

**Molecular and Genetic Influence of HMGA1 Proteins on Nucleotide
Excision Repair**

By

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To the faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of
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Chair

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MOLECULAR AND GENETIC INFLUENCE OF HMGA1 PROTEINS ON NUCLEOTIDE EXCISION REPAIR

Abstract

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The family of architectural transcription factors known as the high mobility group (HMG) A1 proteins are both positive and negative gene regulators that bind to DNA and chromatin substrates in a structure-specific manner. It has previously been demonstrated that over expression of HMGA1 is a naturally occurring phenotype of many cancers. It has also been shown that over expression of transgenic HMGA1 proteins promotes neoplastic transformation and progression to malignant phenotypes. The following thesis describes the effects of HMGA1 over expression on functional nucleotide excision repair (NER) pathways, providing a potential link between HMGA1 abundance and the observed genetic instability of many human cancers. Specifically, evidence that over expression of HMGA1 proteins (1) decreases overall NER efficiency and (2) disrupts coordinate transcriptional regulation of genes involved in NER is described. In addition, the following thesis will describe preliminary evidence indicating that HMGA1 proteins also compromise base excision repair (BER) pathways. As NER and BER comprise the major cellular mechanisms by which genomic integrity is maintained, these results implicate HMGA1 proteins as a target for adjuvant chemotherapy in the treatment of cancer.

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CHAPTER 1

INTRODUCTION TO HMGA1 PROTEINS AND EXCISION REPAIR

High Mobility Group (HMG) proteins, so named due to their rapid migration in polyacrylamide gels under an electric field, can be divided into three readily identifiable families; (1) HMGA, (2) HMGB, and (3) HMGN. While all three families share many biochemical and biophysical features, each has their own characteristic functional motifs and is expressed naturally in distinctive ways in different mammalian cells and tissues (Bustin and Reeves, 1996). Each of these families also induces specific changes in DNA and chromatin, therefore differentially affecting cellular processes required in particular cells or stages of development in which these proteins are naturally expressed (Bustin, 1999).

All three HMG protein families are classified as ‘architectural transcription factors’ due to their ability to both positively and negatively regulate gene transcription by binding to DNA or chromatin in a structure-specific, rather than sequence-specific, manner. Upon HMG binding, alterations in DNA and chromatin substrates are observed, and because of this, HMG proteins have also been implicated in a diverse array of additional nuclear processes (Bianchi *et al.*, 2000; Postnikov *et al.*, 1999; Reeves, 2000; Reeves, 2001; Thomas, 2001; Bustin, 2001; Agresti *et al.*, 2003; Reeves and Beckerbauer, 2003). These include chromatin and nucleosome remodeling events, cell cycle-related chromosomal changes, genetic recombination, DNA replication and repair, apoptosis, and molecular chaperoning. Abnormal nuclear events in which HMG proteins have also been shown to play a role include cancer, retroviral integration, and cytokine signaling of tissue damage when released by necrotic and inflammatory cells.

While all three HMG protein families have been shown to influence DNA damage recognition and repair processes, each does so in a markedly different way. Again, characteristic differences in substrate specificity between families are thought to be the most prominent reason for these differential effects. Importantly for the work presented in this thesis are the observations that (1) HMGA proteins bind to the minor groove of AT-rich, B-form DNA and induce bends or other distortions in these substrates, and (2) HMGA proteins selectively bind to bulky, helix-distorting DNA structures, including Holliday junctions, supercoiled plasmids and UV-induced lesions. Both of these binding characteristics occur with a higher binding affinity than either the HMGB or HMGN family members.

HMGA1 Proteins

There are three members of the HMGA protein family, two of which are encoded for by the *HMGA1* gene (**Figure I-1**), located on chromosome 6p21 in humans. HMGA1a and HMGA1b are two distinct isoforms that result from alternative messenger RNA splicing, producing a 33 nucleotide, and thus, an 11 amino acid deletion in the latter. The third family member, known as HMGA2, is the product of the *HMGA2* gene. Each HMGA1 isoform is characterized by three DNA binding domains termed ‘AT-hooks’, which consist of consensus PRGRP sequences and are positioned at various distances from one another depending on the isoform (**Figure I-2**). There are also numerous protein binding regions within the primary sequence of the HMGA1 proteins, allowing them to interact with both DNA and other proteins, either simultaneously or separately.

Solomon *et al.* first described the *in vitro* ability of HMGA proteins to preferentially bind to the minor groove of any stretch of six or more AT bases in duplex DNA (Solomon *et al.*, 1986). Moreover, in solution, HMGA1 proteins exhibit little, if any, secondary structure and are able to undergo a variety of biochemical modifications, including, but not limited to, phosphorylation, acetylation, methylation and ADP-ribosylation (Elton, 1986; Elton and Reeves, 1986; Edberg *et al.*, 2004).

HMGA1 Proteins and Cancer

The HMGA1 proteins (formerly known as HMGI/Y) were first reported in 1983 by Lund *et al.* and in 1984 by Varshavsky *et al.*, in their respective analyses of rapidly proliferating HeLa and African Green Monkey cells. Both of these cell lines greatly over express HMGA1 proteins in contrast to the low or undetectable expression levels observed in most normal cell types. According to Ostvold *et al.*, the HMGA1 proteins are among the most highly phosphorylated proteins in the nucleus of HeLa cells, with marked differences in phosphorylation states between interphase and metaphase cells (Ostvold *et al.*, 1985). Additionally, Varchavsky *et al.* (1984) demonstrated that HMGA1 proteins were associated with AT-rich α -satellite sequences in African green monkey cells. Subsequent studies further deduced that HMGA1 proteins were responsible for positioning or ‘phasing’ nucleosomes on these AT-rich sequences (Wu *et al.*, 1983; Strauss and Varshavsky, 1984). These early studies prompted investigations demonstrating that over expression of HMGA1 proteins is one of the most consistent biochemical features observed in human cancers from various tissues (reviewed in Reeves and Beckerbauer, 2001), including but not limited to pulmonary hamartomas (Kazmierczak *et al.*, 1996; Rogalla *et al.*, 1996; Xiao *et al.*, 2001), uterine leiomyomas

(Sornberger *et al.*, 1999), lipomas (Tkachenko *et al.*, 1997; Kazmierczak *et al.*, 1998), and breast carcinoma (Dal Cin *et al.*, 1997; Baldassarre *et al.*, 2003), as well as thyroid, prostate and ovarian neoplasias (Chiappetta *et al.*, 1998; Tamimi *et al.*, 1993; Tallini *et al.*, 1999, and Galande *et al.*, 2002; Masciullo *et al.*, 2003).

At present, more than 160 reports describing the effects of HMGA1 proteins in cancer biology have been published, providing a growing body of evidence that these proteins influence cellular transformation processes, including acquisition of growth advantage, ability to avoid induced cell death or apoptosis, and, most importantly regarding the work presented here, decreased genomic integrity. In fact, previous work has demonstrated that transgenic mice over expressing HMGA1 develop aggressive lymphoid malignancies in a tissue-specific manner (Xu *et al.*, 2004). Furthermore, over expression of HMGA1 proteins induces neoplastic progression of MCF-7 human mammary epithelial cells to more malignant phenotypes, as assayed by ability to grow in an anchorage-independent manner in soft agar, as well as by the ability to form tumors in nude mice (Reeves *et al.*, 2001). Additionally, inhibition of HMGA1 protein translation prevented neoplastic transformation induced by either myeloproliferative sarcoma virus or Kirsten murine sarcoma virus (Berlingieri *et al.*, 1995) and caused death in thyroid anaplastic carcinoma cell lines ARO and FB1 (Scala *et al.*, 2000). Most importantly, in 2004, L.M. Resar and colleagues provided evidence that antisense inhibition of HMGA1 proteins prevented transformation of Burkitt's lymphoma cells, indicating HMGA1 as a potential oncogene *in vivo*.

From a molecular standpoint, there are several hypotheses which support the role of HMGA1 in neoplastic transformation and malignant progression. First, transcriptional

activation of other oncogenes and repression of tumor suppressor genes either through direct or indirect (i.e. DNA binding or protein-protein interactions, respectively) is possible given the nature of HMGA1 as an architectural transcription factor. This is supported by evidence implicating HMGA1 in the down-regulation of tumor suppressor genes BRCA1 in breast carcinoma and mouse embryonic stem cells (Baldassarre *et al.*, 2003) and caveolin-1 in breast carcinoma (Treff *et al.*, 2003). Additionally, HMGA1 has been shown to alter transcription of estrogen-responsive genes in breast cancer via a protein-protein interaction mediated pathway involving the estrogen receptor (Massaad-Massade *et al.*, 2002). Furthermore, recent evidence indicates that growth advantage, one of the three main characteristics of transformed cells, is stimulated by HMGA1 through positive regulation of the KIT-ligand promoter and sensitization to EGF activation of Ras/ERK signaling in breast cancer cells (Treff *et al.*, 2003; Treff *et al.* 2004). It is important to note that a majority of HMGA1-induced transcriptional regulation results from HMGA1 proteins contributing to the activity of numerous transcription factors, so-called ‘fine-tuning’ transcriptional events. This effect, while causing transcriptional changes that may not be overly dramatic, is highly reproducible and includes target pathways, such as NER, in which even minor disruption of tightly coordinated gene regulation, causes abnormal pathway functioning.

A second hypothesis involves disruption of normal DNA and chromatin processing caused by direct binding of HMGA1 proteins to AT-rich DNA regions. This hypothesis is supported by work demonstrating that HMGA1 participates in Topoisomerase II-mediated chromosomal rearrangements (Takaha *et al.*, 2002).

HMGA1 Proteins and DNA Repair

The first evidence implicating HMGA1 proteins in DNA repair included cDNA microarray analyses comparing transcript profiles of stably-transfected MCF-7 human mammary epithelial cells over expressing the HMGA1b protein with their non-expressing counterparts. These data indicated that a number of genes involved in excision repair and mismatch repair processes, as well as some genes involved in homologous recombination, non-homologous end-joining and double strand break repair were transcriptionally repressed in the presence of HMGA1 proteins (Reeves *et al.*, 2001; Treff *et al.*, 2004; **Figure I-3**). However, whether the observed down-regulation of these genes is a result of direct or indirect HMGA1 action on their respective promoter sequences has yet to be determined.

Interestingly, only one of the genes directly involved in NER, *xeroderma pigmentosa complementation group A (XPA)*, was found to be significantly down-regulated in HMGA1 over expressing MCF-7 cells. Coincidentally, *xeroderma pigmentosum (XP)* patients deficient in XPA protein expression display the most severe phenotype of this disease (Kraemer *et al.*, 1975). Additionally, other genes shown to be peripherally involved in NER efficiency, including *x-ray cross complementing gene 1 (XRCC1)*, *excision repair cross complementing group 1 (ERCC1)*, *ataxia telangiectasia mutated (ATM)*, and *breast cancer gene 1 (BRCA1)* were also down-regulated in the presence of HMGA1 proteins (Treff *et al.*, 2003; Baldessarre *et al.*, 2003). Importantly, decreases in these repair factors have been associated with compromised DNA repair in several systems (Caldecott *et al.*, 2003; Borrmann *et al.*, 2003; Shiloh and Kastan, 2001; Lombard *et al.*, 2005).

Furthermore, as mentioned earlier, the HMGA1 proteins preferentially bind to the minor groove of AT-rich regions of the DNA both *in vivo* and *in vitro*. NER, which presides over repair of bulky, helix distorting lesions, including those induced by ultraviolet (UV) light, often targets lesions in AT-rich regions. Thus, the ability of the HMGA1 proteins to bind to both AT-rich regions, as well as non B-form DNA presents a second possible mechanism for compromising NER efficiency by blocking repair factor access to lesion sites. This is supported by *in vitro* evidence indicating that HMGA1 binding is not affected by the presence of UV-induced DNA damage (Adair *et al.*, 2005). Moreover, repair of these HMGA1-bound fragments in *Xenopus* oocyte nuclear extracts was delayed significantly when compared to repair of the same UV-damaged fragment in the absence of HMGA1 proteins (Adair *et al.*, 2005).

Classically, cell lines and organisms deficient in a specific type of DNA damage recognition or repair are sensitive to DNA damaging agents whose induced lesions are normally processed by that particular pathway (Friedberg *et al.*, 1995). Coincidentally, both MCF-7 cells induced to express HMGA1 proteins, as well as naturally HMGA1-expressing Hs578T human breast tumor cells are more than twice as sensitive to UV-induced killing than are their non-expressing counterparts (Adair *et al.*, 2005).

Knockdown of HMGA1 protein levels in these cells by an RNA-interference-based method has been shown to abrogate this sensitivity. Moreover, Baldassarre *et al.* have also shown that over expression of HMGA1 proteins in both murine embryonic stem (ES) cells and stably-transfected MCF-7 cells sensitizes those cells to cisplatin-induced cell death (Baldassarre *et al.*, 2005). Intriguingly, as it has been previously established that normal HMGA1 protein expression occurs during embryonic development in both

humans and mice, it has also been demonstrated that murine and human embryonic stem (ES) cells are more sensitive to cisplatin, bleomycin and UV-irradiation (Ikeuchi *et al.*, 1981; Baldassare *et al.*, 2005). Furthermore, despite the fact that in the great majority of cases homozygous HMGA1 knockout is embryonic lethal, HMGA1^{-/-} ES cells have been extracted from pre-death embryos and grown in culture to show that sensitivity of ES cells to these damaging agents is reduced when HMGA1 is absent (Baldassarre *et al.*, 2005).

Together, these data provide substantial evidence that HMGA1 proteins interfere with normal NER processing. These data, in combination with recently published work describing increased proliferation of cells expressing HMGA1 proteins, indicate that expression of HMGA1 proteins in human tumor cells may not only be a marker of malignancy and metastatic progression, but may also play a role in the carcinogenic process.

Nucleotide Excision Repair

Maintenance of genomic integrity in human cells is the responsibility of several endogenous cellular mechanisms of DNA repair. Of these different mechanisms, the excision repair pathways are thought to be responsible for the majority of lesion removal, estimated to repair more than 10,000 lesions per cell per day (Ames *et al.*, 1993). The two pathways that constitute cellular excision repair are nucleotide excision repair (NER; **Figure I-4**) and base excision repair (BER; **Figure I-5**). In humans, repair of bulky, helix-distorting lesions, such as those induced by exposure to cisplatin or UV radiation, is carried out by the process of NER, while small base modifications, such as alkylations and some oxidative lesions, are repaired by BER. Additionally, there are designated

subpathways of both NER and BER, each being responsible for repair of lesions within a particular context, for example, transcriptionally active genes (Friedberg *et al.*, 1995).

NER, which includes more than 20 different proteins, involves 6 basic steps: (1) damage recognition, (2) binding of a multi-protein complex at the damaged site, (3) double incision of the damaged strand both 5' and 3' of the lesion site, (4) removal of approximately 24-32 nucleotides containing the damaged bases, (5) filling in the gap by DNA polymerase, and (6) ligation. The two subpathways of NER are transcription coupled repair (TCR) and global genomic repair (GGR), and while most repair factors are associated with both pathways, it is thought that those proteins involved in damage recognition are mutually exclusive (Friedberg *et al.*, 1995).

The basic NER machinery consists of the CSA and CSB proteins, proteins XPA-XPG, and several other factors such as basal transcription factor IIH (TF_{II}H). Both XPC (in complex with the hHR23B protein) and XPE are GGR-specific factors, while CSA and CSB are TCR-specific proteins. In each case, these mutually exclusive factors are involved in lesion recognition. While XPC-hHR23B lesion binding initiates GGR, TCR is thought to be initiated when RNA polymerase II stalls at a lesion site in the transcribed strand, and CSA and CSB are believed to contribute to displacement of the RNA polymerase to make room for the NER machinery (Friedberg *et al.*, 1995). Several groups have demonstrated that repair of damage in actively transcribed strands of genes occurs more rapidly than damage in non-transcribed strands or in non-gene regions (Bohr *et al.*, 1985; Balajee *et al.*, 1997; reviewed by Mitchell *et al.*, 2003). In both TCR and GGR, following damage recognition, TF_{II}H, which contains helicases XPB and XPD, binds and unwinds the DNA flanking the lesion site. Replication protein A (RPA), a

single-stranded DNA binding protein, then joins the NER machinery to stabilize the unwound strands. It is then thought that the XPA protein binds and ‘double checks’ the damage, aiding in lesion recognition (Lombard *et al.*, 2005). However, recent evidence indicates that first a single XPA molecule binds, followed by a second XPA molecule to form a homodimer which stabilizes the NER machinery and facilitates lesion recognition (Yang *et al.*, 2002; Liu *et al.*, 2005). Next, two structure-specific endonucleases, XPG and XPF (the latter in complex with ERCC1), make single strand incisions on either side of the lesion to release a 24-32 nucleotide fragment (Friedberg *et al.*, 1995). Repair is completed by filling in of the resulting gap by template-dependent DNA polymerization and ligation.

Importantly, deficiencies in NER factors confer various phenotypes including predisposition to cancer, premature aging and in some cases, neurological diseases (reviewed by Lombard *et al.*, 2005). Defects in CSA and CSB are associated with the inherited disorder Cockayne’s Syndrome (CS) in humans, a disease phenotypically characterized by mental retardation, severe postnatal growth failure, cachectic dwarfism, retinal degeneration, deafness and premature death (de Waard *et al.*, 2005). Patients deficient in the XPD protein suffer from trichothiodystrophy (TTD), a disease characterized by brittle hair and skin, some photosensitivity and a shortened life span (Bergmann and Egly, 2001). Most prominently, defects in the XP proteins XPA-XPG, all display phenotypes of xeroderma pigmentosum (XP), a severely debilitating disease characterized by extreme sensitivity to sunlight, greatly increased risk of skin cancer and, in some cases, neurodegeneration (Lindahl and Wood, 1999; Rapin *et al.*, 2000).

Furthermore, NER has been implicated in the removal of some oxidative DNA lesions, indicating complimentary activity with the BER pathway (Lombard *et al.*, 2005; de Waard *et al.*, 2003). Considering the ubiquitous occurrence of oxidative lesions, the biological impacts of this type of damage are significant. Specific evidence for this comes from work by de Waard and colleagues, who demonstrated that both *CSB*^{-/-} and *XPA*^{-/-} mice displayed cell type-specific hypersensitivity to oxidative damage (de Waard *et al.*, 2003). Furthermore, two different groups have published work demonstrating that *XPA*^{-/-} cells are deficient in repair of oxidative base lesions induced by free oxygen radicals formed via exposure to fluorescent light, hydrogen peroxide (H₂O₂) and γ -radiation (Sato *et al.*, 1993; Lipinski *et al.*, 1999).

The work presented in this thesis addresses whether or not NER is compromised in living cells in the presence of HMGA1 proteins. In Chapter II, data indicating that NER of cyclobutane pyrimidine dimers (CPDs) is indeed sub-maximal in human mammary epithelial cells induced to over express HMGA1 proteins, compared to their non-HMGA1-expressing counterparts, is presented. Additionally, evidence demonstrating that repair of the second most common UV-induced lesion, the 6-4 photoproduct, is delayed, but not incapacitated, is also presented. Chapter III then goes on to present work indicating that reduced levels of the NER factor XPA are exhibited by HMGA1-expressing cells, suggesting that overall NER inefficiency could be due to sub-optimal intracellular concentrations of this repair factor. Finally, Appendix I highlights preliminary data suggesting that BER of methylated bases may also be compromised in the presence of HMGA1 proteins in the human mammary epithelial cell line described above. Together, these data indicate that HMGA1 proteins may be contributing to the

increased rate of mutation displayed by various cancer cells that commonly over express these proteins via interference with genome maintenance processes.

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FIGURE LEGENDS

Figure I-1. (A) Intron/Exon organization of the human *HMGA1* gene. Functional domains of the translated protein, indicated by DNA binding domains (DBD) 1, 2 and 3 are separated into discrete regions. Black boxes represent exons, solid horizontal line represents intron sequences. (B) Diagram of the human HMGA1a protein. Horizontal black line represents full-length HMGA1a protein with rectangular boxes indicating the AT-hook (DNA binding) domains I, II and III, respectively. The site of alternative splicing is indicated at the bottom by a bracket, demonstrating the region of 11 amino acid residues that are deleted in the HMGA1b isoform.

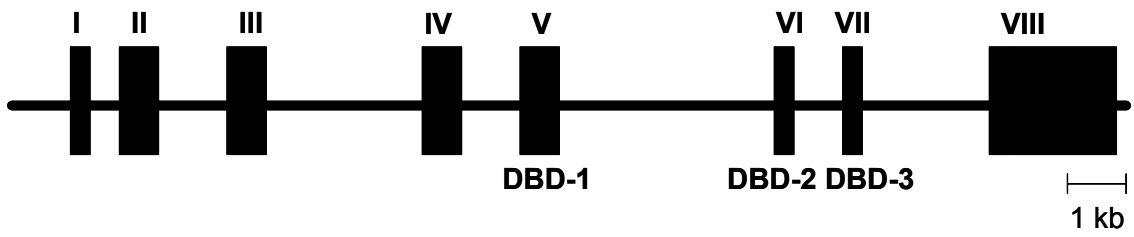
Figure I-2. Table of genes involved in DNA damage response, repair or recombination which are down-regulated by HMGA1-protein over expression. Abbreviations: ATRX, α -thalassemia/mental retardation syndrome X-linked (helicase II); BRCA2, breast cancer gene 2; CIB1, calcium and integrin binding 1 (calmyrin) gene; DSB, double-stranded break; GADD45gamma, growth arrest and DNA-damage inducible gene gamma; GS, genome stability; HR, homologous recombination; HUS1-like, human homolog of HUS1 (hydroxyurea sensitive; *S.pombe*); KIAA0658; gene of unknown function potentially involved in genome stability; MBD4, methyl-CpG-binding domain protein 4 DNA glycosylase; PRR, post-replication repair; RAD50, human homolog of *S. cerevisiae* RAD50 gene; RAD54, human homolog of *S. cerevisiae* RAD54 gene; RECQL4, RecQ-like protein 4 ATP-dependent DNA helicase gene; RECQL5, RecQ-like protein 5 ATP-dependent DNA helicase gene; TCR, transcription-coupled repair; UBE2B (RAD6B), ubiquitin-conjugating enzyme 2B gene; XPA, xeroderma pigmentosa group A complementing gene; XRCC1, x-ray repair cross complementing gene 1. (*Adapted from Reeves and Adair, 2005*).

Figure I-3. Alternative NER pathways. (A) Transcription-coupled repair (TCR) is characterized by damage recognition via a stalled RNA polymerase. TCR-specific repair proteins, Cockayne syndrome group A (CSA) and Cockayne syndrome group B (CSB), are recruited to the stalled polymerase and stabilize the open helical complex to allow for subsequent NER processing via helicase proteins XPB and XPD (components of transcription factor IIH (TF_{II}H)), XPA and single-stranded DNA binding protein, replication protein A (RPA), endonucleases XPG and XPF (in complex with excision repair cross complementing gene 1 (ERCC1)), which, collectively, catalyze removal of a 24-32nt stretch of single stranded DNA containing the lesion site. Synthesis and ligation to complete repair are then completed by DNA polymerase δ or ϵ , and DNA ligase I. (B) global genomic (GG) NER is characterized by damage recognition via the XPC-hHR23B protein complex. Upon damage recognition, subsequent NER processing occurs via the same mechanisms as those described above for TCR.

Figure I-4. Alternative BER pathways. (A) Short-Patch BER initiated by either bifunctional or monofunctional glycosylases results in an unmodified abasic (AP) site. Recognition of the unmodified AP site results in a one-nucleotide repair patch generated by a common ligation step requiring interaction of the scaffold protein x-ray cross complimenting protein 1 (XRCC1). (B) Long-Patch BER initiated by a monofunctional glycosylase resulting in a reduced or oxidized abasic site. Processing of this modified abasic site occurs via a proliferating cell nuclear antigen (PCNA)-dependent pathway and involves cleavage of a 2-8 nucleotide flap by the flap endonuclease 1 (FEN1) protein. In both pathways, initial incision of the damaged DNA strand is accomplished by either human AP endonuclease 1 (HAP1) or by DNA Polymerase β .

Figure I-1.

A



B

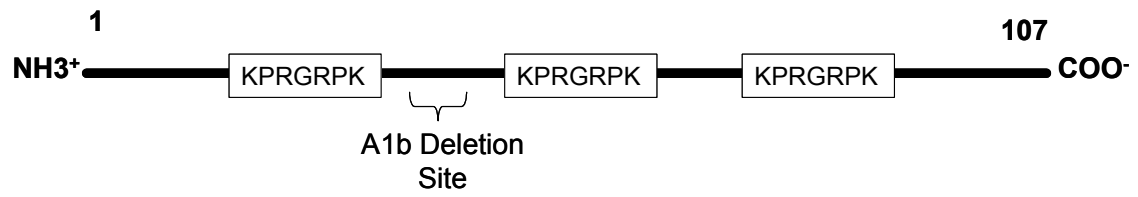


Figure I-2.

Gene	Fold Decrease	Affected Process/Mechanism
Affymetrix Human U133A Microarray Chip Triplicate Analyses (Treff & Reeves, 2003):		
XRCC4	-3.4	DSB/EJ repair
XPA	-2.6	NER (+/- TCR)
RAD54	-2.5	HR
BRCA2	-2.2	DSB repair via HR
RECQL5	-2.1	HR/GS?
MBD4	-1.9	BER
CIB1	-1.8	DSB repair/HR
XRCC1	-1.7	BER/SSB repair
HUS1	-1.7	BER (long patch)?
UBE2B (RAD6B)	-1.7	PRR
ATRX	-1.6	HR
RAD50	-1.6	DSB repair/HR
RECQL4	-1.5	HR/GS?
Results from Clonetech Atlas Human Cancer cDNA Expression Array (Reeves et al., 2001):		
DNase X	-7.5	DNA repair and recombination
DNA methyltransferase	-6.0	Reversal of O6-alkylguanine lesions
DNA ligase III	-4.8	BER (short patch)
ATM	-3.9	DSB response/repair

***(Taken from Reeves and Adair, 2005).**

Figure I-3.

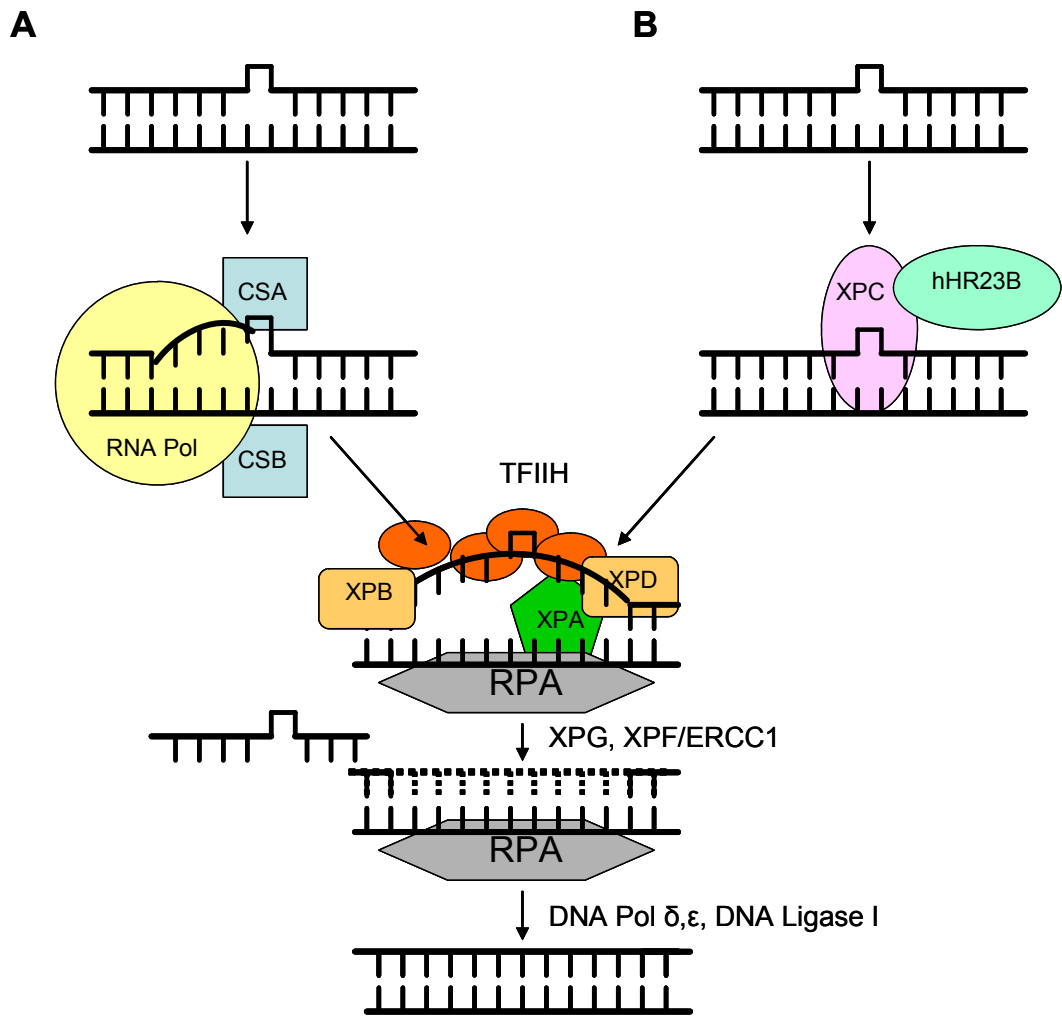
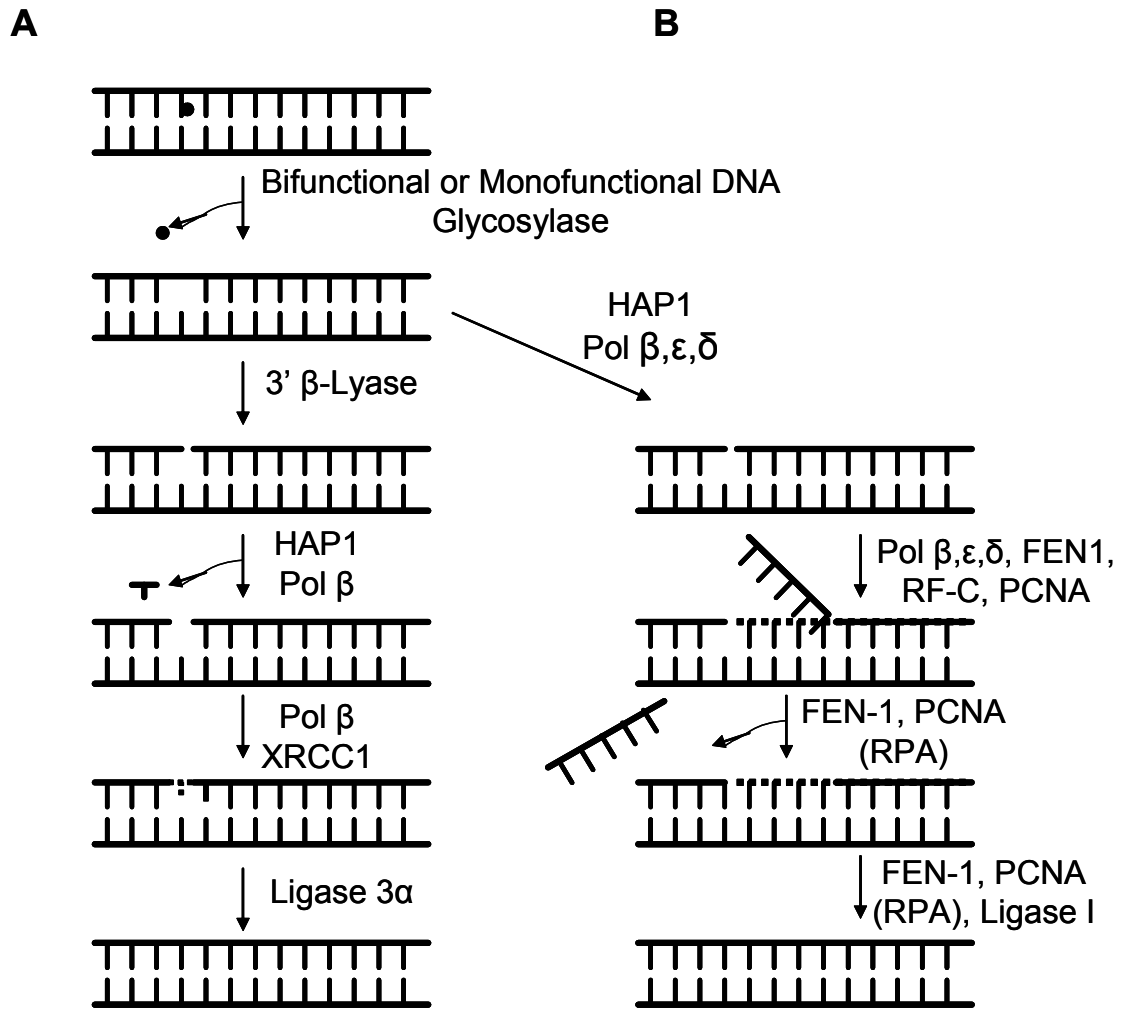


Figure I-4.



CHAPTER 2

HMGA1a NEGATIVELY INFLUENCES NUCLEOTIDE EXCISION REPAIR

Some data reported in this chapter are taken from accepted publication, and are in the format required for the specific journal in which they were accepted. (See figure references where noted)

Abstract

The mammalian non-histone “high mobility group” A (HMGA) proteins are the primary nuclear proteins that bind to the minor groove of AT-rich DNA. They may, therefore, influence the formation and/or repair of DNA lesions that occur in AT-rich DNA, such as cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts induced by UV radiation. Recent work, including cDNA microarray analyses demonstrating reduced levels of DNA repair protein transcripts in HMGA1-expressing cells, have implicated that these architectural transcription factors may also transcriptionally influence DNA repair processes, and thus maintenance of genomic integrity. Employing both stably transfected lines of human MCF7 cells containing tetracycline-regulated HMGA1 genes and human Hs578T breast tumor cells which naturally over express HMGA1 proteins, we show that cells over-expressing HMGA1a protein exhibit increased UV sensitivity. Moreover, we have demonstrated that knockdown of intracellular HMGA1 concentrations via two independent methods, tetracycline-mediated gene transcription knockdown and RNA interference (RNAi) targeting, abrogates this sensitivity. Most significantly, we observe that HMGA1a over expression inhibits global genomic nucleotide excision repair (NER) of UV-induced CPD lesions in MCF-7 cells. Together, these results suggest that HMGA1 directly influences repair of UV-induced DNA lesions in intact cells. Given the commonly observed abundance of HMGA proteins in a variety

of human cancers, these findings have important implications for the role that HMGA protein over expression might play in the accumulation of mutations associated with many types of human cancers.

INTRODUCTION

In most organisms, bulky helix-distorting DNA lesions, such as those caused by UV irradiation or treatment with DNA adducts, are repaired by the excision and replacement of 24-32 nucleotides of the damaged DNA strand through a process known as nucleotide excision repair or NER (de Laat *et al.*, 1999). Many factors, such as (a) the type of DNA damage, (b) the DNA sequence surrounding the lesion, (c) the position of the lesion within chromatin and (d) the interactions of the damaged DNA with DNA binding proteins, are known to affect the efficiency of NER (Pfeifer *et al.*, 1997; Balajee *et al.*, 2000; Smerdon and Conconi, 1999). For instance, nucleosomes have been shown to interfere with NER both *in vitro* and in intact cells (Liu and Smerdon, 2000; Kosmoski *et al.*, 2001; Wang *et al.*, 2003; Wellinger and Thoma, 1997; Hara *et al.*, 2000), presumably reflecting limited access of NER proteins to DNA lesions within these chromatin structures (Gontijo *et al.*, 2003). Certain DNA binding proteins such as transcription factor IIIA and HMGB1 proteins are also known to repress NER at their cognate sequences in a similar manner (Huang *et al.*, 1994; Conconi *et al.*, 1999; Malina *et al.*, 2002). Conversely, repair of transcribing genes is enhanced by association of both transcriptional activators and the RNA polymerase II elongation complex (Frit *et al.*, 2002; Svejstrup, 2002). The third family of HMG proteins, HMGN, has been shown to enhance repair of UV-induced DNA lesions *in vivo* via a DNA ‘unpacking’ mechanism that facilitates access of NER factors to lesion sites (Birger *et al.*, 2003).

Stable DNA photoproducts result from UV-induced covalent linkage between adjacent bases and are prototypes of helical distorting, bulky lesions (Douki *et al.*, 2000). Cyclobutane pyrimidine dimers (CPDs) are the most abundant, stable form of UV-induced DNA damage and, if unrepaired, are known to cause mutations and skin cancer (Pfeifer *et al.*, 1997; de Gruijl *et al.*, 2001). CPDs result from saturation of 5,6 double bonds on adjacent pyrimidines within the DNA sequence (Friedberg *et al.*, 1995). These lesions can alter DNA structure, causing bending of the helical axis and disruption of Watson-Crick base pairs at lesion sites (Park *et al.*, 2002) and have been shown to impede both RNA and DNA polymerases (Tornaletti and Hanawalt, 1999; Cordonnier and Fuchs, 1999). 6-4 photoproducts, which result from covalent linking of the 4 position on one pyrimidine to the 6 position on an adjacent pyrimidine, severely alter the DNA helix, and result in a T→C transition 85% of the time (LeClerc *et al.*, 1991; Kamiya *et al.*, 1998). Additionally, both of these UV photoproducts can inhibit transcription by out-competing normal promoters for transcription factors (Vichi *et al.*, 1997) or possibly by interrupting formation of the transcription initiation complex (Tommasi *et al.*, 1996; Liu *et al.*, 1997).

The HMGA1 proteins are non-histone chromosomal proteins that mediate a variety of biological functions, including regulation of gene transcription, modulation of chromatin structure, and induction of neoplastic transformation and malignant progression (reviewed by Reeves, 2001). HMGA1a and HMGA1b (alternatively spliced forms of the HMGA1 proteins) are members of the HMGA family and share distinct structural properties, such as three separate DNA-binding domains (called ‘AT-hooks’) and an acidic C-terminal domain (Reeves, 2001). The HMGA1 proteins have been shown

to bind a variety of DNA and chromatin substrates including the minor groove of AT-rich sequences (Solomon *et al.*, 1986), nucleosomes (Reeves and Nissen, 1993), supercoiled DNA (Nissen and Reeves, 1995), and four way junctions (Hill *et al.*, 1999).

Interestingly, the structure of AT-rich DNA is thought to be important for HMGA binding as opposed to sequence. HMGA binding has also been shown to induce changes in DNA structure which, during transcriptional activation, leads to formation of multi-nucleoprotein complexes referred to as “enhanceosomes” (Thanos and Maniatis, 1995) on regulatory promoter elements. Furthermore, HMGA1a is a proto-oncogene (Wood *et al.*, 2000) whose over expression is commonly observed in a large number of different naturally occurring cancers (Reviewed in Reeves and Beckerbauer, 2001) and whose experimental up-regulation induces neoplastic transformation and increased malignancy of neoplastic cells (Reeves, Edberg and Li, 2001; Xu, Y., 2004). Moreover, inhibition of HMGA1 protein translation prevented neoplastic transformation induced by either myeloproliferative sarcoma virus or Kirsten murine sarcoma virus (Berlingieri *et al.*, 1995) and caused death in several carcinoma cell lines (Scala *et al.*, 2000).

Consideration of these characteristics of CPDs and HMGA proteins, as well as evidence that levels of XPA and BRCA1, two proteins involved in NER, are reduced in HMGA1-expressing cells, led us to suspect that these proteins might participate in the process of NER or UV-induced damage formation at AT-rich sequences. To investigate this possibility, we examined the consequences of HMGA1 over-expression on the repair of 6-4 photoproduct and CPD lesions in intact cells. The UV sensitivity and efficiency of global genomic repair was investigated in stably-transfected MCF-7 human breast epithelial adenocarcinoma cell lines containing an inducible HMGA1 gene. The results

of these studies demonstrated that HMGA1 over-expression in human cells inhibits their ability to survive exposure to UV light and significantly reduces their efficiency of global genomic nucleotide excision repair of CPD lesions (Adair *et al.*, 2005).

Additionally, these effects were abrogated after tetracycline-mediated HMGA1 knockdown. Importantly, UV-sensitivity results were verified in a naturally HMGA1-expressing cell line of Hs578T human epithelial adenocarcinoma cells before and after RNA-interference (RNAi)-mediated HMGA1 knockdown.

Previously, a complex of the HMGA1 protein with the PRRII region of the IL-2R α gene promoter (John *et al.*, 1995) was employed as a model for studying the effects of these proteins on both DNA damage and repair in a cell free system. This investigation of the possible mechanism(s) involved in HMGA1 inhibition of NER included *in vitro* repair assays and electrophoretic mobility shift assays (EMSAs), and demonstrated that UV photoproducts do not markedly affect HMGA1 binding to lesion-containing DNA, but do change the CPD yield (Adair *et al.*, 2005). Most importantly, and in agreement with the results in living cells, it was found that repair of CPDs in the PRRII DNA-HMGA complex by *Xenopus* oocyte nuclear extracts is strongly inhibited by HMGA1 binding (Adair *et al.*, 2005). Taken together, these findings suggest that inhibition of DNA repair processes by HMGA proteins may contribute to the genetic and chromosomal instabilities commonly found in cancerous cells which, almost universally, over-express these proteins. Significantly, acquisition of mutations is related to activation of oncogenes and silencing of tumor suppressor genes, resulting in uncontrolled cell growth and ability to avoid apoptosis, two hallmarks of cancer cells. While previous evidence has indicated HMGA1 proteins in growth stimulation via

transcriptional regulation of caveolin 1 and Kit ligand, as well as stimulation of Ras/ERK signaling (Treff *et al.*, 2003; Treff *et al.*, 2004), this data provides a role for HMGA1 in promoting neoplastic transformation and malignant progression via interference of normal genome maintenance.

MATERIALS AND METHODS

Cell culture and cell cycle distribution analysis

The human breast adenocarcinoma cell line MCF7-tet (i.e., MCF7/Tet-OFF) (BD Biosciences) was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, and 100 µg/ml G418 (Research Products International). Clonal cell line MCF7-7C-Cs is a derivative of MCF7/Tet-OFF cells that has been stably transfected with a tetracycline-regulated pTRE vector encoding HA-tagged HMGA1a cDNA (Reeves *et al.*, 2001). This stably transfected line was maintained in the presence of 100 µg/ml hygromycin. The MCF7-7C-Cs cell line expresses high levels of HA-tagged HMGA1a protein when grown in medium lacking tetracycline (these are referred to as 'ON' cells). To prevent expression of the HA-tagged HMGA1 genes, the MCF7-7C-Cs cells were cultured in media containing 2 µg/ml tetracycline (and are referred to as 'OFF' cells). The Hs578T line of human mammary epithelial adenocarcinoma cells (ATCC Number HTB-126; American Type Culture Collection) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2mM L-glutamine. Prior to all experiments the levels of endogenous and HA-tagged HMGA1 proteins in test plate cell cultures were determined by Western blot analysis of cell extracts prepared with TRIzol (Life Technologies), using

either a monoclonal antibody against the HA-tag of transfected HMGA1 proteins (Reeves et al., 2001) or a specific polyclonal antibody against HMGA1 proteins (Reeves and Nissen, 1999). Western blots reacted with a polyclonal HRP-conjugated anti-actin rabbit antibody (Sigma, Co.) served as loading controls. Once over expression of HMGA1 protein was confirmed in test plates, parallel experimental cell cultures were harvested and fixed in cold absolute ethanol. Cells were then stained with 0.01 mg/mL propidium iodide (PI) for DNA content analysis by flow cytometry using a Becton Dickinson FACS caliber cytometer and Cell Quest Software.

Analysis of survival and colony forming ability of HMGA1-overexpressing cells following UV irradiation

Ten days post-induction of HMGA1 protein expression, cell cultures were analyzed for survival following UV irradiation. For UV exposure, media was removed from culture plates and cells were irradiated using low pressure Hg lamps (Sylvania, Model G30T8) at doses of 2, 10, 25, 50, and 100 J/m² (measured with a Spectroline DM-254N short wave UV meter) (Spectronic Corp.), with non-irradiated cells serving as a control. Cell survival post-irradiation was monitored via Trypan Blue (0.4%) exclusion and cell viability counting using a hemacytometer. Briefly, cells were first treated with trypsin/EDTA to remove monolayers, followed by centrifugation. Cell pellets were then resuspended in limited volumes of growth media and diluted 1:1 in 100 µl of 0.4% Trypan Blue dye. After 5 min, 20 µl of cell suspension was loaded onto a Levy Ultra Plane hemacytometer and the number of viable cells determined. Percent survival was calculated by comparison to non-UV-irradiated controls.

To evaluate the ability of cells to proliferate after UV exposure, approximately 2.5 x 10⁵ stably-transfected MCF-7 cells were plated in 100-mm cell culture dishes

containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, and 100 µg/ml G418. Cells in the exponential growth phase were then either irradiated at one of several UV doses (2, 10, 25, 50 or 100 J/m²) or left unirradiated (control). Following irradiation, cells were harvested and re-plated in the appropriate medium at a concentration of 1000 cells per 100-mm dish. After 10 days, colonies were stained with crystal violet (0.1% in 95% ethanol) and scored using a light microscope. Colony counts (number of colonies containing 50 cells or more) in UV treated samples were compared to number of colonies formed in non-UV-irradiated plates to determine percent of colony formation.

RNAi-mediated HMGA1 knockdown analyses

All cell cultures were maintained as described above for survival determination. Once cells reached 50% confluence, media was replaced with antibiotic free medium for 24 hours. Cells were then transfected with 20nM siRNA targeting the HMGA1 gene (Santa Cruz Biotechnology) using Lipofectamine 2000[®] (Invitrogen) according to the manufacturer's instructions. 48 hours post-transfection, cells were assayed for HMGA1 protein levels via Western blotting and survival analyses after UV exposure were repeated following the protocol described above. Again, percent survival was calculated by comparison to non-UV-irradiated controls.

Global Genomic Repair of CPD Lesions in Living Cells

Both MCF-7-tet ('OFF') and MCF7-7C-Cs ('ON') cells were cultured as described above. Once cells reached confluence, as determined by light microscopy, 2 mM hydroxyurea (HU) was added to each plate for 1 hr to suppress residual cell cycle

progression. After HU treatment, media was removed and cells were irradiated at 10 J/m². Media was then replaced and cells were incubated under standard conditions described above (see survival determination methods) for various times post irradiation to allow repair to occur. At the desired time post irradiation, genomic DNA was isolated from cells by published methods (Sambrook *et al.*, 1989). Briefly, cells on each plate were resuspended in 1 ml of 0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, 2 mM EDTA, 0.1% NP-40, and 60 mM Tris-HCl (pH 8.0). The mixture was transferred into a microcentrifuge tube and incubated on ice for 5 minutes. Nuclei were then pelleted by centrifugation at 1000 g for 5 min, washed once with buffer without NP-40, and resuspended in 1.5 ml of 150 mM NaCl and 5 mM EDTA (pH 8.0). Nuclei were incubated with proteinase K (final concentration: 0.3 mg/ml) at 37°C for 1 hr, after which 0.15 mg DNase-free RNase A was added and incubation continued for an additional 30 min. Finally, DNA was isolated by phenol extraction and ethanol precipitation.

DNA samples (5 µg) were treated with T4 endonuclease V (T4 endo V) to generate single-strand breaks at CPD sites and ~5 µg of DNA per sample were separated on 1% alkaline agarose gels at 20 V for 20 hours at room temperature. Gels were neutralized in 1 M Tris, 1.5 M NaCl, pH 7.0 for several hours at room temperature before DNA was depurinated (0.25 N HCl for 30 min) and transferred to nylon membranes in 0.4 N NaOH overnight. Radioactive probes were generated from *Eco*RI-digested MCF-7 cell genomic DNA followed by random priming (RadPrime™, Invitrogen). Quantification of CPDs was performed on data from phosphorimages of the Southern

blots, using ImageQuant software and number-average DNA length analysis (Bespalov *et al.*, 2001).

Immunocytochemical analyses

For immunocytochemical analyses, MCF-7 cells both induced to over express HMGA1a proteins as well as parental MCF-7 cells were cultured on sterile 22mm x 22mm glass coverslips under standard culture conditions, as described above, until 50-60% confluent. Coverslips were then irradiated at 20J/m² and were either fixed immediately, or were replaced in culture media and incubated to allow repair to occur. At various times post-irradiation coverslips were fixed in ice cold absolute methanol for 20 minutes at -20°. Methanol was then removed and coverslips were washed in 1X PBS for five minutes before permeabilization using 1X PBS + 0.1% Triton X-100 for five minutes. Coverslips were washed three times in 1X PBS and subjected to 30 minutes in 2M HCl to depurinate and denature double-stranded DNA. This was followed by a five minute wash in 1M Borate Buffer (pH 8.0) and three washes in 1X PBS for five minutes each. Coverslips were blocked by adding 5% Biotin in PBS and incubating at room temperature for 30 minutes. A primary antibody against CPDs (TDM2) or 6-4 photoproducts (TDM1) was added to blocking solution in a 1:500 dilution and incubated for one hour at room temperature. In co-localization assays, MR19 antibody (against HMGA1) was also added to the primary incubation at a dilution of 1:500. Coverslips were then washed three times for five minutes each with 1X PBS + 0.1% Tween 20 before addition of anti-mouse Oregon Green-conjugated secondary antibody (and anti-rabbit Texas Red-conjugated secondary antibody in co-localization assays) was added at a 1:1000 dilution in blocking solution, and incubated at room temperature for one hour.

Coverslips were then washed again for five minutes each in 1X PBS + 0.1% Tween 20 before mounting on sterile glass microscope slides in mounting media containing DAPI. Confocal microscopy was used to visualize cell nuclei via DAPI fluorescence, as well as staining of 6-4 photoproducts and HMGA1.

RESULTS

Over expression of HMGA1 Proteins Inhibits NER in MCF7 Cells

Mammalian cells that are deficient in various aspects of DNA repair exhibit, in most cases, increased sensitivity to DNA-damaging agents (Friedberg *et al.*, 1995). To gain insight into whether or not the HMGA1 proteins play a role in NER of UV-induced lesions *in vivo*, the UV sensitivity of genetically engineered human cell lines was examined by two methods, colony forming ability and dye exclusion after UV exposure. For these studies, human MCF-7 mammary epithelial cell lines were used that contain stably integrated, tetracycline-regulated genes coding for either the HMGA1a or HMGA1b isoform proteins (Reeves *et al.*, 2001). The endogenous levels of HMGA1 proteins in these MCF-7 cells are very low (Reeves *et al.*, 2001), and induction of transcriptional expression of the HMGA1 genes is controlled by removing tetracycline from the cell culture medium. Western blot analysis has demonstrated that, within 10 days of inducing the cells to express stably integrated HMGA1 gene products, the intracellular concentration of HMGA1a is greatly increased (>10-fold) in the HMGA1a 'ON' cells, compared to the concentration in HMGA1a 'OFF' cells. Quantitative assessments have also demonstrated that, after longer periods of induction, the amount of HMGA1a protein in the 'ON' cells can reach up to ~40-fold higher than in the 'OFF' cells (Treff *et al.*, 2004). It should be emphasized that the range observed in many

naturally occurring human tumors is between 15 to >50-fold more than normal cells (Bustin and Reeves, 1996) and thus data presented here represent effects seen within the physiological range of HMGA1 protein over expression found in various cancer cells.

The viability of cell lines was monitored by colony forming assay after exposure to various doses of UV radiation and the % colony forming ability determined using non-irradiated cell colonies as controls (**Figure II-1, Panel A**). At UV doses from 2 J/m² to 100 J/m², MCF-7 cells over expressing HMGA1a proteins were less capable of forming viable colonies (50 cells or more) when compared to MCF-7 cells not over expressing these proteins (i.e. ‘OFF’ cells). The greatest differences in viability occurred at UV doses of 10 and 25 J/m², which induce the physiological dose equivalent of CPDs observed in human cells after only a few minutes exposure to solar UVB on a bright summer day (e.g., see Douki *et al.*, 2000 and references therein).

Given previous data demonstrating induction of cell cycle progression by HMGA1 proteins (Treff *et al.*, 2004), flow cytometric analysis was employed to determine if cell cycle distributions between the two cell lines were different (**Figure II-1, Panel B**). No significant differences in the number of gated cells at each cell cycle stage were observed, indicating that while HMGA1 over expressing cells do exhibit a faster doubling time, the cell cycle distribution of ‘ON’ cells in a random population is the same as that of ‘OFF’ MCF-7 cells containing only very low levels of endogenous HMGA1 proteins.

To further investigate UV sensitivity in these cell lines, a trypan blue dye exclusion assay was employed to monitor cell viability (**Figure II-2, Panel A**). With this assay, cells are not required to attach to culture dishes and traverse the cell cycle as in

colony forming assays, but are simply required to survive a UV exposure (as assessed by exclusion of the dye). Similar to the colony forming assay, cells were cultured to induce expression of the HMGA1 gene and then irradiated at different doses of UV (2-100 J/m²). After UV irradiation, cells were given 96 hours to ‘recover’ before survival was measured and compared to non-irradiated cells as a control. At all UV doses examined, cells expressing HMGA1a displayed decreased survival after UV exposure (**Figure II-2, Panel A**). Indeed, between doses of 10 and 100 J/m², ‘ON’ cells were as much as 50% more sensitive to UV damage compared to ‘OFF’ cells. Furthermore, survival of ‘OFF’ cells was the same as for the empty-vector transfected parental MCF-7 cells (**Figure II-2, Panel C**). These results indicate that MCF-7 cells over-expressing HMGA1 proteins exhibit a phenotype characteristic of mammalian cells (partially) deficient in NER of CPD lesions (Friedberg *et al.*, 1995). Additionally, to investigate the generality of these results, this same experiment was repeated using the human carcinoma cell line, Hs578T, that naturally expresses high levels of endogenous HMGA1 proteins (Reeves *et al.*, 2001), and these cells also exhibit increased sensitivity to UV exposure (**Figure II-2, Panel A**). It is important to note that, while considered a less stringent estimate of cell viability, the UV sensitivity of HMGA1-expressing cells determined by trypan blue exclusion was similar to that observed using the colony forming assay.

To determine if knockdown of HMGA1 proteins in over-expressing cells could reverse the UV sensitivity effects observed, both an RNAi-based approach as well as a tetracycline depletion approach were employed (**Figure II-2, Panels B and C**). Hs578T cells were transfected with 20 nM commercially available siRNAs targeting the HMGA1 transcript and cell survival after UV irradiation was again monitored. Western blot

analysis after transfection revealed a 2.5-fold decrease in the concentration of HMGA1a protein in these cells. This corresponded to an expression level equivalent to 2-fold over normal MCF-7 cells. Importantly, this modest decrease in the amount of intracellular HMGA1a in over expressing Hs578T cells nevertheless resulted in a significant increase in their ability to survive moderate dose UV exposures (**Figure II-2, Panel B**).

To verify survival results obtained in Hs578T cells using RNAi-mediated knockdown of HMGA1, a tetracycline depletion approach was used as an independent means of knocking down HMGA1 levels in stably transfected cells (**Figure II-2, Panel C**). In this set of experiments, HMGA1-transfected MCF-7 cells were cultured in the presence of 2.5ug/mL tetracycline to suppress HMGA1 expression. As a control to insure that observations were not due to tetracycline-related effects other than HMGA1 knockdown, normal MCF-7 cells were also cultured in the presence of tetracycline. Ten days post tetracycline treatment, when Western blot analysis demonstrated a complete depletion of HA-tagged HMGA1 proteins from the cells, cell survival after UV irradiation was again monitored. The UV survival of HMGA1-transfected MCF-7 cells (after knockdown) was equivalent to survival of normal MCF-7 cells (**Figure II-2, Panel C**), and no significant effects from adding tetracycline were observed in normal MCF-7 cells.

The ability of the 'OFF' and 'ON' cell lines to repair UV-induced CPDs was directly examined by adapting a sensitive, quantitative method developed for measuring the efficiency of global genomic DNA repair in yeast (Bespalov *et al.*, 2001) for use with human MCF-7 cells. In this assay, a CPD-specific endonuclease (T4 endo V) that makes single-strand cuts 5' to CPD sites (Latham and Loyd, 1994) is employed to monitor

strand breaks remaining in genomic DNA as a function of repair time. As shown by the representative blots in **Figure II-3, Panel A** for ‘OFF’ and ‘ON’ cells, respectively, at each time point genomic DNA is isolated from cells and treated (+) or not treated (-) with T4 endo V, separated on denaturing agarose gels and transferred to a nitrocellulose membrane by a standard southern blot protocol. Random hybridization probes are then used to detect all genomic DNA sequences with minimal sequence bias. Analysis of scans of the phosphorimage signal profiles of the heterogeneous populations of DNA fragments on the membrane can be integrated to determine the number-average size of DNA fragments in each lane (Bespalov *et al.*, 2001). As illustrated in **Figure II-3, Panel A**, genomic DNA isolated from cells immediately after UV irradiation (0 hr repair) and digested with T4 endo V (+ lane) migrates as a smear of heterogeneous single stranded fragments whose ensemble average size [indicated by an asterisk (*)] can be accurately determined (see Bespalov *et al.*, 2001 and references therein). This is in contrast to the DNA not treated with T4 endo V, which migrates only a short distance into the gel (**Figure II-3, Panel A**, 0 hr T4 endo V (-) lanes). Importantly, at various times following irradiation, when the cells undergo global genomic repair, the number average size of T4 endo V digested DNA fragments increases as a consequence of the removal of CPD lesions (**Figure II-3, Panel A**, compare + T4 endo V lanes). Moreover, repair is nearly complete by 24 hr post-irradiation and the DNA fragments approach the migration of undamaged DNA near the top of the blot (**Figure II-3, Panel A**, compare the dashed lines connecting the asterisks to the UV- lanes).

The representative scans of gel lanes (0 hr and 3 hr post-irradiation) show that the rate of NER removal of CPD lesions from genomic DNA is significantly retarded in the

'ON' cells over expressing HMGA1 protein (**Figure II-3, Panel B**). In fact, differences in repair capacities between 'ON' and 'OFF' cells were evident at all time points examined (**Figure II-3, Panels B and C**). The difference in repair capacities, however, is most obvious at 3 hr post UV exposure (**Figures II-3, Panel C**), where 'OFF' cells have repaired approximately 66% of induced CPDs, while 'ON' cells have repaired < 5% of these lesions. Furthermore, in contrast to *in vitro* repair results (Adair *et al.*, 2005), the observed difference in repair completion at 24 hr (~17%) remains for at least 96 hr post UV exposure, reaching the limit of repair detection by this method. In addition, based on the average number of CPDs per kb calculated at time 0 hr by this method (0.95 ± 0.17 for 'OFF' cells and 0.91 ± 0.20 for 'ON' cells), there is no indication that over expression of HMGA1 proteins alters the amount of DNA damage induced by this UV dose (10 J/m^2). This was also shown by immunocytochemical analyses employing a CPD-specific antibody, wherein there were no statistically significant differences in the average fluorescence intensity of HMGA1 'OFF' and 'ON' cell nuclei immediately after a 10 J/m^2 dose of UV radiation (**Figure II-4**).

As the previously described method for quantitating removal of CPDs does not account for 6-4 photoproduct removal, disappearance of these lesions was also monitored via immunocytochemical analyses. As indicated in **Figure II-5**, repair of 6-4 photoproducts does appear to be slightly delayed in HMGA1-expressing cells, albeit not nearly as exaggerated as in CPD removal (**Figure II-4**). Moreover, no 6-4 photoproducts were detectable by this method at six hours post-UV exposure in either cell line, indicating that complete 6-4 photoproduct repair was achieved.

To preliminarily assess whether HMGA1 was associated with either of these lesion types in MCF-7 cells over expressing these proteins, co-localization assays were undertaken (**Figure II-6, and Figure II-7**). In these studies, it appeared that HMGA1 was associated with some 6-4 photoproduct foci, but not with CPDs, in HMGA1 ‘ON’ cells. This suggests that HMGA1 may be bound to 6-4 photoproduct lesions, but not CPDs, in living cells exposed to UV radiation. Nevertheless, these data provide the first evidence linking over expression of HMGA1 proteins with compromised repair efficiency in the chromatin of living cells.

CONCLUSIONS

In this study, we demonstrate that a stably transfected line of human MCF-7 cells, as well as a human adenocarcinoma cell line (Hs578T), over expressing HMGA1 proteins exhibit an increase in UV sensitivity and a decrease in cell viability after UV exposure, phenotypes characteristic of cells deficient in NER. Over expression of even relatively low levels of HMGA1 proteins in these cells increases their sensitivity to UV-induced killing (**Figures II-1 and II-2**). Moreover, removal of CPDs from total genomic DNA of the MCF-7 cells over expressing stably transfected HMGA1 is significantly repressed (**Figures II-3 and II-4**). Together, these results demonstrate for the first time that over expression of HMGA1 proteins in cells inhibits repair of UV-induced DNA lesions in intact cells.

Regarding the potential molecular mechanism of NER inhibition by HMGA1, previous *in vitro* analyses using *Xenopus* oocyte extracts to repair a known HMGA1-binding site on the Positive Regulatory Region II (PRRII) of the Interleukin 2 receptor alpha (*IL2R α*) gene, have demonstrated a lag in NER when HMGA1 is bound (Adair *et*

al., 2005). The results from these *in vitro* experiments strongly suggest that the observed inhibition of global genomic repair of UV-irradiated intact cells that are over-expressing HMGA1 (**Figures II-3 and II-4**) is likely due, at least in part, to direct binding of these proteins to UV-induced lesions, inhibiting access of repair-associated proteins (Adair *et al.*, 2005). This is further supported by additional immunocytochemical data indicating that HMGA1 proteins co-localize with 6-4 photoproduct foci within MCF-7 nuclei (**Figure II-6**). Nevertheless, we cannot exclude the possibility that HMGA1 proteins in these cells may be directly interacting with repair factors thereby sequestering them from repair activities. Likewise, the HMGA1 proteins may negatively regulate transcription of certain genes which code for repair factors, also resulting in decreased repair efficiency (Reeves *et al.*, 2001). Consistent with this latter possibility, microarray and western blot analyses indicate that a pivotal NER factor, XPA, is down-regulated in stably transfected MCF-7 cells over expressing HMGA1 proteins, compared to their non-HMGA1-expressing counterparts (**See Chapter III, Figure III-3**). These data provide some clarity to an apparent discrepancy between *in vitro* repair assays (Adair *et al.*, 2005), in which all CPDs were eventually removed from HMGA1-DNA complexes, and repair analyses performed in living cells (**Figures II-3 and II-4**). In the latter case, cells that are over-expressing HMGA1 exhibit not only a delay in the onset of CPD repair following UV exposure, but also lag behind control, non-over-expressing cells in their extent of CPD repair even 24 hours after irradiation. Interestingly, based on immunocytochemical data, repair of 6-4 photoproducts also exhibits a delay in HMGA1-expressing MCF-7 cells, but does, in fact, reach completion within six hours, as does repair of 6-4 photoproducts in non-HMGA1-expressing cells (**Figure II-5**). Previously,

Cleaver *et al.* demonstrated that decreased levels of functional NER factors, such as XPA, repair of 6-4 photoproducts is successful while repair of CPDs is compromised (Cleaver *et al.*, 1995). It is generally thought that 6-4 photoproducts induce greater helical distortion, and promote higher mutation frequencies, than do CPDs, and thus, are recognized and repaired at a faster rate (LeClerc *et al.*, 1991; Friedberg *et al.*, 1995; Cleaver *et al.*, 1995). This proposed extra-helical distortion induced by formation of 6-4 photoproducts could provide a possible explanation for co-localization analyses indicating that HMGA1 is associated with these lesions in living cells, but not with CPDs, given the ability of HMGA1 to bind to non-B-form DNA (**Figures II-6 and II-7**).

HMGA1 has been proposed to be involved in alterations of chromatin structure that may occur by competing with linker histones (H1) for DNA binding and/or by helping to recruit chromatin remodeling complexes (Reeves, 2001). It is therefore possible that HMGA1 can modulate DNA repair in a different fashion than observed here when DNA damage is folded into a nucleosome. Indeed, a recent report by Birger *et al.* shows that the HMGN1 protein (a member of a separate HMG family) actually *promotes* NER in intact cells, possibly by facilitating access to chromatin DNA. Like HMGN1, HMGA1 also has a high affinity for nucleosome core particles (Reeves and Nissen, 1993) and, upon binding, induces localized changes in chromatin structure that could facilitate gene transcription (Reeves and Wolffe, 1996). However, the types of alterations in chromatin structure induced by these structurally distinct HMG proteins may be quite different from each other since they have opposite effects on the efficiency of NER in chromatin of living cells. Thus, our finding that HMGA1 inhibits NER needs to be investigated further using nucleosomes and chromatin as binding substrates of HMGA1.

Moreover, HMGA1 is subject to extensive posttranslational modification during apoptosis and the cell cycle (Reeves and Nissen, 1995; Diana *et al.*, 2001). Since modification of AT-hook domains is known to decrease binding affinity for its cognate DNA binding sites (Nissen *et al.*, 1991), it will be important to also examine how modification of HMGA1 proteins modulates DNA repair.

Finally, greatly increased levels of expression of both HMGA1 transcripts (Nacht *et al.*, 1999) and proteins (Reeves, 2001) are consistently observed features of many naturally occurring cancers (including breast, prostate, lung, thyroid, cervical, colorectal and pancreatic cancers and leukemias), with increasing HMGA1 levels directly correlating with increasing malignancy and metastatic potential of the tumors (reviewed in Reeves and Beckerbauer, 2001). Additionally, chimeric proteins with AT-hook domains are produced as a result of chromosomal rearrangements in a variety of benign mesenchymal tumors including lipomas, leiomyomas, and endometrial polyps as well as others (Tallini and Dal Cin, 1999). The results of the present study suggest that the elevated levels of HMGA1 found in cancer cells may significantly increase their likelihood of accumulating genomic damage over time. Thus, the association of DNA repair and HMGA1 could be important for the acquisition of genetic and chromosomal instabilities that are frequently associated with tumor progression and increased metastatic potential of cancers. In this connection, it has recently been suggested that elevated levels of endogenous HMGA1 proteins are closely associated with the appearance of chromosomal rearrangements in prostate cancer cells (Takaha *et al.*, 2002).

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FIGURE LEGENDS

Figure II-1. MCF-7 cells over expressing HMGA1a proteins are less able to form colonies after UV exposure, despite normal cell cycle distribution. (A) Individual plates of MCF-7 cells either induced or not to over express HMGA1 proteins were exposed to a dose of UV radiation between 2 and 100J/m² and then harvested and replated to allow viable cells to form colonies. Colonies were visualized and counted 12 days after exposure and were compared with non-irradiated colonies as a control. Data represent the mean (\pm 1 S.D.) of three independent experiments for each cell line. (B) Cell cycle distributions of MCF-7 cells over expressing (ON, open bars) or not over expressing (OFF, solid bars) HMGA1 proteins. Bars represent the mean of three independent fluorescence-activated cell sorter analyses of each line (\pm 1 S.D.).

Figure II-2. Cells over expressing HMGA1 proteins are more sensitive to UV irradiation. (A) Confluent HMGA1-transfected MCF-7 cells, cultures under experimental conditions to either produce (ON) or not produce (OFF) HMGA1 proteins, as well as naturally HMGA1-expressing Hs578T cells, were exposed to UV radiation at various doses. Surviving cells were counted 96 hours post-irradiation, and the fraction of viable cells (as determined by trypan blue exclusion) relative to the non-irradiated controls was determined as a function of UV dose. Data represent the mean (\pm 1 S.D.) of six independent experiments with OFF (black bars), ON (white bars), and Hs578T (gray bars) cells at UV doses from 2-100J/m². (B) RNA interference-mediated knockdown of HMGA1 proteins in Hs578T tumor cells decreases UV sensitivity at moderate UV doses. HMGA1 concentrations in short interfering RNA-transfected (20nM) cells were 2.5-fold lower than in non-transfected Hs578T cells. Solid line and filled squares represent original survival data for Hs578T cells (from Panel A), and dashed line and open squares represent the mean (\pm 1 S.D.) of three independent short interfering RNA-transfected cell experiments. (C) Tetracycline-mediated depletion of HMGA1 proteins in transfected MCF-7 cells abrogates UV sensitivity at all UV doses examined. No HMGA1 proteins were detectable by western blot analysis after depletion. Solid line and filled squares represent normal MCF-7 cells cultured in the presence of tetracycline (2.5ug/mL), dashed line and open squares represent tetracycline-depleted MCF-7 cells. Both lines represent the mean (\pm 1 S.D.) of three independent experiments for each cell line.

Figure II-3. Global genomic NER of CPD lesions is inhibited in MCF-7 cells over expressing HMGA1 proteins. (A) Southern blot of genomic DNA isolated from non-HMGA1-expressing (OFF) and HMGA1-expressing (ON) MCF-7 cells after UV irradiation (10J/m²). Time indicates the number of hours, post UV exposure, when cells were harvested and assayed for repair. Positions of the ensemble average size of DNA in each lane are shown with asterisks and are connected by a dotted line. The lanes marked MW are standard molecular weight markers derived from HindIII-cut lambda phage DNA. (B) Representative scans of specific lanes from Southern blot analysis of HMGA1-ON and HMGA1-OFF cells at 0 and 3 hours after repair. Fractional intensity was normalized to the total intensity of each lane as well as to control lanes. (C) Plot of removal of CPD lesions following different repair times in HMGA1-ON and HMGA1-OFF cells. Data represent the mean (± 1 S.D.) of four independent experiments for each cell line.

Figure II-4. Global genomic NER of CPD lesions is inhibited in MCF-7 cells over-expressing HMGA1 proteins. Immunocytochemistry using Oregon Green-conjugated anti-CPD antibody and MCF-7 cells induced to either express (bottom panel) or not express (top panel), HMGA1a proteins. UV- panels were used as a background control. Each figure is representative of the average total fluorescence observed in five random fields for each treatment at 60X magnification.

Figure II-5. Global genomic NER of 6-4 photoproduct lesions is delayed in MCF-7 cells over-expressing HMGA1a proteins. Immunocytochemistry using Oregon Green-conjugated anti-6-4 photoproduct antibody and MCF-7 cells induced to either express (bottom panel) or not express (top panel), HMGA1a proteins. UV- panels were used as a background control. Each figure is representative of the average total fluorescence observed in five random fields for each treatment at 60X magnification.

Figure II-6. HMGA1 occasionally co-localizes with 6-4 photoproduct foci in MCF-7 cells. Immunocytochemistry using Texas Red-conjugated anti-HMGA1 antibody (left panel), Oregon Green-conjugated anti-6-4 photoproduct antibody (center panel) and MCF-7 cells induced to express HMGA1a proteins. Right panel represents merged image, in which co-localization of HMGA1 and 6-4 photoproducts is visualized as yellow fluorescence. These panels represent the average number of yellow foci observed in 5 random fields at 60X magnification.

Figure II-7. HMGA1 does not co-localize with CPDs in HMGA1-expressing MCF-7 cells. Immunocytochemistry using Texas Red-conjugated anti-HMGA1 antibody (left panel), Oregon Green-conjugated anti-CPD antibody (center panel) and MCF-7 cells induced to express HMGA1a proteins. Right panel represents merged image, in which co-localization of HMGA1 and CPDs is visualized as yellow fluorescence. These panels represent the average fluorescence intensity observed in 5 random fields at 60X magnification. No co-localized yellow fluorescence was observed in any random field at this time post UV exposure.

Figure II-1. (Taken from Adair *et al.*, 2005)

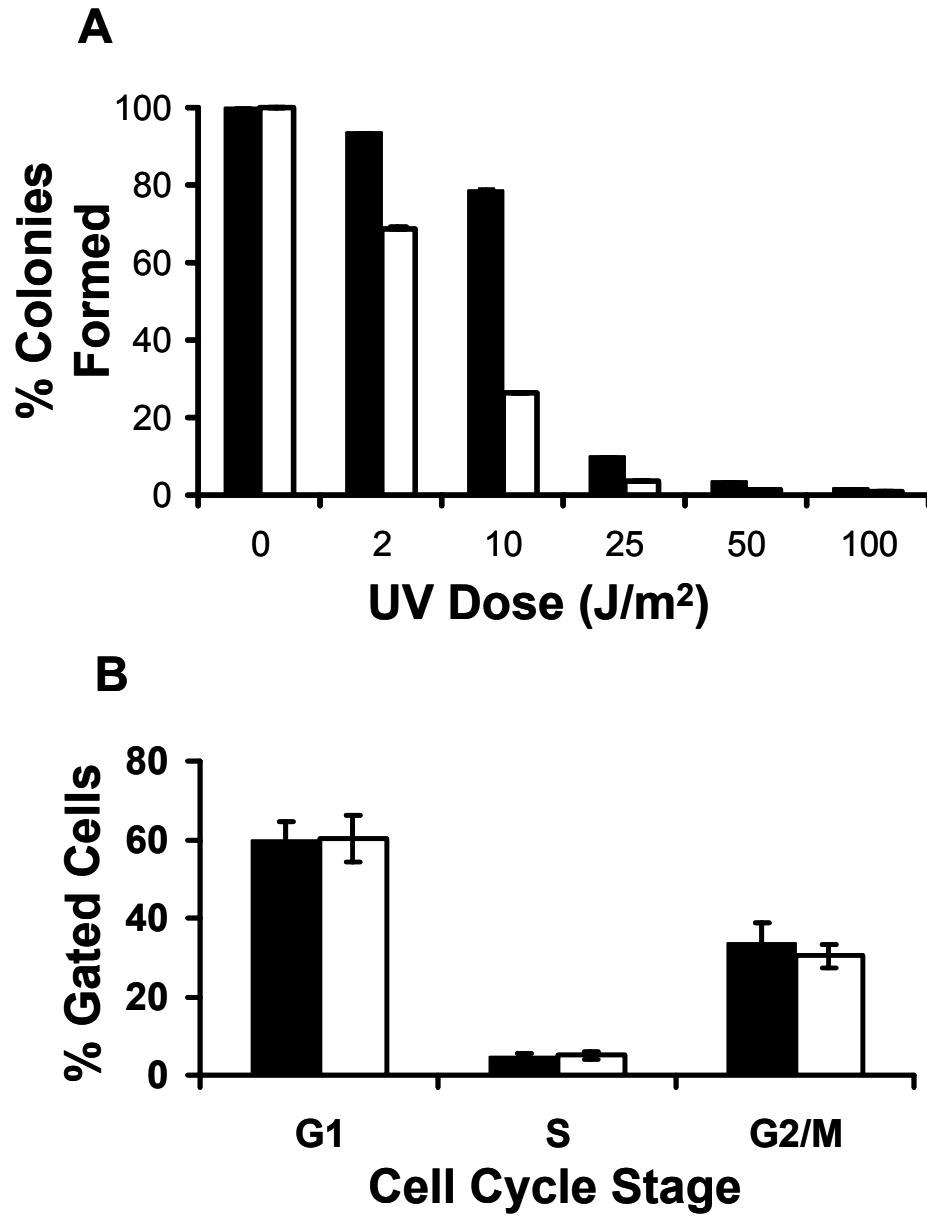


Figure II-2. (Taken from Adair *et al.*, 2005)

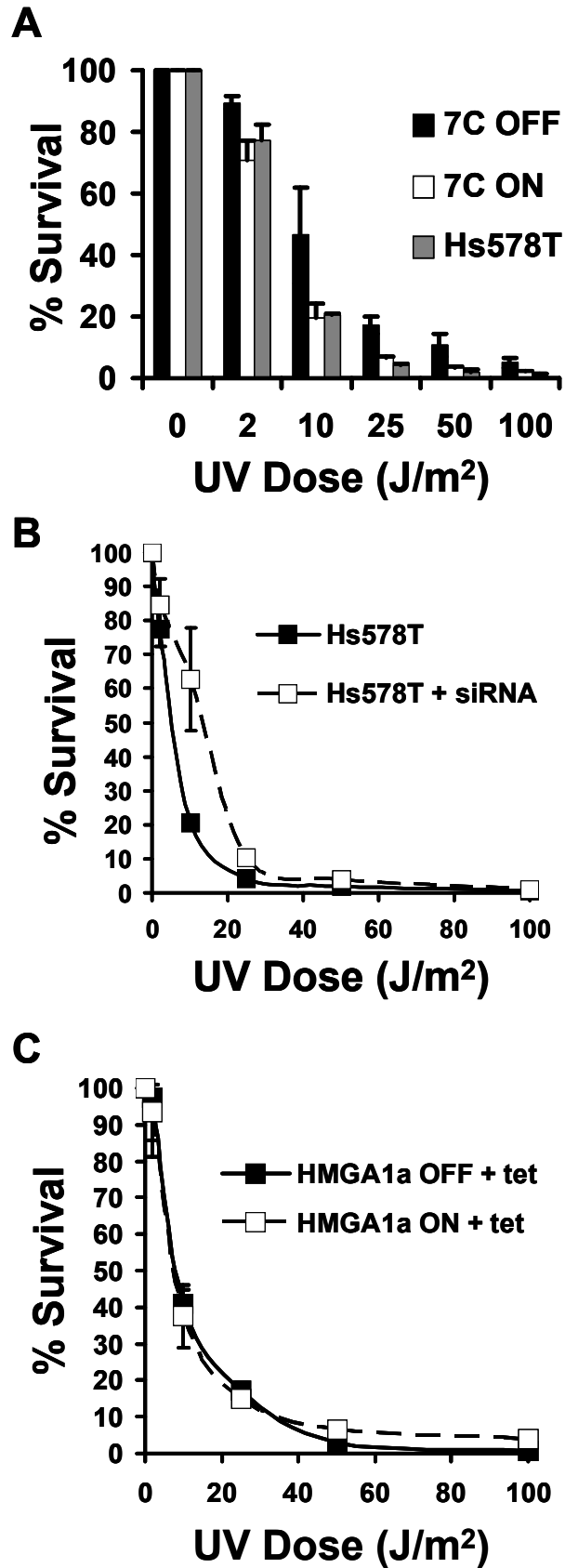


Figure II-3. (Taken from Adair *et al.*, 2005)

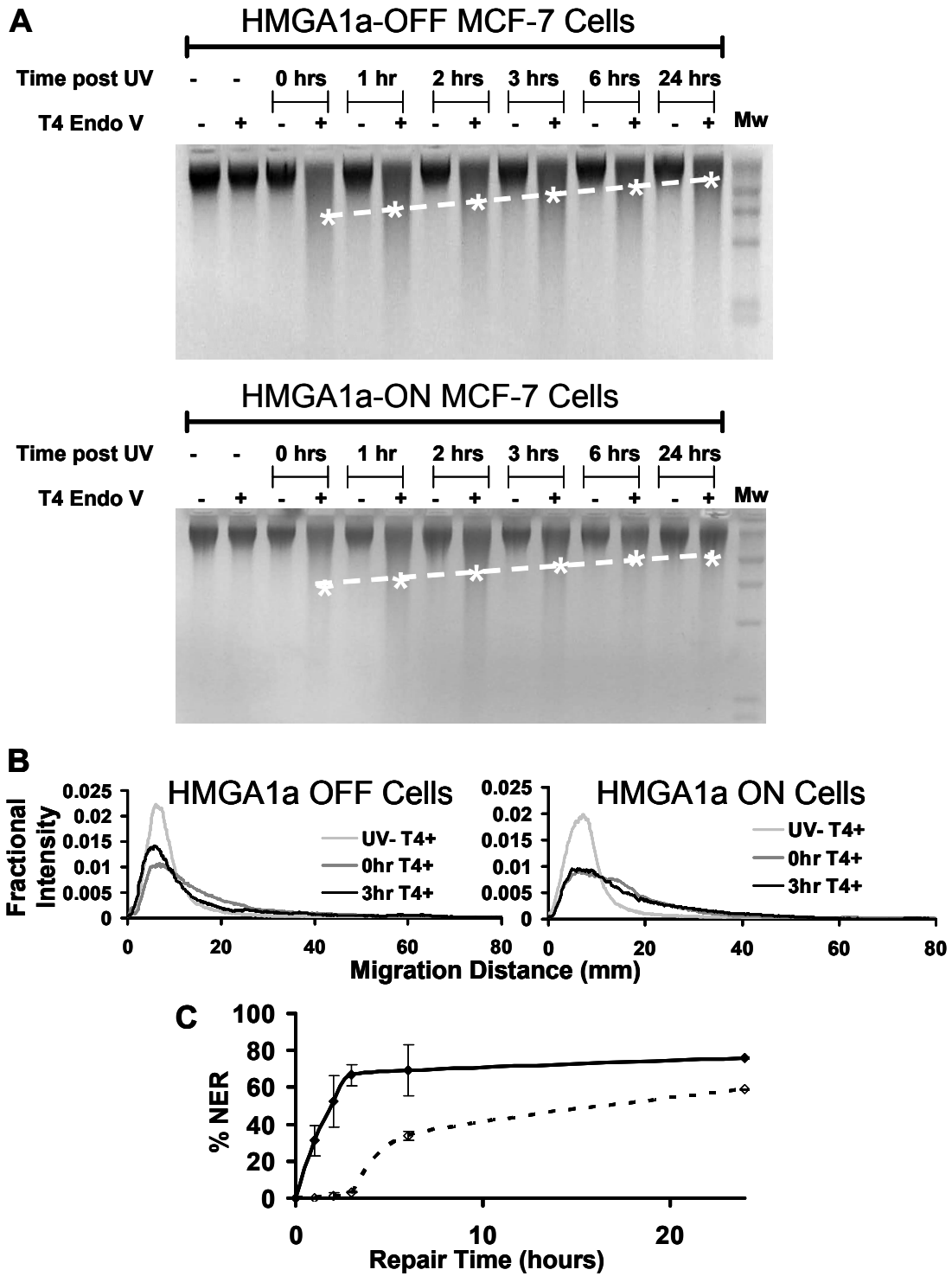


Figure II-4.

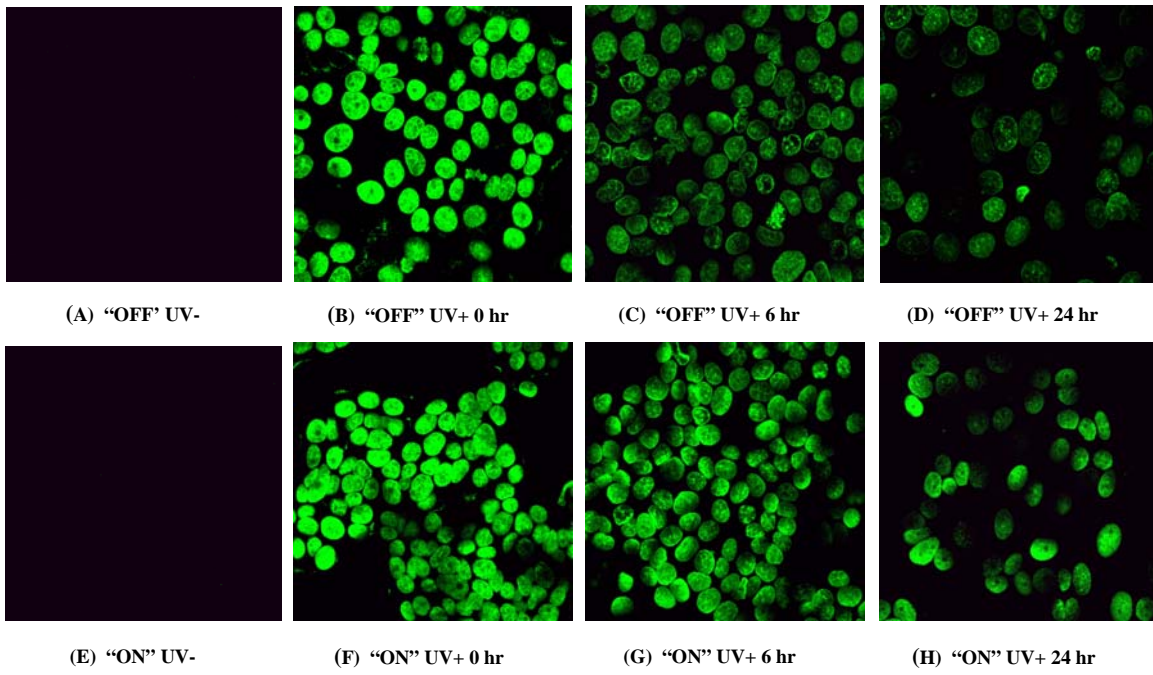


Figure II-5.

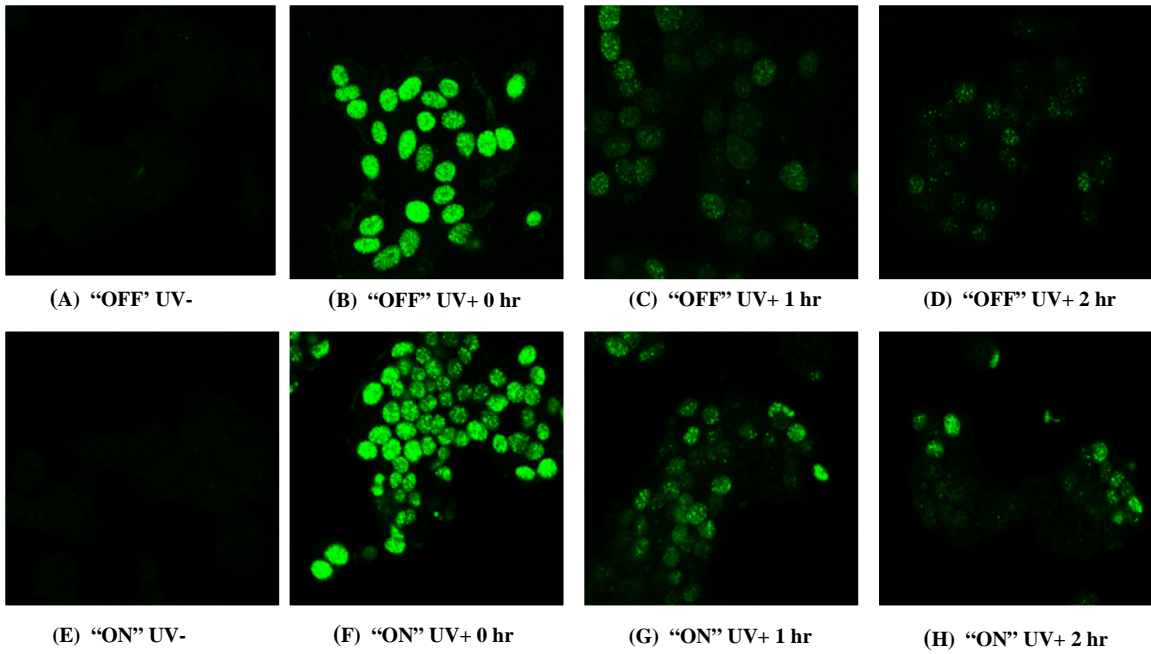


Figure II-6.

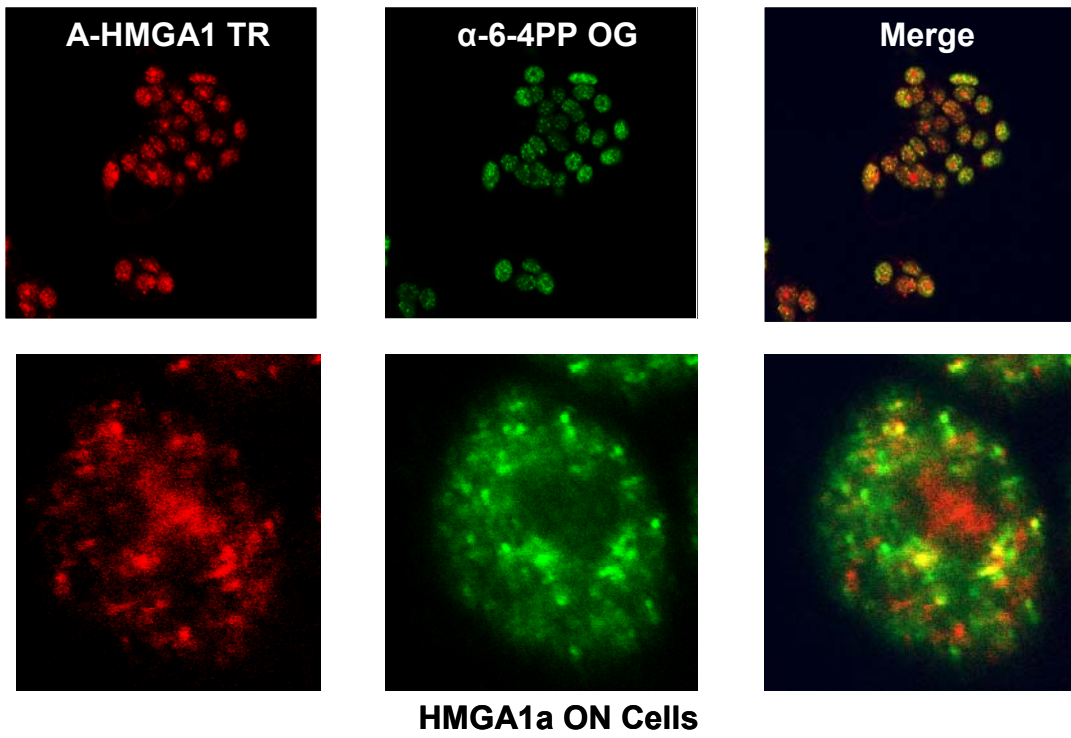
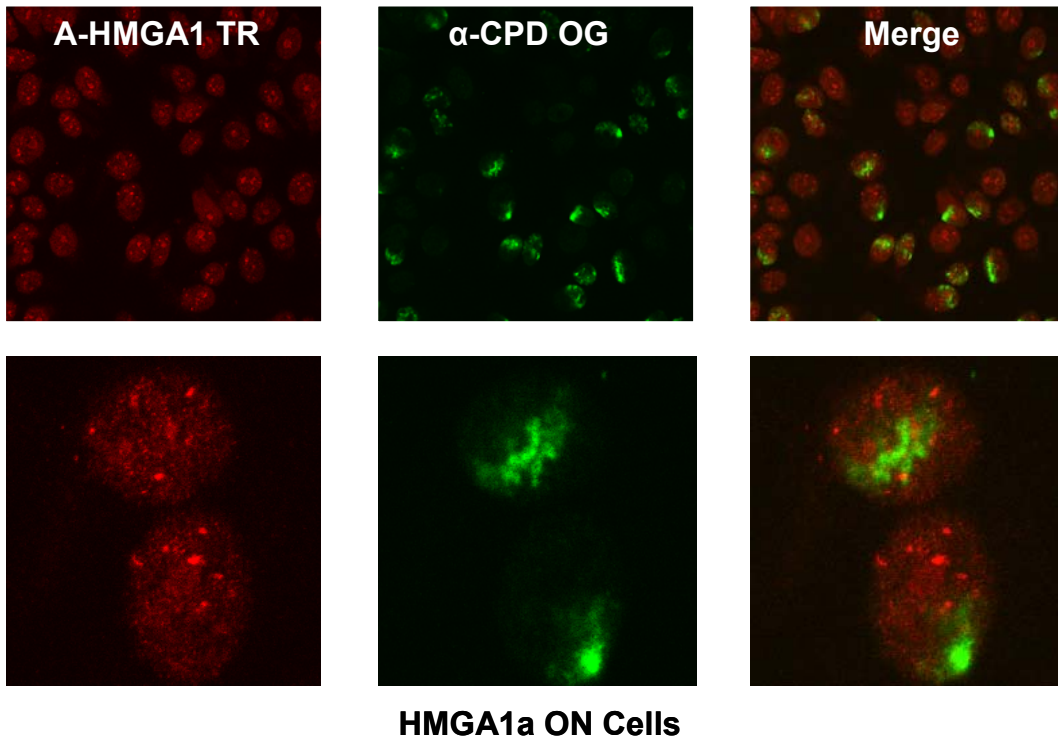


Figure II-7.



CHAPTER 3

XPA PROTEIN IS DOWN-REGULATED IN THE PRESENCE OF HMGA1a PROTEINS

Abstract

Recent work has demonstrated that stably-transfected MCF-7 cells induced to over express HMGA1 proteins exhibit deficient NER after exposure to UV light. One possible molecular mechanism for this NER inhibition is down-regulation of factors involved in the NER pathway, such as the repair protein xeroderma pigmentosa complementation group A (XPA). Microarray data indicates a 2.6-fold decrease in intracellular XPA mRNA in MCF-7 cells expressing HMGA1 proteins compared to non-HMGA1 expressing cells. Additionally, XPA protein levels are approximately 3-fold lower in HMGA1-expressing MCF-7 cells. Moreover, while a 2-fold induction of XPA proteins is observed in normal MFC-7 cells thirty minutes after a 20J/m² dose of UV, no apparent induction of XPA protein is observed in MCF-7 cells expressing HMGA1. Preliminary reporter analyses employing a firefly luciferase construct containing 2.2kb of the XPA promoter indicate that this region of the DNA is similar in HMGA1-expressing MCF-7 cells compared to their non-HMGA1-expressing counterparts. However, chromatin immunoprecipitation (ChIP) analyses have established that HMGA1 is associated with the endogenous XPA promoter. These data provide strong preliminary evidence that HMGA1 proteins are involved in down-regulation of *XPA* gene expression, a phenomenon associated with compromised NER and carcinogenesis.

INTRODUCTION

The ability to recognize and respond to DNA damage is a ubiquitous property of living organisms, from mycoplasma to multicellular vertebrates. The two DNA repair pathways responsible for a majority of day-to-day genomic maintenance are BER and NER, also known as the excision repair pathways. Of these, NER is considered to be responsible for detecting and repairing large, helix-distorting lesions such as CPDs and cisplatin adducts. BER, however, is thought to repair small, single-base modifications, most of which arise from endogenous damage such as oxidation. These two pathways are further distinguished by the separation of damage recognition from catalytic activity. While, in BER, the protein responsible for recognizing and binding to the damaged base also catalyzes cleavage of the glycosyl bond, in NER, the recognition proteins have no observed catalytic activity and simply act as binding sites for catalytically active proteins. Interestingly, evidence supporting a role for NER factors in BER and vice versa has indicated that compromise in one pathway can lead to inefficient repair by the other (Clayton *et al.*, 1974; Satoh *et al.*, 1993; Driggers *et al.*, 1996). Specifically, several groups have demonstrated that in the absence of XPA, a non-catalytic member of the NER pathway, repair of certain types of oxidative damage is also repressed (Satoh *et al.*, 1993; Driggers *et al.*, 1996; Lipinski *et al.*, 1999).

Interestingly, deficiencies in many of the proteins involved in NER result in severe sensitivity to DNA damaging agents, such as UV light and cisplatin, whose lesions are normally repaired via NER (Kraemer *et al.*, 1987). It was through the identification of patients exhibiting this phenotype that many of the factors involved in NER were, in fact, identified. In particular, patients deficient in functional XPA protein display the

most severe symptoms of the human disease xeroderma pigmentosa (XP), for which eight of the NER factors are named.

With regard to the work presented here, stably-transfected MCF-7 cells induced to over express HMGA1 proteins exhibit both UV-sensitivity, as well as compromised NER of CPDs, and delayed repair of 6-4 photoproducts (**See Chapter II**, Adair *et al.*, 2005). Additionally, recent work by Baldassarre *et al.*, has shown that ES cells over expressing HMGA1 proteins are also more sensitive to treatment with cisplatin, a chemotherapeutic agent which forms bulky DNA adducts (Baldassarre *et al.*, 2005). Furthermore, it has been shown that MCF-7 cells induced to over express HMGA1 proteins are also more sensitive to dimethylsulfate (DMS), an alkylating agent whose base modifications are typically repaired via BER (**See Appendix I, Figure A-2**). Given these data, in combination with microarray analyses indicating decreased concentrations of XPA mRNA in cells expressing HMGA1, a feasible hypothesis would be that observed NER (and potentially BER) defects are the result of decreased intracellular XPA protein concentrations. The data presented in this section test the hypothesis that HMGA1 proteins affect intracellular XPA concentrations via transcriptional repression of the *XPA* promoter.

XPA

The XPA protein is a highly conserved, 38-42kDa polypeptide containing three separate protein-protein interaction domains, a nuclear localization signal and a zinc finger domain (**Figure III-1**). While nuclear localization of XPA is observed under many different experimental conditions, nuclear sequestration of this protein is not required for functional participation in NER (Miyamoto *et al.*, 1992). XPA has been shown to bind

both to damaged as well as undamaged DNA, albeit with different affinities (Jones *et al.*, 1993). While previous work has demonstrated that XPA alone can bind to 6-4 photoproducts with reasonable affinity, binding of XPA to the RPA protein greatly increases its affinity for damaged DNA, and CPDs, in particular (He *et al.*, 1995; Li *et al.*, 1995). Thus, lesion specificity of XPA can be enhanced by heterodimerization with other proteins, including RPA. Moreover, binding of XPA to RPA is considered essential for NER function and the binding domains within the polypeptide have been well characterized (Li *et al.*, 1995; **Figure III-1**). XPA has also been demonstrated to interact with NER proteins ERCC1 (in complex with XPF), as well as TF_{II}H (**See Chapter I, Figure I-4**).

XPA is thought to play several roles in NER. Several groups have reported a ‘verification’ role for this protein, indicating that while XPA may not be the first protein associated with a lesion site, its interaction with the damaged site verifies that damage is present and stabilizes the initial complex to allow other NER factors to bind (Tanaka *et al.*, 1989; He *et al.*, 1995; Nagai *et al.*, 1995; Saijo *et al.*, 1996). There are conflicting reports as to whether the proofreading and stabilization functions of XPA are accomplished as a monomer, homodimer or both (Cleaver and States, 1997). However, it is apparent based on discovery of a cancer-predisposing human disease characterized by lack of functional XPA, that this protein is essential for cellular processes of genome maintenance.

Kraemer and colleagues demonstrated that XPA was one of the eight genes underlying the human disorder xeroderma pigmentosum (XP) and that patients lacking functional XPA displayed some of the most severe phenotypes of this disease (reviewed

by Cleaver and Kraemer, 1995). XP is characterized by any one of a number of mutations in the XPA gene, all of which render the encoded gene product non-functional (**Figure III-1**). In the absence of functional XPA, patients exhibit complete loss of UV-damage repair capacity, and a 10^3 - 10^4 increase in the age-specific incidence of skin cancer, as well as neurodegeneration (Maher *et al.*, 1979; Kraemer *et al.*, 1987).

The *XPA* gene, located on human chromosome 9q34.1, spans approximately 23kb and consists of six exons. Layher and Cleaver reported in 1997 that the expression of the *XPA* gene in human fibroblasts was extraordinarily low, with only five to eight molecules of mRNA per cell. Accordingly, two different groups have reported that the intact *XPA* promoter is extraordinarily weak in human cells, as assayed by (CAT)-reporter activity (Satokata *et al.*, 1993; Topping *et al.*, 1995). Furthermore, like other NER genes, the *XPA* promoter lacks common basal transcriptional signals, such as TATA, CCAAT and G/C boxes (**Figure III-1, Panel B**) (van Duin *et al.*, 1988; Weeda *et al.*, 1991; Topping *et al.*, 1995). A 110bp negative regulatory element has been characterized and is conserved between mice and humans (Satokata *et al.*, 1993; van Oostrom *et al.*, 1994; Topping *et al.*, 1995). It is important to note that deletion of this conserved negative regulatory element results in only a 2-fold increase in *XPA* transcription, reiterating the notion that the *XPA* gene is among the class of genes with the lowest expression levels (Topping *et al.*, 1995). Also of importance is the finding that XPA mRNAs are expressed at different levels in a variety of cell types, indicating that cell-type-specific transcriptional regulation may exist (Layher and Cleaver, 1996).

Pertinent to the work presented in this thesis, is the demonstration that intracellular concentrations of XPA are rate-limiting for NER, and that modest increases

of XPA have dramatic effects on NER efficiency (Cleaver *et al.*, 1995). In fact, Cleaver *et al.* demonstrated that the UV-sensitivity of human fibroblast cells was a linear function of intracellular XPA concentrations in experiments in which *XPA* cDNA was expressed from a conditional promoter. Moreover, while mutations in the XPA gene result in normal translation of a non-functional gene product, it has been demonstrated that low levels of functional XPA allow repair, albeit delayed, of 6-4 photoproducts, but not of CPDs (Cleaver *et al.*, 1995). Intriguingly, this repair phenotype was observed in HMGA1a-expressing MCF-7 cells (See Chapter II, Figures II-4 and II-5).

Given this evidence, the hypothesis that HMGA1-expressing MCF-7 cells, which display sensitivity to UV light and inefficient NER of UV-induced lesions, also display decreased intracellular concentrations of XPA compared to their non-HMGA1-expressing counterparts was tested. To assess this, western blot analysis was used, in combination with reporter assays and chromatin immunoprecipitation analyses, to indicate whether XPA levels were, in fact, decreased, and whether transcriptional repression involving HMGA1 was associated.

MATERIALS AND METHODS

Cell culture

The human breast adenocarcinoma cell line MCF7-tet (i.e., MCF7/Tet-OFF) (BD Biosciences) was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, and 100 µg/ml G418 (Research Products International). Clonal cell line MCF7-7C-Cs is a derivative of MCF7/Tet-OFF cells that has been stably transfected with a tetracycline-regulated pTRE vector encoding HA-

tagged HMGA1a cDNA (Reeves et al., 2001). This stably transfected line was maintained in the presence of 100 µg/ml hygromycin. The MCF7-7C-Cs cell line expresses high levels of HA-tagged HMGA1a protein when grown in medium lacking tetracycline (these are referred to as ‘ON’ cells). To prevent expression of the HA-tagged HMGA1 genes, the MCF7-7C-Cs cells were cultured in media containing 2 µg/ml tetracycline (and are referred to as ‘OFF’ cells).

Western blot analysis

Levels of intracellular XPA and HMGA1 proteins were determined by Western blot analysis of cell extracts prepared with TRIzol (Life Technologies). 100µg total protein from either HMGA1 ON or HMGA1 OFF cells was loaded onto a 12% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS). Proteins were separated electrophoretically at 100V for approximately 2.5 hours before transferring to PVDF membrane (Millipore) via tank transfer. Membranes were probed for XPA and HMGA1 using either a monoclonal antibody against XPA (Santa Cruz) or a specific polyclonal antibody against HMGA1 proteins (Reeves and Nissen, 1999). Western blots reacted with a polyclonal HRP-conjugated anti-actin rabbit antibody (Sigma, Co.) served as loading controls. Films were then scanned and quantitated densitometrically using ImageQuant software to determine fold differences observed.

For UV-induction, cells were cultured as described above until 90-95% confluence. Media was then removed from culture plates and cells were irradiated using low pressure Hg lamps (Sylvania, Model G30T8) at a dose of 20 J/m² (measured with a Spectroline DM-254N short wave UV meter) (Spectronic Corp.). Media was then added and cells were incubated for 30 minutes to allow induction of XPA. Total proteins were

then isolated as described above using TRIzol reagent (Invitrogen) as recommended by the supplier.

Construction of a reporter vector containing the XPA promoter

2.2kb of the 5' untranscribed region (UTR) of the *XPA* gene (Accession #U16815) was amplified from BAC clone bA54606 (Sanger-Wellcome Institute) by PCR using primers XPA 1 MluI S1 (sense:5'caggcccacgcgtcccatgctgctcaggtagc3') and XPA 5'UTR MluI AS1 (antisense:5'ccgaggcacgcgtctccgcggttgctctaa3'). Cloning of this PCR product into the pGL3-Basic firefly luciferase reporter vector (Promega) was accomplished via MluI digestion of the vector, followed by gel purification in 1% agarose and subsequent overnight ligation of digested vector and amplified insert at 16°C in the presence of T4 Ligase (Invitrogen). Ligation products were immediately transformed into CaCl₂-competent DH5α *E. coli* cells and plated on Luria broth (LB) agar plus 100µg/mL Ampicillin. Colonies formed after 12 hours were subjected to insert verification by drop-colony PCR using the following primers: RVprimer3 (sense: 5'ctagcaaaataggctgtccc3') (Promega) and XPA Neg AS1 (antisense: 5'tgtgtggacctcaggcaaatgcag3'). Clones containing vector plus insert in the correct orientation produced a 1.4kb PCR product under these reaction conditions, and were then subjected to plasmid miniprep (Quiagen) and subsequent DNA sequencing. Two resulting reporter constructs were produced by this method, and were named pGX2 and pGX9, for pGL3-Basic vector containing the *XPA* promoter from clones 2 and 9, respectively.

Luciferase reporter assays

MCF-7 cells, both HMGA1-expressing and non-expressing, were plated in 24-well plates in duplicate at 1×10^5 cells per well in 0.5mL DMEM containing 10% FBS without any antibiotics. Plates were incubated for 24 hours or until confluence was 90-95%. Transient transfection of cells was accomplished using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For each cell line, in separate transfections, either pGX9 or promoter-less pGL3-Basic was added to cells at 200ng per well, such that 24 wells of each were obtained. 20ng per well pCMV-Ren, encoding the *Renilla* luciferase gene under the control of a minimal cytomegalovirus (CMV) promoter was also added as a control for transfection efficiency. Cells were then incubated overnight, whereupon media was removed, cells were washed once with sterile PBS, and plates were either subjected to a 20J/m^2 dose of UV, or sham irradiated. Media was then replaced and plates were returned to the incubator. At 1, 3, 6 and 24 hours post treatment (either UV irradiation or sham), triplicate wells of both pGX9- and pGL3-Basic transfected cells were subjected to PBS washing and subsequent harvest by the addition of 100 μL passive lysis buffer (Promega) and incubation at room temperature for 30 minutes. 25 μL of each lysate was then added to a luminometer test tube and dual luciferase readings were taken after addition of substrate for firefly (Promega) and *Renilla* luciferase (Stop and Glow; Promega), respectively.

Chromatin immunoprecipitation analyses

To determine whether HMGA1 was associated with the endogenous XPA promoter, MCF-7 cells over expressing HMGA1 proteins were cultured as indicated above until complete confluence was obtained. Approximately 4×10^6 cells were then

trypsinized and resuspended in 10mL serum-free DMEM containing 1% formaldehyde and incubated for 10 minutes at 37°C. Glycine was then added to a final concentration of 125 mM to quench the cross-linking reaction, and cells were pelleted by centrifugation. Cell pellets were washed once in ice cold PBS containing protease inhibitors and then resuspended in SDS-lysis buffer (1%SDS, 10mM EDTA, 50mM Tris-HCl, pH 8.0) and placed on ice for 30 minutes. Volumes were increased to 1mL by addition of ChIP dilution buffer (0.01%SDS, 1.1%Triton-X-100, 1.2mM EDTA, 16.7mM Tris-HCl, pH 8, 16.7mM NaCl, 100µM protease inhibitors) and samples were sonicated to produce average size DNA fragments of 450-500bp, as determined by electrophoresis and ethidium bromide staining in 1% agarose. Cellular debris was then removed and supernatants were divided into four separate microcentrifuge tubes. Pre-clearing of samples was accomplished by addition of 30µL Protein Sepharose A beads and 1µg salmon sperm DNA, followed by rotation of samples at 4°C for one hour. Beads were then removed and two of four samples were incubated overnight at 4°C with rotation in the presence of MR19 (titer 2/10/99), a polyclonal antibody with high specificity for HMGA1 proteins. Additional samples were incubated in the presence of either non-specific Rabbit IgG or no antibody as controls. After incubation, 30µL Protein Sepharose A beads and 1µg salmon sperm DNA were added to each and pull-down was accomplished by incubation at 4°C with rotation for at least one hour. Beads were spun down and washed once each with Buffers 1 (0.1%SDS, 1%Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8, 150mM NaCl), 2 (0.1%SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8, 500mM NaCl) and 3 (0.25M LiCl, 1%NP40, 1% sodium deoxycholate), followed by two washes in TE buffer (1mM EDTA, 10mM Tris-HCl, pH

8) for five minutes each wash at 4°C with rotation. Samples were then eluted by addition of ChIP elution buffer (1% SDS, 0.1M NaHCO₃) and two additional 15 minute incubations at room temperature with rotation. To reverse formaldehyde cross-links and remove RNA, samples were incubated in the presence of 6µg/mL DNase-free RNase A at 65°C for five to six hours. This was followed by overnight incubation at 37°C in the presence of 30µg/mL proteinase K to remove proteins. DNA remaining was then purified by phenol:chloroform extraction and ethanol precipitation.

To evaluate whether regions of the XPA promoter were endogenously associated with HMGA1, PCR of immunoprecipitated DNA was performed using primers specific to a 450bp region of the XPA promoter, which consists of one identified potential HMGA1 binding site and the only conserved regulatory element described within this promoter, a 100bp negative regulatory region. Primers used were XPA New Neg S1 (sense; 5'aactgtcaagagctcagacttaacc3') and XPA 5' UTR AS1 (antisense; 5'ctccgcggttgctctaa3'). Additional PCR of a portion of the kit ligand (KL) promoter, to which HMGA1 is known to be bound, was performed as a control for effective immunoprecipitation. Reactions were resolved by electrophoresis in 1% agarose in the presence of ethidium bromide.

RESULTS

Previous work has demonstrated that intracellular concentrations of XPA mRNA were decreased 2.6-fold in MCF-7 cells induced to over express HMGA1 proteins, compared to non-HMGA1 expressing MCF-7 cells, as assayed by microarray analysis (Reeves and Adair, 2005; **Chapter 1, Figure I-3**). Interestingly, and in accordance with phenotypes observed in human cells expressing low levels of functional XPA protein

under a variety of experimental conditions, defective NER of CPDs is observed in HMGA1-expressing MCF-7 cells, but not parental MCF-7 cells (Adair *et al.*, 2005). Moreover, as previously noted, immunocytochemical data examining removal of 6-4 photoproducts in these cells indicates that removal of these lesions does occur, albeit at a slower rate than that of non-HMGA1-expressing MCF-7 cells (**Compare Figure II-4 to Figure II-5**). This latter finding is in accordance with published findings by Cleaver *et al.*, which demonstrated that under conditions of low XPA expression, removal of 6-4 photoproducts was successful, while removal of CPDs was limited to transcriptionally active regions of the genome in human fibroblasts (Cleaver *et al.*, 1995). Furthermore, co-localization studies employing antibodies against HMGA1 proteins, 6-4 photoproducts and CPDs indicated that HMGA1 does, in fact, co-localize with some 6-4 photoproduct foci in the nucleus, but does not appear to be associated with CPDs (**See Chapter II, Figures II-6 and II-7**).

To assess whether decreased XPA transcript levels in MCF-7 cells expressing HMGA1 proteins corresponded to a decrease in translated full-length XPA protein, western blot analysis of total proteins from both parental and stably-transfected, HMGA1-expressing MCF-7 cells were performed (**Figure III-2**). In these experiments, cells were either UV-irradiated at a dose of 20J/m², or left unirradiated. Since microarray analyses indicated that basal transcript levels of XPA were compromised in cells expressing HMGA1 proteins compared to parental MCF-7 cells, basal protein levels were also examined and compared between these two cell types. However, with regard to NER of UV-induced DNA damage, it is also important to determine the UV-inducibility of XPA, since previous reports indicate that upon exposure to UV irradiation,

intracellular concentrations of XPA proteins increase substantially (Cleaver *et al.*, 1995). In both cases, reduced amounts of intracellular XPA were observed in cells expressing HMGA1 proteins, both before and after UV irradiation, when compared to total Actin and histone H1 as loading controls (**Figure III-2**). **Figure III-3** is a graphical representation of multiple experiments, in which western blots were densitometrically analyzed, indicating a 3-fold decreased basal XPA protein level in HMGA1-expressing MCF-7 cells. This correlates with the observed 2.6-fold reduction in basal XPA transcript levels exhibited in microarray analyses (**Chapter 1, Figure I-3**). Moreover, intracellular XPA protein levels exhibited a 6-fold decrease in HMGA1-expressing cells 30 minutes after a 20J/m² dose of UV, compared to XPA levels in parental MCF-7 cells (**Figure III-3**).

To assess whether observed deficiencies of XPA transcript and protein in the presence of HMGA1 proteins were the result of transcriptional down-regulation, reporter assays employing a construct containing the firefly luciferase gene under the control of 2.2kb of the wild-type XPA promoter were performed. Despite the extraordinarily weak promoter activity reported by Topping *et al.*, as well as Satokata *et al.*, basal promoter activity was detectable in parental MCF-7 cells over 24 hours, compared to promoter-less controls, which displayed no detectable luciferase activity (**Figure III-4, Panel A**). Interestingly, when transiently transfected cells were irradiated at 20J/m², no apparent changes in luciferase activity were observed, compared to basal promoter activity (**Figure III-4, Panel B**). When promoter activity was assessed in MCF-7 cells expressing HMGA1 proteins under the same conditions, no significant difference in luciferase activity was observed in three of four time points, both before and after UV-

irradiation, indicating no change in transcriptional activity of this promoter in these cells (**Figure III-4, Panel C**).

Given the chromatin remodeling function of HMGA1 in living cells, and considering that transient reporter assays do not examine promoter activity within the context of chromatin, chromatin immunoprecipitation analyses were employed to determine whether or not HMGA1 proteins are associated with this region of the DNA endogenously. Briefly, MCF-7 cells induced to over express HMGA1 proteins were subjected to formaldehyde, to cross-link interacting proteins as well as proteins interacting with DNA, followed by immunoprecipitation with HMGA1-specific antibodies to pull down regions of the genome that HMGA1 interacts with, either directly or via protein-protein interactions. Sonication of the DNA to an average fragment size of 500bp prior to pull-down ensures some specificity in subsequent PCRs used to identify interacting sequences. In this experiment, as indicated in **Figure III-5**, a 450bp region of the XPA promoter containing the only putative regulatory element within the 5' UTR did immunoprecipitate under these experimental conditions. Immunoprecipitated DNA samples were also subjected to PCR amplification of a 350bp region of the KL promoter, to which HMGA1 has previously been reported to interact, as a control to verify antibody pull-down (**Figure III-5**). These results indicate association of HMGA1 with the endogenous XPA promoter in living cells, either through direct DNA-binding or interaction with proteins factors bound to this promoter region.

CONCLUSIONS

Adair *et al.*, have previously demonstrated that MCF-7 cells over-expressing HMGA1 proteins are compromised in their ability to repair UV-induced CPD lesions,

when compared to non-over expressing MCF-7 cells (Adair *et al.*, 2005). Baldassarre *et al.*, have also demonstrated that cells over expressing HMGA1 proteins are more sensitive to treatment with cisplatin (Baldassarre *et al.*, 2005). In combination, these data suggest that cells expressing HMGA1 proteins are compromised in their ability to carry out NER efficiently. Given the structure-specific binding nature of the HMGA1 proteins, it is possible that these peptides are bound to DNA at lesion sites, preventing repair factor access to these lesions. However, given the bimodal repair profile for removal of CPD lesions within HMGA1-expressing MCF-7 cells, defined by a complete lack of induction four hours after UV irradiation, and sub-maximal induction between four and twenty-four hours post UV, multiple mechanisms of NER interference are considered likely.

One additional possibility is limited availability of NER factors, a likely mechanistic candidate given the well-established role of the HMGA1 proteins as both positive and negative regulators of transcription, as well as their ability to interact with a wide host of other proteins. Evidence supporting a role for HMGA1 proteins in down-regulation of repair factors includes microarray analyses employed to compare basal transcriptome profiles of normal MCF-7 cells and MCF-7 cells over-expressing HMGA1 proteins (Reeves and Adair, 2005; Figure I-3). In these experiments, a large number of transcripts were up-regulated in the presence of HMGA1 proteins, however, of those that were found to be down-regulated, a remarkable number were involved in DNA damage recognition and repair, as well as in homologous recombination (Figure I-3). Of these, one gene known as *XPAC*, for *xeroderma pigmentosa group A complimenting gene*, or *XPA* for short, was found to be 2.6-fold decreased in MCF-7 cells over expressing HMGA1 proteins. Given the fact that persons deficient in functional XPA comprise the

most severely affected patients diagnosed with XP disease, and that previous reports have indicated UV-sensitivity as a function of intracellular XPA concentrations, it was perceived as a likely possibility that XPA protein levels are decreased in cells expressing HMGA1 proteins, and this is, in part, responsible for the increased UV sensitivity observed in MCF-7 cells over expressing HMGA1 proteins (Kraemer *et al.*, 1987; Cleaver *et al.*, 1995).

The work described here indicates that not only are XPA transcript levels decreased in MCF-7 cells expressing HMGA1 proteins, but proteins levels are proportionately decreased as well in these cells (**Figures III-2 and III-3**). Moreover, this decrease in intracellular XPA protein concentrations is exaggerated even further following exposure of cells to UV irradiation at 20J/m², a dose considered to be within the physiological range of sunlight exposure (**Figures III-2 and III-3**). These data provide evidence that, in the presence of HMGA1 proteins, intracellular concentrations of functional XPA protein are decreased, indicating that XPA deficiency should be explored as a cause of the sub-maximal NER observed in these cells previously (Adair *et al.*, 2005).

There are several possible mechanisms by which HMGA1 proteins could be negatively influencing intracellular XPA concentrations. First, while previous evidence for this has not been reported in the literature, it is possible that HMGA1 is destabilizing XPA protein via protein-protein interactions or activation of pathways which target XPA for destruction. Secondly, it could also be possible for HMGA1 proteins to stimulate destabilization of XPA transcripts in the same manner, a mechanism supported by recent evidence that HMGA1 interacts with several mRNA processing factors (Sgarra *et al.*,

2005). Third, HMGA1 could regulate translation of the XPA transcript via inhibition of factors involved in this process either directly or indirectly, however, as is the case with the two previous possibilities, there is limited support for this proposed role of HMGA1 proteins in the literature. What has been well-defined as a role for HMGA1 proteins in the regulation of a number of gene products is transcriptional activation and repression via interactions with promoter regions themselves, as well as through interactions with other proteins bound to endogenous promoters of those genes (Reviewed by Reeves and Beckerbauer, 2001).

Other arguments for transcriptional control of the *XPA* gene include evidence that the half-life of the XPA protein is greater than 12 hours long (Reviewed by Cleaver and States, 1997). Given that induction of intracellular XPA protein concentrations is observed within 30 minutes after UV exposure as assayed by western blot analysis, well before the expiration of one half-life, induction is more likely the result of transcriptional activation, stabilization of mRNA or increased message translation. Furthermore, microarray results indicate that basal transcript levels are, in fact, different in cells expressing HMGA1 proteins compared to non-HMGA1-expressing MCF-7 cells, indicating either mRNA destabilization or decreased transcription in HMGA1-expressing cells.

To assess transcriptional repression of the *XPA* gene, reporter assays were performed using the pGX9 construct containing 2.2kb of the endogenous human XPA promoter upstream of a gene encoding firefly luciferase. Interestingly, despite reports indicating that the XPA promoter is one of the weakest in the human genome, basal promoter activity is detectable in normal MCF-7 cells transiently transfected with the

pGX9 reporter (**Figure III-4, Panel A**). Furthermore, observed reporter activity was not significantly altered in parental MCF-7 cells upon UV irradiation over a 24-hour time course (**Figure III-4, Panel B**). While this suggests that increased intracellular concentrations of XPA observed after UV irradiation are not the result of a transcriptional activation event, it does not exclude the possibility that activation requires another portion of the genome not included in the reporter construct, or that regulatory elements included within the construct itself prevent UV-activation. Additionally, comparative transfections in MCF-7 cells over expressing HMGA1 proteins revealed no significant difference in reporter activity, both before and after UV irradiation, compared to that observed in normal MCF-7 cells (**Figure III-4, Panel C**). This suggests that the promoter is not repressed in the presence of HMGA1 proteins, although whether the endogenous promoter is influenced by HMGA1 within the context of chromatin in the nucleus cannot be determined from these experiments. Given the established role of HMGA1 as a chromatin remodeling factor within the nucleus, experiments to determine whether an association between HMGA1 and the endogenous XPA promoter exists could provide more interpretable information regarding action of this promoter in living cells expressing HMGA1 proteins.

Chromatin immunoprecipitation assays have been used frequently in the literature to determine association of various proteins with specific endogenous DNA sequences using a variety of cross-linking methods. By incubating MCF-7 cells expressing HMGA1 proteins in the presence of formaldehyde, covalent cross-linking of proteins to proteins and proteins to DNA wherever interactions between the two exist within the cellular context occurs. Utilizing this method, it is possible to delineate whether or not a

particular protein, like HMGA1, is associated with a particular region of the genome, for example, the *XPA* promoter. However, it is not possible to delineate whether this association is the result of HMGA1 binding to DNA directly or if HMGA1 is interacting with other proteins which, in turn, are bound to the endogenous DNA sequence examined. Importantly, characterization of the *XPA* promoter has revealed 23 potential HMGA1 interacting sites, as defined by a stretch of six or more adenines and thymines in a sequence (**See Figure III-1, Panel B**). Furthermore, several transcription factors known to interact with HMGA1 proteins have consensus binding sequences within the 2.2kb region upstream of the *XPA* transcriptional start point (*tsp*) (Zhong *et al.*, 2000; reviewed by Reeves and Beckerbauer, 2001).

Employing ChIP analyses, it was determined that HMGA1 is associated with at least one 500bp region of the endogenous *XPA* promoter, containing the defined negative regulatory region and one potential HMGA1 binding site that lies within this putative regulatory region (**Figure III-5**). While this provides preliminary evidence linking HMGA1 physically to the endogenous *XPA* promoter sequence, it does not preclude the possibility that the protein may be bound to other regions of the upstream promoter, or to other proteins bound to the endogenous gene sequence. Furthermore, previous work has demonstrated a role for HMGA1 in the formation of complex DNA structures known as transcriptional ‘enhanceosomes’ on promoter regions of genes such as interleukin 2 receptor α (IL2-R α) (Reviewed by Reeves and Beckerbauer, 2001). In the case of the *XPA* gene, HMGA1 could be serving a similar, ‘molecular glue-like’ role in the formation of a transcriptional ‘repressosome’. Thus, it can be postulated that HMGA1 could be bound at an upstream region of the *XPA* promoter, inducing bending of the

DNA via a number of protein-protein interactions, resulting in immunoprecipitation of a connected DNA fragment within this network.

In conclusion, the evidence provided in this body of work demonstrates that XPA protein levels are decreased in cells expressing HMGA1 proteins, and that HMGA1 is associated with the 5' flanking region of the *XPA* gene. While evidence supporting an association of HMGA1 with the endogenous *XPA* promoter is presented, more work will need to be done to characterize the action of HMGA1 with respect to this gene product. Additionally, in order to make the connection between the observed decreases in available XPA protein within HMGA1-expressing MCF-7 cells, and the previously described NER deficiencies characterized in these same cells, complementation analyses will need to be performed. In these experiments, complementing XPA expression using wild-type cDNAs should rescue some of the UV-sensitivity of these cells, if not all, provided the XPA protein decrease, as a result of HMGA1, is responsible for the observed repair defects. Nevertheless, evidence provided supports a continuously developing role for the HMGA1 proteins in decreasing genomic integrity commonly observed in cancer cells which naturally over express these proteins.

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FIGURE LEGENDS

Figure III-1. (A) XPA protein map with known mutations and clinical phenotypes. Linear amino acid sequence is indicated by a horizontal black bar. Amino acid numbers are indicated above vertical lines at the end of each terminus and structural features are indicated by open boxes. Light blue boxes indicate known protein-protein interaction domains. The types and positions of mutations associated with XP group A disease with complete loss of function are indicated at the top, while mutations occurring in mild XP group A disease where partial function is retained are indicated at the bottom. A (+) sign indicates an insertion, while a (-) sign indicates a deletion. (B) Schematic diagram of the *XPA* gene promoter. Horizontal blue bar represents 2.2kb 5' flanking region of the *XPA* gene. Red bar indicates the conserved 107bp negative regulatory region. Bent arrow indicates transcriptional start point (*tsp*) and bracketed 827bp section represents the minimal promoter sequence described by Satokata *et al.* (Satokata *et al.*, 1993). Vertical black bars represent potential HMGA1 binding sites, consisting of 6-9 (thin bars) or >10 (thick bars) successive adenine and/or thymine bases in sequence.

Figure III-2. XPA protein levels are decreased in MCF-7 cells over expressing HMGA1 proteins. Western blot analysis of total protein samples from normal MCF-7 (HMGA1 ‘OFF’) cells as well as MCF-7 cells expressing HMGA1 proteins (HMGA1 ‘ON’). Top panel indicates results using a monoclonal antibody against the XPA protein, middle panel indicates results using a polyclonal antibody against HMGA1 proteins, and bottom panel indicates total actin loading controls. Lanes 1 and 3 correspond to proteins isolated from untreated cells, while lanes 3 and 4 represent proteins isolated from cells 30 minutes following a 20J/m^2 dose of UV radiation.

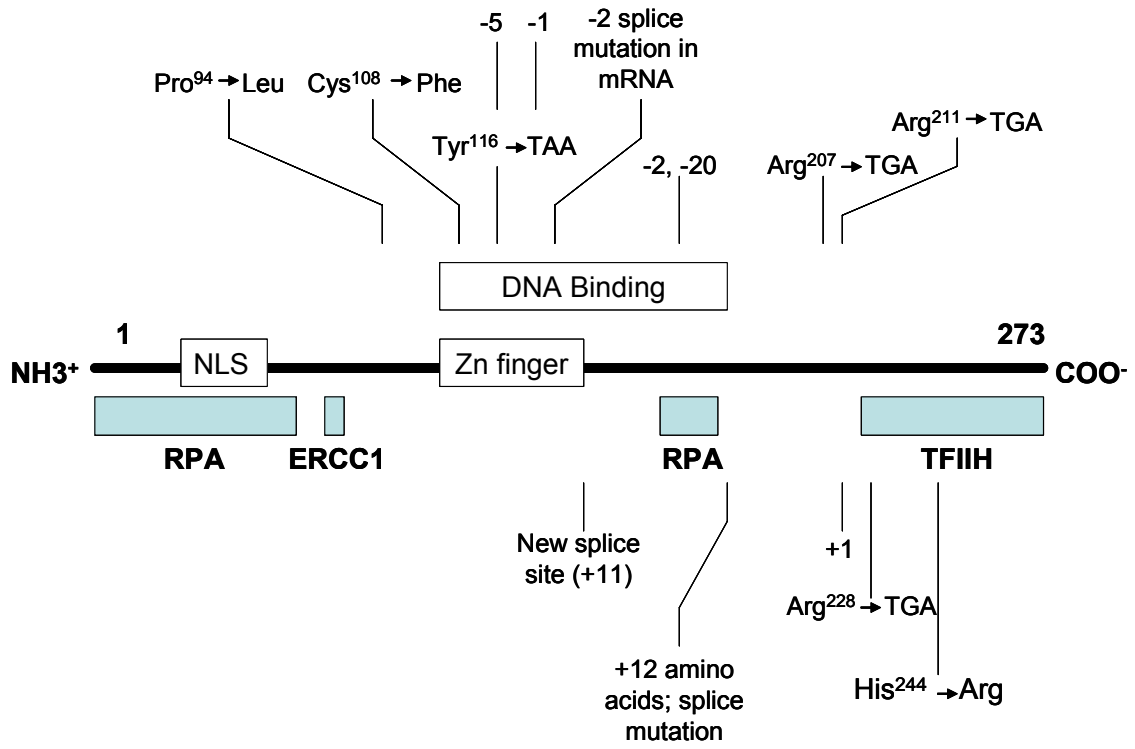
Figure III-3. XPA proteins are decreased 3-fold in MCF-7 cells expressing HMGA1 proteins before UV irradiation and 6-fold after UV irradiation compared to non-HMGA1 expressing MCF-7 cells. Graphical representation of densitometric analysis of four separate western blot experiments probing total protein for XPA using a monoclonal antibody. Black bars represent parental MCF-7 cells (HMGA1 'OFF') and white bars represent MCF-7 cells induced to over express HMGA1 proteins (HMGA1 'ON') 20-fold more than parental cells. Error bars indicate ± 1 standard deviation for this set of experiments.

Figure III-4. *XPA* promoter activity appears normal in MCF-7 cells over expressing HMGA1 proteins. Graphical representation of results obtained from transient transfections of reporter construct pGX9 into parental MCF-7 cells (HMGA1 OFF) and MCF-7 cells expressing HMGA1 proteins (HMGA1 ON). (A) Basal promoter activity is detectable in HMGA1 OFF cells compared to cells transfected with promoter-less reporter vector. Black bars represent luciferase activity observed in cell lysates from HMGA1 OFF cells transfected with 200ng/well pGX9 reporter construct over 24 hours post mock UV irradiation. White bars represent luciferase activity observed in cell lysates from HMGA1 OFF cells transfected with 200ng/well promoter-less pGL3-Basic. Error bars represent ± 1 standard deviation. (B) pGX9 reporter construct does not appear to be activated upon exposure to UV radiation. Black bars represent luciferase activity observed in cell lysates from HMGA1 OFF cells transfected with 200ng/well pGX9 reporter construct over 24 hours post mock UV treatment. White bars represent luciferase activity observed in cell lysates from HMGA1 OFF cells also transfected with 200ng/well pGX9 reporter construct for 24 hours after a 20J/m^2 dose of UV radiation. Error bars represent ± 1 standard deviation. (C) Reporter activity in MCF-7 cells over expressing HMGA1 proteins is similar to that in non-HMGA1-expressing MCF-7 cells. Black bars represent ratio of firefly luciferase activity to Renilla luciferase activity observed in cell lysates from HMGA1 OFF cells transfected with 200ng/well pGX9 reporter construct and 20ng/well CMV-Renilla construct as a control for transfection efficiency over 24 hours post mock UV treatment. White bars represent ratio of luciferase activities observed in cell lysates from HMGA1 ON cells transfected as above for 24 hours post mock UV treatment. Error bars represent ± 1 standard deviation.

Figure III-5. HMGA1 protein is associated with the endogenous *XPA* promoter sequence in MCF-7 cells over expressing HMGA1 proteins. Top panel represents PCR amplification of a 450bp fragment of the *XPA* promoter corresponding to nucleotides -50 to +400 in reference to the transcriptional start point (*tsp*). Bottom panel represents PCR amplification of a 350bp fragment of the KL promoter previously reported to interact with HMGA1. Lane 2 represents reactions in which input DNA from before immunoprecipitation was used as template for each primer set. Lanes 3 and 4 represent reactions in which DNA immunoprecipitated using an HMGA1 antibody was used as template. Lanes 1, 5 and 6 represent no template, non-specific antibody immunoprecipitated, and no antibody immunoprecipitated reaction controls, respectively.

Figure III-1.

A



B

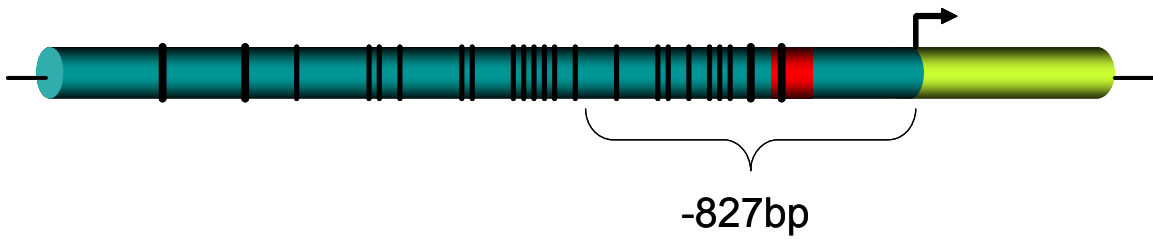


Figure III-2.

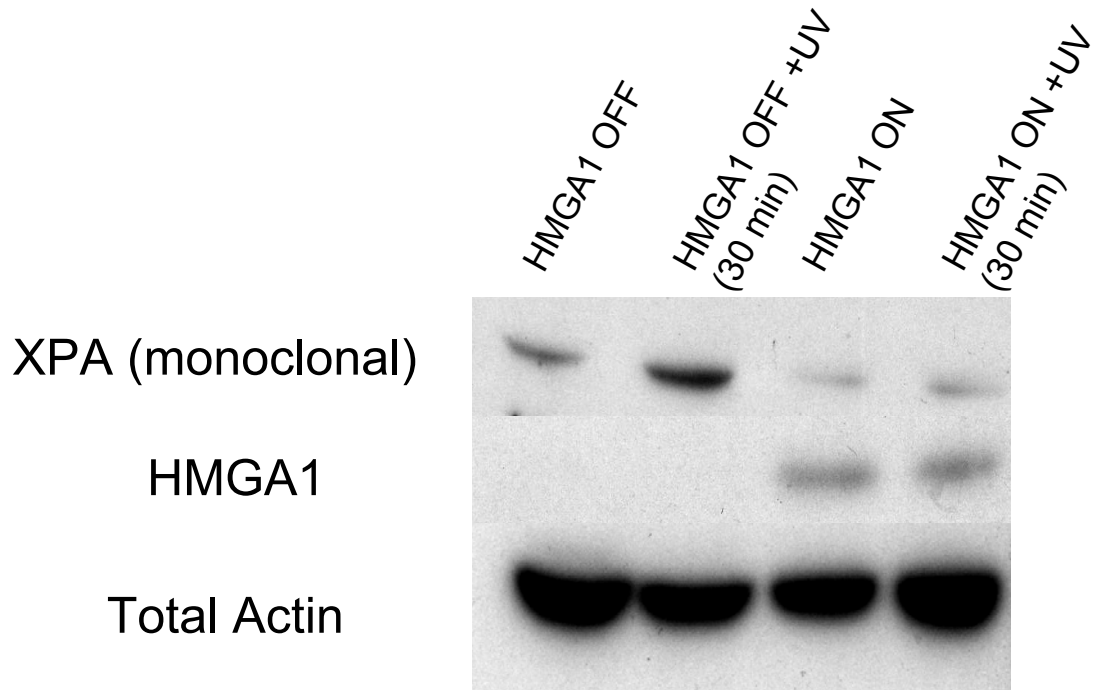


Figure III-3.

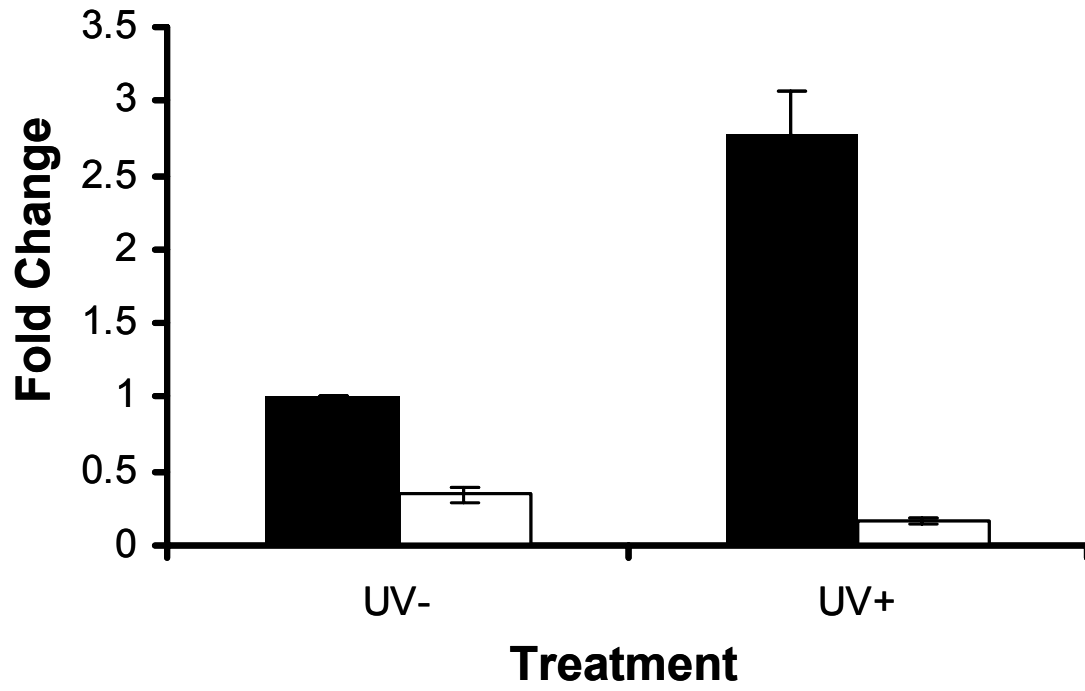


Figure III-4.

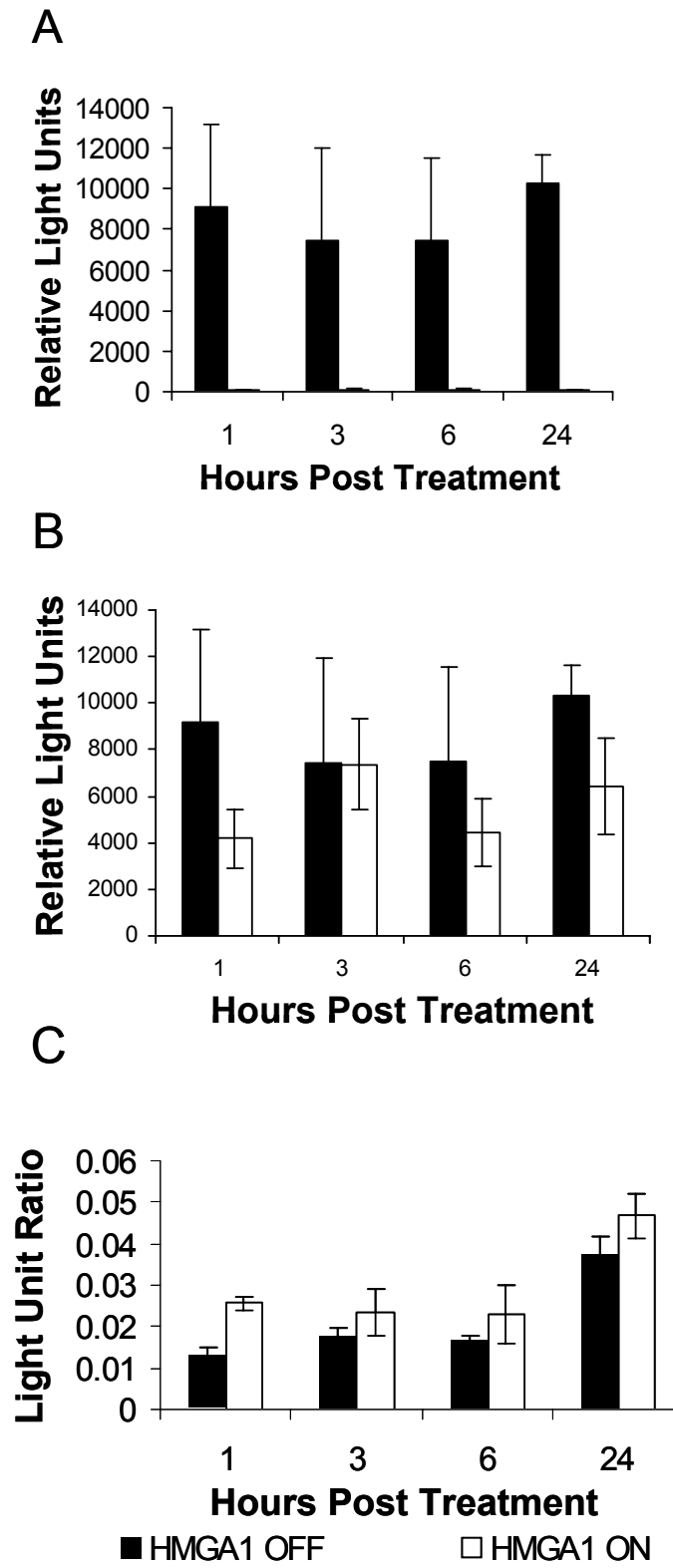
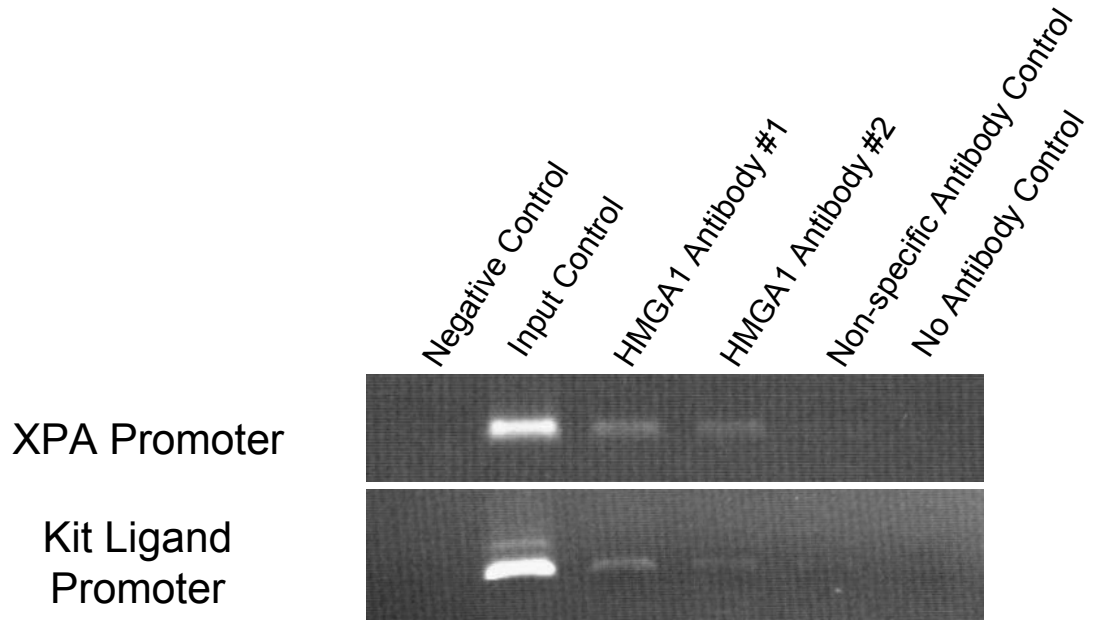


Figure III-5.



CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

Abstract

Over expression of HMGA1 proteins is one of the most commonly observed phenotypes in a variety of different cancers. Moreover, the degree of over expression of the HMGA1 proteins has been correlated to degree of malignancy and metastatic potential within cancer tissues that express them. Recent evidence has supported various roles for HMGA1 proteins in carcinogenesis, a process characterized by three distinct yet inter-related phenotypes including: (1) increased cellular proliferation, (2) ability to avoid programmed cell death, and (3) increased frequency of mutation. Work presented in this thesis supports a role for HMGA1 proteins in interference of normal nucleotide excision repair (NER) in a human mammary epithelial cell line induced to over express HMGA1 proteins, as well as a human mammary adenocarcinoma cell line (Hs578T) which naturally expresses HMGA1 proteins. Further stably transfected MCF-7 cells over expressing HMGA1 proteins exhibit decreased intracellular concentrations of a pivotal protein in NER processing, xeroderma pigmentosa group A complementing (XPA). Preliminary evidence indicates that decreased levels of this important NER factor may result from transcriptional repression involving HMGA1 action on the *XPA* promoter, however additional work must be done to confirm or refute this hypothesized phenomenon. Regardless of the mechanism, these results emphasize the complexity of HMGA1 activity within cells that express these proteins and support the growing body of evidence linking HMGA1 proteins with carcinogenic progression, making HMGA1 a potential target for diagnosis and treatment of a number of different cancers.

DISCUSSION

Since their discovery in 1983, the HMGA1 proteins have been reported to be involved in or influence an ever increasing number of molecular and cellular processes including cellular proliferation, viral integration, DNA replication, transcription, cellular differentiation and death, to name a few (reviewed by Reeves and Edberg, 2004). By the late 1980's, several groups reported increased HMGA1 mRNA and protein in a variety of cancers, procuring interest in the HMGA1 proteins as diagnostic markers of cellular transformation and malignant potential (Elton and Reeves, 1986; Johnson *et al.*, 1988; Bussemakers *et al.*, 1991; Giancotti *et al.*, 1991; Manfioletti *et al.*, 1991; Ram *et al.*, 1993; Chiappetta *et al.*, 1995). However, in 1995, Kazmierczak and colleagues described HMGA1 as the target of 6p21.3 chromosomal rearrangements in various benign mesenchymal tumors, providing the first evidence that HMGA1 proteins possessed oncogenic potential and causal involvement in tumorigenesis (Kazmierczak *et al.*, 1995). Later that same year, Schoenmakers *et al.* described involvement of HMGA2 proteins in rearrangements on human chromosome 12q15, an event frequently observed in benign solid tumors (Schoenmakers *et al.*, 1995). Since these findings were reported, the number of studies analyzing the role of the HMGA protein family in carcinogenesis has expounded, and, likewise, the body of evidence supporting a role for these proteins in neoplastic transformation and malignant progression.

It is important to note that the process of neoplastic transformation, or conversion into a cancer cell, is complex, involving failure of multiple cellular controls with corresponding activation of other cellular processes to produce a cell with three general phenotypic characteristics: (1) increased proliferation, or uncontrolled cell cycle

progression, (2) the ability to defy programmed cell death, also known as apoptosis, and (3) increased rate of mutation. To acquire all of these characteristics requires multiple molecular events, some of which are inherited, while others result from environmental experiences and still others result from aberrant regulation of normal day to day cellular functions. Consequently, several theories exist to explain how these characteristics might be acquired.

One such theory, referred to as the ‘mutator phenotype theory’, suggests that these characteristics are acquired when mutations occur in the DNA, rendering genes responsible for maintaining normal cellular control (tumor suppressor genes) non-functional, and activating genes responsible for cellular growth and apoptosis avoidance (oncogenes) (reviewed by Rockwell, 2001; Loeb and Loeb, 2003). This theory is supported by the existence of carcinogenic agents whose mechanism of action involves damage to DNA, exposure to which is linked to cancer development. These include agents such as ultraviolet light (UV), ionizing radiation, and dimethylsulfate (DMS) (reviewed by Vogelstein and Kinzler, 1993). Additional evidence in support of this theory comes from human diseases characterized by deficiencies in inherent cellular mechanisms of DNA repair, such as xeroderma pigmentosa, Bloom’s syndrome, Werner’s syndrome, and Cockayne’s syndrome, all of which render patients more susceptible to certain cancers (reviewed by Lombard *et al.*, 2005). Lastly, inherited mutations in certain genes have been shown to increase an individual’s predisposition to certain cancers, suggesting that there are fewer additional molecular events that need to occur to neoplastically transform cells in individuals harboring such mutations (Loeb, 1996; Tomlinson, 1999; Loeb and Loeb, 2000; Bignold, 2002; Bignold, 2003).

As previously mentioned, there are several inherent cellular mechanisms in place to maintain genomic integrity. These processes, collectively known as DNA repair, constitute multiple pathways, two of which are responsible for a majority of genetic insult response (Friedberg *et al.*, 1995). These two pathways are referred to as the excision repair pathways, each of which involves excision of single-stranded DNA harboring the damaged base from the genome, followed by synthesis of undamaged DNA complementary to the remaining undamaged DNA strand. Differences between these two pathways occur in their respective mechanisms of damage recognition, the type of DNA damage each repairs, the length of the single-stranded oligonucleotide excised, and the protein factors involved in the repair process.

The base excision repair (BER) pathway, discovered by Tomas Lindahl in 1974, responds to small base alterations, including methylation, alkylation and oxidative DNA damage (reviewed by Krokan *et al.*, 2000). Upon induction of DNA damage, the multistep BER process is initiated by recognition of the lesion by a damage-specific glycosylase enzyme which acts catalytically to release the damaged base, resulting in an apurinic or apyrimidinic (AP) site. An AP endonuclease then recognizes the abasic site and initiates subsequent BER processing including excision, resynthesis and ligation (**Chapter I, Figure I-5**; Krokan *et al.*, 1997; Mol *et al.*, 1999).

The process of nucleotide excision repair (NER) is responsible for recognition and removal of covalent DNA alterations that distort the DNA helix, initiation of which is characterized by an invariant damage-recognizing multiprotein complex with no catalytic activity (Lindahl and Wood, 1999). Following damage recognition, additional multiprotein complexes are recruited to the damaged site, resulting in cleavage of the

damaged strand, releasing the damaged base(s) as an oligonucleotide and resynthesizing and ligating the newly made DNA (**Chapter I, Figure I-4**).

Recent reports have established a role for HMGA1 proteins in the inhibition of both NER and BER processes (Adair et al., 2005; Baldassarre et al., 2005; reviewed by Reeves and Adair, 2005). These include studies demonstrating that sensitivity to UV light and cisplatin is increased in stably-transfected MCF-7 cells expressing HMGA1 proteins, as well as in murine ES cells, which, like human ES cells, naturally over express HMGA1 (Adair *et al.*, 2005; Baldassarre *et al.*, 2005). Moreover, both studies demonstrated that removal of HMGA1 from these cells, by three independent methods, abrogated sensitivity to both UV light and cisplatin.

Specifically, work presented in this thesis describes inhibition of nucleotide excision repair in human mammary epithelial cells over expressing HMGA1 proteins and potential molecular mechanisms of this inhibition. Chapter II describes published work (Adair *et al.*, 2005) regarding the increased sensitivity to UV light displayed by HMGA1-expressing cells, including both stably-transfected (MCF-7) and human (Hs578T) cell lines, as well as knockdown data demonstrating that resistance is recovered after removal of HMGA1 via two different methods (**Chapter II, Figures II-1 and II-2**). Given previously published work demonstrating that HMGA1-expressing cells are more proliferative than non-HMGA1-expressing cells (Treff *et al.*, 2003; Treff *et al.*, 2004), differences in UV sensitivity could be attributed to cell line differences in cell cycle distribution. However, data showing no significant distinction in the cell cycle distribution of random populations from both cell lines refutes this possibility (**Chapter II, Figure II-1, Panel B**). Paramount to published work presented in Chapter II is

quantitative analysis of CPD removal from MCF-7 cells induced to over express HMGA1 proteins, as well as from non-HMGA1-expressing MCF-7 cells (**Chapter II, Figures II-3 and II-4**). These data indicate that, upon exposure to physiologically relevant UV doses, MCF-7 cells expressing HMGA1 exhibit no induction of CPD removal until four to six hours post UV, when sub-maximal CPD removal is initiated (**Chapter II, Figure II-3, Panel C**). These data suggest that repair of CPDs is delayed and compromised in the presence of HMGA1 proteins within the context of a living cell. However, in these studies examining CPD removal, the formation and relevance of 6-4 photoproducts, which are also produced upon UV exposure, albeit to a lesser degree than CPDs, to these observations must also be considered. Regarding the molecular mechanism of NER inhibition, preliminary evidence indicating whether the observed inefficiency of CPD removal is due to HMGA1 association with UV-induced lesion sites, or to interference with NER factor expression or activity is addressed in the remainder of this thesis.

Unpublished immunocytochemical data presented in Chapter II addresses whether removal of 6-4 photoproducts is also hindered in MCF-7 cells expressing HMGA1 proteins (**Chapter II, Figure II-5**). While initiation of 6-4 photoproduct removal, like that of CPDs, is also delayed in these cells, complete removal of these lesions, as indicated by no detectable fluorescence, was achieved in a time comparable to that of non-HMGA1-expressing cells (**Chapter II, compare Figure II-4 to Figure II-5**). Thus, while removal of CPDs is hindered in cells expressing HMGA1 proteins, repair of 6-4 photoproducts does not appear to be completely impeded, although initiation is delayed.

Since HMGA1 has been shown to bind to non-B-form DNA, including naked DNA fragments containing CPDs and Holliday junctions (reviewed by Reeves and

Adair, 2005; Adair *et al.*, 2005), immunocytochemical analyses were employed to determine if HMGA1 associated with either 6-4 photoproducts or CPDs in living cells after UV exposure (**Chapter II, Figure II-6 and II-7**). Based on merged images from these experiments, HMGA1 is capable of co-localizing with 6-4 photoproducts, but not with CPDs in MCF-7 cells induced to over express HMGA1. This could be explained by differences in the structural nature of 6-4 photoproducts and CPDs, which is thought to be the underlying reason for observed differences in mutagenicity and the rate of repair of these lesions in normal cells (Friedberg *et al.*, 1995; Cleaver *et al.*, 1995). While 6-4 photoproducts result from covalent linkage of the 6 position of one pyrimidine residue to the 4 position of an adjacent pyrimidine residue, CPDs result from saturation of respective 5,6 double bonds between adjacent pyrimidines (Friedberg *et al.*, 1995). *In vitro* data demonstrated that the spectrum of CPDs formed in a DNA fragment bound by HMGA1, before exposure to UV light, was altered (Adair *et al.*, 2005). Thus, it could be possible that 6-4 photoproducts are more likely to be induced at locations within the genome where HMGA1 is bound, however, based on the average per cell fluorescence intensity in **Figure II-5** immediately following UV exposure (compare panels labeled ‘Time 0 hours’), there does not appear to be an increase in the number of 6-4 photoproducts induced in HMGA1-expressing cells. Coincidentally, this was also observed for CPD induction, indicating that while *in vitro* data suggested modulated CPD formation in DNA bound by HMGA1, no evidence for this phenomenon in living cells was demonstrated (Adair *et al.*, 2005).

It could be hypothesized that HMGA1 prefers binding to the more helically distorting UV-induced lesion, the 6-4 photoproduct, rather than the CPD, and that this

binding could prevent repair factors from having access to 6-4 photoproducts, thus causing the observed delay in repair initiation. Further evidence to support HMGA1 binding to 6-4 photoproduct lesions comes from comparison of time course data from immunocytochemical analyses and quantitative CPD removal (**Chapter II, Figures II-3 and II-5**). While initiation of 6-4 photoproduct repair is delayed in HMGA1-expressing cells, complete repair is achieved between four and six hours post UV, which incidentally, corresponds to the initiation point of sub-maximal CPD repair in the same cells. Thus, one mechanistic hypothesis for these observed effects is that HMGA1 binds to 6-4 photoproducts, preventing repair factors from having initial access, thus delaying repair of these lesions. As with all protein-DNA interactions, this dynamic interaction is eventually interrupted and successful repair of 6-4 photoproducts is completed, freeing available repair factors to resolve CPD lesions. This hypothesis, however, does not explain the sub-maximal efficiency of CPD removal observed once repair is initiated. Additionally, it could also be possible for HMGA1 proteins to interact with NER factors, either freely, or while simultaneously interacting with DNA lesions, but data presented here does not address this possibility.

The work presented in Chapter III describes evidence for HMGA1-mediated down-regulation of a pivotal NER factor, xeroderma pigmentosa complementation group A (XPA), as one of many possible molecular mechanisms for the observed deficiency in CPD removal. Pertinent to this hypothesis, Cleaver *et al.* have demonstrated that sub-normal levels of functional XPA in cells results in efficient removal of 6-4 photoproducts, but not of CPDs (reviewed by Cleaver *et al.*, 1995). In accordance with previous microarray data, indicating a 2.6-fold decrease in XPA transcript levels in

HMGA1-expressing MCF-7 cells, western blot analysis revealed a concomitant 3-fold decrease in intracellular XPA protein levels in these same cells (**Chapter III, Figure III-2**). Moreover, non-transfected MCF-7 cells displayed characteristic intracellular increase in XPA protein thirty minutes following exposure to a physiologically relevant dose (20J/m²) of UV radiation, while HMGA1-expressing MCF-7 cells exhibited no detectable change in intracellular XPA concentrations (**Chapter III, Figures III-2 and III-3**). This decrease in intracellular XPA protein could be responsible for the defective CPD removal also exhibited by MCF-7 cells expressing HMGA1, but data to support this hypothesis is not presented in this thesis.

Further unpublished work presented in this thesis addresses the mechanism by which XPA may be down-regulated in MCF-7 cells induced to over express HMGA1 proteins. Several possibilities, as discussed in Chapter III, include destabilization of XPA protein or mRNA, or inhibition of mRNA translation, although none of these roles has been well-described for HMGA1 proteins in the literature (Manabe *et al.*, 2003; Sgarra *et al.*, 2005). An alternative, and more obvious, mechanism involves transcriptional repression of the *XPA* gene, a role that has been well-characterized for HMGA1 with respect to other genes in the human and murine genomes (Baldassarre *et al.*, 2003; Treff *et al.*, 2003; Treff *et al.*, 2004; reviewed by Reeves and Beckerbauer, 2001). This mechanism is further supported by western blot data from non-HMGA1-expressing MCF-7 cells indicating that XPA protein levels dramatically increase within 30 minutes after exposure to UV radiation. Given that the reported half-life of the XPA protein is >12 hours, the observed increase in intracellular protein concentrations after 30 minutes, approximately 1/24 the half-life, suggest either transcriptional activation, mRNA

stabilization or increased message translation (Cleaver *et al.*, 1995). Given that basal transcript levels, in the absence of UV, exhibited a significant difference between HMGA1-expressing cells and non-HMGA1-expressing cells via microarray analyses, the possibilities were further narrowed to transcriptional repression or mRNA destabilization.

To investigate the possible mechanisms of XPA transcriptional down-regulation in HMGA1-expressing cells, reporter assays utilizing a vector encoding the firefly luciferase reporter gene under the control of 2.2kb of the XPA promoter were employed (**Chapter III, Figure III-4**). In these studies, it was demonstrated that basal transcriptional activity of this reporter construct could be detected in both HMGA1-expressing MCF-7 cells as well as their non-expressing counterparts, compared to cells transfected with promoter-less vector (**Chapter III, Figure III-4, Panels A and C**). Interestingly, however, basal transcriptional levels appeared to be the same in MCF-7 cells over expressing HMGA1 proteins as indicated by statistically similar luciferase activities in cell lysates from both HMGA1-expressing and non-expressing cells (**Chapter III, Figure III-4, Panel C**). Furthermore, no induction of luciferase activity, and thus promoter activity, upon UV irradiation was observed, indicating that the included 5' untranslated region (UTR) used in these reporter assays was not UV-responsive (**Chapter III, Figure III-4, Panel B**). It is possible that an additional sequence, not included in this reporter construct, is required for UV-induction of promoter activity. Likewise, it is also possible that some portion of the included 5'UTR is repressing UV-induction, although neither of these hypotheses was tested within the scope of the work presented here. Basal reporter activities in the two cell lines examined provide no preliminary evidence indicating that HMGA1 is capable of either directly or

indirectly regulating transcription of the *XPA* gene in MCF-7 cells. However, it is important to note that these reporter assays do not examine promoter activity within the context of chromatin, since the transfected construct is not integrated into the genome. Thus, as HMGA1 serves a structural role in chromatin remodeling during transcriptional control of many other genes, this may be pertinent to observed reporter assay results in MCF-7 cells over expressing these proteins.

To assess whether HMGA1 is associated with the endogenous *XPA* promoter in MCF-7 cells over expressing these proteins, chromatin immunoprecipitation analyses were employed (**Chapter III, Figure III-5**). In these experiments, HMGA1-expressing cells were exposed to 1% formaldehyde, which forms covalent cross-links between proteins as well as proteins and DNA, as long as they are interacting with one another within the cellular context. Regions of the genome with which HMGA1 is associated, either via protein-protein interactions or direct DNA binding, can then be immunoprecipitated by incubating in the presence of an HMGA1-specific antibody. Polymerase chain reaction (PCR) can then be used to identify fragments pulled down with the protein-specific antibody. Importantly, a 450bp region of the endogenous *XPA* promoter, corresponding to nucleotides -448 to +2, including the conserved negative regulatory region and one potential HMGA1-binding site, was pulled down using this method (**Chapter III, Figure III-5**). In these experiments, successful amplification of a 350bp fragment of the kit ligand (KL) promoter was used as a positive control for immunoprecipitation, since this endogenous promoter was previously reported to interact with HMGA1 in stably-transfected MCF-7 cells (Treff *et al.*, 2004). These data indicate that HMGA1 is associated with the endogenous *XPA* promoter, although they do not

distinguish between association via DNA binding or protein-protein interactions. Also, given the ability of HMGA1 to stimulate formation of complex DNA structures, such as transcriptional enhanceosomes, it is possible that HMGA1 may be serving a molecular glue-like function on the *XPA* promoter in the formation of a transcriptional ‘repressosome’, however, evidence for this is not presented in this thesis.

Regarding the work presented here, HMGA1 clearly affects NER functioning in response to UV-induced DNA damage in MCF-7 cells induced to over express these proteins. Evidence presented provides some preliminary mechanistic information indicating that HMGA1 may interfere with NER via association with UV-induced 6-4 photoproducts, as well as through repression of the NER factor XPA. Furthermore, Appendix I describes a study demonstrating sensitivity of MCF-7 cells over expressing HMGA1 proteins to dimethylsulfate, a DNA methylating agent whose base modifications are typically repaired by BER processing (**Appendix I, Figure A-2**). This, in combination with microarray and quantitative real-time PCR data indicate that HMGA1 proteins may also interfere with the process of BER, although much more work needs to be done to establish this proposed effect.

While much remains to be investigated to establish HMGA1-mediated excision repair compromise in human tumors, these reports provide important preliminary evidence supporting HMGA1 as an oncogenic factor contributing to the increased mutation frequency observed in cancers that almost universally over express these proteins. Moreover, they emphasize the potential advantage of targeting HMGA1 proteins as a means of diagnosing and treating many different cancer types.

FUTURE DIRECTIONS

Based on data presented in this thesis, future work follows two obvious directions: (1) Elucidating direct HMGA1-binding to UV-induced DNA damage and the effect of this direct interference on NER efficiency, and (2) Further characterization of HMGA1-mediated XPA repression and the effect of this down-regulation on NER efficiency. While both mechanisms are likely contributing to observed NER deficiencies in HMGA1-expressing cells, each pathway, and its contribution to the overall NER defect must first be elucidated before combinatorial studies can be done with confidence. Furthermore, it may very well be the case that multiple mechanisms, other than those indicated here are also responsible, to some degree, for the observed NER deficiency, and those proposed must be eliminated as contributing factors before other, less obvious alternatives, can be elucidated.

Regarding the direct interaction of HMGA1 with UV-induced 6-4 photoproducts, and not CPDs, additional *in vitro* experiments using DNA fragments containing both types of lesions will be useful. While reported work using fragments containing CPDs has demonstrated that HMGA1 is capable of binding to these lesions in cell-free systems, evidence from living cells does not indicate this possibility (**Chapter II, Figures II-6 and II-7**; Adair *et al.*, 2005). Given that UV exposure induces formation of both 6-4 photoproducts in living cells, and not just CPDs, *in vitro* experiments should be repeated using DNA containing both of these lesions to determine if HMGA1 prefers structurally distinct 6-4 photoproduct lesions. Moreover, binding of HMGA1 to these lesions may also be affected in the context of chromatin, thus *in vitro* experiments examining binding

of HMGA1 to both CPDs and (6-4) photoproducts in chromatin reconstitution systems must also be pursued.

The hypothesis that HMGA1 proteins may be bound to lesion sites, preventing repair factors from having access to the damage must also continue to be studied. This hypothesis is one of a few that will be indicated if HMGA1-induced deficiencies in XPA, when corrected, result in only partial recovery of UV resistance and NER efficiency. However, it is also of importance to note that complete recovery of UV resistance and NER function in HMGA1-expressing MCF-7 cells after XPA complementation does not exclude this hypothesis, given *in vitro* evidence for HMGA1 binding to damaged DNA fragments both before and after UV damage induction (Adair *et al.*, 2005). It is also possible that in binding to damaged sites, HMGA1 proteins maintain interactions with repair factors recruited to those damaged sites, not only preventing them from catalyzing repair reactions, but also sequestering them from non-HMGA1-bound lesions. This hypothesis can be pursued via immunocytochemical analyses utilizing antibodies against known NER factors to determine if these factors co-localize with HMGA1 associated with lesion sites. Protein immunoprecipitation studies could also be useful in demonstrating interactions between HMGA1 and proteins involved in NER, if these proposed interactions exist in the cellular milieu. Given the large number of previously characterized protein-protein interactions HMGA1 participates in, it will also be necessary to perform immunoprecipitation before and after UV exposure to determine if damage induces HMGA1-NER factor interactions.

Additionally, if interaction of HMGA1 with either UV-induced lesions or NER factors is indicated, various mutational assays can be employed to elucidate binding

mechanisms. However, it should be emphasized that given results associating HMGA1 with endogenous DNA sequences in the *XPA* promoter, inhibiting certain DNA-binding or protein-protein interactions could also interfere with proposed transcriptional repression and its associated effects on NER, providing results which could be difficult to interpret.

While transcriptional activity of the *XPA* promoter does not appear to be repressed in MCF-7 cells over expressing HMGA1 proteins, further analysis of concomitant decreases in XPA transcript levels before and after UV-irradiation should be performed. In this respect, knockdown of HMGA1 in these cells should result in recovery of normal XPA transcript levels and, subsequently, protein levels. This can be analyzed via various methods of measuring intracellular mRNA levels, such as reverse transcriptase (RT)-PCR, Northern blotting, and quantitative real-time PCR. Moreover, reporter analysis of promoter activity in MCF-7 cells over expressing HMGA1 proteins before and after UV irradiation needs to be performed.

Importantly, to determine whether HMGA1 associated with the endogenous *XPA* promoter elicits transcriptional activity, further studies to characterize the nature of this interaction should be performed. Foremost, using ChIP samples already collected and tested for both *XPA* and *KL* pull-down, the remainder of the 2.2kb 5'UTR of the *XPA* gene must be analyzed for association with HMGA1. These studies may provide inference as to which regions of the promoter, and thus, which known transcription factors or potential HMGA1-binding sites, may be involved in the association. Additionally, various cross-linking agents, including the FR and FK pro-drug classes which have been shown to cross-link HMGA1 to the DNA where minor groove

interactions exist, can be employed to distinguish between direct HMGA1 binding to promoter regions versus association via protein-protein interactions (reviewed by Reeves and Edberg, 2004). However, there are certain time constraints resulting from use of these cross-linking agents, including their requirement for in-cell activation over several hours, which could inhibit studies examining HMGA1-promoter interactions after UV exposure.

Generally, with respect to observed XPA deficiencies and NER inhibition in MCF-7 cells over expressing HMGA1 proteins, it will be important to evaluate to what degree this repression of XPA contributes to the observed NER phenotype. To determine this, rescue experiments utilizing full-length *XPA* cDNA under the control of a non-HMGA1-regulated, constitutive promoter can be pursued. If this repression contributes to the observed NER deficiency, as proposed, recovery of intracellular XPA concentrations should rescue, at least in part, removal of CPDs and overall UV-sensitivity previously described in MCF-7 cells over expressing HMGA1 proteins.

In conclusion, while the specific studies described above will be useful in elucidating mechanisms of HMGA1-mediated NER inhibition in human cell lines, further studies in murine and human tissue samples must be done to establish this effect in cancers which naturally over express these proteins. In this regard, it is important to note that UV sensitivity was also observed in the human mammary epithelial adenocarcinoma cell line (Hs578T), which naturally over expresses HMGA1 proteins (**Chapter II, Figure II-2**). Additionally, other groups are pursuing mouse models for decreased repair in the presence of HMGA1 proteins, and some preliminary evidence to this end is available in the literature (Baldassarre *et al.*, 2005).

Pertinent to this proposed role for HMGA1 proteins, it must be established that over expression of these proteins not only leads to compromised excision repair, but that tissues expressing HMGA1 proteins also have an increased frequency of mutation. In this regard, mouse models of HMGA1 over expression will also be useful. Additionally, flow cytometric assays for microsatellite instability are available and clonal expansion of MCF-7 cells over expressing HMGA1 proteins for analysis by this method is currently underway (Vo *et al.*, 2005).

Finally, work presented here represents the first preliminary evidence linking HMGA1 proteins, which are naturally over expressed in a variety of human cancers, to the decreased genomic integrity associated with those cancers. However, as outlined above, much more extensive investigation is necessary to define the molecular mechanisms of this linkage, and solidify the role of HMGA1 proteins as instigators of increased mutation frequency.

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APPENDIX I

POTENTIAL ROLE FOR HMGA1 PROTEINS IN DISRUPTION OF BASE EXCISION REPAIR

Abstract

The majority of DNA damage has endogenous origin, including oxidative damage, methylation and base deamination, all of which are thought to be repaired by base excision repair pathways. The widely accepted model for base excision repair (BER) includes five steps: (1) base removal, (2) incision of the abasic site, (3) processing of strand break termini, (4) DNA synthesis, and finally (5) ligation. Several reports have indicated that defects in the late steps of BER are lethal, while no significant phenotypes have been observed in early gene defects. Here, preliminary evidence to support a role for HMGA1 in down-regulation of BER genes BRCA2, XRCC1 and DNA Ligase III, as indicated by microarray analyses and quantitative real-time PCR, is presented.

Additionally, MCF-7 cells induced to over express HMGA1 proteins are more sensitive to dimethylsulfate (DMS), a DNA damaging agent whose resulting lesions are repaired by BER pathways. Together, these data indicate that HMGA1 may inhibit BER, as well as NER, dually compromising the two DNA repair pathways responsible for the majority of genome maintenance. This, too, supports a role for HMGA1 proteins in promoting acquisition of mutation, a phenomenon associated with cancer cells, which commonly over express these proteins.

INTRODUCTION

Cells are exposed to a variety of genetic insults on a daily basis from both environmental and endogenous sources. Insults of endogenous origin, including (i) oxidation of DNA by reactive oxygen species (ROS) generated in cells by aerobic metabolism, (ii) spontaneous deamination of bases, and (iii) methylation of bases by S-adenosylmethionine (reviewed by Fortini *et al.*, 2003; Friedberg *et al.*, 1995). Most of these lesions can cause stalling of replicative DNA polymerases, however, when read through, an alarming number cause miscoding. For instance, O6-methylguanine and 8-oxoguanine mispair with thymine and adenine, causing GC>AT and GC>TA transversions, respectively (Friedberg *et al.*, 1995; Krokan *et al.*, 1997; Mol *et al.*, 1999). Moreover, it is estimated that 2×10^4 oxidative DNA lesions are created and processed per cell per day in the human body (Foray *et al.*, 2003). Sohal and Weindruch proposed in 1996 that these lesions are, in fact, a source of persistent, chronic DNA damage which could contribute to aging (Sohal and Weindruch, 1996; reviewed by Balaban *et al.*, 2005).

Most of these damaged bases are removed by a single-strand DNA excision repair pathway known as base excision repair (BER), while a small fraction are repaired by direct damage reversal (Lindahl and Wood, 1999). BER consists of three characterized subpathways, all of which can be simplified into five basic steps: (1) base removal by a damage-specific glycosylase, (2) incision at the resulting abasic site by an AP (apurinic or apyrimidinic, depending on the base removed) endonuclease, (3) processing of the resulting blocked termini to remove a 2-8 nt stretch, (4) DNA synthesis to fill in the gap, and (5) resealing of the DNA strand (Friedberg *et al.*, 1995; reviewed by Fortini *et al.*,

2003). Sub-pathways of BER are classified as being short-patch or long-patch, depending on the number of nucleotides removed during the excision, and further sub-pathway distinction is characterized by the type of enzyme that recognizes and removes the damaged base (**See Chapter I, Figure I-2**; reviewed by Krokan *et al.*, 2000).

The first step, catalyzed by a DNA glycosylase enzyme, involves recognition of the damaged base and cleavage of the N-glycosidic bond to produce the AP site. The AP site itself is considered to be a DNA lesion, commonly referred to as a single-strand DNA interruption (SSI), which can result in cytotoxicity if left unrepaired (Loeb, 1985). These SSIs are then repaired via the long-patch or short-patch BER pathways, which utilize different DNA polymerases and DNA ligases (**See Chapter I, Figure I-2**; Erick *et al.*, 2003). Each pathway uses either an AP endonuclease or an AP lyase to generate a single-strand break (ssb) via cleavage of the phosphodiester bond (Friedberg *et al.*, 1995). In short-patch BER, resynthesis of a single nucleotide at the lesion site is accomplished via the action of DNA polymerase β (Pol β). Long-patch BER involves resynthesis of two to eight nucleotides by either Pol β or replicative polymerases δ or ϵ . Displacement of the strand containing the lesion involves flap endonuclease 1 (FEN1) and proliferating cell nuclear antigen (PCNA), which recognize and cleave the base of the flap structure of the damaged oligonucleotide and act as a loading clamp for replicative polymerases, respectively (reviewed by Fortini *et al.*, 2003). Ligation of newly synthesized strands falls to two enzymes, DNA ligase I and III. DNA ligase I has been shown to interact with Pol β and PCNA, and is thought to be involved in nick ligation during both DNA replication and repair (Li *et al.*, 1994; Prasad *et al.*, 1996; Levin *et al.*, 1997). DNA ligase III has two isoforms, designated α and β , which result from alternative splicing,

and differ in their C-termini (Mackey *et al.*, 1997). DNA ligase III α has been demonstrated to interact with the DNA repair protein XRCC1, providing a physical link between this DNA ligase and DNA pol β (Nash *et al.*, 1997; Cappelli *et al.*, 1997). Cappelli *et al.* demonstrated that this interaction allows DNA ligase III α to participate in the repair of direct and indirect SSBs during short-patch BER (Cappelli *et al.*, 1997).

Since its discovery by Thomas Lindahl in 1974, BER has been characterized as the DNA repair pathway which is highly conserved as the cellular defense mechanism against spontaneous DNA decay, as well as environmental threats (Eisen and Hanawalt, 1999). Considering this, it was surprising to discover that absence of each of the DNA glycosylases separately results in only small or moderate changes in spontaneous mutation frequencies and no gross phenotypical changes (reviewed in Krokan *et al.*, 2000, and Fortini *et al.*, 2003). However, defects in late-BER steps (after base removal) are considered lethal and are hypothesized to be the result of BER genes like Pol β , APE and XRCC1 being involved in developmental processes more so than in DNA repair, although no work to support this hypothesis has been published (reviewed in Fortini *et al.*, 2003).

In this section, initial data indicating that transcript levels of BER genes XRCC1, and DNA ligase III, as well as breast cancer gene 2 (BRCA2), which is thought to be involved in transcriptional regulation, chromatin remodeling, cell cycle checkpoint and control, as well as BER, are lower in cells over expressing HMGA1 proteins. Additionally, as is the case in cells deficient in BER, MCF-7 cells are more sensitive to the methylating agent dimethylsulfate (DMS), whose methylations of hydrogen-bond accepting ring nitrogens on adenine, guanine and cytosine bases are normally repaired by

this pathway. The work presented here provides preliminary evidence that BER, as well as NER, is compromised in MCF-7 cells over expressing HMGA1 proteins.

MATERIALS AND METHODS

Quantitative real-time PCR

MCF-7 cells, either induced to over express HMGA1 proteins (HMGA1 ON) or un-induced (HMGA1 OFF), were cultured as described in chapters 2 and 3, materials and methods, in 100mm culture dishes until 80% confluent. Cells were then harvested for total RNA using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. 2µg total RNA was reverse transcribed into cDNA using an AMV First Strand Synthesis Kit (Promega) according to the manufacturer's recommended protocol. 2µL of the first-strand synthesis reaction was used with TaqMan Universal Master Mix (Applied Biosystems) and either HPRT1 (assay ID-Hs99999909_m1) or DNA Lig III (assay ID-Hs00242692_m1) assays-on-demand solutions as recommended by the supplier (Applied Biosystems). Relative quantitation was carried out using the comparative C_T method as described in User Bulletin #2: Relative Quantitation of Gene Expression (P/N 4304859) (Applied Biosystems).

Analysis of survival of HMGA1-expressing cells after exposure to DMS

Ten days post-induction of HMGA1 protein expression, cell cultures were analyzed for survival following exposure to DMS at varying concentrations. Media was removed from culture plates and cells were trypsinized to remove monolayers. Samples were centrifuged and resuspended in serum-free media containing no antibiotics. DMS was added at varying concentrations (0, 0.1, 0.5, 1, 2.5 and 5mM) and cells were incubated on ice for 30 minutes in suspension. Following incubations, cells were centrifuged and

DMS-containing media was removed. Cells were resuspended in complete media without antibiotics and re-plated for 48 hours to allow one full cell cycle progression to occur. Cell survival post-DMS treatment was monitored via Trypan Blue (0.4%) exclusion and cell viability counting using a hemacytometer. Briefly, cells were first treated with trypsin/EDTA to remove monolayers, followed by centrifugation. Cell pellets were then resuspended in limited volumes of growth media and diluted 1:1 in 100 μ l of 0.4% Trypan Blue dye. After 5 min, 20 μ l of cell suspension was loaded onto a Levy Ultra Plane hemacytometer and the number of viable cells determined. Percent survival was calculated by comparison to non-DMS-treated controls.

RESULTS

Previous microarray data indicated that mRNA levels of BER genes XRCC1, BRCA2 and DNA Ligase III were decreased 1.7, 2.2 and 4.8-fold, respectively, in MCF-7 cells over expressing HMGA1 proteins compared to non-HMGA1-expressing cells (**See Chapter I, Figure I-3**). While time did not permit confirmation of these results for XRCC1 and BRCA2 genes, quantitative real-time PCR was used to verify these results for DNA Ligase III transcript levels (**Figure A-1**). Based on this data, DNA Ligase III transcript levels were down-regulated 2.2-fold in the presence of HMGA1 proteins. While this confirms down-regulation in MCF-7 cells expressing HMGA1 proteins, these data, representing three independent experiments, demonstrate only about half the down-regulation observed in array experiments. The reason for this discrepancy between the cDNA and oligonucleotide microarray results is unknown. Nevertheless, these results importantly demonstrate, by two independent techniques, the negative influence of HMGA1 protein expression on the final, and most crucial, stage of BER.

To determine if BER was, in fact, compromised in HMGA1-expressing MCF-7 cells, sensitivity to DMS was assessed. As Friedberg *et al.* described, if cells are deficient in a particular DNA repair pathway, then those cells will be more sensitive to DNA damaging agents whose lesion would normally be repaired by that pathway (Friedberg *et al.*, 1995). Both HMGA1 ON and HMGA1 OFF cells were exposed to DMS doses from 0 to 5mM, and at all doses $\geq 0.5\text{mM}$ DMS, HMGA1-expressing cells were as much as 50% more sensitive to this mutagen (**Figure A-2**).

CONCLUSIONS

Limiting intracellular concentrations of functional DNA repair factors has been shown to decrease repair pathway efficiency in a number of different studies (Friedberg *et al.*, 1995; reviewed by Cleaver and States, 1997 and Fortini *et al.*, 2003). Given work presented in chapters one through four of this thesis demonstrating that NER is repressed in the presence of HMGA1 proteins and that this repression may be induced by direct, or perhaps indirect, transcriptional down-regulation of XPA, an important NER factor, it is possible that BER could be compromised in the same manner. Microarray data indicating that several important BER factor transcripts are reduced in HMGA1-expressing cells supports this possibility (**Chapter I, Figure I-3**).

DNA Ligase III is the enzyme thought to be responsible for sealing BER nicks in short-patch BER, and has been shown to be important in homologous recombination (Caldecott *et al.*, 1994; Kubota *et al.*, 1996). Furthermore, ligase III is the only enzyme capable of catalyzing the formation of phosphodiester bonds in mitochondria, where consistent production of reactive oxygen species (ROS) induces a large amount of spontaneous DNA damage on a daily basis (Pinz *et al.*, 1998; Lakshimpathy and

Campbell, 2001). In addition, Lakshimpathy and Campbell demonstrated in 2001, that antisense-mediated reduction of DNA Ligase III levels reduced integrity of mitochondrial DNA (Lakshimpathy and Campbell, 2001). Finally, two reports have indicated that Chinese hamster ovary-derived EM-9 cells, which are deficient in XRCC1 and DNA ligase III, are defective in homologous recombination and sensitive to DNA alkylating agents (Hoy *et al.*, 1987; Thacker, 1989).

XRCC1 has been shown to interact with DNA pol β as well as DNA ligase III (Caldecott *et al.*, 1994; Kubota *et al.*, 1996). Marintchev *et al.* also demonstrated that XRCC1 binds to gapped DNA complexed with pol β (Marintchev *et al.*, 1999). Furthermore, XRCC1 has been shown to interact with, and stimulate the catalytic activity of, APE1, suggesting that once DNA is nicked by APE1, XRCC1 mediates positioning of pol β to perform its lyase activity (Vidal *et al.*, 2001; Sobol *et al.*, 2003). In fact, several sources have hypothesized that XRCC1 plays a role in every stage of BER, acting as a scaffold for assembly of repair factors and efficient lesion processing (Rice, 1999; Wilson and Kunkel, 2000; Mol *et al.*, 2000; Thompson and West, 2000). This complex role for XRCC1 is supported by evidence that deletion of this gene in mice is lethal at embryonic day 6.5 (Tebbs *et al.*, 1999).

Lastly, the observed deficiency in BRCA2 can also be related to inefficient BER. Previously, Baldassarre *et al.* demonstrated that BRCA1 transcript levels were decreased in both MCF-7 cells and murine ES cells over expressing HMGA1 proteins (Baldassarre *et al.*, 2003). While BRCA1 and BRCA2 have been implicated in homologous recombination and transcription-coupled repair, they have also been shown to interact with RPA and PCNA during replication-associated repair (reviewed by Venkitaraman,

2002; Scully *et al.*, 1997; Wang *et al.*, 2000; Choudhary and Li, 2002). Moreover, defects in BRCA1 and BRCA2 have been correlated to increased incidences of breast cancer and are classified as tumor suppressor genes (reviewed by Venkitaraman, 2002).

These data, along with reported studies on the down-regulation XRCC1, DNA Ligase III and BRCA2, implicate another mechanism by which HMGA1 decreases BER efficiency, thus decreasing genomic integrity. Considering the impact of a BER deficiency on daily genomic maintenance, establishing a link between HMGA1 expression and BER deficiency would further support a role for these proteins in promoting mutation, a common feature of the cancers known to over express HMGA1.

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FIGURE LEGENDS

Figure A-1. MCF-7 cells expressing HMGA1 proteins contain less DNA ligase III transcript than do non-HMGA1-expressing MCF-7 cells. Graphical representation of triplicate quantitative RT-PCR results for DNA ligase III transcripts in HMGA1 OFF and HMGA1 ON cells as a function of fold change. Results were normalized to HPRT threshold of amplification. Error bars represent ± 1 standard deviation.

Figure A-2. MCF-7 cells expressing HMGA1 proteins are more sensitive to DMS than non-HMGA1-expressing MCF-7 cells. Black bars represent % survival for HMGA1 OFF cells at each indicated DMS dose. White bars represent HMGA1 ON cell survival at the same DMS concentrations. Error bars represent ± 1 standard deviation.

Figure A-1.

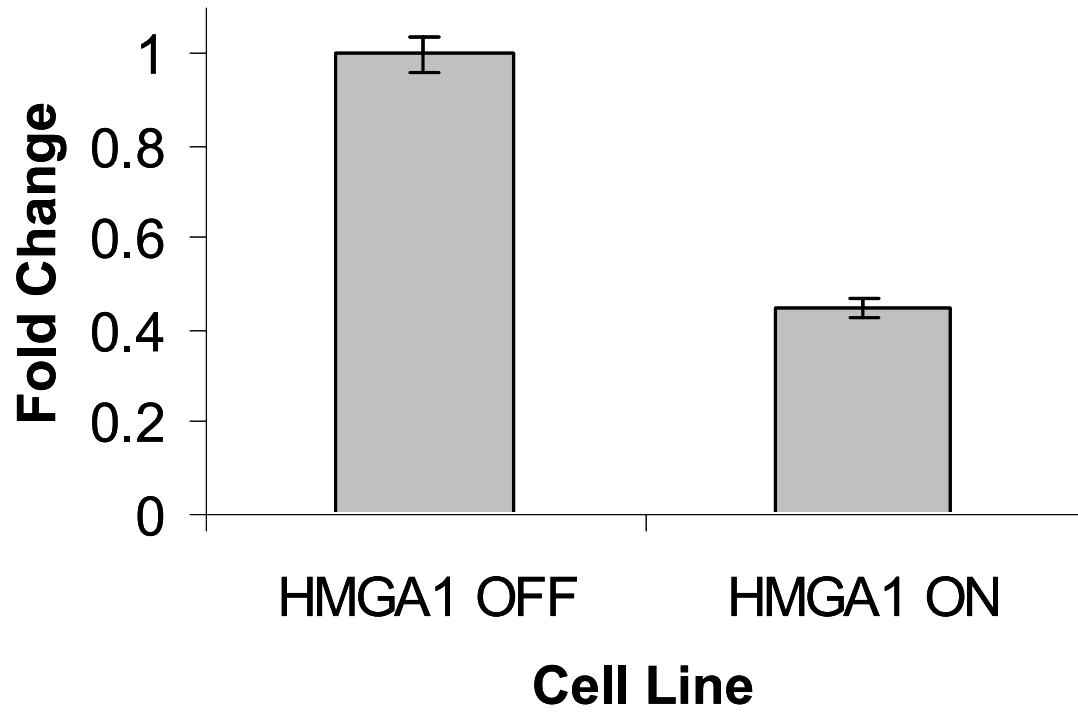


Figure A-2.

