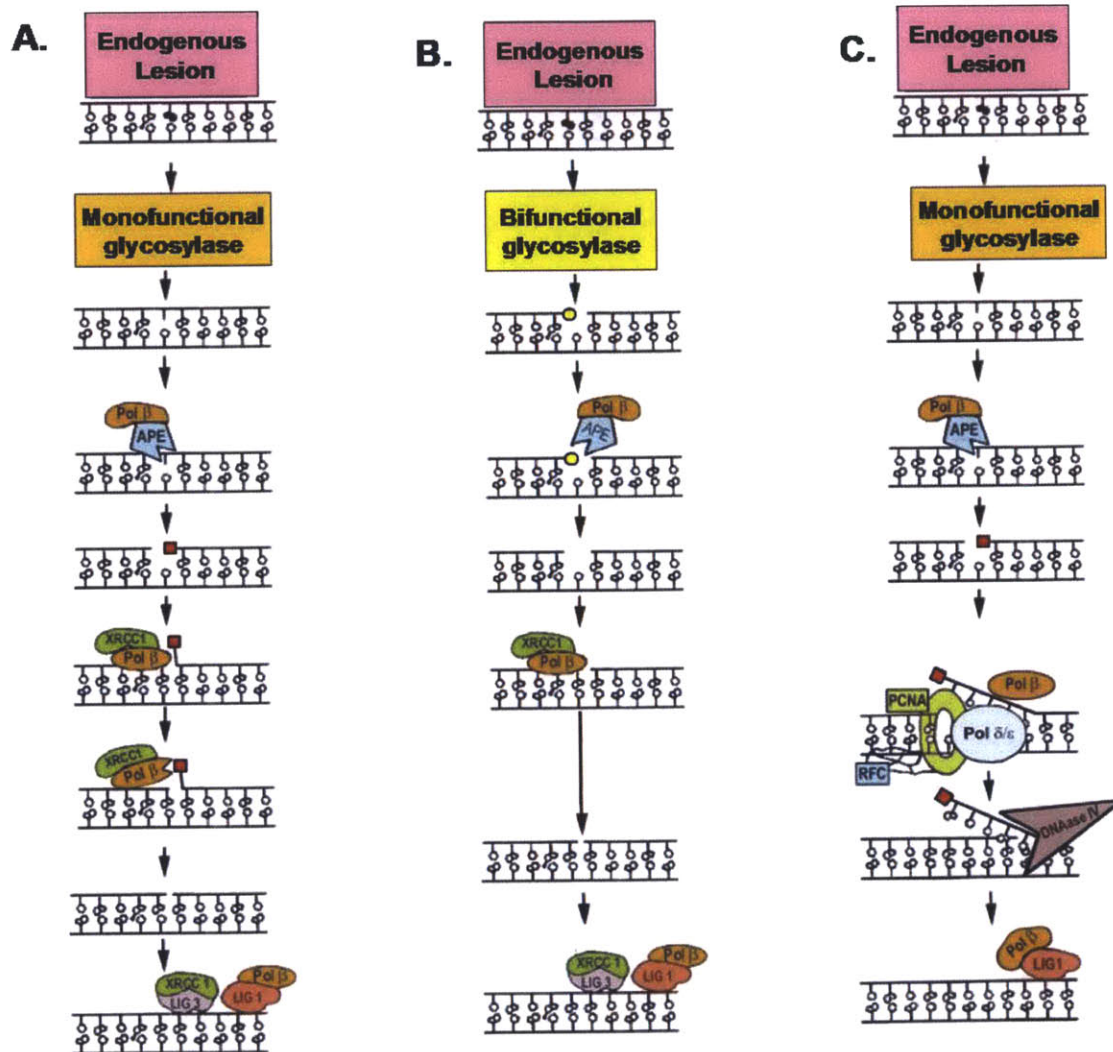


Figure 1-3. Base excision repair pathway schematic. A. Short patch—monofunctional glycosylase, **B.** Short patch—bifunctional glycosylase and **C.** Long Patch—monofunctional glycosylase BER. (modified from Frosina, 2000). See text for details.

0. Facilitative Functions		1. Lesion Excision	2. Strand Scission	3. Gap Tailoring	4. DNA Synthesis	5. Ligation
PARP 1 & 2	XRCC1	UNG				
		AAG (MPG)				
		SMUG1	APE 1&2	Polβ (5'drP) Lyase		
		TDG				Lig IIIα
		MBD4				
		MYH	Artemis		Polβ Polλ	
		NTHL	NTHL Lyase	PNKP (3'-phosphodiesterase)		
		OGG 1&2	Ogg1 AP Lyase			
		NEIL 1,2&3	NEIL AP Lyase	TDP1		Lig I
PCNA				FEN1	Polδ/ε	
RF-C						

Table 1-2. BER pathway protein components and functions. BER consists of 5 major functions: Lesion excision, strand scission, gap tailoring, DNA synthesis and ligation. These functions are carried out by specific proteins in two distinct sub-pathways: monofunctional glycosylase-initiated and bifunctional glycosylase-initiated pathways that can progress via short (single nucleotide) or long patch (2-7 oligonucleotide) repair. There are also facilitative proteins associated with multiple sub-pathway components, *e.g.* XRCC1, or associated with a certain sub-pathway *e.g.* PCNA in long patch repair. Colors indicate participation of specified protein in one or potentially multiple sub-pathways.

Protein Enzymatic Class	Protein Family/ Sub-type	<i>E. coli</i>	<i>S. cerevisiae</i>	<i>H. sapiens</i>
Glycosylase	Uracil–DNA glycosylase superfamily	Ung Mug	Ung1p	UNG TDG SMUG1
	Nth superfamily	Nth MutY	Ntg1p, Ntg2p	NTHL1 MUTYH OGG1
		AlkA	Ogg1p Mag1p	
	Fpg superfamily	Tag Fpg, Nei		NEIL1, 2 & 3
	Other	Aag		AAG (MPG)
AP Endonucleases	Xth family	Xth	Apn2p	APE1 & 2 (APEX, Hap-1, Ref-1)
	Nfo family	Nfo	Apn1p	
	PALF family			PALF
DNA Polymerases	Family A	Pol I	Pol γ	Pol γ
	Family B		Pol δ , Pol α , Pol ϵ	Pol δ , Pol α , Pol ϵ , Pol ζ
	Family X			Pol β , Pol λ
DNA Ligases	NAD ⁺ -dependent	LigA		
	ATP Dependent		Cdc9p	Lig I, Lig III α
Other	3'-phosphodisterase		Rad1p-Rad10p, Mus81p-Mms4p	PNKP
	Flap endonuclease		Rad27p	FEN1
	XRCC1			XRCC1
	PARP			
	PCNA		Pol30p	PCNA
	9-1-1 complex		Ddc1p-Rad17p- Mec3p	RAD9B-RAD1-HUS1
	RFC			RFC

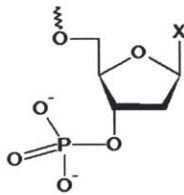
Table 1-2. Proteins involved in different steps of BER in *E.coli*, budding yeast and human cells (modified from Zharkov, 2008). See text for details.

Gene	Protein	Common Name	Cognate Substrate(s)	Mono- or Bifunctional
UNG	UNG	Uracil DNA glycosylase	Uracil (U)	Monofunctional
SMUG1	SMUG1	SMUG DNA glycosylase	U, 5-hydroxymethyluracil	Monofunctional
MBD4	MBD4 (MED1)	Methyl-Binding domain glycosylase 4	U or T opposite G at CpG, T opposite O ⁶ -methylguanine	Bifunctional
TDG	TDG	Thymine DNA glycosylase	U, T, or etheno-C opposite G (Preferably CpG sites)	Monofunctional
OGG1	OGG1	8-oxo-G DNA glycosylase	Oxidized & ring-opened purines e.g. 8-oxoG & FaPy-G	Bifunctional
MYH	MYH	MutY Homolog DNA glycosylase	A opposite 8-oxoG, 2-OH-A opposite G	Monofunctional
NTHL	NTHL (NTH1)	Endonuclease III	Ring-saturated or fragmented pyrimidines	Bifunctional
AAG	AAG (MPG, ANPG, MDG, MID1)	Alkyladenine DNA Glycosylase	3-alkylpurines, hypoxanthine, etheno-A	Monofunctional
NEIL1	NEIL1	Endonuclease VIII-like DNA glycosylase 1	Same as NTHL; FaPy-A and 8-oxoG	Bifunctional
NEIL2	NEIL2	Endonuclease VIII-like DNA glycosylase 2	Oxidized or fragmented pyrimidines	Bifunctional
NEIL3	NEIL3	Endonuclease VIII-like DNA glycosylase 3	?	Bifunctional

Table 1-4. Human DNA glycosylases and their cognate substrate(s) (modified from Friedberg, 2006). See text for details.

3' Blocking Lesions:

3'-phosphate



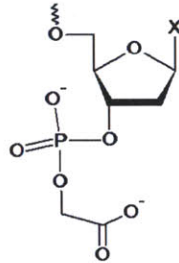
Formation:

- ROS attack of deoxyribose
- Bifunc. glycosylase activity (β, δ elimination) e.g. NEIL1&2

Repair processing:

- PNKP
- APE1(SSB)
- TDP1

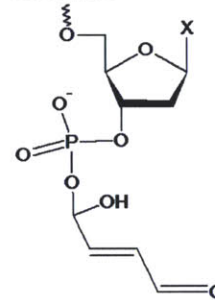
3'-phosphoglycolate ester



- ROS attack of deoxyribose

- APE1(SSB), TDP1(DSB)
- APE2, Artemis

3'-phospho α, β -unsaturated aldehyde



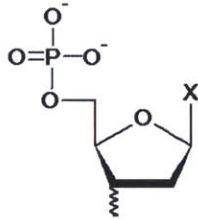
- Bifunctional glycosylase e.g. hOGG1, NTH1
- Spontaneous cleavage of AP site (β elimination)

- APE1

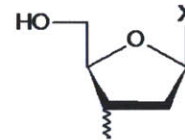
Figure 1-4A. Base excision repair 3' replication blocking lesions. X= OH for bifunctional glycosylase and APE-induced strand scission, following bifunctional glycosylase step (modified from Wilson, 2007).

5' Blocking Lesions:

5'-deoxyribose phosphate



5'-OH



Formation:

- AP endonuclease cleavage of AP site

- ROS attack of deoxyribose

Repair processing:

- **Pol β** (lyase activity)

- **PNKP**

Figure 1-4B. Base excision repair 5' replication blocking lesions. X= OH for APE-induced strand scission in BER, following monofunctional-glycosylase step. (modified from Wilson, 2007).

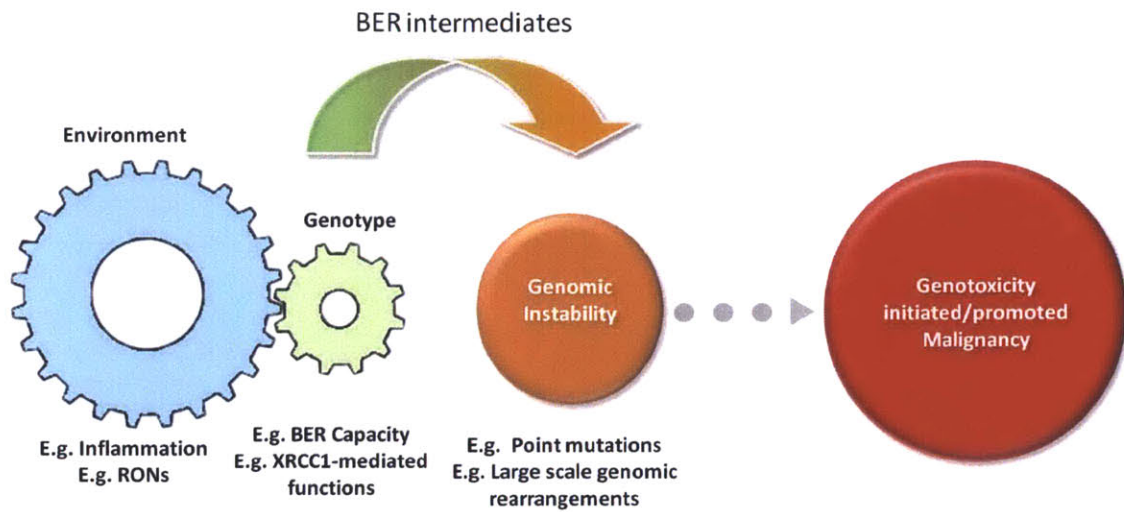


Figure 1-5. Hypothetical model for inflammatory genotoxin mediated malignancy. Inflammation-mediated carcinogenesis is, in part, due to a genotype/environment interaction with genotype exemplified by repair capacity and environment by inflammatory RONS. By modulating the levels of BER intermediates and toxicity that result from RON-mediated DNA damage, BER modulates the inflammation-mediated genomic instability that may potentiate malignancy.

Chapter II

Role of XRCC1 in response to nitric oxide and peroxynitrite-mediated DNA Damage

2.1 Abstract

Inflammation mediated cancer accounts for upwards of 15% of all malignancies, strongly necessitating investigation of the molecular interactions at play. Inflammatory reactive oxygen and nitrogen species (RONS), including peroxynitrite (ONOO^-) and nitric oxide (NO^\cdot), potentially play a role in the toxicity and large scale genomic rearrangements associated with many malignancies. We hypothesize that base excision repair modulates susceptibility to malignancy by modulating the levels of BER-intermediates, genomic instability and toxicity resulting from exposure to RONS. Surprisingly, few studies have examined the functional role of BER deficiencies on exposure to reactive nitrogen species. Here, we exploited both CHO and human glioblastoma cell lines engineered to carry deficiencies in BER to study repair of DNA damage induced by RONS. In particular, we focused on XRCC1 for two reasons. First, XRCC1 is a scaffold protein critical for BER. Second, it has been established that certain polymorphisms in the XRCC1 gene are associated with either increased or decreased risk of cancers associated with inflammation, suggesting a possible link between inflammation-induced cancer and BER. Cytotoxicity and SSB-intermediate levels were evaluated in repair proficient and deficient cells following exposure to the peroxynitrite donor, SIN-1 and to gaseous NO^\cdot . We found that the XRCC1 null cells are somewhat more sensitive to killing by SIN-1 when compared to WT, as assessed by clonogenic survival. In addition and as expected, XRCC1^{-/-} cells are deficient in repair of methyl methane sulfonate-induced SSBs. Unexpectedly however, we found that XRCC1 does not significantly impact repair of SIN-1 induced SSBs. Furthermore, using small scale bioreactors to expose cells to NO^\cdot , delivered in the gaseous form by perfusion into media via silastic tubing, we found that the SSB levels measured in XRCC1 null cells were not

increased relative to WT cells (and if anything were decreased). This result is consistent with a model in which XRCC1 plays a role in facilitating strand scission of NO[•]-induced damage, perhaps via facilitation of AAG glycosylase activity. To assess these dynamics of BER-mediated repair in human cells, we exploited engineered human cell lines with varied levels of AAG and XRCC1. Our findings suggest a difference in the repair dynamics and damage thresholds between CHO and glioblastoma. For human glioblastoma cells, balanced levels of both XRCC1 and AAG are required to suppress NO[•]-induced SSBs and toxicity. Additionally, XRCC1 is acting predominantly downstream of AAG glycosylase in mediating repair of genotoxic insult. This work is some of the first to assess the functional role of XRCC1, and other BER components, in response to nitric oxide and peroxynitrite and suggests unexpected complexities in the role of XRCC1 in response to reactive nitrogen species.

2.2 Introduction

The association between chronic inflammation and cancer is now well established with upwards of 15% of all malignancies being driven by chronic inflammation (Balkwill, 2001; Coussens, 2002; Kuper, 2000; Ohshima *et al.*, 2003; Pisani *et al.*, 1997). Of particular interest is the role played by reactive oxygen and nitrogen species (RONS) in the initiation and promotion of malignancy. During inflammatory states, neutrophils and macrophages become activated and release RONS including nitric oxide (NO^\cdot) and superoxide (Dedon, 2004; Lonkar and Dedon, 2011). These reactive oxidants serve to kill invading bacteria and parasites, but often proximal host cells incur collateral damage. While NO^\cdot -derived species are likely to have pleiotropic effects in mediating malignancy, we hypothesize that DNA damage caused by NO^\cdot and NO^\cdot -derived chemicals can cause toxicity and genomic instability that initiates and promotes malignancy.

NO^\cdot is an important signaling molecule at lower concentrations (Bredt and Snyder, 1994; Cary *et al.*, 2006; Thomas *et al.*, 2008), regulating processes in the nervous, immune and cardiovascular systems (Bredt and Snyder, 1994; Cary *et al.*, 2006; Thomas *et al.*, 2008). However, at higher concentrations NO^\cdot can be genotoxic (Burney *et al.*, 1997; Wink *et al.*, 1991). Indeed, via its reactions with superoxide and oxygen to form peroxynitrite (ONOO^-) and nitrous anhydride (N_2O_3) respectively, NO^\cdot has been shown to make numerous DNA lesions both *in vitro* (Burney *et al.*, 1999b; Wink *et al.*, 1991) and *in vivo* (Gal and Wogan, 1996). For example, ONOO^- can oxidize guanine making 8-nitro-guanine (Yermilov *et al.*, 1995), which can spontaneously depurinate leaving an abasic site, or be further oxidized to 8-oxo-guanine (8-oxo-G) (Dedon, 2004; Epe *et al.*, 1996; Kennedy *et al.*, 1997; Uppu *et al.*, 1996), which can then also spontaneously depurinate (Dedon, 2004). Furthermore, ONOO^-

can make direct DNA single strand breaks (SSBs) in the presence of CO₂ via the action of nitrosoperoxycarbonate (Tretyakova, 2000). In contrast, N₂O₃ primarily creates base deamination products; converting guanine to xanthine, adenine to hypoxanthine and cytosine to uracil (Burney *et al.*, 1999a). Additionally, RONS can cause lipid peroxidation which creates secondary metabolites that can then alkylate DNA, creating mutagenic alkylation lesions like 1, N⁶-ethenoadenine (εA), for example (El Ghissassi *et al.*, 1995; Nair *et al.*, 1998). εA blocks replicative polymerases (Frick *et al.*, 2007), is mutagenic causing AT→GC transitions and A:T→T:A and A:T→T:A transversions (Basu *et al.*, 1993; Pandya and Moriya, 1996) and is a signature p53 and K-ras mutation observed in rat and human liver cancers, following exposure to vinyl chloride (Barbin, 1998; Barbin, 2000).

The DNA lesions described above, resulting from exposure to RONS, can promote a number of deleterious phenotypes including toxicity (Szabo *et al.*, 1996; Zhang *et al.*, 1994) elevated DNA double strand breaks (DSBs) (Clemons *et al.*, 2007) and genomic instability via point mutation (deRojas-Walker *et al.*, 1995; Gal and Wogan, 1996) and large-scale genomic rearrangements (Kiziltepe, 2005; Li, 2006). Consequently, the repair of RON-mediated DNA damage may be an important mechanism for mitigating the toxic and tumorigenic effects of these lesions. Indeed, an individual's capacity to repair inflammation-mediated DNA lesions may represent a hitherto poorly-defined risk factor for inflammation-mediated malignancy (Fortini, 2003; Frosina, 2007; Meira LB, 2008; Sweasy, 2006; Tudek).

In mammalian cells, base excision repair (BER) is responsible for repairing many of the inflammation-mediated lesions described above. Briefly, this pathway consists of five, enzyme-catalyzed steps: lesion excision, strand scission, gap tailoring, DNA synthesis and ligation. For a detailed review of BER, the authors refer the reader to section 1.3 of this

thesis and elsewhere (Fortini, 2003; Hegde *et al.*, 2008; Zharkov, 2008). Importantly, all five enzymatic steps in the BER pathway are putatively facilitated by the x-ray repair cross-complementing group 1 (XRCC1) protein. Indeed, XRCC1 is a scaffold protein that, although lacking its own enzymatic activity, facilitates the enzymatic roles of numerous other BER pathway proteins. For example the alkyl adenine DNA glycosylase (AAG), that carries out excision of 3-methyl adenine (3me-A) (Campalans *et al.*, 2005), hypoxanthine (Hx) (Campalans *et al.*, 2005), ϵ A (Meira LB, 2008) and other alkyl purines (Friedberg, 2006), has been shown to be physically associated and functionally associated with XRCC1 (Campalans *et al.*, 2005). Indeed, XRCC1 deficient cells have been shown to be deficient in the repair of alkylation (Taylor, 2002; Wong and Wilson, 2005) and ionizing radiation-induced (Schwartz, 1987; Thompson, 1990; Vanankeren, 1988) DNA damage and are acutely sensitive to killing by alkylating agents like methyl methane sulfonate (MMS) (Horton, 2008; Thompson *et al.*, 1982). Furthermore, XRCC1 deficient cells have higher baseline and post-MMS-exposure sister chromatid exchanges (SCEs) (Thompson, 1985; Zdzienicka *et al.*, 1992), which are a measure of large scale genomic rearrangements and thus represent a genomic instability mutator phenotype.

Surprisingly, little is known about the susceptibility of XRCC1 deficient cells to inflammatory genotoxins. Previous studies have shown that XRCC1 deficient Chinese hamster ovary (CHO) cells have elevated levels of SCEs on exposure to NO \cdot . XRCC1 deficient mouse (Horton, 2008) and CHO (Dianova *et al.*, 2004) cells are only moderately sensitive to hydrogen peroxide, but XRCC1 does protect against the lethality of oxidative damage in non-dividing neural cells (Kulkarni *et al.*, 2008). Collectively, these findings suggest that XRCC1 may be important in the repair of RON-mediated DNA damage and may modulate

toxicity and cancer risk. Indeed, various polymorphic variants in XRCC1 have been shown to be either increasingly (Duell *et al.*, 2001) or decreasingly (Lee *et al.*, 2001; Ratnasinghe, 2001; Stern *et al.*, 2001) associated with various inflammation-associated malignancies. These findings underscore the importance of defining the role of XRCC1 in response to inflammatory genotoxins, both for cancer risk assessment and for therapeutic intervention.

Here we show that XRCC1 is important for mitigating peroxynitrite induced toxicity by a BER-independent mechanism. Unexpectedly, we also show that in CHO cells, XRCC1 does not protect cells from NO[•]-induced toxicity. Furthermore, while XRCC1 facilitates AAG-mediated excision of inflammation associated lesions εA and hypoxanthine, XRCC1 does not protect cells from NO[•] induced damage. Instead, XRCC1 plays a role downstream of the glycosylase for repair of alkylation-induced BER-intermediates.

2.3 Materials and Methods

2.3.1 Cells and Cell Culture

AA8 and EM9 CHO cells were obtained from ATCC (Manassas, VA) and the human complemented EM9 cells were engineered, and kindly provided by the lab of Larry Thompson (Thompson, 1990). CHO cells were cultured in 10% FBS (Atlanta Biologics, Lawrenceville, GA) in DMEM (Cat#11965, Invitrogen, Carlsbad, CA) and penicillin/streptomycin (100U/ml;100ug/ml) (Sigma, St. Louis, MO). CHO cells were cultured in 150mm dishes (Falcon, BD) and were kept growing exponentially, being passaged approximately once every three days. Cell passaging was conducted by aspirating media, rinsing in warm PBS and treating cells with trypsin (Invitrogen, Carlsbad, CA) for 5-10min,

quenching with 10mL media and then transferring 1mL of the resultant cell suspension to 30mL fresh media.

Engineering of glioblastoma LN428 cells has previously been published (Tang *et al.*, 2009; Tang *et al.*, 2011). Briefly, the cell line LN428 is an established glioblastoma derived cell line with mutations in p53 and deletions in p14ARF and p16 and is WT for PTEN (Tang *et al.*, 2009). Human AAG expressing cells were generated by transfection using FuGene 6 transfection reagent (Roche Diagnostic Corp.) according to the manufacturer's protocol. Transfected cell lines were cultured in G418 and/or puromycin for 2 wk, and individual clones stably expressing human AAG were selected. The shuttle vectors (control: pLKO.1-puro-turbo green fluorescent protein [GFP]) were purchased from Sigma, as was the XRCC1 shRNA oligonucleotide (Gene ID: NM_006297, Clone ID: NM_006297.1-1489s1c1, Target sequence: CCAAGTGTCCAGGAAGATATA, Oligonucleotide Sequence: CCGGCCAGTGTCCAGGAAGATATACTCGAGTATATCTTCTGGAGCACTGGTTTTT). The WT cells were cultured in alpha MEM (MediaTech, Manassas, VA), 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), antibiotic/ antimycotic (Sigma, St. Louis, MO), gentamycin, L-glutamine (Sigma, St. Louis, MO). The engineered glioblastoma cells including the XRCC1KD cell line, the AAGOE/XRCC1KD cell line, the AAGOE and the GFP cell lines, were cultured in the same media as the WT media described above except with 1µg/mL puromycin (Sigma, St. Louis, MO).

2.3.2 Cell extract preparation and Western Blot

CHO whole cell extracts were prepared using RIPA according to manufacturer's instructions (Pierce, Rockford, IL). Protein concentration in extracts was done by BCA

quantification (Pierce, Rockford, IL) according to the manufacturer's instructions. 20-100µg of protein was loaded into a precast 4-20% gradient Tris-HCl gel (BioRad, Hercules, CA). Protein was transferred onto nitrocellulose membrane and XRCC1 protein was probed using a rabbit polyclonal primary antibody against XRCC1 (Abcam, Cambridge, MA) and an HRP-conjugated secondary goat antibody against rabbit IgG. For the loading control a rabbit monoclonal antibody (Cell Signaling, Beverly, MA) was used.

LN428 nuclear extracts were prepared and protein concentrations were determined as described previously (Tang *et al.*, 2009). 20 µg of protein was loaded on a precast 4–20% Tris-glycine gel (Invitrogen) or 25 mg of protein was loaded onto a precast 4–20% Mini-PROTEAN TGX gel (Bio-Rad). The following primary antibodies were used in the

Western blot assays: anti-human MPG (Mab; clone 506-3D)(Tang *et al.*, 2009); anti PCNA (Santa Cruz Biotechnology, Santa Cruz, CA).

2.3.3 CHO MMS and SIN-1 Exposures for Comet Assay

Growing CHO cells were rinsed with warm PBS and were passaged as described above. Approximately 24 hours later cells were removed from their dishes by trypsin treatment for 5-10 min. Cells were then counted and re-suspended in 1% low melting point (LMP) agarose (Invitrogen, Carlsbad, CA) at 37°C at a density of 2.5×10^5 cells/ mL. A volume of 500µL (1.25×10^5 cells) of gel containing re-suspended cells was then pipeted onto 37°C pre-warmed, agarose-precoated glass slides and cover-slipped. The cells were allowed to settle onto the slides for 10 minutes and were then moved to 4°C to allow the gel to solidify.

Cover-slips were removed and the cells were then exposed to either MMS or SIN-1 at the specified concentrations in phosphate buffered saline (PBS) (Invitrogen, Carlsbad, CA). For the repair experiments, following exposure to the specified dose of MMS or SIN-1, the slides were rinsed with PBS and were transferred to CHO media for the specified period of time before being lysed for the alkaline comet assay (See Section 2.3.6).

2.3.4 CHO and Glioblastoma NO[•] Exposures

For NO[•] exposures, 1×10^6 CHO or glioblastoma cells were seeded in media into 60mm tissue culture dishes (BD Falcon, Franklin Lakes, NJ) and allowed to adhere. Approximately 24 hours later, cells were placed in 100mL and were exposed to gases for specified periods of time, as previously described (Kiziltepe, 2005; Li *et al.*, 2006; Wang *et al.*, 2003). Following exposure, a sample of media was taken for nitrite quantification and the cells were removed from the dish by trypsin treatment for 5-20 min at 37°C. The cells were then counted and were split into two batches for subsequent analysis: one batch for the clonogenic survival assay and the other batch for analysis by alkaline comet assay.

2.3.5 Clonogenic Survival Assay

Clonogenic survival assay was conducted as reported elsewhere (Freshney, 2005). Briefly, treated (long term exposure) or untreated (dose response and repair experiments) CHO and glioblastoma cells were seeded at known numbers and allowed to adhere for

approximately 24 hours. Untreated cells were then exposed for SIN-1 or MMS for 30min at 37°C. The cells were then rinsed with PBS and incubated in media for 10-14 days to form colonies. The colonies were then fixed in 1:1 Methanol:PBS and stained with 0.5% crystal violet in 1:1 Methanol:PBS for 10min. Colonies were rinsed with de-ionized water and allowed to dry and were then counted. Survival for each cell type was calculated as a ratio of plating efficiency for each cell type vs. the WT plating efficiency. *P values* were calculated by student's t-test on the mean of at least three replicate experiments.

2.3.6 Alkaline comet assay

To measure the accumulation of base excision repair intermediates on exposure to a given genotoxin, a modified version of the alkali comet assay (single cell gel electrophoresis) was employed (Olive, 2006). Briefly, cells immobilized in 1% LMP agarose were lysed for at least one hour in lysis buffer containing 2.5M NaCl, 100mM Na₂EDTA, and 10mM tris and 1% triton X-100 at pH 10. Following lysis, cells in gel are held in electrophoresis buffer (0.3M NaOH, 1mM Na₂EDTA) for 40 minutes for alkali unwinding. Then, following the alkali unwinding incubation, electrophoresis was performed at 1V/cm, 300mA for 30min. Slides were neutralized in 0.4M tris, before staining with 500µg/mL ethidium bromide. Image analysis was carried out by Metafer v.3.6.7 (Metasystems, Waltham, MA). At least 100 nucleoids per condition were analyzed and the median calculated. Each experiment was replicated at least 3 times and the mean of all replicates plotted. *P values* were calculated by student's t-test.

2.4 Results and Discussion

2.4.1. Role of XRCC1 in response to NO[•] and peroxynitrite exposure in CHO cells

Reactive oxygen and nitrogen species released by activated immune cells can cause myriad DNA lesions, which in turn can result in a number of deleterious phenotypes. Among these potentially deleterious reactive species are NO[•] and superoxide, which can combine to form peroxynitrite (Fig. 1a)—one of the dominant reactive nitrogen species formed under physiological conditions. Our first objective was therefore to investigate whether cells deficient in XRCC1 are susceptible to peroxynitrite and NO[•].

To facilitate this investigation, Chinese hamster ovarian (CHO) cells were exposed to SIN-1-derived peroxynitrite, and gaseous NO[•]. Specifically, we exploited EM9 CHO cells, deficient in XRCC1; AA8 CHO cells wild type (WT) for CHO XRCC1; and H9T3-6-3 and H9T3-7-1, EM9 CHO cells complemented with human XRCC1 (Fig. 1b). EM9 cells are effectively null for XRCC1 as they carry a frameshift mutation that results in expression of only a third of the XRCC1 protein (Shen, 1998). The EM9 mutant cells were initially isolated from their parental AA8 CHO cell line by a generalized mutant screen for sensitivity to DNA damaging agents and are sensitive to killing by EMS, MMS and ionizing radiation (Schwartz, 1987; Thompson *et al.*, 1982; Vanankeren, 1988). Additionally, EM9 cells display defective base excision repair (Thompson *et al.*, 1982; Thompson, 2000) and have elevated frequency of baseline (Dillehay *et al.*, 1984; Thompson *et al.*, 1982) and post-genotoxin exposure sister chromatid exchanges (SCEs). As controls, we employed H9T3-6-3 and H9T3-7-1 cells, kindly provided by Larry Thomson. These cells were made by complementing XRCC1-deficient EM9 cells via

cosmid transformation with a human gene, subsequently cloned and identified as XRCC1 (Thompson, 1990). The H9T3-6-3 and H9T3-7-1 cells are therefore human XRCC1-complemented EM9 CHO cells.

As a point of reference in these studies, XRCC1 cells were exposed to the alkylating agent methyl methanesulfonate (MMS). XRCC1 deficient EM9 CHO cells (Thompson *et al.*, 1982; Thompson, 1990; Thompson, 2000; Wong and Wilson, 2005) and indeed other XRCC1 deficient cell types (Brem, 2005; Taylor, 2002; Wong and Wilson, 2005) have previously been demonstrated to be unable to efficiently repair DNA lesions formed following MMS exposure. Exposure of cells to MMS therefore serves as a positive control for the repair deficiency in XRCC1 deficient cells, and represents a suitable reference against which to benchmark the relative toxicity and base excision repair intermediate levels in XRCC1-deficient cells on exposure to inflammatory RONS.

Following exposure to SIN-1, gaseous NO[•] and MMS, CHO cells were assessed for survival via clonogenic survival assay. As previously established (Thompson *et al.*, 1982; Thompson, 1990; Wong and Wilson, 2005), XRCC1 deficient CHO EM9 cells were significantly more susceptible to MMS exposure than XRCC1 WT AA8 cells and human XRCC1-complemented H9T3-6-3 and H9T3-7-1 cells (Fig. 1c). In contrast, XRCC1 deficient cells were only moderately more sensitive to SIN-1 ($p < 0.05$ for doses over 5mM) when compared to WT cells or human XRCC1-complemented cells to SIN-1 exposure (Fig.1c). These results are consistent with studies showing XRCC1 deficient cells are mildly sensitive to oxidizing agents (Horton, 2008). Unexpectedly, however, EM9 cells were no more sensitive than XRCC1 WT AA8 cells on exposure to gaseous NO[•] exposure (Fig. 1c), even at doses that are toxic to WT cells.

The observation that XRCC1 cells are susceptible to killing by SIN-1 and not by NO[•] is surprising and may be due to the difference in the spectrum of lesions made by SIN-1 versus NO[•]. While SIN-1 and NO[•] may make many of the same lesions via peroxynitrite, including direct DNA SSBs (Doulias, 2001; Tretyakova, 2000) and oxidized base lesions including 8-oxo-7, 8-dihydro-2'-deoxyguanosine (Inoue and Kawanishi, 1995), 8-nitro-2'-deoxyguanosine (Yermilov *et al.*, 1995; Yermilov *et al.*, 1996) and 5-guanidino-4-nitroimidazole (Gu *et al.*, 2002), NO[•] exposure can result in other chemistries. For example, NO[•] can cause base deamination via the action of nitrous anhydride (N₂O₃). Deamination of DNA by nitrous anhydride can form xanthine and hypoxanthine from guanine and adenine, respectively. Therefore, while the deamination products of NO[•] may be more readily tolerated by XRCC1 deficient cells, the excision products of the oxidative lesions and direct SSBs induced by ONOO⁻, may result in BER intermediates that are more toxic. Indeed, it has been demonstrated that the intermediates of BER, following excision of a damaged base, and not the damaged bases themselves, may mediate the toxicity associated with an exposure to a DNA damaging agent (Sobol, 2000; Sobol *et al.*, 2003; Spek *et al.*, 2002)

As an alternative approach for evaluating the biological role of XRCC1 in response to RONS, we investigated XRCC1's impact on BER capacity following RONS-induced DNA damage. XRCC1 deficient cells and their WT and complemented counterparts were assessed for BER capacity, using the alkaline single cell gel electrophoresis (comet assay). The alkaline comet assay is a well-established, gel-electrophoresis based assay that has been used to measure DNA SSBs, apurinic/apyrimidinic (AP) sites and alkali-labile base lesions in single cells (Collins, 1997; Olive, 2006; Singh, 1988). The assay can thus be used to measure the levels of DNA base excision repair intermediates, following exposure to inflammatory RONS.

XRCC1 deficient cells trend towards a greater accumulation of base excision repair intermediates compared to WT cells as the dose of MMS was increased (Fig. 2a), which is consistent with previous studies (Brem, 2005; Horton, 2008; Kubota, 2003; Taylor, 2002; Wong and Wilson, 2005). XRCC1 deficient EM9 cells were also deficient in the repair of MMS induced BER-intermediates (Fig. 2a insert). XRCC1 deficient cells displayed equivalent levels of BER intermediates as WT cells on exposure to increasing doses of SIN-1 (Fig. 2a) and the XRCC1 null EM9 cells repair SIN-1-induced BER-intermediates with the same efficiency as the AA8 WT cells (Fig. 2a insert). This indicates that, at least in CHO cells, XRCC1 does not play a significant role in the repair of SIN-1-induced DNA damage. This finding suggests that the XRCC1-dependent protection against SIN-1 toxicity may be independent of the role of XRCC1 in BER.

To explore the possibility that XRCC1 modulates repair of NO[•]-induced DNA damage, we used the comet assay to assess the BER intermediate levels in EM9 and AA8 CHO cells on exposure to NO[•]. In contrast to SIN-1 exposure, on increasing duration of exposure to NO[•] the WT AA8 cells showed higher levels of NO[•]-induced BER intermediates than the XRCC1 deficient EM9 cells. The levels of NO[•]-induced BER-intermediate levels in the WT AA8 cells then fell to below the levels demonstrated by the XRCC1 null EM9 cells after 8 hours of exposure to NO[•] (Fig. 2b).

The observation that the XRCC1 WT cells have higher levels of BER intermediates than the XRCC1 null EM9 cells may at first glance seem anomalous. However, since the comet assay detects AP sites *and* frank DNA SSBs, the comet assay effectively detects DNA species in between the glycosylase step and the ligation step of the BER pathway. Therefore, the increase in BER intermediates in WT cells relative to the XRCC1 deficient cells on

exposure to NO[•] may be due to an XRCC1-facilitated removal or scission of NO[•]-induced base damage. Specifically, XRCC1 may facilitate a glycosylase that acts specifically on NO[•]-induced DNA damage and not SIN-1-induced damage, *i.e.* deamination products like xanthine and hypoxanthine. Indeed, other groups have shown that AAG can excise deamination products like Hx (Asaeda *et al.*, 2000; Miao *et al.*, 1998; Saparbaev, 1994), and that XRCC1 can facilitate this activity (Campalans *et al.*, 2005). We therefore set out to more directly explore the impact of XRCC1 on AAG-mediated excision of NO[•]-mediated DNA damage.

2.4.2 Role of XRCC1/ AAG interaction in human (glioblastoma LN428) cell extracts

Two key mutagenic lesions created by NO[•] and excised by AAG are hypoxanthine (Hx) and ethenoadenine (εA). Hx is the deamination product of adenine that is formed by NO[•] via the nitrosative action of nitrous anhydride (N₂O₃)—an auto-oxidation product of the reaction between NO[•] and molecular oxygen (Dedon, 2004). Hx mispairs with cytosine, causing A:T to G:C transition mutations. In contrast, εA can mispair with adenine, guanine or cytosine (Speina *et al.*, 2003) and is a DNA damage product of RON-derived lipid peroxidation (Chung *et al.*, 1996; El Ghissassi *et al.*, 1995; Marnett, 2000). Furthermore, εA inhibits DNA replication (Tolentino *et al.*, 2008) and is thus a toxic base lesion that may potentiate malignancy (Bartsch and Nair, 2004). We therefore investigated whether, and to what extent, XRCC1 facilitates AAG excision of the inflammation-associated Hx and εA DNA lesions. To do this, we used LN428 glioblastoma cells that were WT (LN428WT) or that were

engineered to be deficient in XRCC1 via lentiviral shRNA knock down (XRCC1KD), or engineered to over-express AAG (AAGOE), or be both deficient in XRCC1 and be over-expressing AAG (AAGOE/XRCC1KD), and cells engineered to express GFP (GFP) as a control for the exogenous constructs in the other cells (Fig. 3a).

Using a fluorescence-based molecular beacon excision assay that has previously been used to measure the efficiency with which glycosylases can excise specific base lesions in real time, *in vitro* (Maksimenko *et al.*, 2004), we assessed the ability of LN428WT cells, XRCC1KD, AAGOE, and AAGOE/XRCC1KD cell extracts, to excise ϵ A and Hx. We found that a control molecular beacon containing no lesion, incubated with extracts from LN428WT, XRCC1KD, AAGOE, and AAGOE/XRCC1KD cells, resulted in minimal increase in fluorescence (Fig. 3a). In contrast, a molecular beacon containing Hx resulted in a time-dependent increase in fluorescence, when incubated with extracts from LN428WT, XRCC1KD, AAGOE, and AAGOE/XRCC1KD cells, which is indicative of base release by the AAG glycosylase (Fig.3c). As expected, cells over-expressing AAG and WT for XRCC1 (AAGOE) yielded the highest increase in fluorescence, indicating that these cells are the most efficient at excising a Hx lesion. However, when XRCC1 is knocked down in the AAGOE cells, as is the case with the AAGOE/XRCC1KD cells, there is a *decrease* in the observed fluorescence (Fig. 3b). This difference indicates that in cell extracts XRCC1 facilitates AAG excision of Hx, which is consistent with previous work (Campalans *et al.*, 2005). Furthermore, consistent with XRCC1 being a scaffold protein that does not directly excise damaged bases, the XRCC1KD cells resulted in only a modest increase in fluorescence, similar to the LN428WT cells. This nominal increase in fluorescence is probably due to the low amounts of AAG expressed in

the LN428WT parental cell line. The levels of AAG in the LN428WT parental cell line are so low as to preclude detection by western blots (Fig. 3a).

We then queried whether ϵ A excision by AAG, like Hx excision, could be facilitated by XRCC1. We incubated cell extracts from the glioblastoma cells with a molecular beacon, this time containing ϵ A. Similar to the Hx molecular beacon, a time-dependent increase in fluorescence was observed on incubation with LN428WT, XRCC1KD, AAGOE, and AAGOE/XRCC1KD cells. Of the cell lines examined, the AAGOE cells yielded the greatest increase in fluorescence and the AAGOE/XRCC1KD cells yielded slightly lower fluorescence (Fig. 3b). The difference in the observed fluorescence between the AAGOE cells and the AAGOE/XRCC1KD cells indicated that, as is the case for Hx, XRCC1 does indeed facilitate AAG excision of ϵ A. Collectively, these data indicate that XRCC1 is capable of stimulating the AAG-mediated excision of the inflammation-associated base lesions, Hx and ϵ A, *in vitro*.

2.4.3 Role of XRCC1/ AAG in response to NO[•] exposure in live human (glioblastoma LN428) cells

While our findings suggested that XRCC1 can facilitate AAG-mediated excision of inflammation-associated base lesions *in vitro*, we queried whether the same dynamics existed in live cells. As a control to the NO[•] exposure protocol that we employed, glioblastoma cells were incubated with a single concentration of 50 μ M of MMS for increasing durations of time. The base excision repair intermediates generated were then measured by alkaline comet assay. We found that there was a time-dependent increase in base excision repair intermediates for all the cell types examined following exposure to

MMS (Fig. 3c). Importantly, we found that the LN428WT, XRCC1KD, GFP, and the AAGOE cells, accumulated similar levels of BER intermediates. This indicates that the increase in base-excision initiation that is the result of over expression of AAG is tolerated in these cells. Additionally, this result indicates that the deficiency in XRCC1 is similarly tolerated. However, when XRCC1 is decreased in cells that are over-expressing AAG, there is a significant accumulation of BER intermediates, as is observed in the case of the AAGOE/XRCC1KD cells (Fig. 3c). The accumulation of BER intermediates in cells that are over-expressing AAG and are deficient in XRCC1 on exposure to an alkylating agent, is consistent with a model in which XRCC1 is primarily facilitating the activity of a BER step downstream of the glycosylase. Thus while AAG is initiating BER and excising damaged bases from DNA to form BER intermediates that can then be detected by the comet assay, the lack of XRCC1 prevents the efficient resolution of these BER intermediates. We speculate that the lack of XRCC1 results in the decreased efficiency of the dRP lyase activity of polymerase β (Pol β), previously shown to be the rate-limiting step in the monofunctional glycosylase initiated BER (Nakamura and Swenberg, 1999; Sobol *et al.*, 1996; Sobol *et al.*, 2003; Srivastava *et al.*, 1998). Indeed, it has been demonstrated that the XRCC1-pol β interaction is important for the repair of both alkylation (Wong and Wilson, 2005) and oxidative (Dianova *et al.*, 2004) DNA damage. Thus, the accumulation of BER intermediates in AAGOE/XRCC1KD cells may primarily be due to an accumulation of BER intermediates occurring in between the glycosylase step and the dRP lyase gap tailoring step of polymerase β . This result demonstrates the importance of XRCC1 in tolerating an imbalance in BER, caused by excess glycosylase activity. Therefore, when considering the potential impact of an observed increase in glycosylase activity, the efficiency of downstream components, including steps facilitated by XRCC1, must be similarly considered.

To explore the extent to which XRCC1 can facilitate AAG-initiated base excision of NO[•]-mediated DNA damage in live cells we exposed glioblastoma cells to two steady state concentrations of 1.74μM and 11.23μM of NO[•], achieved by exposure to 10% and 100% NO[•] respectively. We assessed cells exposed to both concentrations of NO[•] for BER intermediates using the alkaline comet assay. Interestingly, the response of the human glioblastoma cells to NO[•] exposure was different to the CHO cell response. This result is consistent with previous findings that different cell types have different thresholds for, and thus respond differently to, NO[•] exposures (Burney *et al.*, 1997; Li, 2006; Wang *et al.*, 2003). Similar to the MMS exposures, BER intermediates in the XRCC1KD cells, AAGOE cells, and GFP control cells exposed to NO[•] were similar to the levels in the LN428WT cells. The AAGOE/XRCC1KD cells, while not statistically different to the WT cells, trend towards modestly elevated BER intermediates compared to the WT cells. This suggests that, following exposure to NO[•], XRCC1 facilitates a step downstream of the glycosylase for AAG-initiated BER, albeit to a lesser extent than following exposure to MMS. The slight increase in BER-intermediates observed in the AAGOE/XRCC1KD vs. the LN428WT cells, may be due to the fact that that NO[•] induced DNA damage that is processed by the bifunctional glycosylase BER sub-pathway (*e.g.* 8-oxo-G), in addition to lesions processed by the monofunctional glycosylase sub-pathway (*e.g.* Hx, εA). Therefore, the relative difference in BER intermediates between AAGOE/XRCC1KD and the LN428WT cells when comparing exposure to MMS vs. NO[•], may indicate that XRCC1 preferentially facilitates a step downstream of monofunctional glycosylases and less so steps downstream of the bifunctional glycosylases. This is consistent with previous observations that XRCC1 null cells are relatively more susceptible to alkylating agents than they are to oxidative DNA damage (Horton, 2008), when compared to WT cells. Importantly, these results suggest that for

MMS exposures, and to a lesser extent NO² exposures, deficiencies in XRCC1 act to reveal imbalances in BER.

In order to determine the susceptibility of the human glioblastoma cell lines to killing by NO², we initially exposed cells to 50 μ M MMS for increasing durations of time and assessed the cells for survival by clonogenic survival assay. We found that the LN428WT, XRCC1KD, GFP, and the AAGOE cells were killed as the duration of exposure to MMS was increased (Fig. 3d). Importantly and as expected, the XRCC1KD cells were more sensitive to killing by MMS than LN428WT cells. This is consistent with previous studies that have found that XRCC1 deficient cells are acutely sensitive to alkylating agents (Horton, 2008) and indeed consistent with the toxicity observed in the CHO XRCC1 knock out cells in this work. Surprisingly however, the AAGOE/XRCC1KD cells that displayed highly elevated BER intermediates on exposure to MMS, were not particularly susceptible to killing by MMS (Fig. 3d). This suggests that the toxicity observed in the XRCC1KD human glioblastoma cells may be independent of BER intermediates, and independent of XRCC1's role in BER. Further, this suggests that the elevated BER intermediates observed in AAGOE/XRCC1KD cells are tolerated and do not result in toxicity.

On exposure to NO², we found that all the glioblastoma cell genotypes examined were increasingly sensitive to killing as the duration of exposure to both the 10% and 100% NO² increased. Furthermore, consistent with the levels of BER-intermediates measured in these cells, none of the glioblastoma cells assessed were relatively more sensitive to killing by NO², when compared to the LN428WT cells (Fig. 3d). This indicates that XRCC1 confers no protection against killing by NO², in contrast to the protection conferred against killing by MMS.

2.5 Conclusions:

Understanding the role that reactive nitrogen and oxygen play in initiating and promoting malignancy is important for risk assessment and therapeutic intervention in inflammation-associated malignancies. Here, we examined the role of a putative risk factor in inflammation-associated malignancy: the role of XRCC1 in the repair of DNA damage by the reactive nitrogen species ONOO^- and NO^\cdot . We found that in CHO cells, XRCC1 is protective against ONOO^- mediated toxicity, but this protection was independent of XRCC1's role in base excision repair. These results suggest that XRCC1 may be modulating toxicity in a BER-independent fashion. Indeed, there is evidence that XRCC1 may play a role in Ku-independent, non-homologous end joining (Ahmed *et al.*, 2010; Audebert *et al.*, 2004; Charbonnel *et al.*, 2010) a mechanism for the repair of DNA double strand breaks which can result in toxicity and other deleterious phenotypes (Helleday, 2007). Additionally there are several lines of evidence that suggest XRCC1 may play a role in homologous recombination, another pathway by which DNA DSBs can be repaired. For example, Taylor *et al.* showed that XRCC1 partially co-localizes with an important homologous recombination protein, Rad51, implicating XRCC1 in homologous recombination (Taylor, 2000). Further, the high levels of XRCC1 observed in testes relative to levels in other cell types (Yoo *et al.*, 1992) and the occurrence of these higher levels specifically in pachytene spermatocytes (Zhou and Walter, 1998b), implicate XRCC1 in meiotic recombination. These examples, and indeed our findings here, are consistent with role(s) for XRCC1, in addition to its role in base excision repair.

While XRCC1 may stimulate AAG excision of the inflammation mediated base lesions Hx and ϵ A *in vitro*, in live human cells XRCC1 primarily acts downstream of the glycosylase for alkylation damage and NO⁺DNA damage, albeit to a lesser extent in the latter. Indeed, consistent with the levels of BER-intermediates in the glioblastoma cell lines, none of the glioblastoma genotypes examined were more sensitive to killing by NO⁺, despite the significant sensitivity displayed by the AAGOE/XRCC1KD cells to MMS exposure. Importantly, we have demonstrated that a deficiency in XRCC1 reveals an otherwise undetectable imbalance in base excision repair, on exposure to MMS.

Collectively, the result of these studies suggest that the association between XRCC1 polymorphic variants and malignancy may be independent of the role of XRCC1 in BER and or may be due to BER imbalances that are revealed by the differential capacity of XRCC1 to facilitate BER steps downstream of the glycosylase initiating step.

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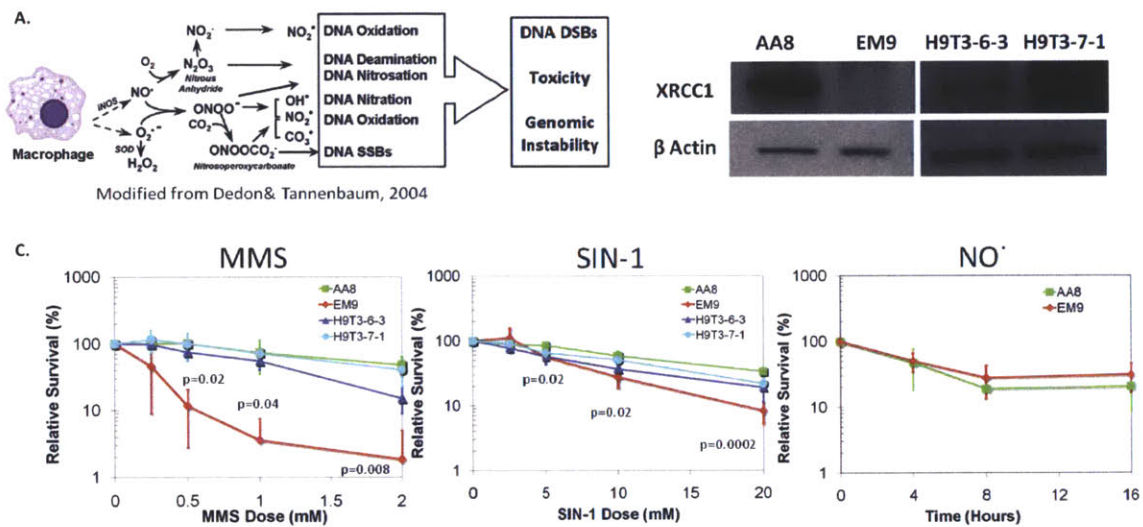


Figure 2-1. Role of XRCC1 in modulating toxicity of peroxynitrite and NO[·] in CHO cells. A. During inflammatory states macrophages become activated and release reactive nitrogen and oxygen species that can cause DNA lesions that result in DNA DSBs, toxicity and genomic instability. **B.** XRCC1 WT (AA8) and null (EM9) CHO cells, as well as human XRCC1 complemented EM9 (H9T3-6-3 and H9T3-7-1) cells were used to assess the **(C)** toxicity of MMS, the peroxynitrite donor SIN-1 and gaseous NO[·]. P values were produced by a two-tailed *t*-test .

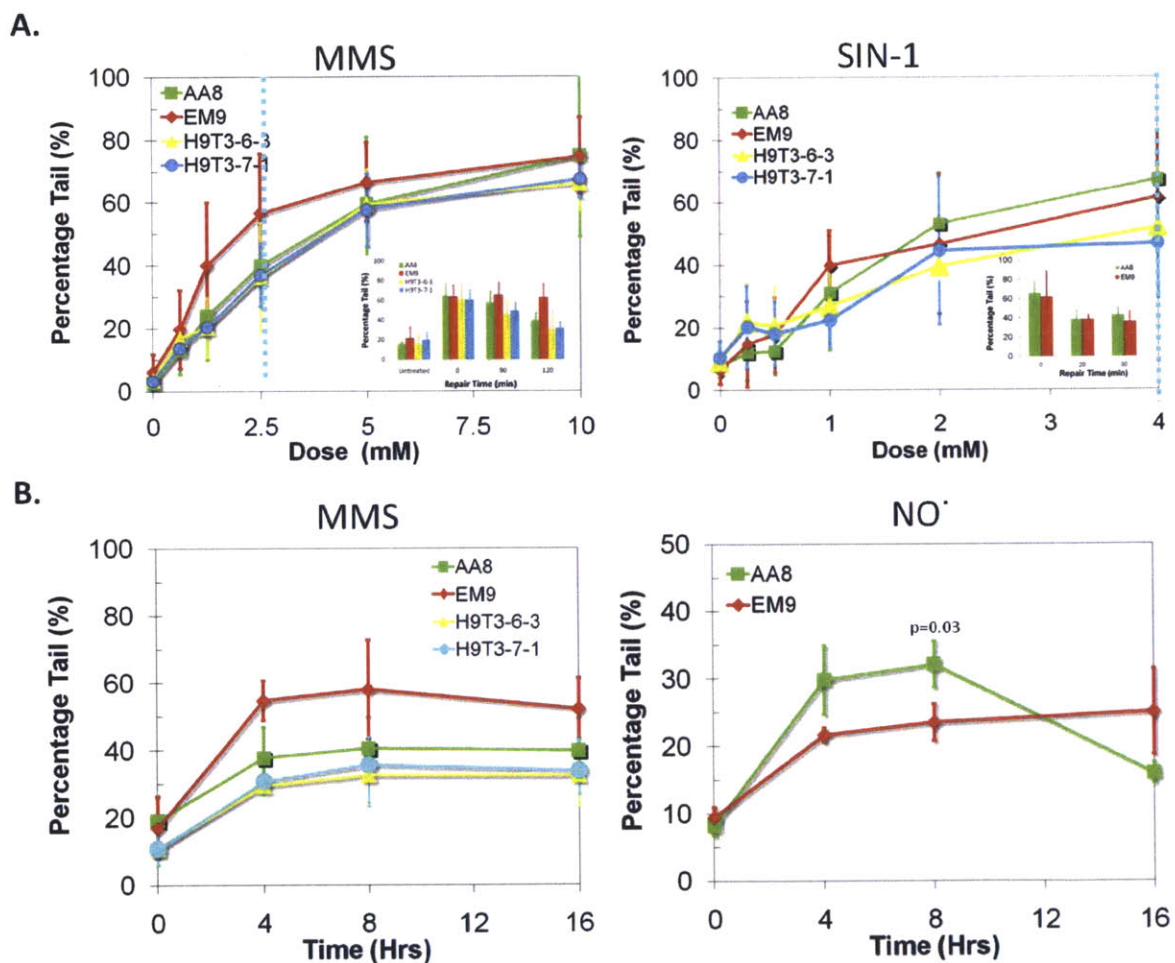


Figure 2-2. Measurement of base excision repair intermediates. Base excision repair intermediates measured in CHO cells exposed to **A)** SIN-1 and **B)** gaseous NO \cdot . Inserts in **A)** are repair kinetics generated by treating the cells at the dose specified (---) dose of the respective agent. MMS exposures serve as experimental controls for comparison with the agent of interest. P values were produced by a two-tailed Mann-Whitney test.

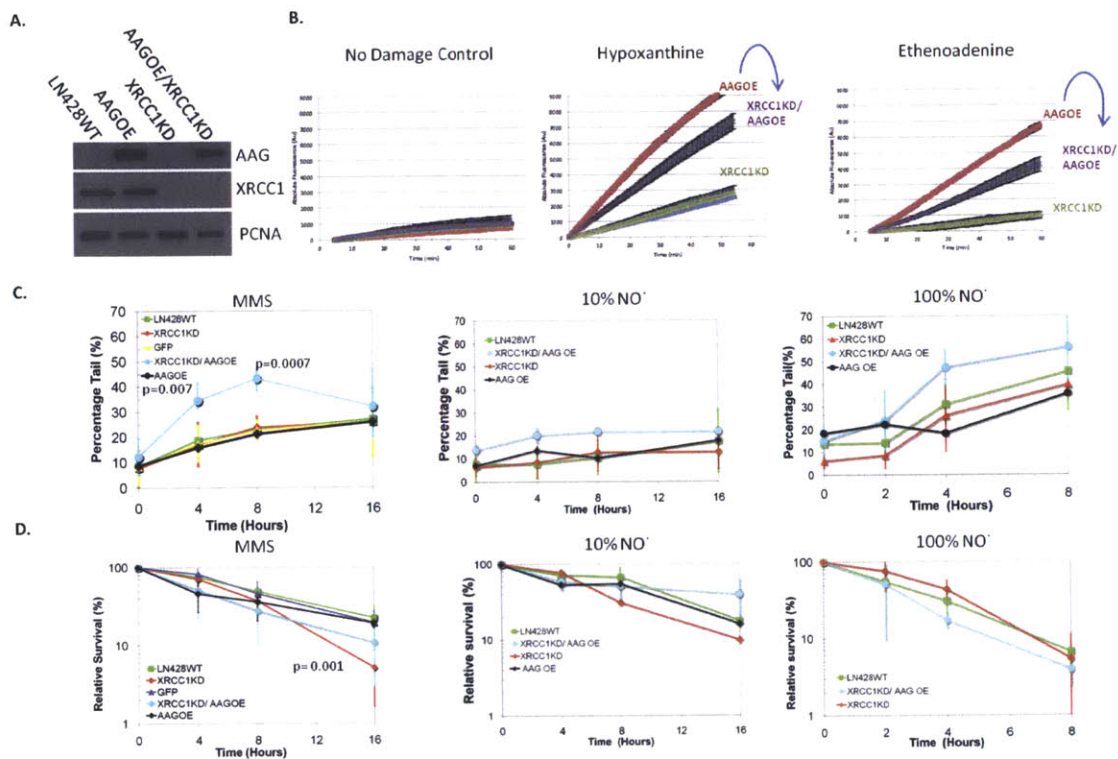


Figure 2-3. XRCC1 facilitation of BER on exposure to NO[•]. **A.** Western blot showing AAG and XRCC1 protein in wild type (LN428 WT), and AAG over-expressing cells (AAGOE) glioblastoma cells, XRCC1 knock down (XRCC1KD), and AAG over-expressing cells with XRCC1 knocked down (AAGOE/XRCC1KD). (Note: LN428 wild-type cells have undetectably low levels of AAG expression). **B.** Fluorescent molecular beacon assay indicating XRCC1 facilitated excision of hypoxanthine and ethenoadenine. Arrow indicates fluorescence shift as a result of decreasing the extent of XRCC1 facilitation by knocking down XRCC1. Cellular responses in glioblastoma cells on exposure to MMS and NO[•] as measured by **C)** base excision repair intermediates (alkali comet assay) and **D)** toxicity (clonogenic survival assay). P values were produced by a two-tailed Mann-Whitney test for the comet assay data and a two-tailed t-test for the clonogenic survival assay.

Chapter III

Development of Tools for Measuring Base Excision Repair

3.1 Abstract

DNA damage by various endogenous and exogenous factors is associated with a number of deleterious phenotypes including cancer, ageing and neurological disorders. The ability to inexpensively, accurately and rapidly measure DNA damage, is therefore of importance for a range of applications including genotoxin exposure monitoring, epidemiological disease risk assessment, drug development and drug screening. This chapter describes our group's initial efforts in developing a tool for the measurement of different kinds of DNA damage, with an emphasis on the measurement of DNA damage repaired by base excision repair. We enumerate the system constraints, design considerations, resultant technical challenges and a few preliminary results using one of the approaches that were employed.

The primary approach utilized now well-established microfabrication techniques to micropattern cells for assessment of DNA damage by alkali single cell gel electrophoresis (comet assay). Micropatterning of cells allows for higher information density and spatial encoding of samples for parallel, high throughput assessment of DNA damage. The comet assay, in contrast, allows for the robust measurement of DNA damage, including damage that is repaired by the base excision repair pathway. Together, these techniques represent the basis of a lab-scale tool that enables the measurement of base excision repair in different cell types. We also present herein, an application of this device, albeit technically fraught, to the measurement of DNA damage in a macrophage coculture system.

3.2 Introduction & Motivation

DNA is constantly damaged by a host of endogenous and exogenous agents (Friedberg, 2006). Nature has therefore evolved many mechanisms by which to prevent, recognize and repair DNA damage. Indeed, failure to adequately repair DNA damage can lead to a variety of deleterious phenotypes, including cancer, aging and neurological disorders (Hoeijmakers, 2009). Furthermore, methods for damaging DNA have found utility, either incidentally or by design, in arenas as varied as warfare, food processing and in healthcare. For instance, the use of mustard gas primarily in World War I (Jowsey *et al.*, 2009; Vogt *et al.*, 1984; Wattana, 2009) and the mounting threat of radiological explosives and dispersion devices (Karam, 2005); radiation sterilization of food (Blumenthal, 1990) and medical implements (Darmady *et al.*, 1961) using ionizing gamma and X-ray irradiation; and the use of radiation and DNA-interacting chemicals, to induce cell death in cancer therapy (Hurley, 2002; Roos and Kaina, 2006). Thus, due to the ubiquity of DNA damaging agents, both those endogenously-derived and as a consequence of our 21st century environment, an understanding of the biological significance of exposure to these agents is warranted. Such an understanding would have applications in disease prevention, disease risk assessment, and may aid in the discovery of improved cancer therapeutics.

An important first step in understanding the biological significance of DNA damage and repair is the ability to detect and quantify DNA damage. Consequently, many techniques have been developed to measure DNA damage and repair. These techniques are as varied as they are numerous, utilizing different modalities and measuring different aspects of, and the response to, DNA damage. As exemplars of such techniques, are methods that detect and measure DNA damage-associated chemistries (*e.g.* aldehyde

reactive probe staining); different chromosomal compositions (*e.g.* sister chromatid exchanges (SCEs) (Perry, 1974) and chromosomal aberrations) and distributions (*e.g.* micronucleus assay (Garriott *et al.*, 1988)); there are methods that detect different protein modifications in response to DNA damage (*e.g.* γ H2AX phosphorylation (Rogakou *et al.*, 1998)); methods that quantify differential electrical and or mechanical properties of DNA after damage (*e.g.* electrical conductivity of DNA with correct base pairing versus mismatches (Guo *et al.*, 2008)); there are methods that measure the rates of incorporation of nucleotides in DNA as it is repaired (*e.g.* unscheduled DNA synthesis (UDS) (Friedberg, 2006)) or indeed even methods that precisely quantify the damaged bases themselves (*e.g.* mass spectrometry (Dawidzik *et al.*, 2003; Taghizadeh *et al.*, 2008; Tretyakova *et al.*, 2001; Zhou *et al.*, 2005)). A detailed review of each of these techniques is beyond the scope of this chapter; however the reader is referred to the literature noted above.

While the techniques described above are useful to varying degrees, each has its limitations. Indeed, some of the barriers that prevent routine assessment of DNA damage are that these existing assays are often laborious, expensive, and technically challenging. We therefore set out to develop a tool (or set of tools) that could measure DNA damage and repair in the context of conditions pertinent to this thesis, *i.e.* inflammation-mediated DNA damage, but that would also have broader applications in measuring DNA damage and repair for epidemiological studies and genotoxicity drug screening. Thus, the criteria used to constrain our approach are as follows:

- 1) *Lesion(s) Specificity: Allow for measurement of base excision repair substrates*
- 2) *Quantitative rigor:*

- a) *Have a low (baseline) damage detection threshold, good sensitivity (approximates linear damage response), and high dynamic range (spanning subtoxic-toxic DNA damage levels)*
- b) *Measure physiologically relevant DNA damage*
- 3) *Throughput: Lab-accessible tool for medium (10-30) to high (>100) sample counts.*
- 4) *Sample Diversity: Allow for different cell types, different parallel conditions*
- 5) *Temporal resolution: Allow for measurements shortly after exposure and at small (5-10min) time intervals*
- 6) *Expense: Allow for relatively low cost per run*

Adapting an existing, validated technology, rather than defining a novel technique was deemed a more expedient approach. Consequently, a systematic assessment of existing techniques for measuring DNA damage, constrained by the above-enumerated criteria, was used to select a base technology upon which to build.

Two techniques were qualitatively deemed appropriate following a non-exhaustive search; the alkaline single cell gel electrophoresis (comet) assay and the polymerase I-mediated biotin-dATP nick-translation (PANT) assay. A discussion of our work in developing a comet assay on a chip (CoaCh) device follows immediately. Then, an application of the most recent CoaCh device in measuring activated macrophage-mediated DNA damage will be reviewed. Our efforts with the polymerase I-mediated biotin-dATP nick-translation (PANT) assay shall not be fully discussed in this thesis. Our preliminary attempts with this technique were not promising and the data quality poor, yielding no dose-dependent

increases in signal. However, this technique is mentioned here for completion and to give the reader an understanding of the scope of this work.

3.3 Comet on a Chip (CoaCh)

3.3.1 Introduction & Background

The Alkaline Comet Assay

The single cell gel electrophoresis (Comet) assay is an electrophoresis-based separation technique used to assess DNA damage and repair, and is based on the relaxation of nucleoid DNA coils as a consequence of sustained damage. Movement of negatively charged DNA in an electric field, away from the central nucleoid body (Suppl. Fig. 3-1.) and toward the positively charged anode through a gel matrix, is proportional to the number of DNA lesions in the nucleoid and is apparent as a 'tail' on visualizing DNA with a fluorescent stain. Initially developed by Ostling and Johanson (Ostling, 1984), and then notably modified by Singh et al. (Singh, 1988) for greater sensitivity; and again by Olive et al. (Olive *et al.*, 1990) for analysis methodology, the comet assay has since been shown to be highly adaptable and useful in the measurement of different kinds of DNA damage, in different cell types, and in cells in different stages of cell cycle.

The traditional alkali comet assay is conducted by immobilizing a single cell suspension of cells of interest in an agarose hydrogel. The agarose is permitted to cool and solidify, trapping cells in place. The cells are then lysed through the gel, by full-gel-incubation in a buffer containing high salt and, depending on the protocol, a non-ionic surfactant *e.g.* triton X-100. The lysed gel-immobilized cells, now termed nucleoids, are then held in alkali buffer for a length of time, allowing the DNA to unwind and for alkali labile sites to be converted to SSBs. The resultant nucleoids are then subjected to an electric field

that causes migration of negatively charged DNA loops to the anode. This results in DNA moving from the center of the nucleoid body, termed the head region, to the tail region, yielding a comet-like morphology in the case of damaged cells (Suppl. Fig. 3-1). Nucleoids, still immobilized in gel, are then brought back to neutral pH (~7.5) and are stained with a nucleic acid stain for analysis *e.g.* ethidium bromide. Nucleoids can be analyzed by eye or computationally to derive metrics of damage from the comet morphology. The most commonly used metrics include tail length, percentage DNA in tail and tail moment. Tail length is sensitive, and thus useful, at low levels of DNA damage but is not particularly useful at higher levels of damage. The comet tail increases in intensity, and not in length, as the DNA damage levels increase (Collins, 2002). The percentage tail is a measure of relative tail intensity compared to the head and thus gives an intuitively descriptive metric of the damage experienced by a nucleus. Percentage tail is linearly related to DNA break frequency and is relatively unaffected by threshold settings as it is a relative metric. The tail moment is the product of the amount of DNA in the tail, expressed as a percentage, and the mean distance of migration in the tail (Olive *et al.*, 1990). The tail moment metric, thus attempts to capture the high dynamic range afforded by the percentage tail and the sensitivity of the tail length. Typically about 100 nucleoids (comets) are analyzed per condition and a mean or median calculated for assessment of DNA damage to that sample.

The alkaline comet assay has been used to assess a wide variety of different types of DNA damage including DNA single strand breaks, double strand breaks, and alkali-labile DNA lesions including apurinic/ apyrimidinic abasic DNA lesions (Rojas *et al.*, 1999). The comet assay can also be coupled with lesion-specific endonucleases and lytic enzymes from various organisms to increase its sensitivity and its range of application to the detection of specific

lesions, otherwise undetected by the comet assay. For example, bacterial endonucleases like endonuclease III (Nth), Formamidopyrimidine DNA glycosylase (FPG) and AlkA, can be used to reveal and detect oxidized pyrimidines, oxidized guanines, and alkylated bases, respectively (Collins, 2011). Bacterial uracil DNA glycosylase (UNG) has also been used to detect uracil in DNA (Duthie and McMillan, 1997). Additionally, a T4 virus endonuclease V has been used to detect cyclobutane pyrimidine dimers (Collins *et al.*, 1997). Furthermore, by challenging cells with appropriate DNA damaging agents, DNA repair capacity can be evaluated for many different repair pathways (nucleotide excision repair; non-homologous end-joining, base excision repair, etc.). Finally, the comet assay can be applied to virtually any tissue (as long as individual cells can be obtained), making it possible to compare the exposure-induced or genetically-induced DNA damage responses of different cell types. Importantly, only very few cells (< 1000) are required for the assay per condition, allowing for sample isolation from even the most cellularly reticent sources and biological compartments, including urine, blood, mucus etc. The comet assay thus represents an amenable and flexible modality for the measurement of base excision repair and inflammation-mediated DNA damage.

Adherent Cell Micropatterning

In order to address the criteria for higher throughput and diversification of samples, we chose an approach that calls for arraying of cells. In addition to maximizing real estate by minimizing the distance between objects of interest, arraying or micropatterning cells allows for spatial encoding: the addressing of specific conditions or samples to specific loci in space. Spatial encoding, therefore allows for the querying of diverse samples in known, specific locations. Indeed, spatial encoding has become an abstraction with tremendous utility in biotechnology, with microarrays that allow for the spatially defined localization and

querying of RNA and siRNA (Mousses S *et al.*, 2003; Wheeler *et al.*, 2004), DNA and cDNA (Butte, 2002), carbohydrates (Wang *et al.*, 2002), proteins (Glökler and Angenendt, 2003; Zhu and Snyder, 2003); and even cells (Xu, 2002) and tissues (Kononen *et al.*, 1998).

While various techniques for micro-patterning cells exist, we considered two broad categories of patterning that were putatively consistent with the comet assay: namely suspended cell patterning and adherent cell patterning (see supplemental table 1). Due, in part, to constraint criterion 2) *b*), and due to culture characteristics of the cells of interest, *i.e.* the requirement to measure physiologically relevant DNA damage and repair in anchorage-dependent cells, we initially pursued adherent cell patterning (See Suppl. Table 3-1 for other approaches considered). Indeed, there is evidence that normal cellular processes, including DNA damage-mediated toxicity, require mechanochemical cues derived from cell adhesion (Chen *et al.*, 1997; Lewis, 2001b; Truong, 2003). Therefore, to measure physiologically relevant DNA damage and repair in adherent cells, an approach utilizing adherent cells was deemed appropriate. However, for information on subsequent exploration of suspended cell patterning the reader is referred to work published by other members of our group (Wood *et al.*, 2010).

Various methods exist for micropatterning cells adherently on a substrate. These methods include methods wherein a cell adhesion substrate is patterned *e.g.* micro-contact printing (Chen, 1998), microstencil printing (Folch, 2000) and photolithographic substrate micropatterning (Bhatia *et al.*, 1997). There are also methods wherein cells themselves are directly patterned *e.g.* direct cell printing (Schiele, 2010), gravitational settling of pre-arrayed cells (Rosenthal *et al.*, 2007) etc. A cursory assessment of these techniques was conducted, with preliminary experimentation and an appreciation of the design constraints

previously specified. Ultimately, we proceeded with photolithographic substrate micropatterning as this method was relatively facile and scalable, while yielding many of the design features required.

3.3.2 Materials and methods

Cells and cell Culture

TK6 Lymphoblastoid cells were a kind gift from the Wogan Laboratory and were cultured as follows. Cells were grown in 150mm tissue culture dishes (Falcon) in 30mL of RPMI (Cambrex, East Rutherford, NJ) media supplemented with 10% heat inactivated horse serum (Cambrex, East Rutherford, NJ), L-glutamine (Invitrogen, Carlsbad, CA) and Pen/ Strep (100U/mL; 100µg/mL) (Sigma, St. Louis, MO) and maintained in log phase at a concentration of $\sim 0.8-1.2 \times 10^6$ cells/mL by passaging every 2-3 days.

HeLa Cells were a kind gift from the Bhatia laboratory and were cultured as follows. Cells were grown in 150mm tissue culture dishes (Falcon) in 30mL of High glucose DMEM (Invitrogen, Carlsbad, CA) media supplemented with 10% fetal bovine serum (Atlanta Biologics, Lawrenceville, GA), L-glutamine (Invitrogen, Carlsbad, CA) and pen/ strep (Sigma, St. Louis, MO) and maintained in log phase at a concentration of $\sim 0.8-1.2 \times 10^6$ cells/mL by passaging every 2-3 days.

Rat hepatocytes were isolated from 2-3 month old female Lewis rats (Charles River, MA) weighing 180-220 g, by a modified procedure described by Seglen (Seglen, 1976). Briefly, the animals were anesthetized in a chamber containing saturated ether. The liver was first perfused through the portal vein with 400 ml of perfusion buffer followed by a 1mM ethylenediamine tetraacetic acid (EDTA) at 30 mL/min. The perfusate was then equilibrated with 1 L/min 95 % O₂ and 5 % CO₂ through silicone tubing that was maintained at 37°C using a heat exchanger. Subsequently, 200mL of 0.05% collagenase in perfusion buffer was perfused with 5 mM of CaCl₂ at a flow rate of 20mL/min for 10 minutes. At this

stage the swollen liver was torn from the ligaments and was transferred to culture dish. The liver capsule was torn apart, and the cell suspension passed through two nylon filter-mesh grids, sized 250 μ m and 62 μ m respectively. The cell suspension was centrifuged for 3 minutes at 500 rpm at 4°C. Non-parenchymal cells (*i.e.* stellate, kupffer, endothelial) cells are more buoyant and float in the supernatant which is aspirated to leave a pellet of primary rat hepatocytes. The pellet is then re-suspended in Krebb's Buffer. A cell count of 150 million - 250 million cells was obtained per isolation and a repeated viability of 88% - 93% was calculated using trypan blue exclusion. Non-parenchymal cells based on the morphology and size (~10 μ m) constituted < 1% of the cells after filtering. Hepatocyte culture medium consisted of Dulbecco's Modified Eagle Medium with high glucose, 0.5 U/mL insulin 1% (v/v) penicillin/streptomycin (100U/ml; 100ug/ml), 7 ng/mL glucagon, 7.5g/mL hydrocortisone, and 10% (v/v) fetal bovine serum.

Chip Microfabrication

Prior to chip fabrication, chrome or transparency masks (Fineline Imaging, Colorado Springs, CO) with the desired 'island' size and pitch (~50 μ m) were generated (opaque spots on clear background). Chips were fabricated using either silicon wafers or borosilicate glass. 50mm (1 inch) diameter wafers, or in the case of glass, 34mm #2 borosilicate coverglass slides (Fisher Scientific Inc.). The substrates were incubated for 45min in 3mL (silicon wafer) or 1.5mL (glass) of 1:1 diluted, 1.2mg/mL collagen: de-ionized distilled water, at 37°C. Excess, non- adsorbed collagen was rinsed off with de-ionized distilled water. The chips were then spin coated with S1813 positive photoresist (Shiplot Corporation). The chips were then baked to evaporate excess solvents and exposed to UV light for 90 sec in a bottom side mask aligner (Karl Suss, Waterbury Center, VT) through the chrome or transparency photo-masks printed at 8000 d.p.i. Exposed photoresist was then developed and solubilized

(Microposit 321 Developer, Shipley), rinsed in de-ionized water and baked for 90 seconds to complete curing. The UV exposure time and the bake time were optimized to allow easy 'lift off' of the photoresist during sonication with acetone. To ensure complete removal of UV-exposed photoresist down to bare glass, slides were exposed to oxygen plasma (oxygen pressure 250 mTorr, base vacuum 80mTorr, 200 Watts for 10 minutes). Chips were rinsed with water and placed in either a 60 mm petri dishes (wafer), or in 35mm (glass) petri dishes or into each well of a 6-well plate. Chips were incubated in 'sea' material *e.g.* BSA or PEG disilane overnight and then placed in an acetone bath and sonicated (Fisher, Pittsburg, PA) at 10 seconds intervals for 2-3 minutes to remove residual photoresist and expose the underlying collagen 'island' in a 'sea' of PEG Disilane. Chips were rinsed several times with water, dried under a stream of air, and stored dry at 40°C for up to 2 weeks prior to use.

Alkaline comet assay

A modified version of the alkali comet assay (single cell gel electrophoresis) was employed (Olive, 2006). Briefly, for the 'traditional' comet assay cells were immobilized in 1% agarose. For the adherent comet assay, after patterning or adhering cells, the cells were overlaid with 1% agarose gel at 37°C. Cells, once immobilized in gel, were lysed for at least one hour in lysis buffer containing 2.5M NaCl, 100mM Na₂EDTA, and 10mM tris and 1% triton X-100 at pH 10. Following lysis, cells in gel are held in electrophoresis buffer (0.3M NaOH, 1mM Na₂EDTA) for 40 minutes for alkali unwinding. Following the alkali unwinding incubation, electrophoresis was performed at 1V/cm, 300mA for 30min. Slides were neutralized in 0.4M tris, before staining with 500µg/mL ethidium bromide. Analysis was carried out either by Metafer v.3.6.7 (Metasystems, Waltham, MA) or Komet 5.5 (Andor Technology, Ireland). At least 100 nucleoids per condition were analyzed and the median

calculated. Each key experiment was replicated at least 3 times and the mean of all replicates plotted. Where applicable, *P values* were calculated by student's t-test.

3.3.3 Results

Micropatterning Cells

As an approach to arraying cells, we employed a photolithographic substrate patterning technique, allowing for differential and controlled adsorption of a cellular adhesion bio-molecule on a glass or silicon wafer. The resultant wafer or chip can therefore be described as having bio-molecule 'islands' for cell adhesion, in a 'sea' of material that precludes cellular adhesion (Fig. 3-1a). This technique of patterning substrates for the adhesion of cells, with various modifications, has previously been used to mediate selective adhesion of a variety of cell-types including immune cells (Kim *et al.*, 2004a), neuroblastoma cells (Matsuda *et al.*, 1992), endothelial cells (Stenger *et al.*, 1992), hepatocytes (Bhatia *et al.*, 1997) and myocytes (Rohr *et al.*, 1991), with micron-scale resolution.

A key consideration in the synthesis of the chip, was the choice of materials for the cell-binding 'islands' and the cell-excluding 'sea'. We therefore crudely tested the ability of commonly used cellular adhesion molecule, collagen I, as the 'island' material, and the ability of naked glass, bovine serum albumen (BSA) and poly ethylene glycol (PEG) disilane as 'sea' materials to exclude cells. We found that HeLa cells adhered more to collagen I-coated slides, than naked glass slides coated in PBS, and that this adhesion increased as a function of incubation time (Fig. 3-1b). We also found that BSA was somewhat effective in blocking against cell adhesion, resulting in less cell depletion from a suspended cell culture.

However, when slides were incubated with both BSA and collagen I, BSA did not prevent adhesion of cells (Fig. 3-1b). Finally, we found that coating glass slides with PEG disilane, was remarkably effective at preventing HeLa cell adhesion. We therefore adopted a protocol employing collagen I 'islands' in a PEG disilane 'sea'.

This protocol proved to be effective, with collagen 'islands' being discrete, regular and somewhat uniform (Fig. 3-2a). Furthermore, cell patterning was effective in at least two cell types that were assessed: HeLa and primary hepatocytes (Fig. 3-2a & 2b). The number of captured cells could be controlled by controlling the size of the collagen 'island', with 20 μ m diameter 'islands' capturing the greatest number of single cells (hepatocytes) (Fig. 3-2c). To test whether cell patterning was robust enough to withstand processing consistent with the comet assay, patterned hepatocytes were overlaid with 1% low melting point (LMP) agarose, and cell patterning was assessed microscopically before and after gel overlay. We found that shear stress exerted by gel overlay did indeed cause loss of cells from the array (Fig. 3-2d). It was determined that the speed with which a coverslip was laid on top of the gel overlay affected this phenomenon. Slower placement of coverslip resulted in better array preservation (data not shown). Thus adherent cell patterning, in a manner that is consistent with the comet assay, was achieved.

Adherent Comet Assay

We then queried whether the alkali comet assay could be run on adherent cells. Running of the comet assay on adherent cells is uncommon, but not novel. Indeed, Singh et al. developed a method for running the comet assay on adherently cultured fibroblasts (Singh *et al.*, 1991). To realize the adherent comet assay, a specialized fenestrated slide was

engineered that allowed for cell adhesion in the center and had frosted edges to hold agarose to the slide. Other groups have utilized custom fenestrated slides for the comet assay as well (Petersen *et al.*, 2000). However, instead of a custom fenestrated slide, we made a fenestrated slide using GelBond, a commercially available agarose adhesion film (see “Fenestrated” GelBond slide, Fig. 3-3b). Even though this technique has previously been employed, our implementation was not without challenges. On attempting to run the comet assay on adherent cells we found comet nucleoids with anomalous morphologies. These morphologies were classified and derived nomenclature from their qualitative features, and in keeping with the celestial theme inherent to the comet assay. Astral morphology group: these were nucleoids that were spindle and had astral projections, strikingly similar to the cytosolic projections of adhered fibroblasts. Flare morphology group: This group, although containing nucleoids, displayed stained genetic material in axes different to the electric field and some projections not associated with nucleoids, resembling solar flares. Nebula morphology group: This group was comprised of stained, granulated genetic material in cloud like patterns, wherein no discrete nucleoids could be detected. Two hypotheses were offered for these anomalous morphologies:

1. DNA adsorption to glass surface resulting in anomalous morphologies,
2. Movement of gel resulting in DNA streaking.

Methods for testing and addressing these potential causes were considered and the second hypothesis, proved the more facile to test.

We tested the hypothesis that anomalous nucleoid morphology groups were generated by gel movement by designing a slide that would allow for gel movement (Fig. 3-

3b). This slide, designated a 'billow' slide, would allow for a lifting or billowing of the agarose from the cells adherent to the glass and would also allow for perpendicular translation of the gel overlay. Following execution of the comet assay on traditional comet assay slides, fenestrated GelBond slides and billow GelBond slides comet, we found that gel movement accounts for the Flare morphology nucleoids (Fig. 3-3c). The billow slide managed to recapitulate different flare morphologies that had previously been observed.

To determine the cause of the astral morphology group, we hypothesized that the astral nucleoid morphology was the result of the adherent cell morphology. That is, we believed that on overlaying cells with gel and allowing the gel to solidify around the adherent cell, a cell void is made in the gel that on lysis of the cell fills with genetic material. The genetic material would therefore take on the shape of the adherent cell prior to electrophoresis. To test this hypothesis, we first asked whether cell morphology could be altered after gel overlay. Cells were seeded on collagen pre-coated slides and allowed to adhere and spread. The cells were then overlaid with 1% LMP agarose and the gel allowed to solidify. The gel-immobilized cells were then incubated at room temperature with PBS or 0.25% trypsin/ EDTA. Trypsin is a serine protease that cleaves peptide chains and will cleave cell adhesion molecules in cell-surface interactions. EDTA is a bivalent cation chelator and prevents inhibition of trypsin as well as chelating Ca^{2+} , which cell integrins use for adhesion. We found that an incubation of 10min with trypsin/EDTA caused spindle-shaped adherent cells in gel to round (Fig. 3-4a). Further we found that on running the comet assay on cells treated with PBS or trypsin, the PBS treated cells yielded the astral group morphology cells while the trypsin/EDTA treated cells yielded normal comet

morphology. This indicated that the anomalous astral morphology was the result of the shape of the adherent cell on overlaying with gel.

We then sought to determine whether the adherently run comet assay would yield similar quantitative measurements of DNA damage as the traditional comet assay. This was an important consideration as we did not want to sacrifice any of the quantitative attributes of the traditional comet assay. TK6 cells were initially used for this assessment, as they are rounded and do not spread, on adhesion to polyD-lysine coated slides via non-specific adsorption, thereby negating the need for a trypsin incubation that may have confounded the radiation-induced damage signal. We found that there was a gamma ionizing radiation dose dependent increase in the damage reported by both the traditional comet assay and the adherent comet assay (Fig. 3-4c). The adherent samples did seem to have slightly elevated median olive tail moment when compared to the traditional samples. This comparison of adherent versus suspended cells was repeated for HeLa cells. More rigorous interpretation of the difference between the traditional and adherent comet assay was deferred, as these data were only preliminary (n=1). However, this assessment was informative in that the adherent comet assay was indeed a feasible approach and could be utilized for DNA damage assessment in different cell types.

To determine the quantitative robustness of the patterned adherent cell comet assay, we patterned primary rat hepatocytes and ran the adherent comet assay on the resultant array. Primary hepatocytes were used primarily to demonstrate the utility of CoaCh on primary cells and due to the importance of liver metabolism in genotoxicity assessment. We found that rat primary hepatocytes patterned reasonably well (Fig. 3-5a) with ~60-70% of the chip collagen 'islands' capturing 1-3 cells (data not shown). Importantly,

a radiation dependent increase in DNA damage was observed for both patterned adherent cells and the traditional comet assay (Fig. 3-5b). Adherent and traditional nucleoids display DNA damage. A comparison of the standard deviations of the patterned adherent comets vs. traditional comets reveals that there is less spread and thus higher precision in the adherent patterned comets. This experiment also allowed for a demonstration of the increased throughput enabled by use of the adherent comet assay chip. Hepatocytes were treated with 5 doses of X-rays on one chip using a sliding lead shield. The footprint of the chip was approximately equivalent to that of one and a half slides and thus, throughput was increased approximately 300%. Additionally all cells were treated concurrently, by virtue of being on the same substrate, allowing for more consistent handling across doses for the entire experiment.

3.3.4 Discussion

We demonstrate a technique for measuring DNA damage that could potentially be applied to measuring base excision repair. This technique is relatively cheap, sensitive, applicable to capturing adherent cell biology, and is scalable for higher throughput. In adapting the comet assay as a high throughput tool for the assessment of DNA damage and repair, we decided to address cell patterning and the application of the comet assay to adherent cells separately, effectively decoupling these variables. In addressing the patterning variable, a now standardly-used (Bhatia *et al.*, 1997; Irimia and Karlsson, 2003) photolithographic technique for micro-patterning cells, and for our purposes using collagen 'islands' on a 'sea' of PEG disilane, proved effective in arraying a variety of adherent cell types, including human HeLa cells and rat hepatocytes. While various materials and surface

functionalizing protocols could potentially have been used—and we indeed demonstrated that non-adherent cells, e.g. TK6 lymphoblastoid cells, could be cultured on poly D-lysine slides (Fig. 3-4)—collagen and PEG disilane are commercially available and relatively cheap and easy to use. We demonstrated that naked glass excludes cells to an extent (Fig. 3-1b), and thus with some optimization and likely for only certain cell types, using a glass ‘sea’ could further reduce cost.

This fabrication process also allowed for the control of spatial parameters including distance between ‘islands’ and the size of ‘islands’. Indeed controlling the spacing between ‘islands’, allows for control of the spacing between nucleoids. This, in turn, allows for maximal use of the chip real-estate. Additionally, allowing for control of ‘island’ size, allows for control of the number of captured cells for analysis: the bigger the ‘island’ the greater the number of cells captured by that ‘island’. This feature has utility. Indeed, studies by other members of our group have assessed the possibility of multiple-cell capture for use in the comet assay (Wood *et al.*, 2010). One application of this feature would be in assessing DNA damage levels in tissue samples, *i.e.* capture of small aggregates of cells.

We also demonstrated that comet nucleoid morphology, an important parameter for damage analysis, is dependent on the shape of the cell prior to lysis and is sensitive to any gel movement post-lysis. Importantly, we demonstrated that, trypsin could be used through the gel to restore rounded cellular morphology to yield spheroid nucleoids. This application of trypsin was utilized primarily as an experimental proof-of-concept method for restoring spherical cellular morphology and would likely not be applicable for actual experimentation due to the fact that trypsin may yield a confounding DNA damage phenotype, and may negate any of the benefits associated with capturing normal adherent cell behavior. We

instead propose an approach wherein controlling the shape of the 'island', controls the spheroid morphology. Techniques for controlling cell morphology based on controlling substrate geometry have previously been used with success (Chen *et al.*, 1997), and are the basis for at least one commercial product.

We demonstrated that the adherent comet assay could be used on a number of cell types including HeLa, TK6 and primary hepatocytes. We demonstrated that throughput could be increased and real estate use optimized, by using lead shielding and a single chip to treat cells with 5 doses of X-rays. The same data could only have been achieved by independent dosing of separate slides resulting in increased opportunity for human and timing errors due to the increased slide handling (1 chip vs. 5 slides per run). While we did not formally attempt to maximize the throughput of our chip, additional work done by our group has further demonstrated the increased throughput afforded by cellular micropatterning (Wood *et al.*, 2010).

We also showed, using HeLa cells, TK6 cells and hepatocytes that the adherent cell response was similar to that of suspended cells, albeit slightly higher. This may be due to a greater sensitivity afforded by adherent cells. However, additional studies will be required to compare the DNA damage response in adherent vs. suspended cells and determine the cause of any difference. One such aspect for further study is assessing to what extent cellular adhesion affects DNA migration. In the case of suspended cells, agarose surrounds the cells allowing for multiple degrees of freedom for nucleic acid migration. However, the adherent cell is limited in this regard, with a basal glass surface that may interact with DNA and modify DNA migration physically and or electrostatic ally (Nanassy *et al.*, 2007). Another aspect for further investigation will be defining why adherent cells display decreased

spread, and thus greater precision, in comet damage signal. This could be biological and or physicochemical and could be useful in improving the comet assay in general. Indeed, whether the higher precision of adherent comet data is biological or physicochemical, defining how general this precision phenotype is across different cell types and species will be an important consideration.

3.3.5 Conclusions

The comet assay represents a well established technique for the measurement of DNA damage and repair. Here, we demonstrated that a photolithographic micropatterning protocol employing collagen and cell exclusion by PEG disilane could be used to array cells for the adherent comet assay. We demonstrated that the cell number captured could be altered as a function of collagen 'island' diameter and provided that shear was kept to a minimum, the patterned cells could be retained for the comet assay. We characterized classes of aberrant nucleoid morphology and established that these morphologies were derived from cell-morphology pre-lysis and gel movement post-lysis. Cell morphology could be rectified by trypsinization through the gel or by controlling 'island' geometry. Additionally, gel movement artifacts could be decreased by an adequate appreciation of the existence of this parameter and the movement of the slide fenestration GelBond further to the slide interior. Finally we demonstrated that patterned adherent nucleoids yield similar quantitative information to the traditional suspended nucleoids, but have greater precision and allow for experiments with higher throughput. While some technical challenges remain, continued work by our group and others may yet provide a relatively cheap, high

throughput, quantitatively rigorous tool for DNA damage assessment and the assessment of base excision repair.

3.4 Measurement of macrophage-induced DNA damage

3.4.1 Introduction

Chapter II addressed investigations of the effects of specific reactive oxygen and nitrogen species (RONs), namely nitric oxide (NO[•]) and peroxynitrite (ONOO⁻) on cells with differential base excision repair (BER) capacity. Here, we attempted to evaluate the effects of a generalized inflammatory environment, approximating physiological exposure conditions. To accomplish this we used a macrophage coculture system. Macrophages provide a putatively more physiological model of inflammation as these cells release many other factors besides RONs that could potentially affect DNA metabolism and thus DNA damage levels.

3.4.2 Materials and Methods

Cells and cell culture

Murine RAW264.7 macrophage-like cells were a kind gift from the lab of Gerald Wogan. AA8 and EM9 cells were obtained from ATCC (Manassas, VA). All cells were cultured in 10% FBS (Atlanta Biologics, Lawrenceville, GA) in DMEM (Cat#11965, Invitrogen, Carlsbad, CA) and Penicillin/ streptomycin (100U/ml;100ug/ml) (Sigma, St. Louis, MO). For passaging, all cells were cultured in 150mm dishes (Falcon, BD) and were kept growing exponentially, being passaged approximately once every three days. CHO Cell passaging was conducted by aspirating media, rinsing in warm PBS and treating cells with trypsin (Invitrogen, Carlsbad,

CA) for 5-10min, quenching with 10mL media and then transferring 1mL of the resultant cell suspension to 30mL media. RAW264.7 cell passaging was conducted by aspirating supernatant, rinsing cells very gently with PBS and then shearing loosely attached cells off the surface and re-suspending these cells for re-plating like the CHO cells.

Contra-surface coculture

Macrophages were seeded at desired densities on the ventral surface (Fig. 3-6A) of the 0.4 μ M polycarbonate transwell membrane inserts (Cat#3419, Corning). The macrophages were allowed to adhere for 2-6 hours and then the insert was returned to its correct orientation and $\sim 1 \times 10^6$ target cells seeded. The cells were then either activated immediately or allowed to acclimate overnight. RAW264.7 cells were activated by the addition of “activating media”: media with 20U/mL IFN γ (Cat#485-MI, R&D Systems, Minneapolis, MN) and 20ng/mL LPS (Sigma, St. Louis, MO). The cells were held for specific durations of time and samples were taken from the target cell compartment at intervals throughout, or at the end of each experiment for assessment of activation by nitrite quantification, using Griess reagent (Promega, Madison, WI) (data not shown).

For iNOS inhibition with L-NMA, RAW264.7 cells were seeded in a 96 well plate and allowed to adhere and acclimate. Cells were then treated with activating media with the specified concentrations of L-NMA (Chem-Biochem Research, Inc., Salt Lake City, UT).

Contra-surface coculture comet assay

Following coculture incubation with either activated or inactivated macrophages, polycarbonate inserts were removed and rinsed with ice cold PBS. The target cells were removed from the membrane by trypsin, quenched in ice cold media and re-suspended in molten, 1% LMP agarose at 37°C. The comet assay was then conducted as described in section 2.3.3.

Transwell CoaCh coculture system

In this setup, the microwell-capturing gel was molded as previously described (Wood *et al.*, 2010). CHO target cells were loaded and captured on the chip. Macrophages were placed in the 0.4µm transwell membrane and activated as described above. The cocultures were held for specified durations of time and on removal of the membrane, a 1% Agarose gel overlay was applied and the comet assay conducted as previously described (Wood *et al.*, 2010).

Direct-CoaCh coculture system

This setup is similar to the transwell CoaCh coculture system, except in this setup the membrane is dispensed with and the activated macrophages are applied direct to the chip, following capture of the target CHO cells.

3.4.3 Results & Discussion

After determining the role of XRCC1 in repairing NO[•] and peroxynitrite-induced DNA damage as described in Chapter II, we next sought to investigate the role of XRCC1 in repairing activated-macrophage induced damage. Activated macrophages putatively represent a more physiological model for inflammation-mediated DNA damage than exposure to NO[•] and peroxynitrite alone as, in addition to RONS, activated macrophages release a number of inflammation-associated factors that can interact with, and thus affect DNA metabolism. These factors, including cytokines and chemokines like TNF α , IL-1 and MIP-3 α /CCL20 can result in DNA damage via reactive oxygen species (ROS) (Davies *et al.*, 2008; Fulton and Chong, 1992) or cause cellular proliferation (Coussens, 2002) that promotes for replication in the presence of DNA damage.

To carry out this investigation we once again used XRCC1 null CHO EM9 cells and XRCC1 WT CHO AA8 cells (See Chapter 2.3.1) and exposed these cells to bacterial lipopolysaccharide (LPS) and IFN γ -activated RAW264.7 cells, in a membrane transwell coculture system. The RAW264.7 cell line is a murine, macrophage-like cell line that releases inflammatory chemicals and NO[•] as a linear function of exposure to increasing concentrations of IFN γ and or LPS (Kim and Son, 1996). Additionally, the transwell membrane system has previously been used in a variety of studies to investigate the effects of diffusible species released from one cell type on another target cell type. For example, for studies investigating immune cell and bacterial communication (Bussière *et al.*, 2005) and for studies involving the communication of genomic instability mediating factors from irradiated cells to un-irradiated bystander cells (Yang *et al.*, 2005). The system, for our purposes, consists of a 75mm tissue culture dish with a suspended, 0.4 μ m porous

polycarbonate membrane. CHO target cells and RAW264.7 cells are kept separate by culturing each cell type on opposite sides of the membrane (Fig 3-6), here termed “contra-surface coculture”.

We found that the RAW264.7 cells release NO[·], measured as nitrite, at a constant rate over a period of ~6-42 hours after activation of the cells with LPS and IFN γ (Fig 3-6B). Nitrite and nitrate are terminal oxidation products of NO[·], and nitrite is therefore used as a proxy for NO[·] release into the media (Wang *et al.*, 2003). The amount of NO[·] release, measured as nitrite, is proportional to the number of macrophages seeded and the rate of NO[·] appears to be, at least qualitatively, related to the number of macrophages seeded (see slopes in Fig. 3-6B). Additionally, the nitrite measured in the media on activation of the RAW264.7 is due primarily to NO[·] production by NO[·] synthases (NOS), since the nitrite detected can be decreased by incubation of RAW264.7 with increasing concentrations of N^G-methyl-L-Arginine monoacetate (L-NMA), a potent inhibitor of NOS (Toutouzas *et al.*, 2008; Zhuang and Wogan, 1997)(Fig. 3-6C). The fact that NO[·] synthases can be effectively inhibited using L-NMA is important as it allows for an assessment of the DNA damage attributable to NO[·], in cocultured target cells .

Preliminarily, we found that when target cells and macrophages are cocultured for ~40 hrs in the transwell coculture system, the nitrite concentrations reproducibly (data not shown) increase as a function of increasing the number of seeded macrophages (Fig. 3-7A). We also found that the DNA of target CHO cells was increasingly damaged on exposure to increasing numbers of activated macrophages. Importantly, we preliminarily found that the XRCC1 deficient EM9 cells incurred greater levels of BER intermediates as measured by the alkali comet assay, on exposure to activated macrophages (Fig. 3-7B). However, on

attempting to replicate these data, it was found that the trends observed in the damage levels in the target cells were not easily reproduced (Fig3-8A). Indeed, even the damage incurred by the target cells at the highest macrophage seeding concentrations used in each subsequent experiment, yielded similarly erratic results (Fig. 3-8B), with neither the AA8 nor the EM9 being systematically more damaged than the other. Closer examination of the damage levels at the single cell level revealed that the inconsistency may have been due to non-heterogeneous damage to the target cell population (data not shown). One potential cause for non-heterogeneous damage to the target cells could be non-heterogeneous adhesion of either the target cells or the macrophages, or both.

In an attempt to address the lack of heterogeneity in the surface coverage of the transwell membrane by the cells, we lowered the degrees of freedom of the target cells in the system by using two variants to the contra surface coculture system. These variants utilize the suspended version of the Comet assay-on-a-Chip (CoaCh) device (Wood *et al.*, 2010). The first variant, named “Transwell-CoaCh Coculture”, uses 14 μm microwells for cell capture in a 1% normal melting point (NMP) agarose chip (Fig 3-9A) and the transwell membrane with or without media headspace between the chip and the membrane (Fig 3-9B). The second variant, named “Direct-CoaCh Coculture”, utilized the suspended CoaCh device without a membrane for separation (Fig. 3-9C). In the Direct-CoaCh Coculture setup, target cells were first captured in the microwells, macrophages were then cultured directly over the target cells. We found that both the Transwell-CoaCh setup and the Direct-CoaCh setup yielded similarly erratic and irreproducible results, even though nitrite levels in the media supernatant indicated that there were increasing amounts of NO^{\cdot} released by increasing numbers of seeded macrophages (data not shown).

Another potential cause for the erratic results we observed could have been due to the polycarbonate transwell membrane quenching or otherwise preventing RONS released by macrophages from damaging the target cells. To investigate this possibility, we treated cells adhered to the membrane with 5 μM hydrogen peroxide (H_2O_2) and compared the DNA damage levels in these cells to gel-immobilized cells exposed to the same dose of H_2O_2 . We found that the gel-immobilized cells showed an increase in DNA damage levels on exposure to 5 μM H_2O_2 . However, the transwell-adherent cells did not show any significant increase in damage on exposure to 5 μM H_2O_2 . One possible reason for an absence of damage signal is repair of damage during the time it takes to prepare samples for the comet assay. To control for any repair that may have occurred during this step, we allowed one sample of gel-immobilized and treated cells to repair any induced damage and only lysed this sample when the transwell adherent cells were lysed. The gel-immobilized cells that were allowed to repair still showed a damage signal by comet assay while the treated transwell-adherent cells showed no damage signal. This observation suggests that repair of damage is not the cause for the low damage levels observed in the transwell-adherent cells treated with 5 μM H_2O_2 . Instead, this suggests that the Transwell-adherent cells were not damaged at all, by a dose of H_2O_2 that does damage gel-immobilized cells. We note and concede that another model that may explain these data is that adherent cells repair H_2O_2 -induced DNA damage faster than suspended cells. Nevertheless these observations suggested that there was some uncertainty associated with measuring the DNA damage levels in transwell membrane associated experiments.

An alternative approach involves the use of a protocol, devoid of the polycarbonate membrane, to expose cells to activated macrophages. We therefore sought to employ the

suspended CoaCh approach as this protocol had benefits (*e.g.* throughput and parallel sample processing) that we did not want to sacrifice (Fig. 3-11). However, to initially avoid the complexity afforded by using a biological source of RONS, *i.e.* by using the RAW264.7 cells, we opted to optimize the ability of the suspended CoaCh setup to measure DNA damage on exposure of target cells to gaseous NO[•]. Gaseous NO[•] dosing via bioreactor could be better controlled and was more reproducible than the NO[•] derived from macrophage activation by LPS and IFN γ (data not shown). We found that as expected, the NO[•] levels (as measured by nitrite) increased as the duration of exposure to NO[•] increased (Fig. 3-11A). However on examination and comparison of the DNA damage levels in the CoaCh target cells vs. traditionally treated cells (see Chapter 2.2.4), we found that the CoaCh exposed cells yielded quantitatively and qualitatively different results to the traditionally exposed cells. While these data are preliminary, we hypothesize that this observed difference is due to the differential kinetics, and thus differential reactivity, of NO[•] through agarose gel. Further studies will have to be done to characterize the treatment of cells through agarose.

3.4.4 Conclusions

We have described herein an approach that has potential utility in measuring, among other DNA repair pathways, base excision repair in target cells exposed to either NO[•] or activated macrophages. While technically challenging, and decidedly preliminary, the protocols described in this work have set the foundation for further development of tools for measuring DNA damage and repair pathways in varied systems. We have demonstrated that a macrophage coculture system could potentially be used to query the role of BER in repairing macrophage-induced DNA damage. We demonstrated that although a transwell

system showed promise, this system displayed poor reproducibility. We hypothesized that this lack of reproducibility was due, in part, to target cell and macrophage heterogeneity in covering the membrane surface and that the membrane itself may have the capacity to quench reactive oxygen species. We demonstrated some putative solutions to this problem using suspended CoaCh, however further characterization of this system is required to resolve what we hypothesize are issues with RON diffusion through agarose gel.

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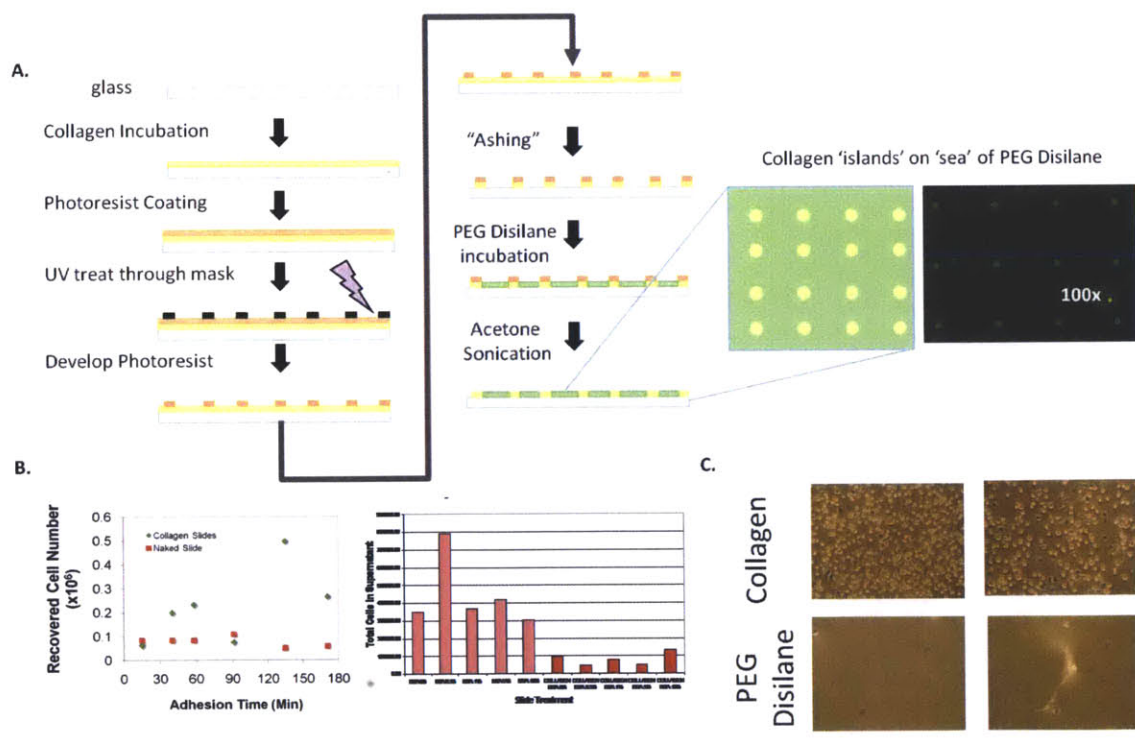


Figure 3-1. Microfabrication protocol and material selection for chip. A, Photolithographic microfabrication process using material information gleaned from **(B)**, cell adhesion quantification on BSA and collagen versus **(C)** adhesion on collagen and PEG disilane.

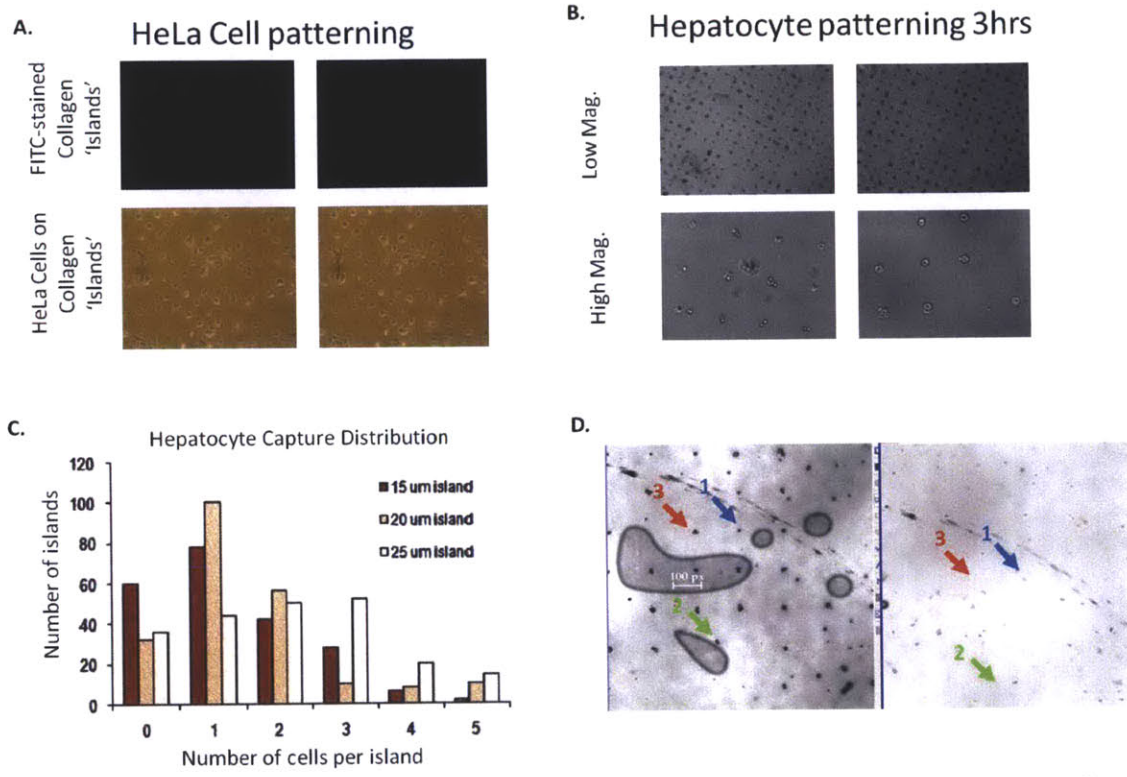


Figure 3-2. Patterning of different cell types for the adherent comet assay. A, HeLa cell patterning and **(B)** hepatocyte patterning 3hrs after adding cells to chip and rinsing with PBS. **C,** Number of hepatocytes captured as a function of collagen 'island' diameter. **D,** Retention of cell patterning following overlay with 1% LMP (low melting point) gel. Cells 1 and 2 are retained after gel overlay, cell 3 is lost.

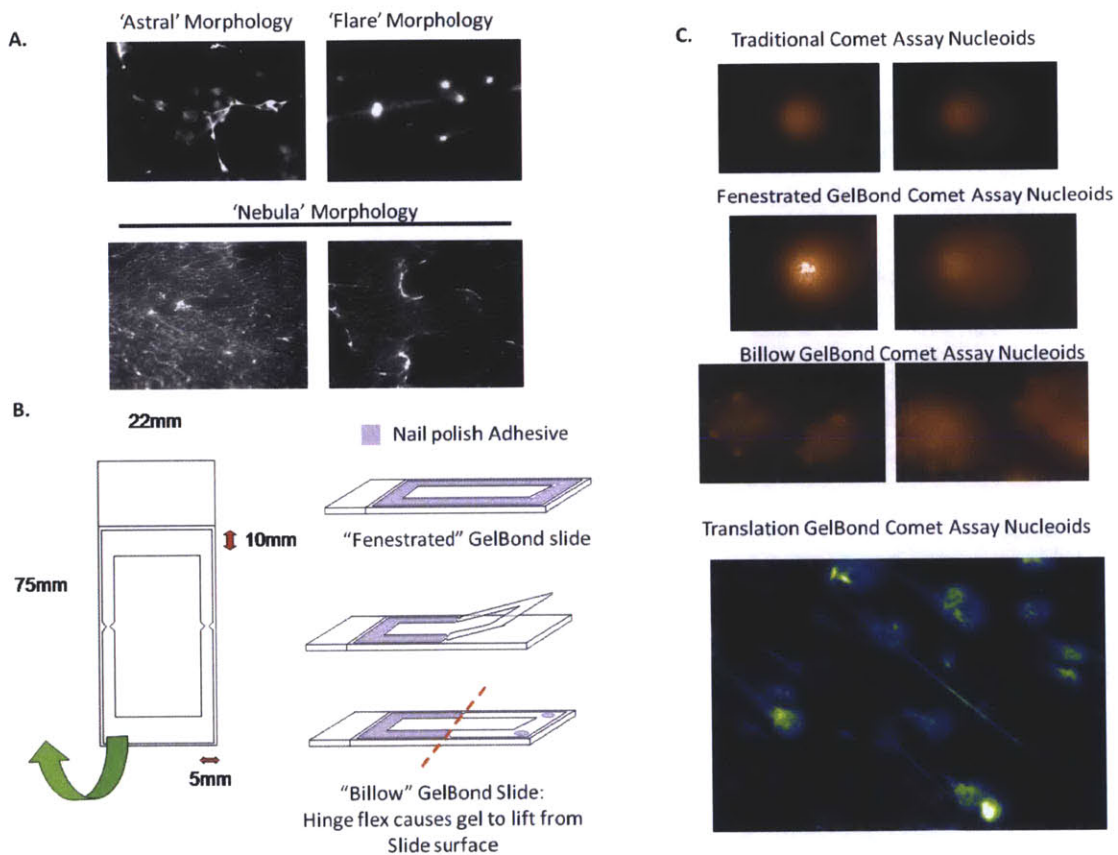


Figure 3-3. Anomalous morphologies of adherent cell nucleoids, following the adherent comet assay. **A,** 'Astral', 'Flare' and 'Nebula' nucleoid morphologies observed after preliminary attempts at the adherent cell comet assay. **B,** Illustration of the billow GelBond slide used to test the effects of gel movement. The experiment generated **(C)** normal nucleoids from the traditional and fenestrated slides and the billow slide recapitulated the 'Flare' morphology.

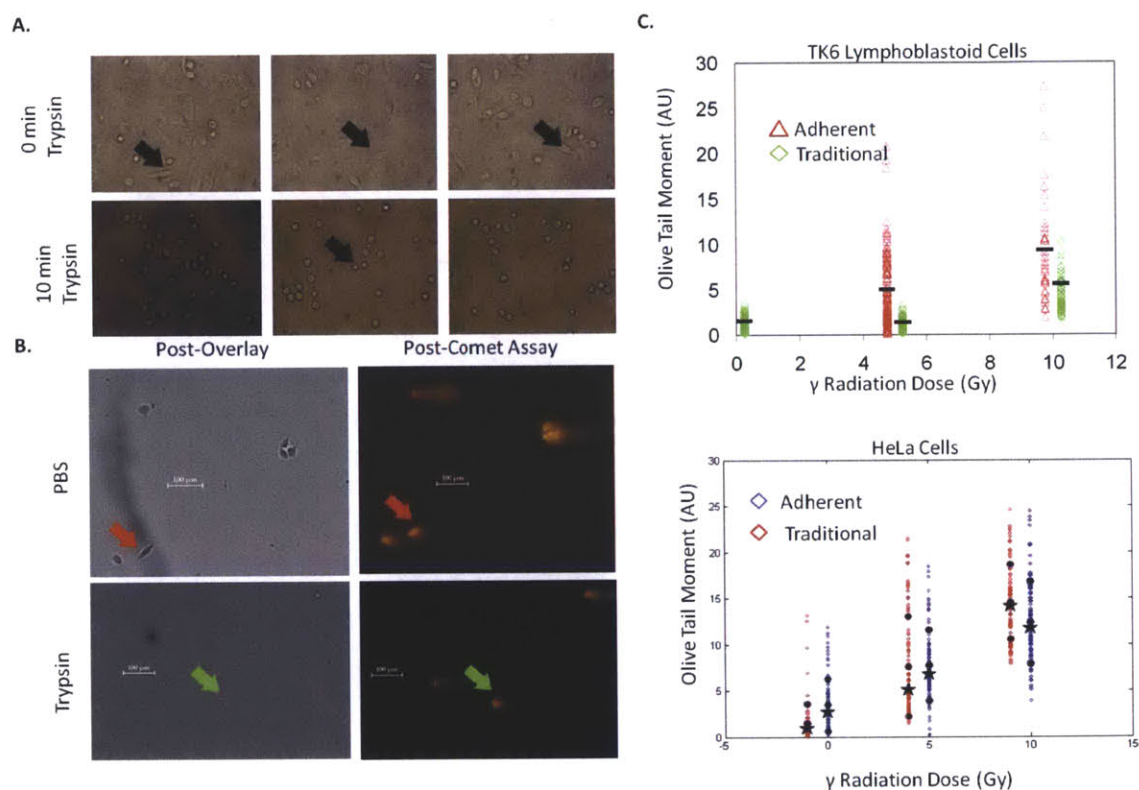


Figure 3-4. Effect of trypsin through gel and cell adhesion on DNA damage assessment. A, Treatment of adherent cells with trypsin, through gel, allows for restoration of spherical cellular morphology. **B,** Nucleoid morphology is dependent on the morphology of the adherent cell pre-lysis. Red arrows indicate spindle shaped cells that formed anomalous nucleoid morphology. Green arrows indicate normal nucleoid morphology. **C.** Quantitative comparison of DNA damage assessment between adherent and suspended (traditional) comet assay nucleoids in TK6 lymphoblastoid cells and HeLa cells.

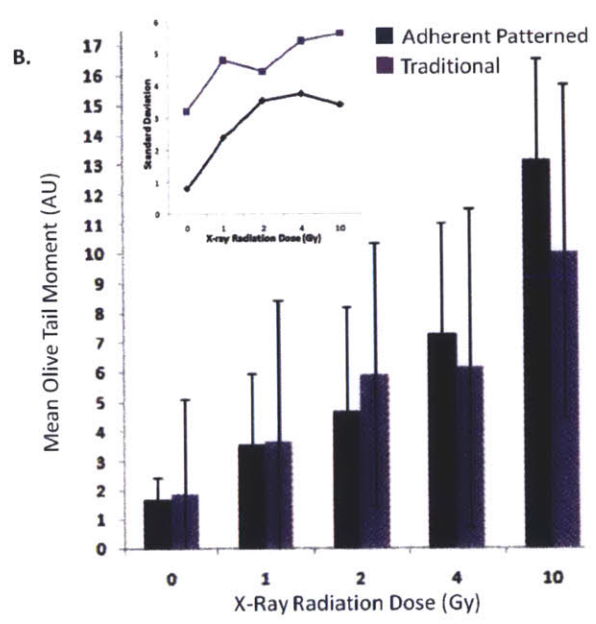
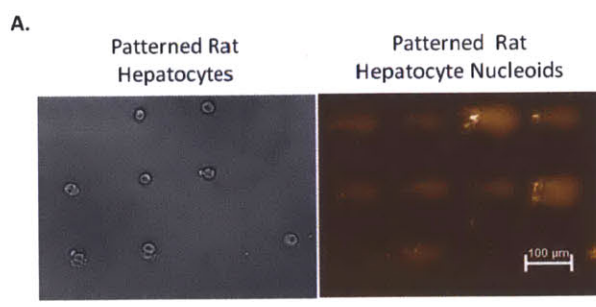


Figure 3-5. Difference between patterned adherent hepatocytes and traditional comet assay. A, Patterned hepatocytes with subsequent patterned nucleoids. **B,** Quantitative comparison of DNA damage and damage spread in adherent patterned comet nucleoids vs. traditionally X-ray irradiated hepatocytes (from thesis of Sukant Mittal).

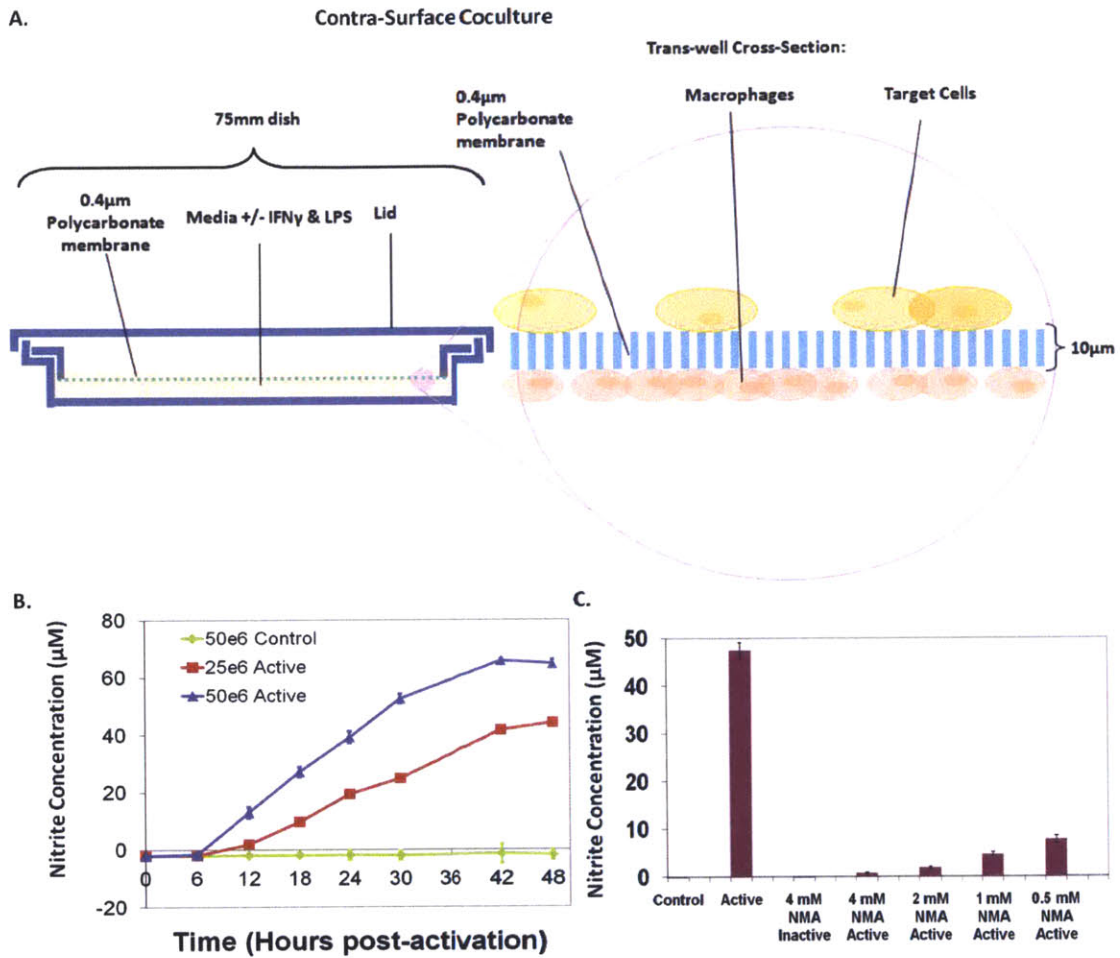


Figure 3-6. Contra-Surface Coculture system. **A**, RAW264.7 cells are cultured on ventral surface of membrane (exterior), target cells are cultured on dorsal surface (interior). **B**, Quantification of nitrite in two different seeding concentrations of macrophages as a function of time post-activation with LPS and IFN γ . **C**, Concentration dependent L-NMA inhibition of RAW264.7 nitric oxide synthases.

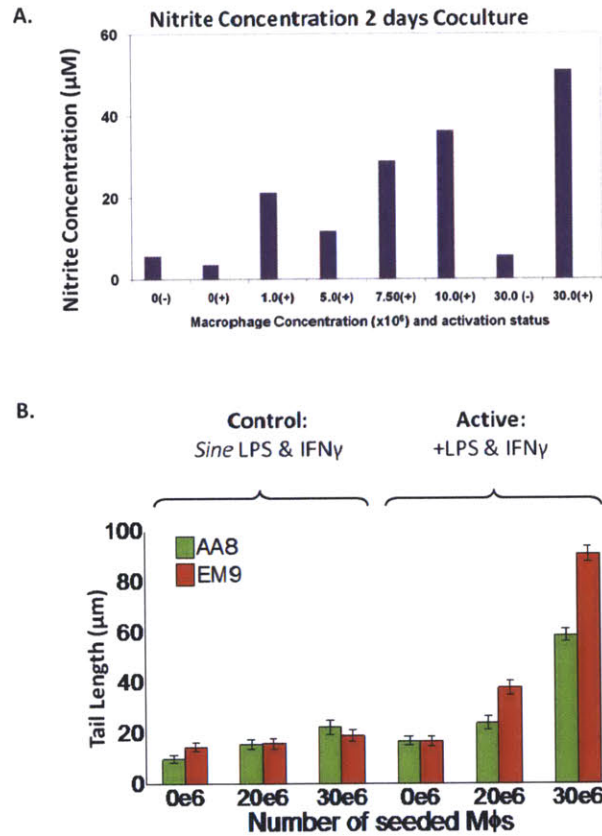


Figure 3-7. Measurement of macrophage dependent NO[•] release and DNA damage. A, Measurement of nitrite concentration in supernatant media following ~40hr CHO cell/RAW264.7 coculture transwell coculture system. **B.** DNA damage assessed by alkaline comet assay in activated (+LPS & IFN γ) and control (Sine LPS & IFN γ) transwell CHO cell/RAW264.7cocultures.

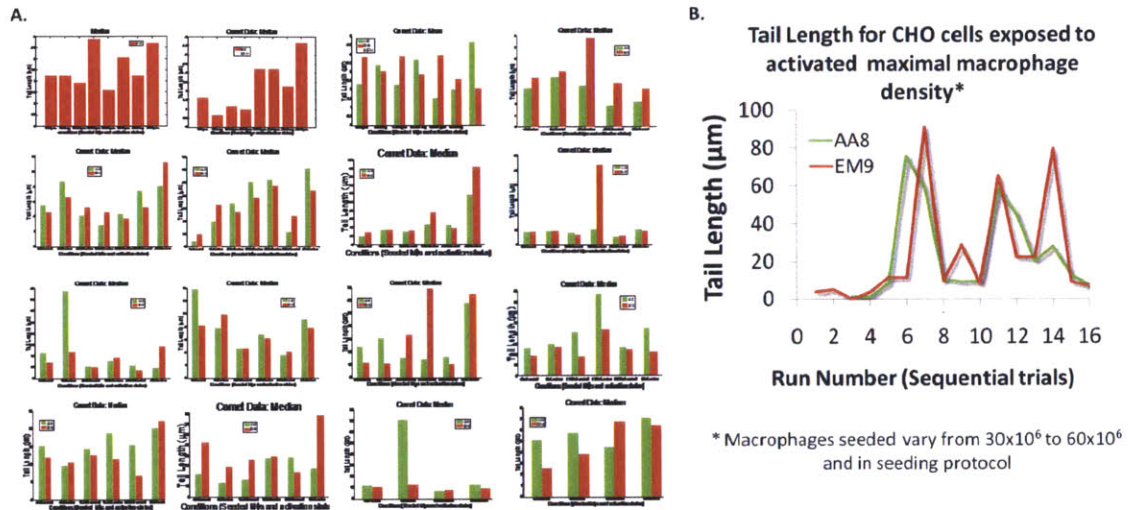


Figure 3-8. Poor reproducibility of damage levels in target CHO cells in tranwell coculture system. **A**, DNA damage in target CHO cells showing multiple replicates of RAW264.7 macrophages/ CHO cells transwell coculture experiments. The number and protocols used to seed macrophages vary. **B**, Plot of the damage levels, as assessed by alkaline comet assay, in the sample with the maximum seeded number of macrophages in a given experiment.

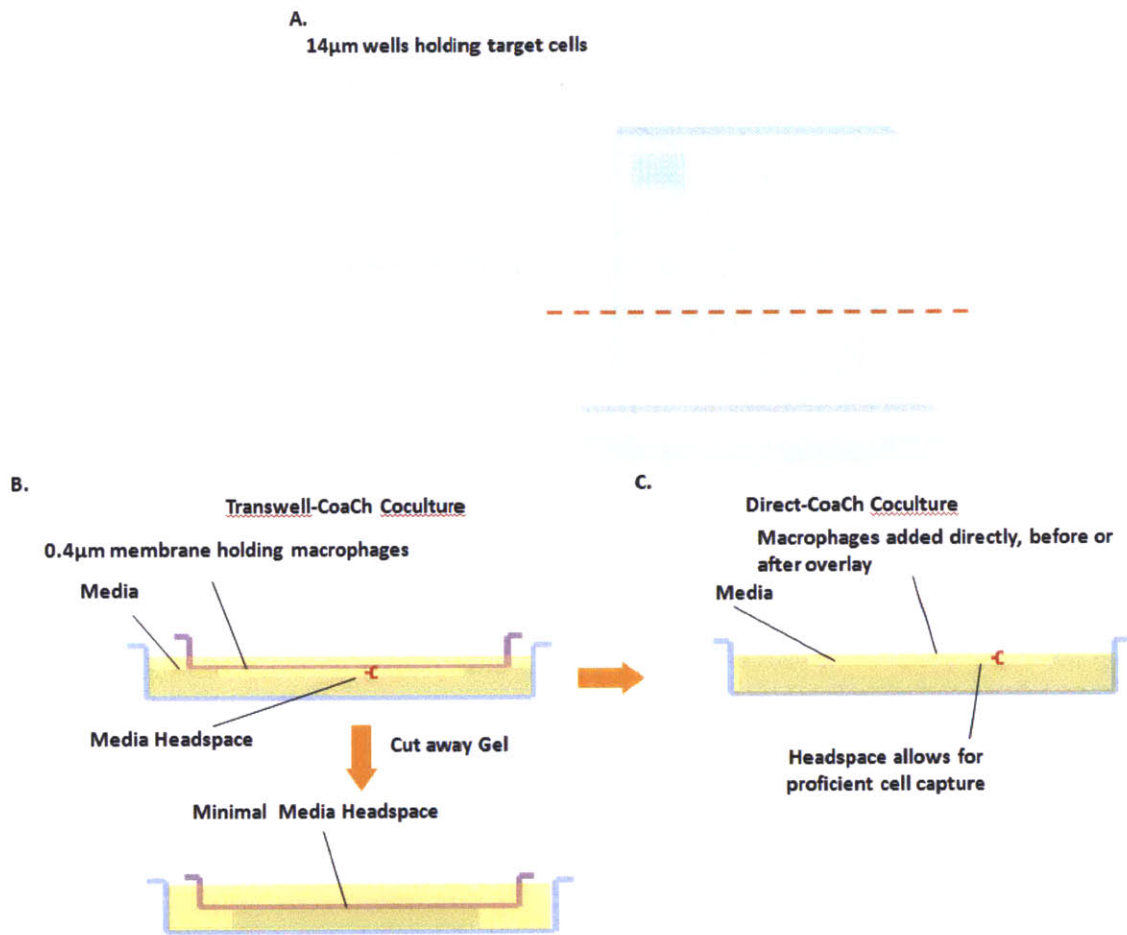


Figure 3-9. Transwell CoaCh System and subsequent variations. **A.** Microwell normal melting point (NMP) agarose chip with 14 μ m diameter microwells is the basis of this setup. Allowing for coculture use with **(B)**, or without **(C)** transwell membrane.

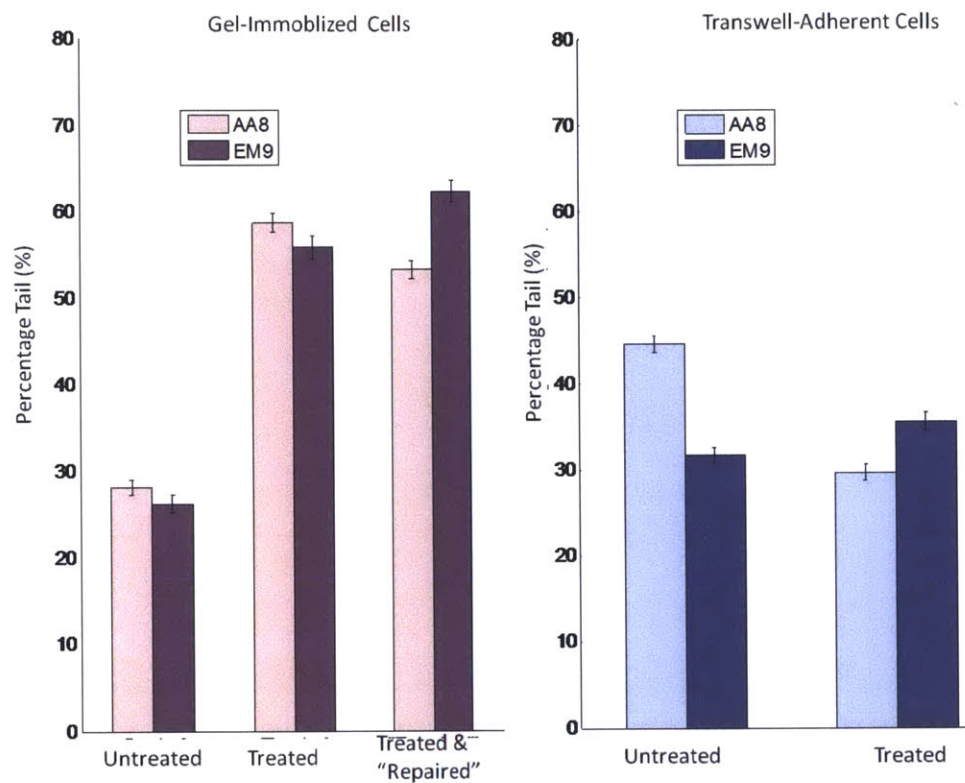


Figure 3-10. Comparison of DNA damage as assessed in Gel-immobilized cells and transwell adherent cells. Cells were exposed to 5 μM H_2O_2 and DNA damage was assessed by the alkaline comet assay. The "repaired" sample is a gel-immobilized sample that was treated with 5 μM H_2O_2 and allowed to repair for the same amount of time as it took to prepare the transwell-adherent samples for the comet assay. "Repaired" sample was lysed for the comet assay at the same time as the transwell-adherent samples.

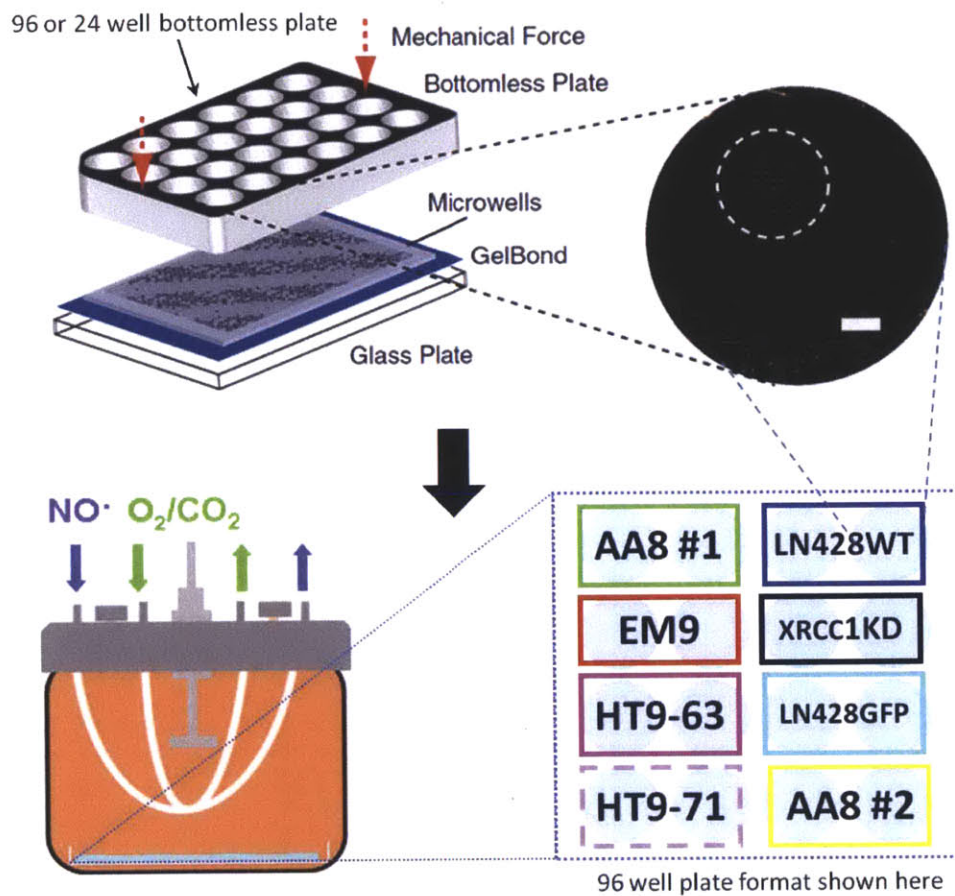


Figure 3-11. Experimental setup for quantification of NO[•]-induced DNA damage using suspended CoaCh. Setup allows for multiple and highly parallel sample treatment and processing. A maximum of 16 samples can be treated per reactor for the 96 well plate format.

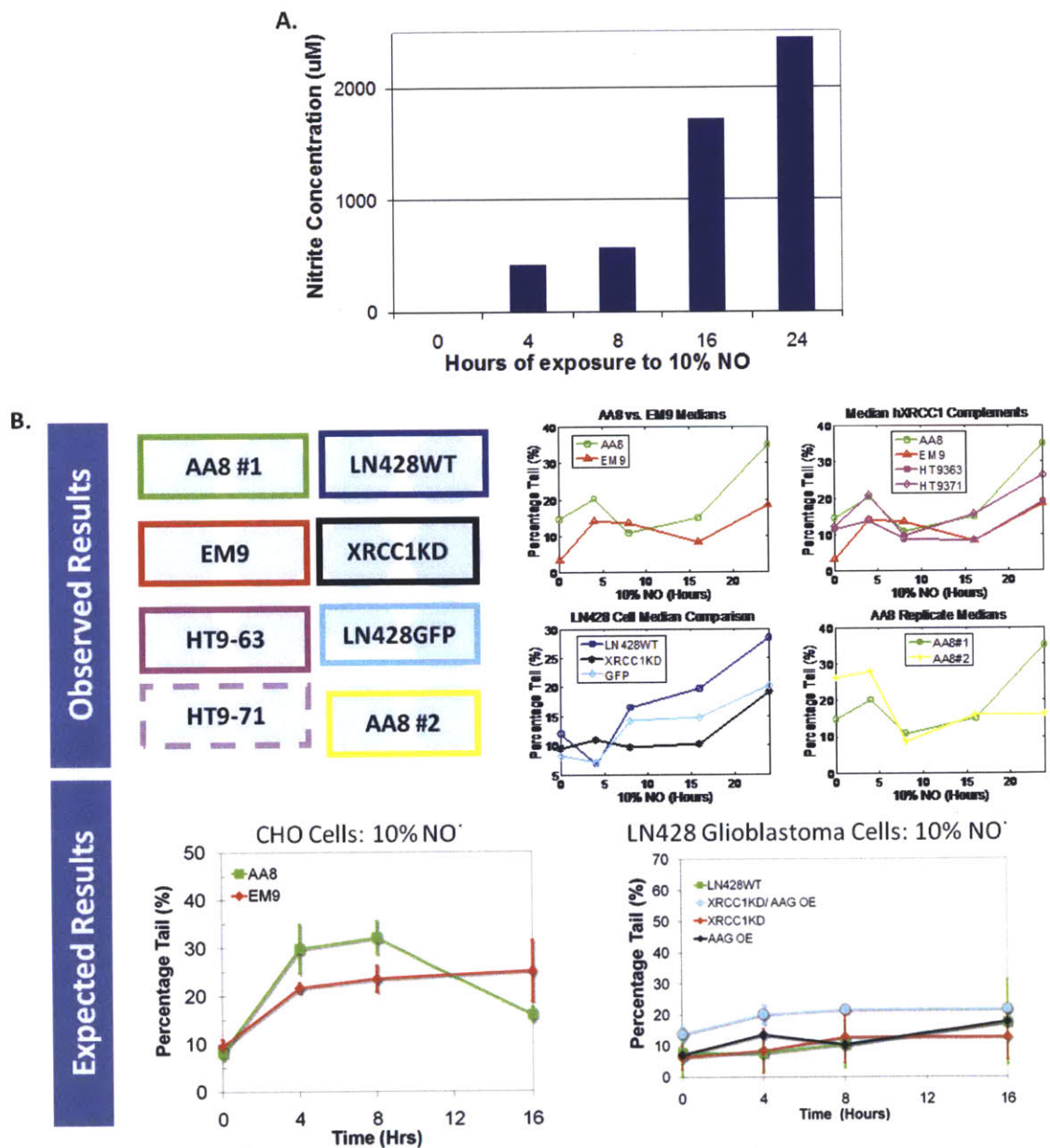
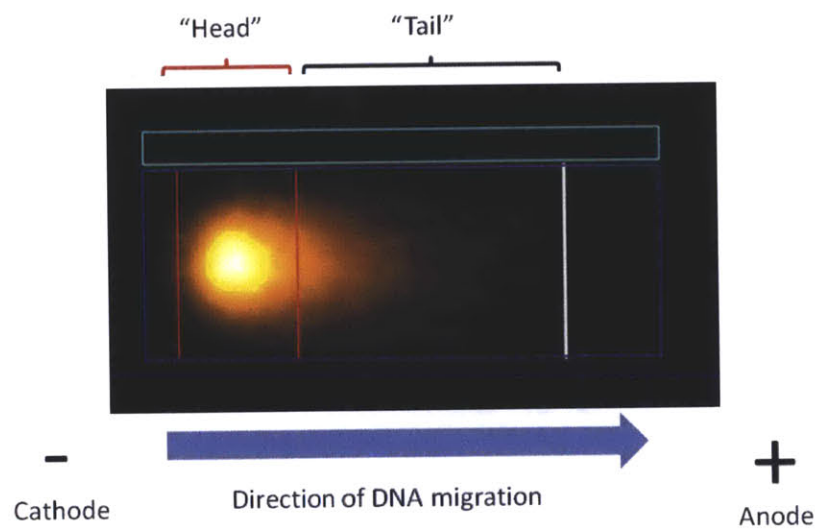


Figure 3-12. DNA damage assessment in CHO and glioblastoma cells treated in suspended CoaCh format or traditional format. A, Nitrite levels measured in CoaCh reactor supernatant, indicating that NO[•] was supplied to the reactors. **B,** Comparison between CoaCh observed results and the expected results as obtained from traditionally treated CHO and glioblastoma cells.



Supplementary Figure 3-1. Representative damaged comet nucleoid with analysis reticule. DNA is pulled from nucleoid head region (red), towards the anode forming the tail (end of tail in white). Cyan box shows region from which background fluorescence is calculated for comparison to nucleoid intensity.

Suspended Cell Patterning	Adherent Cell Patterning
BioFlip Chip	BioFlip Chip
Dielectrophoresis	Dielectrophoresis
Microfluidic placement	Microfluidic placement
Stencil patterning	Direct patterning/ cell printing
Microwell cell capture	Substrate micro-patterning

Supplementary Table 3-1. Alternative approaches for cell patterning for the comet assay. Options include suspended and adherent cell patterning modality. Some approaches allow for either modality.

Chapter IV

Methyltransferases mediate cell memory of a genotoxic insult

4.1 Abstract

Characterization of the direct effects of DNA damaging agents shows how DNA lesions lead to specific mutations. Yet, serum from Hiroshima survivors, Chernobyl liquidators, and radiotherapy patients can induce a clastogenic effect on naive cells, showing indirect induction of genomic instability that persists years after exposure. Such indirect effects are not restricted to ionizing radiation, as chemical genotoxins also induce heritable and transmissible genomic instability phenotypes. While such indirect induction of genomic instability is well described, the underlying mechanism has remained enigmatic. Here, we show that mouse embryonic stem (ES) cells exposed to γ -radiation remember the insult for weeks. Specifically, conditioned media from progeny of exposed cells can induce DNA damage and homologous recombination in naive cells. Notably, cells exposed to conditioned media also elicit a genome destabilizing effect on their neighbors, thus demonstrating transmission of genomic instability. Moreover, we show that the underlying basis for the memory of an insult is completely dependent on two of the major DNA cytosine methyltransferases (MTases), *Dnmt1* and *Dnmt3a*. Targeted disruption of these genes in exposed cells completely eliminates transmission of genomic instability. Furthermore, transient inactivation of *Dnmt1*, using a tet-suppressible allele, clears the memory of the insult, thus protecting neighboring cells from indirect induction of genomic instability. We have thus demonstrated that a single exposure can lead to long-term, genome destabilizing effects that spread from cell to cell and we provide a specific molecular mechanism for these persistent bystander effects. Collectively, our results impact current understanding of risks from toxin exposures and suggest modes of intervention for suppressing genomic instability in people exposed to carcinogenic genotoxins.

4.2 Introduction

It is well established that DNA damaging agents, such as ionizing radiation and chemical genotoxins, can directly induce mutations that in turn promote cancer and ageing (Friedberg, 2006; Hoeijmakers, 2009). Less well understood, but increasingly appreciated, are the indirect effects of such exposures on genomic stability. For example, cells can suffer a persistent, increased frequency of mutations, many cell generations after the original exposure (Kadhim, 1992; Little *et al.*, 1990). Additionally, naïve cells cultured in the presence of the descendants of exposed cells similarly display an increased frequency of genetic changes (Huo, 2001; Nagasawa, 1992; Zhou *et al.*, 2000). These indirect effects of exposure to DNA damaging agents are conventionally described as persistent or bystander effects (Bender, 1962; Morgan, 2003).

A variety of phenotypes have been observed to persist, long after an initial genotoxic exposure. A classic example is delayed reproductive cell death, and reduced plating efficiency, which can persist for more than fifty generations after exposure (Chang and Little, 1992). In addition, *de novo* genetic changes occur many cell divisions after exposure (Kadhim, 1992; Pampfer, 1989; Seymour and Mothersill, 2004). As with persistent effects, many different phenotypes have been associated with the bystander effect. Naïve bystander cells cultured in the presence of either cells that have been previously exposed to a genotoxic agent, or to media from exposed cultures, are prone to genomic instability, toxicity and malignant transformation (Huo, 2001; Lewis, 2001a; Little, 2003; Nagar, 2003; Nagasawa, 1992; Zhou *et al.*, 2000).

An understanding of the mechanisms involved in persistent and transmissible responses to genotoxins is clearly important to human health, given the ubiquitous presence of DNA damaging agents endogenously, in our environment, and in the clinic. Indeed, since the initial discovery of genotoxicity-associated persistent and bystander phenotypes, the underlying causes, physiological impact, and mechanistic etiology of these responses have been intensively studied (Morgan and Sowa, 2005; Mothersill and Seymour, 2005; Mothersill, 2006). Traditionally, persistent and bystander phenotypes have been studied in response to high doses of ionizing radiation (Mothersill, 2001). However more recently, these phenotypes have also been generated by non-ionizing radiation *e.g.* ultra violet (UV) radiation (Limoli, 1998; Mothersill, 1998), reactive oxygen and nitrogen species (Azzam, 2002; Dickey, 2009), cytokines (Dickey, 2009) and other genotoxic, chemical exposures (Rugo, 2005). Thus, because endogenously generated chemical species (*e.g.* cytokines and reactive oxygen and nitrogen species) and exogenous agents to which cells are physiologically exposed (*e.g.* UV and low dose IR radiation), are capable of initiating persistent and bystander phenotypes alike, it is reasonable to posit that these responses represent normal, physiologically relevant, cellular responses to stressors. Consistent with this view-point, are observations of persistent and bystander phenotypes not only at the cellular, but at the tissue (Goldberg, 2002; Koturbash, 2006; Mothersill, 2002; Pant, 1977; Watson *et al.*, 2000a) and even organism level of organization (Mothersill *et al.*, 2007). Further, these responses appear to be evolutionarily conserved across different kingdoms and species (Yang *et al.*, 2008).

Intense interest in the underlying mechanism of the bystander effect has prompted studies that have revealed many of the agents capable of inducing persistent and bystander

phenotypes, as discussed above. Much less is known, however, about the mechanism by which cells retain and consequently transmit 'memory' of an insult, becoming genomically unstable for a long time after exposure. Earlier work in our laboratory showed that genomic instability was transmissible from one cell to the next (*i.e.*, a bystander can induce genomic instability in a naïve cell over multiple generations) (Rugo, 2005). This transmission of genomic instability, while implying heritability, clearly is not consistent with genetic inheritance. Thus our findings, and related observations by others (e.g., (Kovalchuk, 2008; Lorimore, 2003), suggest that persistent and bystander effects might be propagated by an hitherto unknown epigenetic mechanism.

Epigenetic mechanisms of heredity include DNA methylation, histone modification and the functions of certain non-coding RNAs (Goldberg, 2007). Importantly, DNA methylation has been implicated in heritable, persistent changes in phenotype. For example, the persistent and heritable change in coat colour, and conferred obesity-resistance in the progeny of female mice that were fed genistein during gestation, were found to be DNA methylation-dependent (Dolinoy, 2006). Here, we show that DNA methyltransferases (DNMTs), the enzymes responsible for the epigenetic methylation of mammalian DNA, mediate the propagation of an instability phenotype on exposure to a genotoxin. Specifically, we find that DNA methyltransferases 1 and 3a mediate murine embryonic stem (ES) cell memory of an exposure to ionizing radiation.

4.3 Materials and Methods

Alkali single cell gel electrophoresis (Comet) Assay.

Trevigen, Inc (Gaithersburg, MD) Comet Assay kit was employed. Briefly, after harvesting, ES cells were immobilized in 1% agarose gel and were then lysed in the provided lysis buffer, held in alkali conditions and electrophoresed @30V (1V/cm), 300mA, as per product instructions. Andor technology (Belfast, Northern Ireland), Komet 5.5 imaging software was used to capture and analyze at least 100 nucleoids per condition

Sister Chromatid Exchange (SCE) assay.

A modification of the giemsa-based differential staining of sister chromatids (Perry, 1974) was employed. Briefly, ES cells were treated with 10 μ M 5-bromo-2-deoxyuridine (BrdU) for 30-36 hours before the addition of 0.1 μ g/mL demecolcine, to arrest the cells in metaphase. Cells were then harvested, held in a hypotonic solution and fixed in Carnoy's fixative, before being dropped, stained and imaged by standard bright field microscopy. At least 30 metaphase spreads per condition were examined a frequency of exchanges per spread was calculated. All experiments were done with at least three independent replicates. Student's T-test was used to determine statistical significance of any observed differences.

4.4 Results & Discussion

Ionizing radiation is of great societal importance, both in the context of the environment and the clinic. To learn if γ -radiation leads to persistent transmissible instability in ES cells, DNA damage was assessed in bystanders. Naive cells, designated

primary bystanders (Fig. 1a), that shared media with cells descended from irradiated cultures showed increased DNA damage (Fig. 1b), while naïve cells that shared media with sham-irradiated cells showed no increase in DNA damage by comet assay. To test for transmission of this DNA damage, a second group of naïve WT cells, designated secondary bystanders, were co-cultured with the primary bystanders (Fig. 1a). These secondary bystanders had increased DNA damage when exposed to media from primary bystanders to irradiated cells (Fig. 1c), thus demonstrating transmission of radiation-induced genomic instability from exposed cells, to naïve cells (primary bystanders), to naïve cells (secondary bystanders).

To determine whether persistent instability results from methyltransferase-dependent epigenetic changes, we exploited the fact that ES cells do not require genome methylation for viability, and are readily cultured, following disruption of the three major DNA MTases: Dnmt1, Dnmt3a and Dnmt3b (Tsumura, 2006). During normal development, the Dnmt3a and Dnmt3b *de novo* MTases catalyze the transfer of a methyl group from S-adenosyl methionine to the 5 position of cytosine at CpG sites (Chen, 1991). Methylation is maintained primarily through the activity of Dnmt1, which efficiently methylates hemimethylated CpG sites (Stein, 1982). Dnmt1 is essential for heritable, epigenetically regulated changes in gene expression that are key to differentiation and development (Li, 1992). To test the possible role of MTases in cellular memory of an insult, we asked if *Dnmt1*^{-/-}; *Dnmt3a*^{-/-}; *Dnmt3b*^{-/-} cells (gift of M. Okano) were able to remember and transmit genomic instability following γ -radiation. Results show that descendants of irradiated *Dnmt1*^{-/-}; *Dnmt3a*^{-/-}; *Dnmt3b*^{-/-} cells were not able to induce DNA damage by comet assay in neighboring cells, when compared to WT cells (Fig. 2a).

One of the earliest descriptions of a bystander effect in cultured cells revealed that when ~1% of nuclei were irradiated, over 30% of the cells had increased homologous recombination, detected as sister chromatid exchanges (SCEs) (Nagasawa, 1992). Furthermore, ionizing radiation induces a persistent increase in homologous recombination (Huang *et al.*, 2007). We therefore asked if SCEs are induced by the progeny of irradiated WT and *Dnmt1*^{-/-}; *Dnmt3a*^{-/-}; *Dnmt3b*^{-/-} cells. Naive cells indeed exhibited a significant increase ($p < 0.0001$) in the frequency of SCEs when they shared media with descendents of irradiated cultures (Fig. 2b). However, there was only a very slight, yet significant ($p = 0.0168$), increase in SCEs when the irradiated cells were *Dnmt1*^{-/-}; *Dnmt3a*^{-/-}; *Dnmt3b*^{-/-} cells, compared to mock irradiated *Dnmt1*^{-/-}; *Dnmt3a*^{-/-}; *Dnmt3b*^{-/-} cells. Interestingly, SCEs were increased in cells that shared media with unirradiated *Dnmt1*^{-/-}; *Dnmt3a*^{-/-}; *Dnmt3b*^{-/-} ES cells, compared to cells that shared media with unirradiated WT ES cells (Fig. 2b). Given that *Dnmt1*^{-/-} cells are genomically unstable (Chen *et al.*, 1998; Kim *et al.*, 2004b), *Dnmt1*^{-/-}; *Dnmt3a*^{-/-}; *Dnmt3b*^{-/-} ES cells may be similarly unstable and may thus elicit transmissible responses, analogous to the effects of irradiation. Regardless, progeny of irradiated *Dnmt1*^{-/-}; *Dnmt3a*^{-/-}; *Dnmt3b*^{-/-} cells are less able than WT cells to induce genomic instability in naive neighbors, showing that one or more MTases are essential for persistent radiation-induced instability.

To discern the roles of individual MTases, we analyzed ES cells carrying targeted disruptions of each MTase (gift of E. Li) (Lei *et al.*, 1996; Okano *et al.*, 1999). The MTase deficient cells have normal sensitivity to radiation toxicity (data not shown). Interestingly, γ -radiation had no effect on SCEs in primary bystanders to irradiated *Dnmt1*^{-/-} cells, when compared to bystanders to unirradiated *Dnmt1*^{-/-} cells (Fig. 3). The inability of the *Dnmt1*^{-/-}

cells to sustain a heritable phenotypic change is consistent with their hypomethylated phenotype (Lei *et al.*, 1996). In addition, *Dnmt1*^{-/-} cells induce homologous recombination in neighboring cells, even without irradiation, which is consistent with their instability phenotype (Chen *et al.*, 1998; Kim *et al.*, 2004b). Although Dnmt1 is a maintenance MTase, it is possible that Dnmt1's ability to perform *de novo* methylation in response to DNA damage (Mortusewicz *et al.*, 2005) contributes to the transmissible instability. Similar to *Dnmt1*^{-/-} cells, *Dnmt3a*^{-/-} cells did not transmit genomic instability (Fig. 3), indicating that Dnmt3a-mediated *de novo* methylation is necessary for cells to remember and transmit an instability phenotype. Interestingly, as with *Dnmt1*^{-/-}, *Dnmt3a*^{-/-} cells caused an increase ($P < 0.0001$) in SCEs in bystanding WT cells in the absence of radiation, when compared to SCE levels in WT bystanders to unirradiated WT cells. Lastly, unlike Dnmt1 and Dnmt3a, Dnmt3b was not essential for transmissible instability, as a deficiency in this gene still resulted in transmission of an instability phenotype (Fig. 3).

The observation that genomic instability is induced by unirradiated *Dnmt1*^{-/-} and *Dnmt1*^{-/-}; *Dnmt3a*^{-/-}; *Dnmt3b*^{-/-} cells suggested a possible threshold that prevents further induction of instability after irradiation. We hypothesized that transient loss of Dnmt1 might prevent memory of genotoxic exposure, while protecting bystanders from the instability due to Dnmt1 loss. To test this hypothesis, we exploited mouse ES cells carrying a tetracycline repressible *Dnmt1* allele (Borowczyk *et al.*, in press). By three days post doxycycline treatment, Dnmt1 was undetectable, and within three days after removing doxycycline, Dnmt1 expression resumed (Fig. 4a). To suppress Dnmt1 expression before, during and after irradiation, we added doxycycline three days before irradiation and sustained it for seven days. Doxycycline was then removed to restore *Dnmt1* expression

(Fig. 4a). Consistent with previous results (Figures 2 and 3), the descendants of irradiated WT cells induced homologous recombination in neighboring cells. However, under conditions where *Dnmt1* was transiently suppressed, descendants of irradiated cells were not able to induce homologous recombination in their neighbors (Fig. 4b). Importantly, unlike the cells that carried disrupted *Dnmt1* alleles, cells transiently suppressed for *Dnmt1* do not induce instability in their neighbors.

The transmissibility of genomic instability through shared media has important implications when considering potential tissue-wide responses *in vivo*. To explore transmissibility, we studied secondary bystanders. Primary bystanders were able to induce homologous recombination in naïve cells only if the irradiated target cells had had normal *Dnmt1* expression (Fig. 4b). Thus, transient suppression of *Dnmt1* prevented transmission of instability both to naïve primary bystanders, and to their secondary bystander neighbors.

Characterizing the underlying causes of genomic instability is fundamental in cancer etiology, prevention of premature ageing, and for understanding the risks of exposures. It is becoming increasingly clear that indirect mechanisms of mutation induction that involve changes in cellular behaviour, in addition to the directly induced DNA lesions, can lead to an increased risk of disease-causing mutations for months or even years after exposure (Lorimore *et al.*, 2003; Maxwell *et al.*, 2008; Morgan, 2003; Mothersill and Seymour, 2001; Pant, 1977). Furthermore, at least one study suggests that the extent of bystander-induced DNA damage can be as great as that of the original exposure (Dickey *et al.*, 2009).

While the studies described here do not query the exact mechanism by which DNA methylation results in persistent bystander phenotypes, it is possible that changes in gene

expression, mediated by DNA methyltransferases (Hermann, 2004), cause cells to secrete factors that impact genomic stability. Specifically, DNA damage is known to alter Dnmt1 and Dnmt3a activity (Maltseva, 2009; Mortusewicz, 2005) and DNA damage can also alter secretion profiles (Rodier *et al.*, 2009). Additionally, it is known that cells that secrete TNF-alpha, NO[•] and TGF-beta can induce DNA damage in nearby cells (Burr, 2010; Dickey, 2009). Thus, as a result of exposure to secreted, genotoxic species, bystander cells could adopt a methylation pattern similar to that of the target cell, and thus both remember and transmit a bystander phenotype. The memory of the genotoxic insult would therefore be stored structurally in DNA in the form of DNA methylation patterns that are created and maintained by DNA methyltransferases (e.g., Dnmt1 and Dnmt3a). Propagation of the bystander phenotype could then be effected by a change in the secretion profile of the insulted cell. Interestingly, in normal tissues, communication among cells helps to control cell behaviour. Bystander effects may similarly reflect a coordinated response.

The observation that genomic instability can be transmitted from cell to cell, both *in vitro* (Lorimore *et al.*, 2003; Mothersill and Seymour, 2004; Nagasawa and Little, 1992) and *in vivo* (Lorimore *et al.*, 2005; Watson *et al.*, 2000b), opens the possibility that there are tissue wide changes in genomic stability following exposure to a genotoxin, and calls attention to the possibility that persistent and bystander effects are critical risk factors for disease. Here, we have demonstrated that two of the three major MTases, Dnmt1 and Dnmt3a, are essential in order for descendants of irradiated cells to become able to transmit genomic instability to naive cells. Furthermore, we have shown that by temporarily turning off expression of Dnmt1, it is possible to completely eliminate transmission of genomic instability. Interestingly, and indeed consistent with these findings, Dnmt1 and 3a

have also recently been shown to play important roles in neurological memory and learning (Feng *et al.*, 2010). This finding, albeit apparent specifically in neurons, may represent a general mechanism by which cells store information on, and adapt to, genotoxic and other stimuli.

In conclusion, knowledge of the molecular basis for transmission of genomic instability opens the doors to novel interventions, including the potential administration of Dnmt inhibitors in conjunction with cancer chemotherapy to preserve tissue-wide genomic stability and thus suppress secondary cancers.

4.5 Acknowledgements

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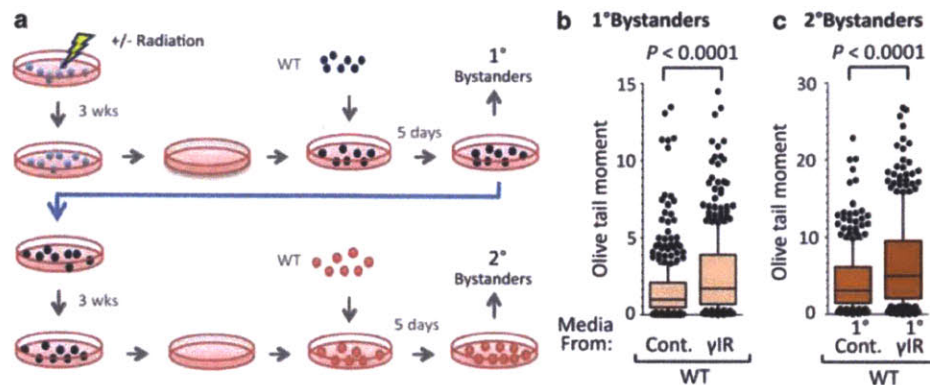


Figure 4-1. Persistent and transmissible induction of genomic instability. **a**, Irradiated (or mock irradiated) target ES cells are cultured for three weeks. During the three weeks, cells were passaged three times a week at densities of $0.5\text{-}2 \times 10^6$ cells per 55mm^2 dish. After three weeks, 6×10^4 naive WT ES cells subsequently shared media with the progeny of target ES cells for 5 days (to create primary bystanders). Primary bystanders were then cultured for another three weeks. Naive WT ES cells then shared media with the progeny of primary bystanders (to create secondary bystanders). Media was shared via co-culture, employing $1\ \mu\text{m}$ transwell inserts (Corning), or by exposure to conditioned media (filtered [$0.25\ \mu\text{m}$]; 1:1, fresh media:conditioned media). ES cells were exposed to ionizing radiation (3 Gy) using a Co-60 source (73 cGy/min). DNA damage was assessed by the alkaline comet assay (Olive, 2006) in primary (**b**), and secondary (**c**), bystanders. For all comet analysis, >100 nucleoids were analyzed per condition using Komet 5.5 (Andor Technology, Ireland) and P values were produced by a two-tailed Mann-Whitney. For comet studies, boxes represent the quartiles, whiskers mark the 10th and 90th percentiles, and the median is indicated. For all studies, data were combined from three or more independent experiments.

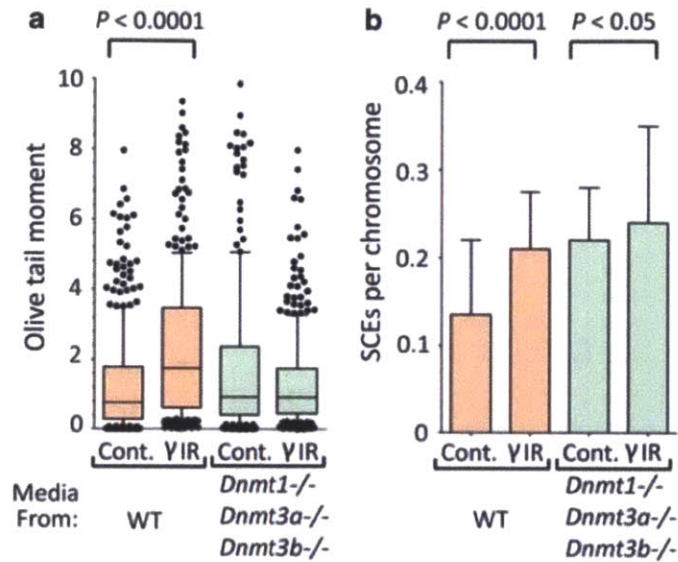


Figure 4-2. γ IR does not lead to the persistent induction of genomic instability in primary bystanders to *Dnmt1*^{-/-} *Dnmt3a*^{-/-} *Dnmt3b*^{-/-} cells. DNA damage by comet assay (a) and SCEs (b) in naive WT ES cells exposed to media from WT and *Dnmt1*^{-/-} *Dnmt3a*^{-/-} *Dnmt3b*^{-/-} cells. See Fig. 1 for experimental design. SCEs were counted for ≥ 80 spreads/condition as previously described (Engelward *et al.*, 1996b). For SCE studies, median with interquartile range is shown and *P* values were produced by a two-tailed t-test.

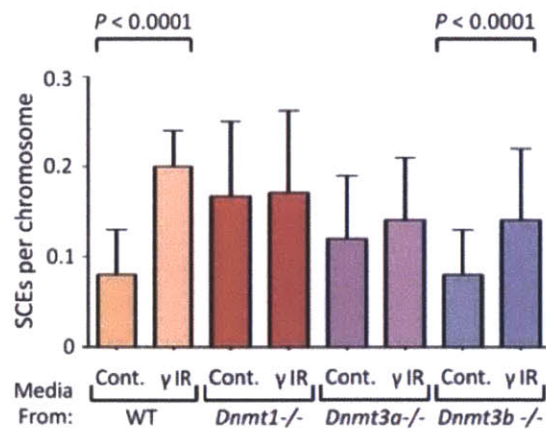


Figure 4-3. Dnmt1 and Dnmt3a are required for persistent induction of homologous recombination in naïve, primary bystander ES cells. SCEs in naïve WT ES cells exposed to media from γ IR (and mock irradiated) WT, *Dnmt1*^{-/-}, *Dnmt3a*^{-/-}, and *Dnmt3b*^{-/-} target ES cell populations. See captions from Figs. 1 & 2 for design and analysis.

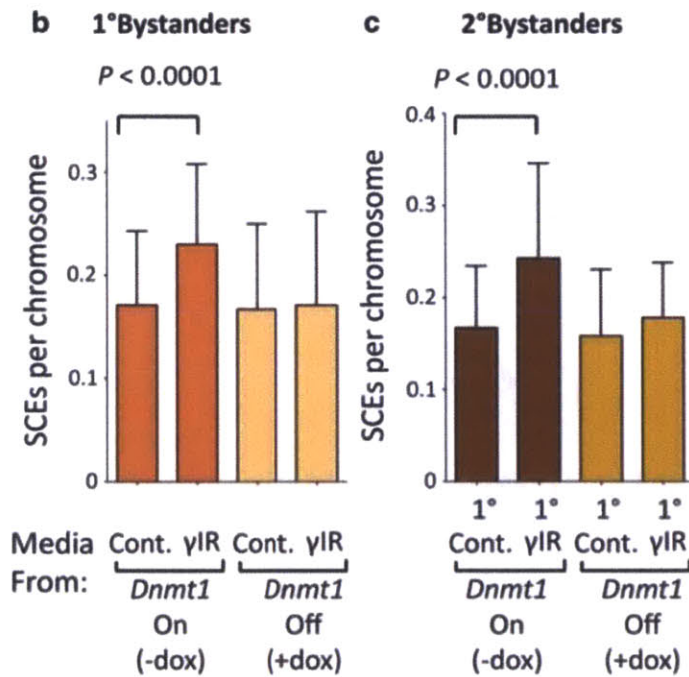
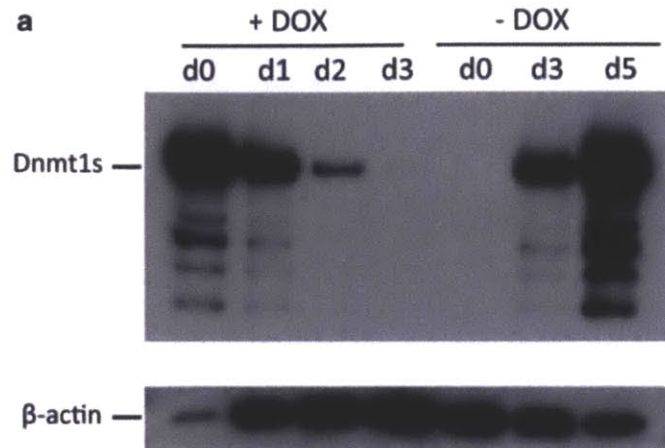


Figure 4-4. Transient suppression of Dnmt1 protects against radiation-induced genomic instability. **a**, Doxycycline-dependent repression of Dnmt1 in mouse ES cells assessed by Western blot. **b**, SCEs in primary bystanders to normal and Dnmt1 transiently-deficient cells. **c**, SCEs in naive (secondary bystander) cells exposed to media from primary bystanders to normal and Dnmt1 transiently-deficient cells. Data analysis as per caption for Fig. 2.

Chapter V

Conclusions, Future Work and Perspective

The role of reactive nitrogen and oxygen species (RONS) in the association between cancer and inflammation is complex (Balkwill, 2001; Coussens, 2002; Philip, 2004; Shacter, 2002). RONS damage DNA, modify the activity of cellular mechanisms to repair this damage, and communicate genomic instability to naive, bystander cells, via epigenetic mechanisms.

In this work, we investigated the role of the base excision repair scaffold protein XRCC1 in the repair of NO[•] and peroxynitrite-induced DNA damage. Further, we explored how XRCC1 interacts with another BER component protein, AAG, to mediate the response to NO[•]-induced DNA damage. We also developed the framework for a tool for the high-throughput measurement of DNA damage and for the measurement of base excision repair. Finally, we investigated the mechanism by which genomic instability can be propagated epigenetically, following exposure of cells to genotoxins.

In our investigation of the role of XRCC1 in mediating the response to RONS-induced DNA damage, we found that XRCC1 protects CHO cells from killing by peroxynitrite, and that this protection appeared to be independent of the levels of BER intermediates. This suggests a role for XRCC1 independent of BER. Indeed, XRCC1 has been implicated in non-homologous end joining (Ahmed *et al.*, 2010; Charbonnel *et al.*, 2010) and homologous recombination (Taylor, 2000; Zhou and Walter, 1998b). However, the fact that no difference is observed in the comet-detectable levels of damage in the XRCC1 WT compared to XRCC1 null cells, suggests there may be a DNA repair-independent mechanism at work. This is a possibility that warrants further study.

We also made observations consistent with previously published (Li *et al.*, 2006) results regarding the differential thresholds of different cell types to NO[•] exposure. We found that CHO cells repaired NO[•] differentially to human glioblastoma cells, resulting in

differential XRCC1-dependent BER-intermediate dynamics. In the CHO cells, the XRCC1 WT cells displayed an initial increase in the formation of BER-intermediates on exposure to increasing cumulative doses of NO[•]. In the human cells, the BER intermediate levels in XRCC1 deficient cells were indistinguishable from the levels in the WT cells, on exposure to increasing cumulative doses of NO[•]. In the human cells, elevated BER-intermediates were observed only in the case of the XRCC1 deficient and AAG over expressing cells. These observations, including the differences between CHO and glioblastoma cells, may be explained by differences in the specifics of interactions between BER proteins in each cell type in mediating responses to NO[•] exposure. Indeed, we found that in human cells, XRCC1 can facilitate AAG-mediated excision of the inflammation associated lesions εA and hypoxanthine, *in vitro*. However, in live cells XRCC1 is likely acting predominantly downstream of its interaction with AAG, in mediating the responses to genotoxins.

The implications for our findings suggest that the associations between polymorphic variants and malignancy may be independent of the role of XRCC1 in BER and or may be due to BER imbalances that are revealed by the differential capacity of XRCC1 to facilitate BER steps downstream of the BER glycosylase initiating step. This warrants more studies of the role of XRCC1 in cell biology more generally and warrants epidemiological studies in which BER pathway functional haplotypes are examined, as opposed to individual variants. For example, epidemiological studies should examine the association between malignancy and XRCC1 *and* Polβ polymorphic variants in individuals. It would therefore be the interaction between the BER pathway polymorphisms, and not any individual variant that would modulate cancer risk. This mode of analysis would be consistent with a model in which BER-intermediates are the mediators of inflammation associated genomic instability. Further

studies will also need to be done to formally test specific XRCC1 polymorphic variants for their ability to repair RON-induced DNA damage.

Additionally, our findings have implications for improved cancer therapeutics. The fact that the cells that displayed the highest BER-intermediates (AAGOE/XRCC1KD) also displayed the highest sensitivity to killing by MMS, suggests that therapeutic strategies that increase BER-intermediates may result in greater toxicity in cancer cells. For example, tumors could be profiled for their BER capacity and then agents, capable of exploiting any observed pathway deficiencies to increase BER-intermediates, could be chosen from preexisting pharmacopeia or rationally developed to generate synthetic lethality in these tumors.

We developed the framework for a tool for the high-throughput measurement of DNA damage and for the measurement of base excision repair. This device consisted of a micro-fabricated chip for cell micropatterning and subsequent single cell gel electrophoresis (comet assay) on arrayed cells. We demonstrated that this device, technical challenges notwithstanding, has potential applications in the measurement of macrophage and NO[•]-induced DNA damage and repair. Continuing work to overcome NO[•] trans-gel diffusion may allow this potential to be realized. Additionally, the adherent CoaCh approach may have utility in a later generation of the device: an operationally integrated chip allowing for cell loading, dosing and assessment of DNA damage and repair on-chip. Nevertheless, our efforts have culminated in a simple and scalable device that provides high throughput, quantitatively robust measurements of DNA damage and repair (Wood *et al.*, 2010).

We also investigated the mechanism by which memory of a genotoxic insult and subsequent genomic instability can be propagated epigenetically, following exposure of cells

to genotoxins, *e.g.* NO[•] and ionizing radiation. We found that two of the three major DNA MTases, Dnmt1 and Dnmt3a are responsible for the propagation of genomic instability following exposure to radiation (Rugo *et al.*, 2010). Continuing work will serve to fully define the precise molecular mechanisms mediated by the DNA methyltransferases, in communicating memory of genotoxic exposures. Nevertheless, knowledge of the molecular basis for transmission of the bystander effect suggests novel interventions, including the potential administration of Dnmt inhibitors in conjunction with cancer chemotherapy to preserve tissue-wide genomic stability and thus suppress secondary cancers. It is also tempting to speculate on the extent to which cellular memory of a genotoxic insult is similar to, and thus gives clues on, the molecular mechanisms of neurological memory.

In toto, this work addressed the role of BER, and specifically the role of the BER scaffold protein XRCC1, in mediating the cellular response to RON-induced DNA damage. This work also described the development of a chip based device for the measurement of DNA damage and repair. Finally, this work revealed a mechanism by which genomic instability can be propagated epigenetically, following exposure to genotoxins. Our findings therefore ultimately suggest ways in which inflammation-mediated cancer can be studied and impacted.

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