HIGH MOBILITY GROUP A1 AND MITOCHONDRIAL TRANSCRIPTION FACTOR A COMPETE FOR BINDING TO MITOCHONDRIAL DNA

By

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The members of the committee appointed to examine the thesis of KELSEY JANEL WERTZLER find it satisfactory and recommend that it be accepted.

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Abstract

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High mobility group A1 (HMGA1) protein functions in the nucleus as a gene regulatory transcription factor. During restricted parts of the normal cell cycle, a sub-fraction of HMGA1 reversibly translocates to mitochondria. However, HMGA1 is found in both the nucleus and mitochondria throughout the cell cycle of cancerous cells, which almost universally over-express this protein. The exact activities of HMGA1 in mitochondria are currently unknown, but the protein has been demonstrated to bind to the regulatory D-loop region of the mitochondrial DNA (mtDNA) and its over-expression is associated with overall mitochondrial dysfunction. Mitochondrial transcription factor A (TFAM) is a multi-functional high mobility group "B-box" (HMGB) type protein that plays an essential role in a number of routine mitochondrial processes by binding to mtDNA. Because previous findings have shown HMGA1 can out-compete HMGB proteins for DNA binding, it was hypothesized that a competition exists between HMGA1 and TFAM for binding to mtDNA. The ability of HMGA1a to bind to mtDNA and compete with TFAM for mtDNA binding was evaluated using electrophoretic mobility shift assays with

B-form mtDNA probes and with synthetic multi-way junctions that mimic proposed D-loop structures. Our findings indicate that HMGA1a has a higher binding affinity for both B-form mtDNA and multi-way junctions as compared to TFAM and that low molar ratios of HMGA1 displace TFAM from these DNA substrates. Chromatin immunoprecipitations verify HMGA1 binding to multiple regions of mtDNA, suggesting this competition likely occurs *in vivo*. These findings demonstrate for the first time that HMGA1 is able to competitively bind throughout mtDNA, and also suggest a dynamic competition between DNA binding proteins occurs in mitochondria. These data have also led to the proposal of a testable model in which over-expression of HMGA1 leads to its accumulation in mitochondria and allows HMGA1 to out-compete TFAM for binding to mtDNA. Because TFAM binding to mtDNA is essential for a number of routine mitochondrial processes, its displacement by HMGA1 would then lead to overall mitochondrial dysfunction, a phenotype that has long been associated with cancer.

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CHAPTER 1

INTRODUCTION

Mitochondria

Mitochondria are involved in a number of cellular functions in addition to ATP production, including an involvement in amino acid biosynthesis, fatty acid oxidation and apoptosis. These double membrane bound organelles house multiple copies of a 16.5 Kb circular genome that codes for 13 essential proteins, 22 tRNAs and 2 rRNAs (**Fig. 1.1**). The two strands of the mitochondrial DNA (mtDNA) are differentiated as the light (L) strand and the heavy (H) strand, due to their relative guanine content and density in CsCl gradients. Nearly the entire mitochondrial genome is made up of gene sequences comprised mainly of protein coding regions that are punctuated by tRNA sequences. Only a 1.1 Kb regulatory region, which contains both H-strand promoters, the L-strand promoter and the H-strand origin of replication, is non-coding. This region is referred to as the Displacement-loop (D-loop), owing to its triple stranded D-shaped structure, which results from premature termination of DNA synthesis about 700 bp downstream of the H-strand origin (**Fig. 1.2**) [1,2].

Replication of mtDNA is coupled with its transcription, as it is dependent on priming by short mRNAs [3]. Replication has been described to proceed via two different mechanisms. In the strand-asynchronous (or strand-displacement) model, replication begins at the heavy strand origin and proceeds unidirectionally to a point two thirds of the way around the genome where the light strand origin is exposed and lagging strand synthesis can then be initiated in the opposite direction [4]. Replication in the strand-synchronous (or strand-coupled) model proceeds bidirectionally, employing classic leading and lagging replication forks that form Y-arcs between both

the heavy and light strand origins [5]. Despite fervent discussion over these two models, a consensus in the field has yet to be reached.

Mitochondrial transcription initiates on both strands of mtDNA in the D-loop and produces pre-cursor mRNAs of varying lengths from several hundred base pairs, to nearly full genome length polycistronic transcripts. These pre-cursor mRNAs are then processed by excision of interspersed tRNAs to give rise to mature mRNAs. Mitochondrial transcription factor A (TFAM), which is described in greater detail below, is required to initiate transcription and is able to recognize promoter sequences in conjunction with a second regulatory protein, either mitochondrial transcription factor B1 or B2 (TFBM1 or TFBM2) [6,7].

The location of mtDNA inside the mitochondrial matrix causes it to be dangerously close to the oxidative phosphorylation machinery on the inner mitochondrial membrane. Mitochondria consume the majority of total cellular oxygen, and as a result are the major source of harmful reactive oxygen species (ROS) [8-11]. This potentially leaves mtDNA exposed to a relatively large mutagen dose which can threaten faithful expression of the essential oxidative phosphorylation proteins themselves. Historically, mtDNA has been thought to be more susceptible to mutation than nuclear DNA due to lack of protection mechanisms such as repair enzymes and chromatin structure. However, it is now generally accepted that mitochondria are indeed able to repair damage using enzymes involved in base excision repair [12-14], non-homologous end joining [15], homologous recombination [16] mismatch repair [17] and alkylation removal [18].

Despite these various repair mechanisms, elevated ROS levels pose a significant problem given that further accumulation of mtDNA mutations perpetuates the cycle of ROS production. Increased ROS levels directly resulting from mtDNA mutations have been demonstrated to contribute to tumor progression and increased metastatic potential [19]. Failure in mitochondrial function resulting from mtDNA mutation has consistently been observed in association with cancers since Otto Warburg's initial findings that mitochondria of cancer cells show reduced capacity for oxidative phosphorylation [20]. Since then, a multitude of studies have demonstrated that mitochondria of various types of cancer tissue suffer from phenotypic changes associated with dysfunction including reduced mitochondrial mass [21], altered membrane potential [22], increased mtDNA transcription [23,24], reduced ATP synthesis [25], and aberrant calcium-dependent apoptotic signaling [25]. Discovery of these types of phenotypic changes have led to an evolving field of study related to the implications of mitochondrial mutation and dysfunction in diseases such as cancer, aging and neurodegenerative disorders [26-30].

High Mobility Group Proteins

High mobility group proteins (HMG) encompass a family of non-histone chromatin proteins that alter chromatin structure. These proteins recognize DNA structure and bind with little or no DNA sequence specificity. Typically, HMG proteins do not necessarily perform a particular function alone, but instead facilitate interactions between other proteins and target DNA. This allows HMG proteins to be involved in a great range of activities with a diverse array of partners. There are three subgroups of

HMG proteins, namely HMGA, HMGB and HMGN, which are categorized mainly according to their distinct DNA binding motifs. Because the work contained in this thesis focuses on HMGA and HMGB proteins, further characteristics of these proteins are described below.

Mitochondrial Transcription Factor A: a High Mobility Group B Protein

TFAM is a mitochondrial DNA binding protein that is a member of the HMGB family of proteins. HMGB proteins, originally characterized in the nucleus, bind DNA by employing B-box peptide domains which insert into the minor groove of B-form DNA (**Fig. 1.3**). B-box domains feature the hydrophobic aromatic amino acids, tyrosine, tryptophan, and phenylalanine, which are signature residues of this highly conserved motif [31]. TFAM has two of these B-box domains as well as a basic C-terminal tail domain that helps it mediate binding to both DNA and other proteins [32]. In addition to B-form DNA, HMGB proteins prefer to bind bent or distorted DNA structures such as four-way junctions, cisplatin adducts, and DNA bulges [33-36]. Once bound, TFAM and other HMGB proteins are able to induce structural changes in DNA like bends, kinks, and loops that aid in various functional regulations [35-37]. In both the nucleus and mitochondrion, HMGB and TFAM proteins are sufficiently abundant to cover a large portion of their respective genomes [33,38].

Although first identified because of its activity as a mitochondrial transcription factor, TFAM has been found to have many distinct functions (for review see [39]). Because mtDNA replication is dependent on transcription, TFAM plays a role in both of these processes [40]. TFAM is also thought to orchestrate the histone-like organization of mtDNA into a nucleoid structure [41-43]. This aspect of TFAM activity is

advantageous not only because it coordinates overall mtDNA arrangement but also because such packaging protects the mtDNA from potential mutagens. Additionally, TFAM has been shown to be involved in DNA repair mechanisms in mitochondria by preferentially binding oxidative lesions and cisplatin damaged DNA [44]. It's unclear what TFAM's exact role in mtDNA repair is, but it has been postulated to facilitate repair by recruiting mitochondrially targeted repair enzymes, like mtOGG1 and mtMYH, to the sites of mtDNA damage [44]. TFAM's involvement in these different aspects of mitochondrial biology is consistent with it being an essential component required for the normal function and maintenance of the mitochondria. Accordingly, reduced expression of TFAM through heterozygous knockout or RNAi causes reductions in mtDNA copy number, mtDNA transcription activity and overall respiratory function [42,45,46]. Total knockout of TFAM in mice is an embryonic lethal mutation [45].

High Mobility Group A Proteins

HMGA proteins are comprised of two functional members, HMGA1 and HMGA2. There are three isoforms of HMGA1 (HMGA1a, HMGA1b, and HMGA1c) that represent mRNA splice variants. HMGA1a is an 11.5 kDa peptide and is the focus of this thesis. HMGA1a, referred to hereafter referred to as HMGA1, is an architectural transcription factor that both negatively and positively regulates a large number of genes by recognizing and binding to promoter elements in a structure-specific manner.

In solution, HMGA1 is quite flexible and lacks a well-defined higher order structure, but upon binding to target DNA, it is able to form three crescent shaped A/T hook peptide domains (**Fig. 1.4**) [47,48]. The A/T hook peptides contain a conserved palindromic amino acid motif of proline-arginine-glycine-arginine-proline (PRGRP), in

which the RGRP is invariant. One, two or all three of these A/T hooks can bind to target DNA, depending on the affinity of HMGA1 for the particular DNA substrate. As their name would suggest, these A/T hooks can interact with short 4-6 base pair stretches of A/T-rich sequences in the minor groove of B-form DNA. Distorted and other non-traditional DNA substrates including supercoiled DNA, certain DNA lesions, and cruciform structures are also readily bound by HMGA1 [49-51]. HMGA1 binds relatively tightly to four-way junctions in vitro and has been demonstrated to out-compete more abundant nuclear proteins, specifically HMGB and histone H1, for binding to this type of structure [52]. This competition is typical of the dynamic flux in chromatin transcription factor binding that is characteristic of nuclear protein-DNA interactions.

In normal, differentiated cells, HMGA1 expression is very low, nearly undetectable. Cancer cells exhibiting uncontrolled growth, however, reliably express significantly elevated levels of HMGA1. HMGA1 is an oncogene, and accordingly, its over-expression consistently leads to neoplastic tumor formation and increasing metastatic potential [53,54]. In fact, characteristic over-expression of HMGA1 has been proposed to be a potential diagnostic indicator of progression in multiple types of cancers including (but not limited to) colon, prostate, pancreatic and breast cancers [53,55-59]. HMGA1 likely contributes to cancer formation and progression through multiple distinct pathways. Previous studies have indicated that HMGA1 over-expression inhibits DNA nucleotide excision repair mechanisms [49,60,61], alters transcription of genes involved in cell growth and proliferation [62], and induces mitochondrial dysfunction associated with cancer [63]. All of these findings suggest potential mechanisms by which HMGA1 might contribute to cancer.

HMGA1 in Mitochondria

Although HMGA1 has been extensively characterized in the nucleus, it has more recently been found to reversibly translocate into mitochondria in a cell cycle-specific manner [64]. It is not yet clear what HMGA1's exact function is in mitochondria, but chromatin immunoprecipitation (ChIP) assays have indicated that it binds to the regulatory D-loop region of the mtDNA [63]. In non-tumor cells expressing low levels of HMGA1 (e.g. NIH3T3 cells), HMGA1 translocation appears to be controlled and predictable, shuttling in and out of mitochondria in the late S/G2 phase of the cell cycle. However, in cells that transgenically over-express HMGA1, its translocation appears to become aberrant and unregulated, directly resulting in dysfunction of several mitochondrial processes including mitochondrial mass, ATP and ROS production, membrane potential and sensitivity to glycolytic inhibition [63]. These findings are also substantiated by similar findings in naturally occurring tumor cells [65].

In genetically engineered cells in which the expression of transgenic HMGA1 protein can be reversibly controlled, several indicators of mitochondrial dysfunction have been shown to be correlated with changing levels of HMGA1 protein [65]. As HMGA1 expression increases, overall mitochondrial mass is reduced, ROS production increases, and the ability to repair oxidative mtDNA damage is reduced. In contrast, after subsequently reducing HMGA1 expression in these cells, the mitochondria begin to recover and show improved functioning of these parameters [65]. It is evident that excess HMGA1 protein is directly related to mitochondrial dysfunction, but the precise mechanism for this dysfunction remains to be elucidated.

The subsequent chapter aims to provide novel insight into the means by which HMGA1 over-expression leads to mitochondrial dysfunction and ultimately contributes to tumor progression. A series of experiments are described that demonstrate three main findings: HMGA1 protein accumulates in the mitochondria of cells in which it is over-expressed; HMGA1binds to multiple regions throughout mtDNA both *in vitro* and *in vivo*; and HMGA1 is able to out-compete TFAM for binding to B-form and non-traditional mtDNA structures. These findings have led to the development of a model proposing that a shift in the expression of HMGA1 can sufficiently alter the ability of TFAM to bind to mtDNA and carry out its many functions. Consequently, compromised TFAM binding of mtDNA leads to decline in mitochondrial function, which ultimately contributes to disease. This model also implies that a competitive and dynamic binding of transcription factor proteins (namely HMGA1 and TFAM) occurs in mitochondria. Although this idea has been widely accepted in the context of nuclear DNA, it has not yet been demonstrated in mitochondria.

Figure 1.1: The Mitochondrial Genome [66]. This detailed map illustrates the genetic and regulatory features of the mitochondrial genome.

Figure 1.1

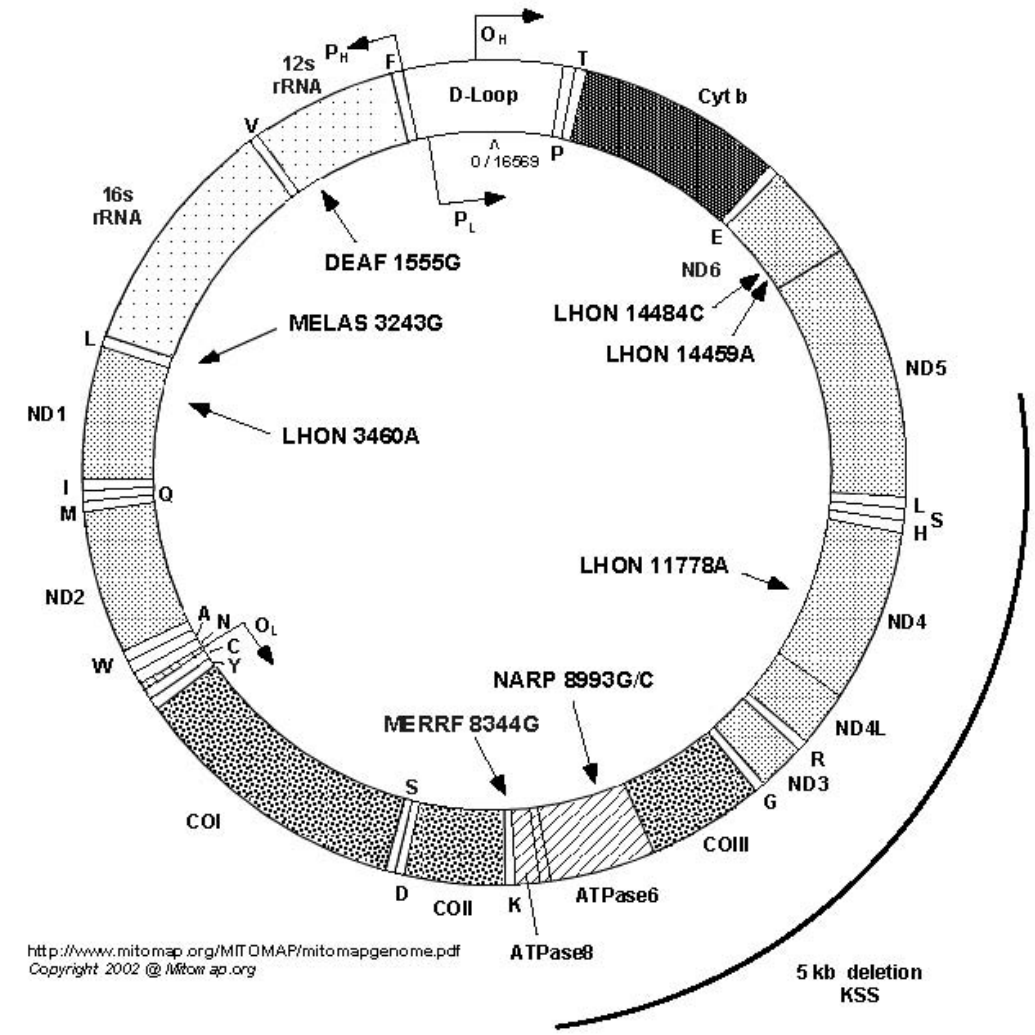

O H
P H
F
12s rRNA
D-Loop
T
Cyt b
V
16016569
P
16s rRNA
P L
DEAF 1555G
E
ND6
LHON 14484C
MELAS 3243G
LHON 14459A
ND5
L
ND1
LHON 3460A
I
M
Q
L
H
S
LHON 11778A
ND2
A
N
O L
C
Y
ND4
W
NARP 8993G/C
MERRF 8344G
ND4L
R
ND3
S
G
COI
COIII
D
COII
K
ATPase6
ATPase8
http://www.mitomap.org/MITOMAP/mitomapgenome.pdf
Copyright 2002 @ Mitomap.org
5 kb deletion
KSS
Complex I genes (NADH dehydrogenase)
Complex III genes (ubiquinol : cytochrome c oxidoreductase)
Transfer RNA genes
Complex IV genes (cytochrome c oxidase)
Complex V genes (ATP synthase)
Ribosomal RNA genes

Figure 1.2: Map of the mitochondrial D-loop region [67]. Heavy and light strands of the mtDNA are shown as heavy and thin lines. The nascent heavy strand is depicted initiating at the heavy strand origin of replication (O_H) which forms the displaced structure of the D-loop. The relative locations of the heavy strand promoter (HSP) and light strand promoter (LSP) are indicated with bent arrows. The light strand origin of replication (O_L) is the only major regulatory element not located in the D-loop region.

Figure 1.2

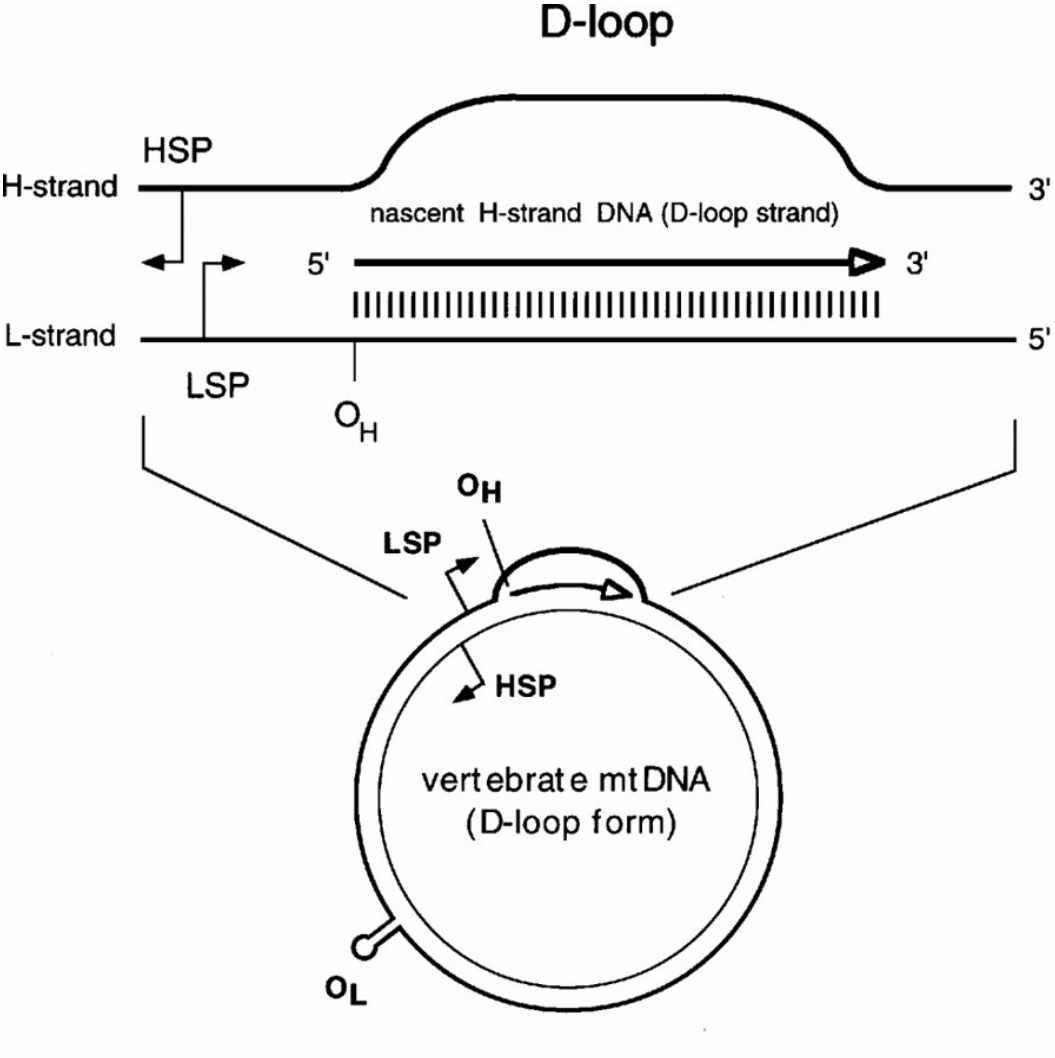

D-loop
HSP
H-strand
3'
nascent H-strand DNA (D-loop strand)
5'
3'
L-strand
5'
LSP
O_H
O_H
LSP
HSP
vertebrate mtDNA
(D-loop form)
O_L

Figure 1.3: HMGB B-box peptide domain [68]. This structure, as determined by NMR illustrates the B-box peptide, indicating the long arm, or extended segment, which binds to the minor groove of the DNA helix.

Figure 1.3

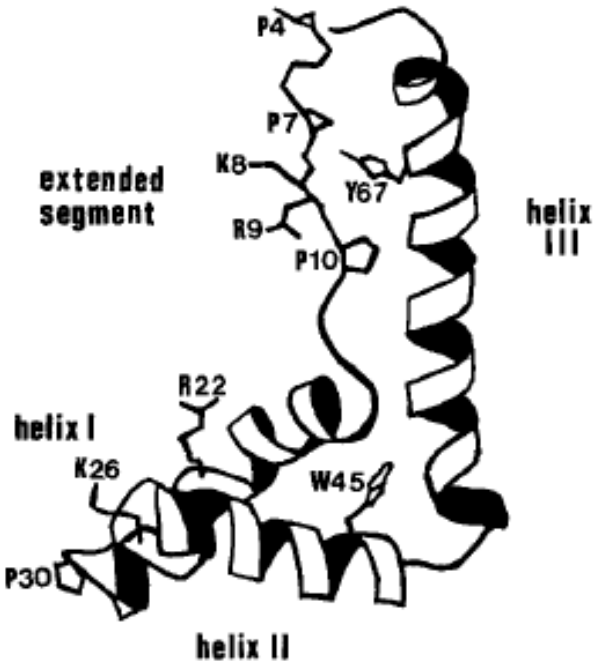
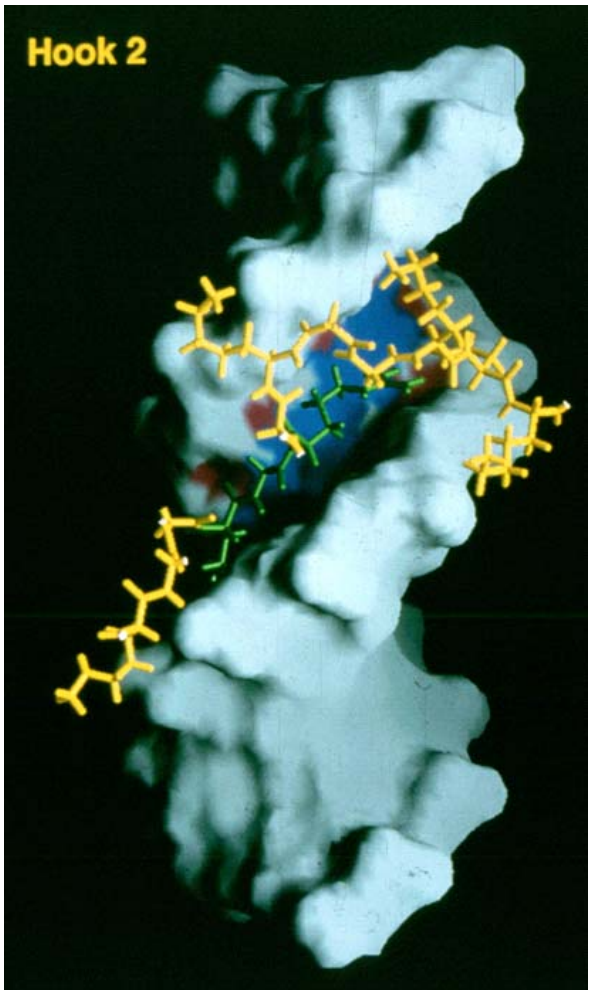

P4
P7
K8
extended segment
Y67
helix III
R9
P10
R22
helix I
K26
W45
P30
helix II

Figure 1.4: HMGA1 A/T-hook peptide domain [69]. The ball and stick representation of HMGA1 is shown in yellow and green binding in the minor groove of A/T-rich DNA, shown in white.

Figure 1.4



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CHAPTER 2:

HIGH MOBILITY GROUP A1 AND MITOCHODNRIAL TRANSCRIPTION FACTOR A COMPETE FOR BINDING TO MITOCHONDRIAL DNA

Abstract

During restricted parts of the normal cell cycle, a sub-fraction of the nuclear transcription factor high mobility group A1 (HMGA1) reversibly translocates to mitochondria. In cancerous cells, which almost universally over-express HMGA1, the protein is found in both the nucleus and mitochondria throughout the cell cycle. In mitochondria, HMGA1 binds to the regulatory D-loop region of the mitochondrial DNA (mtDNA), but the exact functions of HMGA1a in this organelle are currently unknown. Mitochondrial transcription factor A (TFAM) is a multi-functional high mobility group "B-box" (HMGB) protein that binds to mtDNA to perform a number of routine mitochondrial processes. Because previous findings have shown HMGA1a can out-compete HMGB-type proteins for DNA binding, we hypothesized that a competition exists between HMGA1a and TFAM for binding to mtDNA. The ability of HMGA1a to bind to mtDNA and compete with TFAM for mtDNA binding was evaluated using electrophoretic mobility shift assays with B-form mtDNA probes and with synthetic multi-way junctions that mimic proposed D-loop structures. Our findings indicated that HMGA1a had a higher binding affinity for both B-form mtDNA and multi-way junctions as compared to TFAM and that low molar ratios of HMGA1a displaced TFAM from these DNA substrates. Chromatin immunoprecipitation verified HMGA1a binding to multiple regions of mtDNA, suggesting that this competition likely occurs *in vivo*. These findings demonstrated for the first time that HMGA1a was able to competitively bind throughout mtDNA, and also suggested a dynamic competition between DNA binding proteins occurs in mitochondria.

Introduction

Mitochondria house a 16.5 Kb circular genome that codes for 13 essential proteins, 22 tRNAs and 2 rRNAs. Only a 1.1 Kb regulatory region of mtDNA is non-coding, containing transcription promoter sites and an origin of replication. This region is referred to as the displacement- or D-loop, owing to its triple stranded D-shaped structure resulting from premature termination of DNA strand synthesis [1,2]. Replication of mtDNA is coupled with its transcription, as it is dependent on priming by short mRNAs [3]. Transcription initiation requires mitochondrial transcription factor A (TFAM) [4,5] and can occur on either strand of mitochondrial DNA (mtDNA) in the D-loop, producing mRNAs of varying lengths from several hundred base pairs to nearly full genome length polycistronic transcripts .

Although first identified because of its activity as a mitochondrial transcription factor, TFAM has been found to have many distinct functions (for review see [6]). TFAM is regarded as a member of the High Mobility Group B (HMGB) family of proteins because it contains two DNA binding domains called “B-boxes” that mediate its binding to mtDNA [7]. B-box peptide domains, which were originally described in the nuclear HMGB family of proteins, recognize DNA structure rather than specific nucleotide sequence and prefer to bind distorted structures such as four-way junctions [8-11]. Once bound, HMGB proteins, including TFAM, are able to induce structural changes in DNA like bends, kinks, and loops that aid in transcriptional regulation [7,10,11]. In mitochondria, TFAM is not only an essential component of transcription initiation [12], but has also been shown to play a role in formation of nucleoid structure through histone-like organization of the mtDNA [13-15]. Furthermore, TFAM is thought to be

involved in DNA repair mechanisms in mitochondria by binding preferentially to oxidative lesions and cisplatin damaged DNA [16].

Recently, it was found that a member of a different high mobility group family, HMGA1, is able to translocate into the mitochondria from the nucleus at a specific point in the normal cell cycle [17]. This non-histone chromatin protein—specifically HMGA1a, or more simply referred to here as HMGA1—commonly acts as a transcription factor in the nucleus, regulating a large number of genes, both positively and negatively (for review see [18]). In normal somatic cells, the expression level of HMGA1 is often nearly undetectable. In tumor cells, however, HMGA1 is frequently very highly expressed, consistent with it being the only bona fide chromatin protein so far demonstrated to be an oncogene. In fact, increasing levels of HMGA1 proteins have been used as an indicator of neoplastic cell transformation and tumor progression in a number of different human cancer types, and have been proposed as a clinical diagnostic marker for metastatic potential [18-25].

While much is known about HMGA1 in the nucleus, its exact role is in mitochondria is not clear. In non-tumor cells, HMGA1 translocation appears to be controlled and predictable, shuttling in and out of mitochondria in the late S/G2 phase of the cell cycle [17]. However, when over-expressed, HMGA1 translocation becomes aberrant and unregulated, directly resulting in dysfunction of several mitochondrial processes [26]. In transgenic cells induced to over-express HMGA1, multiple indicators of mitochondrial dysfunction have been observed [26,27]. When HMGA1 protein expression is reduced to minimal levels in these same cells, mitochondrial function

returns to normal parameters [27]. These findings imply that changing levels of HMGA1 protein have a direct role in mitochondrial function.

HMGA1 binds to DNA via three peptide domains, called A/T hooks, that confer a particular affinity for the minor groove of short stretches of A/T rich B-form DNA and also bind tightly to other non-traditional forms of DNA such as supercoiled substrates, certain DNA lesions, and four-way junctions [28-30]. Additionally, HMGA1 has been demonstrated to displace other nuclear proteins (including histone H1 and HMGB proteins) when binding to synthetic four-way junctions *in vitro* [31]. Considering that mtDNA has a substantial amount of A/T rich sequence, is supercoiled, possesses a D-loop with a non-traditional structure and that many of its routine functions involve the HMGB-type protein TFAM, the entire mitochondrial genome appears to be a particularly attractive target for HMGA1 binding.

Here we take a closer look at how and where HMGA1 interacts with mtDNA. Our working hypothesis is that in HMGA1 over-expressing cells, aberrant binding of HMGA1 throughout the nucleoid partially displaces TFAM and contributes to mitochondrial dysfunction which, in turn, is ultimately linked to tumorgenesis and metastasis. Our experimental results are the first to report that HMGA1 bound regions of mtDNA other than the D-loop *in vivo*. Also, mobility shift assays demonstrated that *in vitro*, HMGA1 could out-compete (and/or) displace TFAM binding to various mtDNA regions and to synthetic three- and four-way junctions (3WJ, 4WJ) that mimic dynamic *in vivo* D-loop structures. Together, the results from these studies provided significant support for our working hypothesis.

Material and Methods

Cell Culture

MCF7 breast adenocarcinoma cells and 7C "On" cells derived from MCF7 were developed and maintained as described in Reeves et al. 2001 [21]. Briefly, MCF7 cells were stably transfected with a tetracycline regulated hemagglutinin (HA)-tagged HMGA1a cDNA transgene to generate the 7C "On" cell line. This cDNA codes for the human HMGA1a (a.k.a. HMG-I) isoform protein which throughout this report will simply be referred to as HMGA1. In the absence of tetracycline, 7C "On" cells produce greatly elevated levels (up to ~40 fold) of recombinant HMGA1 protein, whereas parental MCF7 cells express very low basal levels of endogenous HMGA1 regardless of the presence of tetracycline. Both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum, 10 mM HEPES, penicillin (100 µg /ml), streptomycin (100 µg /ml) and G418 (100 µg/ml). 7C "On" cell medium was additionally supplemented with hygromycin (100 µg/ml) to select for the HA-HMGA1a transgene.

Recombinant Proteins

Recombinant HMGA1 protein was produced and purified by previously described methods [32]. Full length recombinant TFAM protein was produced and purified as previously described [33]. Briefly, a DNA fragment encoding mature full length TFAM (43-246 amino acids residues) was inserted between BamHI and EcoRI sites of the pPRO-EX-HTb (Invitrogen, Carlsbad, CA, USA). The recombinant TFAM protein was expressed in Escherichia coli BL21 cells. The recombinant protein was recovered from a soluble fraction after disruption of the cells by sonication. Then the recombinant

protein was purified sequentially with three columns: Ni^{+}-bound chelating Sepharose resins, Heparin Sepharose, and SP Sepharose (Amersham Biosciences, Piscataway, NJ, USA). The recombinant proteins was dialyzed in phosphate buffered saline (PBS) containing 20% glycerol and stored at –80^{o} C.

Mobility Shift Assays

B form mtDNA probes for electrophoretic mobility shift assays were generated by PCR amplification using primer sets designed to multiple regions throughout the mitochondrial genome (**Table 2.1**). After PCR amplification, DNA fragments were gel purified and ^{32}P end labeled using PNK enzyme (New England Biolabs, Ipswich, MA). Synthetic junctions were formed by annealing oligonucleotide legs 1, 2, 3, and 4 (4WJ) or legs 1, 4, and 5 (3WJ) after ^{32}P end-labeling of leg 4. The oligonucleotide sequences used were:

Leg 1- CCCTATAACCCCTGCATTGAATTCCAG TCTGATAA;

Leg 2- GTAGTCGTGATAGGTGCAGGGGTTATAGG;

Leg 3- AACAGTAGCTCTTATTCGAGCTCGCGC CCTATCACGACTA;

Leg 4- TTTATCAGACTGGAATTCAA GCGCGAGCTCGAATAAGAGCTACTGT; and

Leg 5- AACAGTAGCTCTTATTCGAGCT CGCGCACGTCCCCAATATCCC.

Radiolabeled probes were mixed with increasing amounts of purified protein in binding buffer containing 10 mM Tris-HCl, pH 8, 1 mM EDTA, 100 nM NaCl, 0.1 ug/ul BSA, 1 mM DTT and poly dG-poly dC oligo added to one half molar equivalent of the DNA probe. Reaction mixes were incubated at room temperature for 20 minutes before adding 1X GE buffer and loading onto a 0.25X TBE, 6.5 % native acrylamide gel. Gels containing duplex, B form DNA probes were run at 50 V for approximately 2.5 hours at

room temperature and 3WJ or 4WJ probes were run for approximately 4.5 hours at 4° C. After electrophoresis, gels were dried and exposed to a phosophoimaging screen. Visualization was carried out with a PhosphorImager 445 SI machine (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software.

Competitive Protein Mobility Shift Assays

EMSAs performed with competing proteins were performed as described above, with the exception of adding a second protein after incubation of the initial protein. The initial protein was incubated with the reaction mixture for 20 min at room temperature, after which the second protein was added to the reaction and allowed to incubate for an additional 20 min at room temperature before electrophoresis.

Chromatin Immunoprecipitations

ChIP assays were performed using 7C "On" cells transgenically over-expressing HMGA1. Approximately 10^7 cells were cross linked with 1% formaldehyde for 10 min, quenched with 0.125 M glycine, harvested, and lysed. Cell lysates were then sonicated to produce 200-400 bp DNA fragments confirmed by gel electrophoresis. Lysates were pre-cleared by incubating for 3 hr with goat polyclonal IgG, poly dG-poly dC competitor DNA, and protein A-sepharose beads. These non-specific complexes were then separated by centrifugation and the supernatants were reserved for specific HMGA1 immunoprecipitation. Pre-cleared lysates were incubated with either total rabbit serum or rabbit α-HMGA1 polyclonal antibody at 4° C overnight. Protein-antibody complexes were then isolated by incubation with protein A sepharose beads in the presence of poly dG-poly dC competitor DNA for 1 hr and collected by centrifugation. Immunoprecipitations were washed and eluted off the protein A sepharose beads.

Formaldehyde cross links were reversed using 300 mM NaCl with 65°C overnight incubation and remaining RNA contaminants and released proteins were degraded with RNAse and proteinase K treatment. Immunoprecipitated DNAs were then purified by phenol-chloroform extraction and ethanol precipitation. DNAs were quantified by ultraviolet absorption assay with a NanoDrop 1000 (Thermo Scientific, Waltham, MA). PCR was performed by using either the COXI or ND2 primer sets (**Table 2.1**). PCR reactions contained 1X Taq polymerase buffer, 5 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 µM of each primer, 100 ng template DNA, and 1 unit of Taq polymerase. Thermalcycler parameters consisted of 94° C for 60 seconds, followed by 29 (ND2) or 32 (COXI) cycles of 30 seconds at 94° C, 50 seconds at 58° C, and 60 seconds at 72° C with a final 60 second elongation at 72° C. PCR products were then visualized on a 2% agarose gel and imaged with a GelDoc XR system (BioRad, Hercules, CA)

Western Blot

Mitochondria from MCF7 and 7C "On" cells were isolated using the Mitochondrial Isolation Kit for Mammalian Cells (Pierce, Rockford, IL) following the manufacturer's suggestion. Control Western blot and real-time PCR experiments demonstrated that mitochondrial preparations isolated by this method were over 90% pure with very low protein or DNA contaminants derived from either the cytoplasm or the nucleus (data not shown). Isolated mitochondria were lysed using an SDS buffer and denatured lysates were electrophoresed using SDS-PAGE before transfer to a PVDF membrane. Rabbit anti-HMGA1 polyclonal immunoglobulins were used as a primary probe of the blot, followed by a secondary probe of HRP-conjugated goat anti-rabbit IgG. SuperSignal

West Pico chemiluminescent substrate (Pierce, Rockford, IL) was used to visualize the bands.

Results

Based on previous findings in our laboratory [17,26,27,31] and the abundance of A/T rich sequence in mtDNA, we hypothesized that HMGA1 would be able to bind throughout mtDNA and potentially compete with the structural HMGB-type protein, TFAM. To evaluate this, we first confirmed that over-expressed HMGA1 protein accumulated in mitochondria. MCF7 breast adenocarcinoma cells expressing basal levels of endogenous HMGA1 were compared with 7C "On" cells over-expressing transgenic HMGA1 protein. Western blot analysis was performed on purified mitochondria isolated from these cell lines (**Fig. 2.1**). While large amounts of HMGA1 remained in the nuclei of 7C "On" cells (data not shown), there was also a marked accumulation of transgenic HMGA1 protein in the mitochondria of these cells. MCF7 cells, however, had considerably lower levels of HMGA1 in both their nuclei and mitochondria as expected. It is interesting to note that MCF7 mitochondria did appear to contain a low level of endogenous HMGA1, which was unlikely to be due to cross-contamination from either the nucleus or cytoplasm (see **Materials and Methods**). The appearance of low levels of endogenous HMGA1 in the mitochondria of these parental cells may reflect either a functional role for the protein in this organelle or, alternately, may be attributable to the adenocarcinoma nature of the MCF7 cells themselves.

***HMGA1a and TFAM readily bind throughout mtDNA in vitro*.**

In order to improve our understanding of how increased amounts of HMGA1a interacted with mitochondria, we investigated the protein's ability to bind to mtDNA *in vitro*. The sequence of mtDNA is exceedingly A/T rich, providing a multitude of potential binding sites for HMGA1 proteins. The relative abundance of A/T sequence in the COXI coding region is representative of A/T sequence throughout mtDNA (**Fig. 2.2**). HMGA1 binding to mtDNA was evaluated *in vitro* using B-form DNA probes corresponding to several regions throughout the mitochondrial genome (**Fig. 2.2**) and 4WJ and 3WJ probes which mimicked proposed structures (but not the sequence) of the regulatory D-loop region [1,34,35].

Electrophoretic mobility shift assays (EMSAs) performed with these B-form mtDNA regions and multi-way junctions titrated with increasing concentrations of HMGA1 protein demonstrated that HMGA1 readily bound to all of these DNA probes (**Fig. 2.3**). As additional proteins bind, DNA probe mobility was reduced. The first shift in band mobility between free DNA and HMGA1-complexed DNA indicates the tightest DNA:protein binding, as the protein will bind to the site of greatest affinity first. Additional proteins successively bind to sites of decreasing affinity as the concentration of protein increases. Appearance of multiple bands within a given lane represent different species of protein:DNA complexes where the lowest band typically indicates one protein bound, the second indicates two proteins bound and so forth. In all of the mtDNA regions analyzed, it was apparent that multiple HMGA1 proteins were capable of complexing with the DNA simultaneously, as reflected by banding patterns of decreasing mobility. This was not surprising, given that the A/T hooks of HMGA1 prefer

to bind A/T rich sequences stretching five or more nucleotides and that our probed regions—in addition to mtDNA in general—contain long stretches of A/T rich sequence, providing numerous potential binding sites (c.f. **Fig. 2.2**).

As expected, **Figures 2.3A** and **2.3B** show HMGA1 bound to both 4WJ and 3WJ synthetic probes that represent proposed D-loop structures *in vivo*. B-form DNA structures were also bound by HMGA1, including regions containing sequence from the D-loop (**Fig. 2.3C**) and regions throughout the mitochondrial genome (**Figs. 2.3D-I**). Overall, the mobility shifts presented in **Fig. 2.3** provided evidence that HMGA1 was capable of binding mtDNA in relatively low protein to DNA molar ratios. These findings were also consistent with previous reports of dissociation constants between 1-3 nM for HMGA1 binding to both stretches of A/T rich B-form DNA substrates [36,37] and 4WJ [29] substrates *in vitro*.

Multiple lines of evidence have established that TFAM plays a role in the packaging of mtDNA into a nucleoid [13-15]. As a result, the structural binding of TFAM throughout mtDNA would be essential for overall mitochondrial genome maintenance. We performed additional mobility shift assays to evaluate the binding ability of TFAM to our B-form and junctional DNA probes. Predictably, the mobility shifts presented in **Fig. 2.4** indicated that TFAM readily bound to our 4WJ probe (**Fig. 2.4A**) at ratios consistent with those previously reported [9] as well as with our 3WJ probe (**Fig. 2.4B**), B-form D-loop mtDNA (**Fig. 2.4C**) and B-form mtDNA outside the D-loop (**Fig 2.4D**). It is noteworthy, however, that significantly greater molar ratios of TFAM were required to shift our mtDNA probes than were necessary for HMGA1, suggesting that

HMGA1 may be able to outcompete TFAM for binding to these types of DNAs in the mitochondrion.

HMGA1 out-competes TFAM for binding to mtDNA and irregular DNA structures.

The question of the relative DNA binding affinities of HMGA1 and TFAM was addressed more directly by performing competitive protein mobility shifts in which the two proteins were allowed to bind to the DNA probes sequentially. After the first protein was allowed to bind to the DNA probe at a fixed protein:DNA ratio (e.g. 9:1, or 9X), the second protein was added at increasing molar ratios of protein to DNA (e.g. 0.5X -5X). Conveniently, the molecular weight of TFAM is approximately twice that of HMGA1, which allowed the differences in the shifting profiles of each specific protein to be more easily visualized.

Figure 2.5A illustrates the competitive binding profile of 4WJ DNA with a fixed concentration of TFAM (allowed to bind first) followed by addition of increasing concentrations of HMGA1 to pre-formed TFAM-4WJ complexes. In the absence of HMGA1 competition, up to four molecules of TFAM protein readily bound the 4WJ probe (lane 3). However, when HMGA1 was added to these TFAM-4WJ complexes, new bands formed that indicated a disruption of the original banding pattern. In **Figure 2.5** and all subsequent competitive protein EMSAs (**Figs 2.6-2.8**) solid arrowheads (►) indicate bands representing only TFAM binding, open arrowheads (>) indicate bands representing only HMGA1 binding, and arrows (→) indicate DNA bands bound by both HMGA1 and TFAM simultaneously. Lane 4 of **Figure 2.5A** shows pre-formed TFAM-4WJ complexes were disrupted at relatively low molar ratios of HMGA1. Because HMGA1 is less than half the molecular weight of TFAM, DNA bound by increasing

amounts of HMGA1 rather than TFAM will migrate more rapidly through, or further down the gel. This was seen by the gradual disappearance of the slowest migrating DNA bands (bound exclusively by multiple TFAM proteins, marked with a solid arrowhead) as HMGA1 displaces TFAM. It was also apparent that HMGA1 could simultaneously bind to DNA already bound to TFAM. This was indicated in bands corresponding to TFAM complexes that showed slightly reduced mobility after addition of HMGA1 (marked by arrows). The relative molar amounts of HMGA1 needed to completely displace TFAM from the 4WJ appeared to be remarkably low, suggesting that HMGA1 was able to bind tighter to 4WJ DNA.

When a reverse experiment was performed (**Fig. 2.5B**) HMGA1 was allowed to bind the DNA probe first at a fixed ratio, followed by addition of TFAM protein at increasing concentrations to the pre-formed HMGA1-4WJ complexes. The addition of TFAM appeared to have little effect on HMGA1-bound complexes until the molar equivalent of TFAM was three to four times that of the DNA probe. Even when TFAM was titrated to a molar ratio seven fold greater than DNA, complexes representing bound HMGA1 (open arrowheads) were still not completely displaced. Taken together, these results provided evidence that HMGA1 strongly out-competed TFAM for binding to cruciform DNA structures.

The mitochondrial D-loop structure can also be mimicked by synthetic 3WJ type DNA structures. Therefore, binding competitions were also performed using a 3WJ DNA probe (**Fig. 2.6**). In such competitions, it was evident that HMGA1 was able to displace TFAM from 3WJs at relatively low molar ratios (**Fig. 2.6A**), while TFAM required much higher concentrations of protein to disturb HMGA1 binding (**Fig. 2.6B**).

In addition to 4WJ DNA, these experiments indicated that HMGA1 was able to out-compete TFAM for binding to 3WJ DNA formations.

Although both HMGA1 and TFAM tended to recognize and bind DNA structure rather than nucleotide sequence, TFAM did seem to exhibit some degree of sequence specificity in the D-loop region where it activates transcription [5,38]. In this case, TFAM might be expected to bind more strongly to this mtDNA site and would be less likely to be displaced by HMGA1. To address this possibility, D-loop mtDNA encompassing known TFAM binding sites was used as a B-form DNA probe in further competitive mobility shifts. **Figure 2.7A** shows that pre-formed TFAM-D-loop complexes were also disrupted by addition of HMGA1. It is evident that the higher molecular weight bands representing exclusive binding of multiple TFAM proteins (indicated by solid arrowheads in lane 3) decreased in intensity as increasing concentrations of HMGA1 were titrated into the reaction as a competitor (lanes 4-9). This reduced intensity and changing banding pattern was interpreted to represent HMGA1 preferentially binding and partially displacing TFAM from the DNA complexes. It is important to note that this apparent displacement occurred when the relative molar ratios of HMGA1 were lower than the relative molar ratio of TFAM. This suggests that HMGA1 protein bound more tightly to D-loop DNA than did TFAM under these conditions. Also consistent with this interpretation was the observation that as increasing ratios of HMGA1 were added to the TFAM-D-loop complexes, new bands appeared (indicated by arrows) that represented *both* HMGA1 and TFAM bound simultaneously to probe DNA, as also seen in **Figs. 2.5** and **2.6**. As HMGA1 ratios increased, these heterogeneous complexes were shifted slightly upward, indicating

multiple HMGA1 proteins were able to bind to a given pre-established TFAM:probe complex.

The results of the reverse experiment, shown in **Fig. 2.7B**, also demonstrated that TFAM was able to simultaneously bind with HMGA1 to D-loop DNA at a relatively low molar ratio (1X, lane 4). In this competition, however, TFAM was required at a ratio of approximately 6-fold molar excess (lane 7) before bands appeared that indicated exclusive TFAM:probe binding (solid arrowhead). While it appears TFAM was more competitive when D-loop probe DNA was used, HMGA1 was, however, still able to displace TFAM at lower molar ratios than were required for TFAM to displace HMGA1. These results provided further evidence that HMGA1 was able to out-compete TFAM for binding to D-loop DNA even when it was in duplex form.

Although competition between HMGA1 and TFAM for binding to mtDNA was of great consequence in the D-loop, it was also significant outside of the control region in the protein coding sequence. We selected a sequence within the NADH dehydrogenase subunit 2 (ND2) gene to evaluate TFAM-HMGA1 binding and observed competitions similar to those seen in the 3WJ, 4WJ, and B-form D-loop DNA experiments (**Figs. 2.5-2.7**). When TFAM was allowed to bind ND2 DNA alone at 8-fold molar excess, it formed bands representing binding of 0 to 4 molecules of TFAM (**Fig. 2.8A**, lane 3). When HMGA1 at just half the molar equivalent of DNA was allowed to bind to these pre-formed complexes, it bound to both DNA unbound by TFAM (indicated by an open arrowhead) as well as DNA already bound by multiple TFAM molecules (indicated by arrows). As observed with 3WJ, 4WJ and D-loop DNA probes, complexes

bound exclusively by TFAM (marked with solid arrowheads) become fainter with increased titration of HMGA1, indicating displacement of TFAM by HMGA1.

In the reverse experiment, TFAM was added to pre-formed HMGA1-ND2 complexes (**Fig. 2.8B**) and TFAM began to displace HMGA1 when molar ratios of TFAM to DNA were 2:1. At 8-fold excess TFAM to DNA (lane 8), HMGA1 was almost completely displaced. These ratios would imply that TFAM had subtly tighter binding to this region of ND2 DNA than to our B-form D-loop and multi-way junction DNAs. However, because HMGA1 was able to disrupt TFAM binding at significantly lower molar ratios than was required for TFAM to displace HMGA1, it can be concluded that HMGA1 was also able to out-compete TFAM for binding to mtDNA in this protein-coding region of the mitochondrial genome.

***HMGA1 binds to mtDNA in vivo*.**

Previous experiments employing chromatin immunoprecipitation (ChIP) assays demonstrated that over-expressed HMGA1 binds to the D-loop region of mtDNA *in vivo* [17]. Given the amount of *in vitro* evidence that HMGA1 was able to bind to mtDNA described above, we selected regions outside the D-loop to test binding by HMGA1 *in vivo* employing ChIP analyses. **Fig. 2.9** shows the ChIP results for the ND2 and cyclo-oxygenase subunit I (COXI) protein coding regions in 7C "On" cells. PCR amplification of these regions indicated that over-expressed HMGA1 in the mitochondria was indeed associated with mtDNA outside the D-loop *in vivo.* This suggested that the type of interactions between HMGA1, TFAM, and mtDNA that we have observed in our competitive EMSA experiments were likely occurring inside mitochondria.

Discussion

The data presented here provide novel evidence establishing that HMGA1 could bind to several representative positions throughout mtDNA, including protein coding regions and the D-loop. Although the structure of the D-loop is somewhat dynamic, we have shown that HMGA1 was able to bind to B-form DNA in addition to 3WJ and 4WJ, which simulate the potential structures of replication initiation thought to exist in the D-loop region [34,35]. Moreover, we have demonstrated that the important multi-functional TFAM protein could be displaced from mtDNA in the presence of low molar ratios of HMGA1. ChIP assays and Western blot experiments have provided *in vivo* evidence that HMGA1 was located in mitochondria and was bound to mtDNA.

Considering that HMGA1 bound with greater affinity than TFAM to various mtDNA substrates *in vitro* (**Figs. 3-8**), if similar competitions occur *in vivo*, even a minor shift in the ratio of HMGA1 to TFAM could potentially cause appreciable interference with TFAM-mtDNA binding. In differentiated cells expressing basal levels of HMGA1, any binding competition would likely be minimal since the relative ratios of HMGA1 to TFAM inside mitochondria should theoretically be quite low. The typical amount of TFAM protein in the mitochondrion is sufficient to cover the entire mitochondrial genome [39] and HMGA1 levels are nearly undetectable in normal cells with only a minor fraction of it entering the mitochondria during a restricted part of the cell cycle. However, when HMGA1 is over-expressed, either transgenically or as a result of advanced tumor progression, the protein aberrantly accumulates in mitochondria (**Fig. 1** and unpublished results), which could plausibly alter the normal balance of mitochondrial HMGA1 and TFAM. The likelihood that cells over-expressing HMGA1

undergo this type of shift in mitochondrial ratios of HMGA1 to TFAM is supported by previous experiments performed in our laboratory using high-stringency analysis of Affymetrix arrays [40]. These experiments have indicated that expression levels of the nuclear-encoded *TFAM* gene remain unchanged between MCF7 cells with low amounts of HMGA1 protein and 7C "On" cells that express an approximately 40-fold increase of HMGA1 protein (unpublished data). These data suggest that increased presence of HMGA1 has little or no effect on TFAM expression at the level of transcription.

TFAM plays a part in many important processes in mitochondria, including transcription, replication, recombination, mtDNA repair, nucleoid formation and ROS protection [6]. TFAM must be able to bind to mtDNA in order to carry out its specific function in each of these processes. If the competitions we have demonstrated *in vitro* indeed occur *in vivo*, the elevated presence of HMGA1 would potentially impede the ability of TFAM to bind to mtDNA, and thus be expected to affect all of these mitochondrial functions to some degree. Indeed, we have previously demonstrated a strong correlation between the level of mitochondrial HMGA1 and reversible changes in overall mitochondrial function using both transgenic cell lines and cells derived from human tumors [26,27]. Interestingly, we have also observed a marked increase in HMGA1 in the mitochondria of naturally occurring cancers, which typically display overall mitochondrial dysfunction (manuscript in preparation).

In contrast to the mitochondrial accumulation of HMGA1 in cancer cells, non-malignant cells that contain only very low endogenous levels of HMGA1 and exhibit contact inhibition of growth (e.g. NIH 3T3 cells) appear to have the ability to regulate both the timing and quantity of HMGA1 protein that is translocated into mitochondria

[17]. This control of HMGA1 movement would allow non-malignant cells to maintain mitochondrial HMGA1:TFAM ratios at levels which preserve global TFAM binding. Controlled translocation of limited amounts of HMGA1 into the mitochondria of normal cells would allow HMGA1 to bind only to sites of the highest affinity, where it likely carries out a specific function. In this case, TFAM would remain associated with its many binding sites on mtDNA, potentially including those that it may share with HMGA1, thereby permitting TFAM to faithfully perform its many essential organellar functions.

In conjunction with previous findings, the data presented here has lead us to propose a model in which aberrant and excessive translocation of HMGA1 into mitochondria displaces TFAM from mtDNA and upsets the numerous functions governed by TFAM, thereby contributing to the mitochondrial dysfunction typically observed in cancer cells. Because non-malignant cells are able to regulate HMGA1 translocation and levels inside mitochondria, TFAM and HMGA1 undergo a controlled competition which is compatible with overall mitochondrial function. The idea of competition between transcription factors for DNA binding is a concept that has been well recognized within the nuclear genome, but has thus far been unidentified in mitochondria. The competitions between TFAM and HMGA1 for mtDNA illustrated in this work provide the first evidence that mitochondria might also be governed by dynamic and competitive transcription factor binding.

Taken together with previously published findings [17,26,27], these results provide direct evidence that HMGA1 proteins are indeed present in mitochondria and influence its biological function. Additional work evaluating HMGA1-TFAM interaction in

mitochondria would undoubtedly provide interesting insight into the *in vivo* relationship of these two proteins.

Acknowledgements

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Table 2.1: PCR primer data. Primer sequences used to amplify mtDNA probes are listed as shown. The 5 prime nucleotide start site of each primer is denoted with 'L' indicating the light strand or 'H' indicating the heavy strand. Nucleotide numbering corresponds to mtDNA sequence as referenced from www.mitomap.org.

Table 2.1

Coding Region	5' nt	Primer sequence	Fragment Length
D-loop	86 L	cattgcgagacgctggagc	305 bp
	390 H	tctggttaggctggtgttag	
ND2	4711 L	ccggacaatgaaccataacc	265 bp
	4975 H	ccacctcaactgcctgctat	
COXI	6229 L	tcctactcctgctcgcatct	286 bp
	6515 H	gcagctaggactgggagaga	
COXII	8113 L	cggacgtctaaaccaaacca	185 bp
	8297 H	gtgggctctagaggggtag	
ATPase6	8612 L	tattgatccccacctccaaa	297 bp
	8909 H	taaggggatggccatggcta	
COXIII	9572 L	ttcgcaggatttttctgagc	392 bp
	9908 H	attagttggcggatgaagca	
ND6	14357 L	acccacagcaccaatcctac	400 bp
	14756 H	ttggggtcattggtgttctt	

Figure 2.1: Isolated mitochondria contain HMGA1 protein. (**A**) Mitochondria were isolated from MCF7 and 7C "On" cell lines and proteins were used to perform Western blot analysis with anti-HMGA1 antibody. (**B**) Coomassie stained blot shows equal loading of total protein.

Figure 2.1

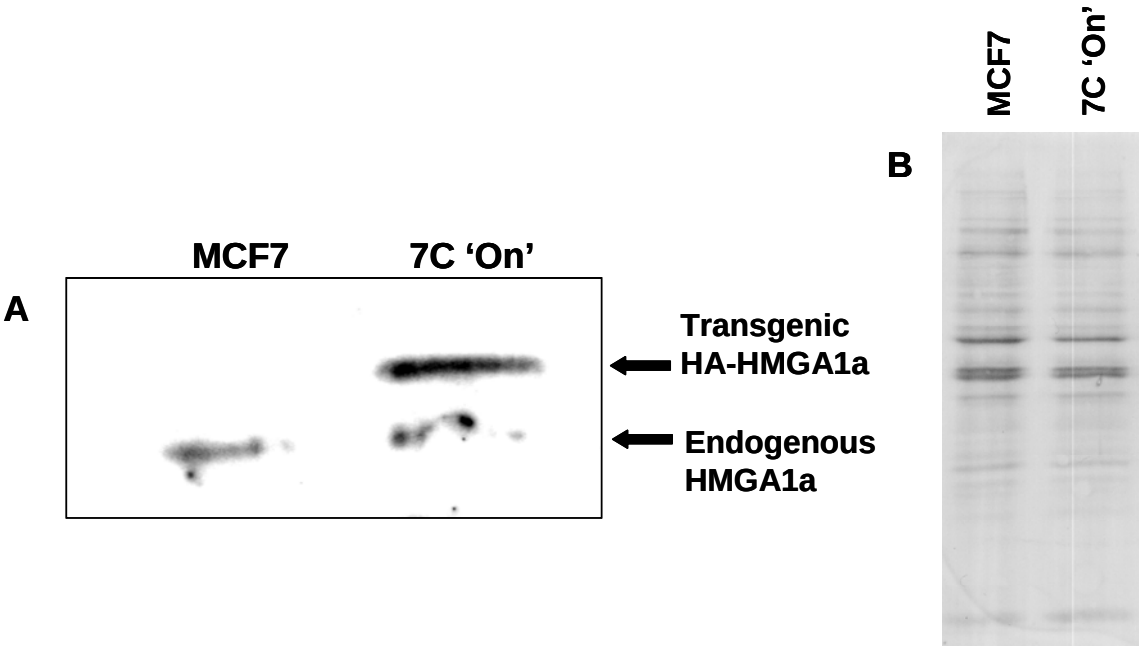

MCF7
7C 'On'
A
Transgenic
HA-HMGA1a
Endogenous
HMGA1a
B
MCF7
7C 'On'

Figure 2.2: **Functional regions of mtDNA and locations of EMSA probes**. The mtDNA light strand sequence is represented by the outer circle and the heavy strand sequence is represented by the inner circle. The D-loop region is shown in white at the 12 o'clock position; tRNAs are interspersed throughout the genome in white with their corresponding one-letter abbreviations; rRNAs are shown purple; NADH dehydrogenase subunit genes are in blue, abbreviated as ND1-6; cytochrome c oxidase subunit genes are in green, abbreviated as COXI-III; ATP synthase subunit genes are in yellow, abbreviated as ATPase6 and 8; and cytochrome b is in red, abbreviated as Cyt b. Selected probe locations are marked inside the heavy strand DNA with black arcs and their exact positions are listed in **Table 1**. The COXI coding region inset illustrates the representative occurrence of A/T rich sequence throughout the mtDNA.

Figure 2.2

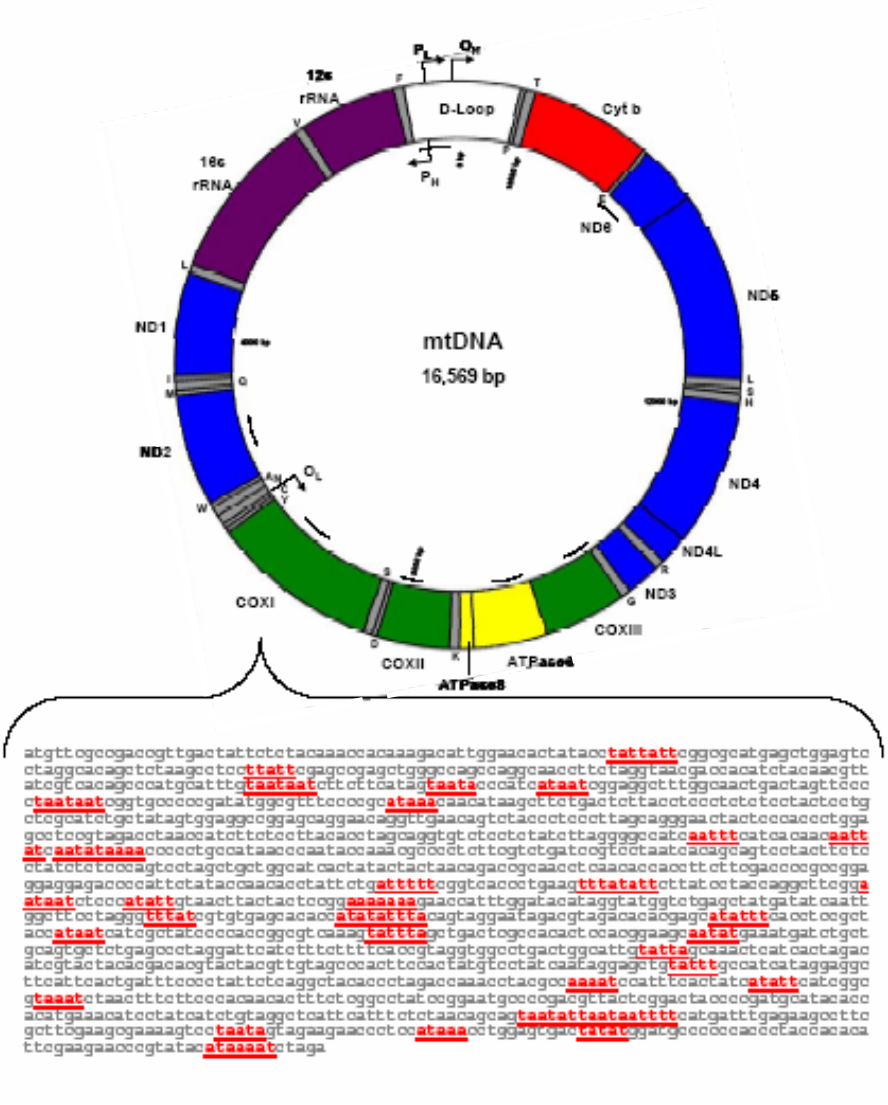

12s
rRNA
16s
rRNA
D-Loop
Cyt b
ND6
ND5
ND4
ND4L
ND3
COXIII
ATPase6
ATPase8
COXII
COXI
ND2
ND1
mtDNA
16,569 bp

Figure 2.3: HMGA1 binds to mtDNA and synthetic D-loop mimics. mtDNA probes are shifted by increasing molar ratios of HMGA1 protein. Molar ratios of protein to DNA are shown as fold excess (i.e. 0.25X indicates a molar ratio of 0.25:1 HMGA1 protein to DNA and 2X indicates 2:1 protein to DNA molar ratio, etc.) DNA probes correspond to synthetic multi-way junctions or to the mtDNA coding regions in which they reside (see **Fig. 2.2**). (**A**) four-way junction (4WJ). (**B**) three-way junction (3WJ). (**C**) D-loop. (**D**) NADH dehydrogenase subunit 2 (ND2). (**E**) Cytochrome c oxidase subunit I (COXI). (**F**) cytochrome c oxidase subunit II (COXII). (**G**) F_0 ATPase subunit 6 (ATPase6). (**H)** Cytochrome c oxidase subunit III (COXIII). (**I**) NADH dehydrogenase subunit 6 (ND6).

Figure 2.3

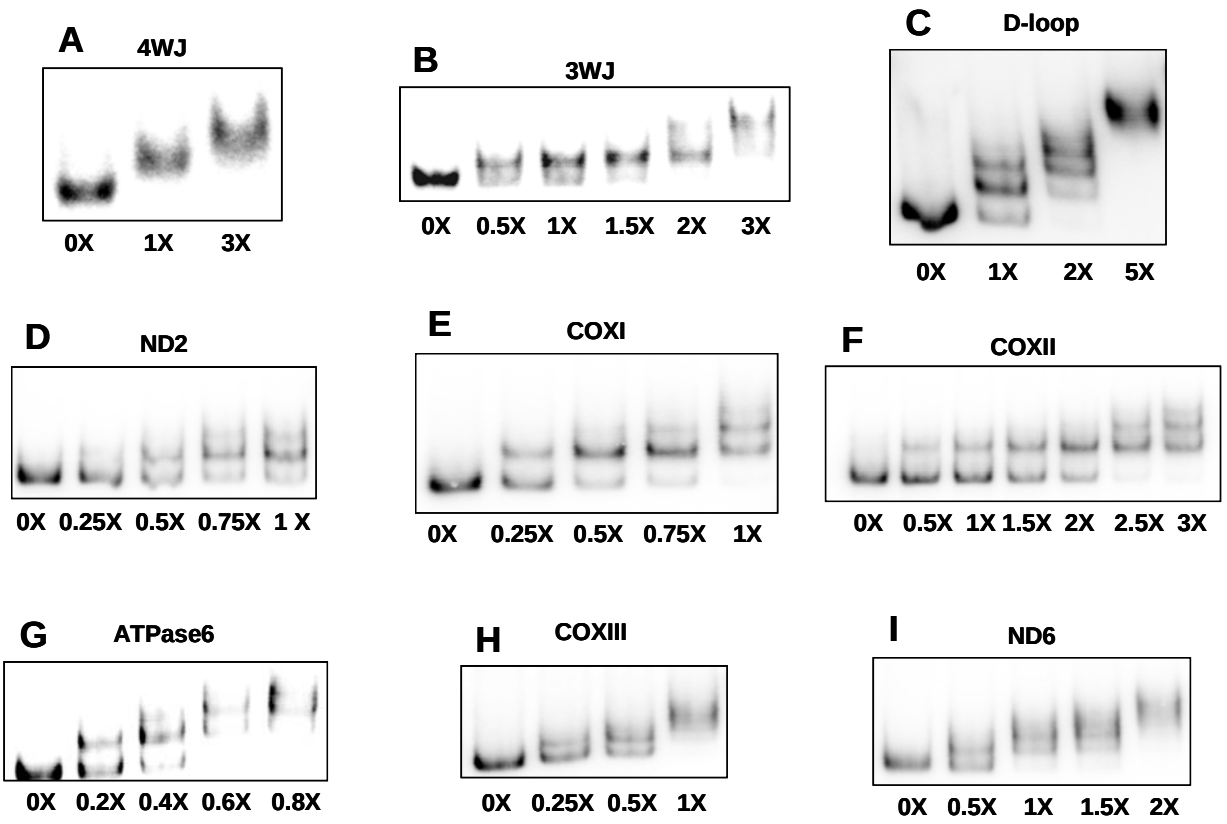

A
4WJ
0X 1X 3X
B
3WJ
0X 0.5X 1X 1.5X 2X 3X
C
D-loop
0X 1X 2X 5X
D
ND2
0X 0.25X 0.5X 0.75X 1 X
E
COXI
0X 0.25X 0.5X 0.75X 1X
F
COXII
0X 0.5X 1X 1.5X 2X 2.5X 3X
G
ATPase6
0X 0.2X 0.4X 0.6X 0.8X
H
COXIII
0X 0.25X 0.5X 1X
I
ND6
0X 0.5X 1X 1.5X 2X

Figure 2.4: TFAM binds to mtDNA and synthetic D-loop mimics. mtDNA probes are shifted by increasing molar ratios of TFAM protein. Molar ratios of protein to DNA are shown as fold excess (i.e. 3X indicates a molar ratio of 3:1 TFAM protein to DNA). DNA probes correspond to synthetic multi-way junctions or to the mtDNA coding regions in which they reside (see **Fig. 2.2**): (**A**) four-way junction (4WJ). (**B**) three-way junction (3WJ). (**C**) D-loop. (**D**) NADH dehydrogenase subunit 2 (ND2).

Figure 2.4

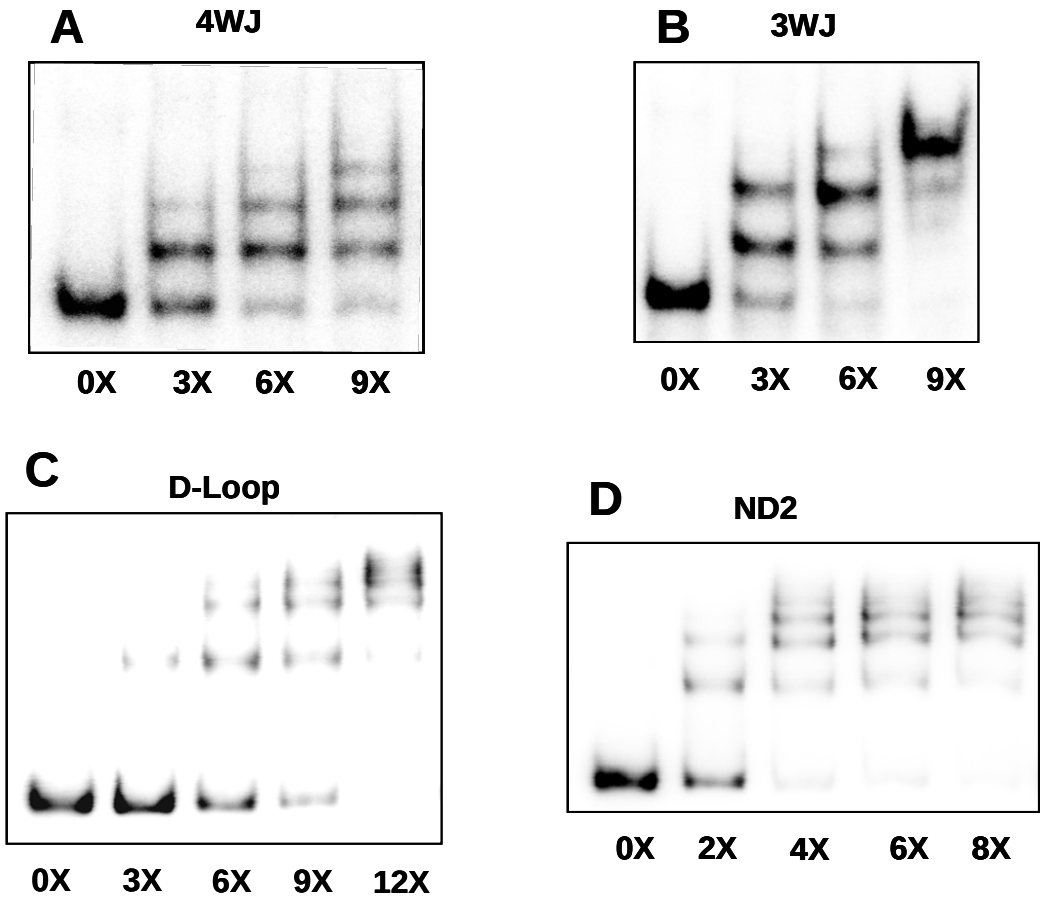
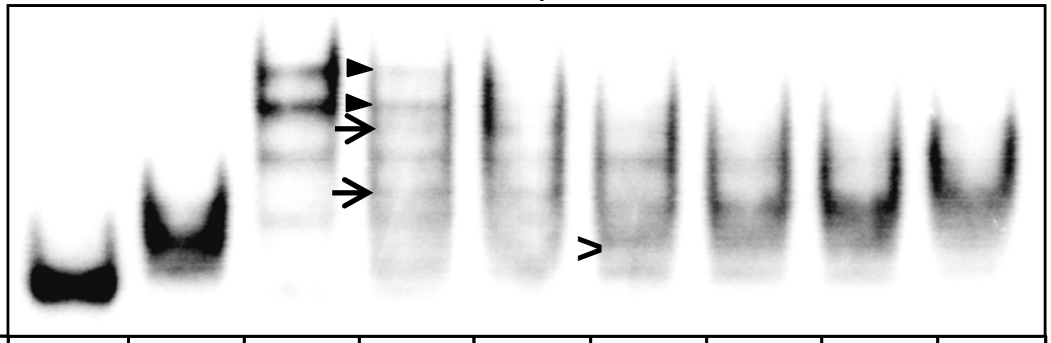

A
4WJ
0X
3X
6X
9X
B
3WJ
0X
3X
6X
9X
C
D-Loop
0X
3X
6X
9X
12X
D
ND2
0X
2X
4X
6X
8X

Figure 2.5: HMGA1 and TFAM compete for binding with 4WJ. (**A**) TFAM protein was allowed to bind with 4WJ DNA at a protein to DNA molar ratio of 9:1 for 20 minutes. After TFAM binding, HMGA1 was added at increasing molar ratios from 0.5 fold to 5 fold excess HMGA1 to DNA (lanes 4-9) and allowed to bind for an additional 20 min. Lane 1 contains free DNA only. Lanes 2 and 3 contain only HMGA1 and TFAM respectively. (**B**) HMGA1 was allowed to bind with 4WJ DNA at equal molar ratios prior to addition of TFAM at increasing molar ratios from 1-7 fold excess TFAM to DNA (lanes 4-10). Lanes 1-3 contain free DNA, only TFAM, and only HMGA1 respectively.

Figure 2.5

4WJ:TFAM, HMGA1

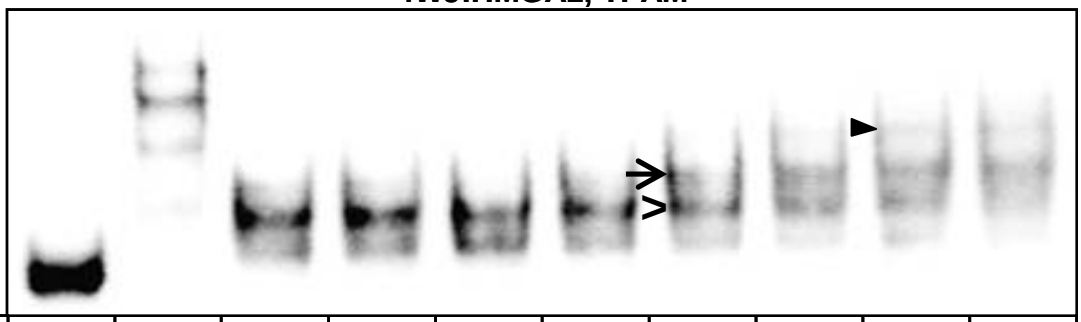
A



Lane	1	2	3	4	5	6	7	8	9
TFAM	0X	0X	9X	9X	9X	9X	9X	9X	9X
HMGA1	0X	2X	0X	0.5X	1X	2X	3X	4X	5X

4WJ:HMGA1, TFAM

B



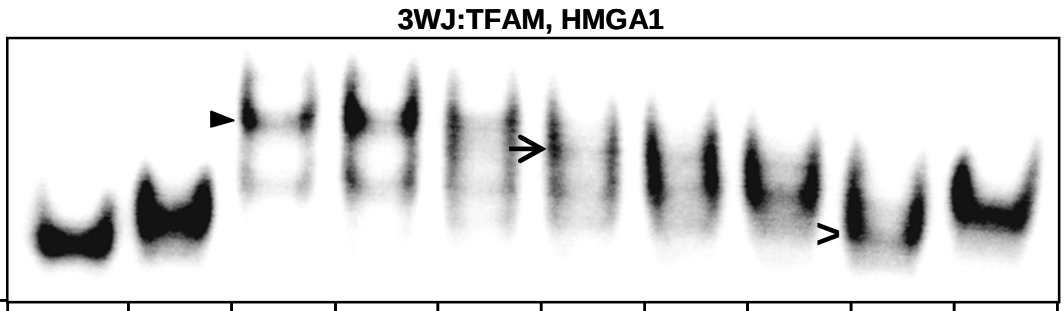
Lane	1	2	3	4	5	6	7	8	9	10
HMGA1	0X	0X	1X	1X	1X	1X	1X	1X	1X	1X
TFAM	0X	7X	0X	1X	2X	3X	4X	5X	6X	7X

► TFAM:4WJ → TFAM/HMGA1:4WJ > HMGA1:4WJ

Figure 2.6: HMGA1 and TFAM compete for binding with 3WJ. (**A**) TFAM protein was allowed to bind with 3WJ DNA at a protein to DNA molar ratio of 10:1. After TFAM binding, HMGA1 was added at increasing molar ratios from 0.5 fold to 10 fold excess HMGA1 to DNA (lanes 4-10). Lanes 1-3 contain free DNA only, only HMGA1 and only TFAM respectively. (**B**) HMGA1 was allowed to bind with 3WJ DNA at a molar ratio of 3:1 prior to addition of TFAM at increasing molar ratios of 3-21 fold excess TFAM to DNA (lanes 4-10). Lanes 1-3 contain free DNA, only TFAM, and only HMGA1 respectively.

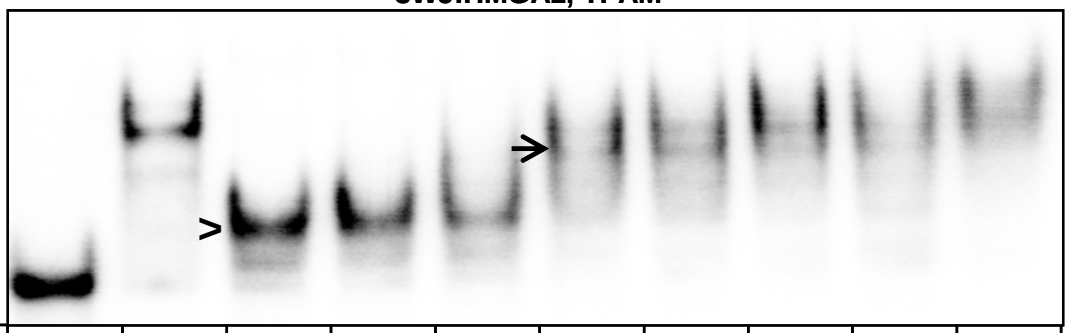
Figure 2.6

A



Lane	1	2	3	4	5	6	7	8	9	10
TFAM	0X	0X	10X	10X	10X	10X	10X	10X	10X	10X
HMGA1	0X	2X	0X	0.5X	1X	2X	3X	4X	5X	10X

B



Lane	1	2	3	4	5	6	7	8	9	10
HMGA1	0X	0X	3X	3X	3X	3X	3X	3X	3X	3X
TFAM	0X	12X	0X	3X	6X	9X	12X	15X	18X	21X

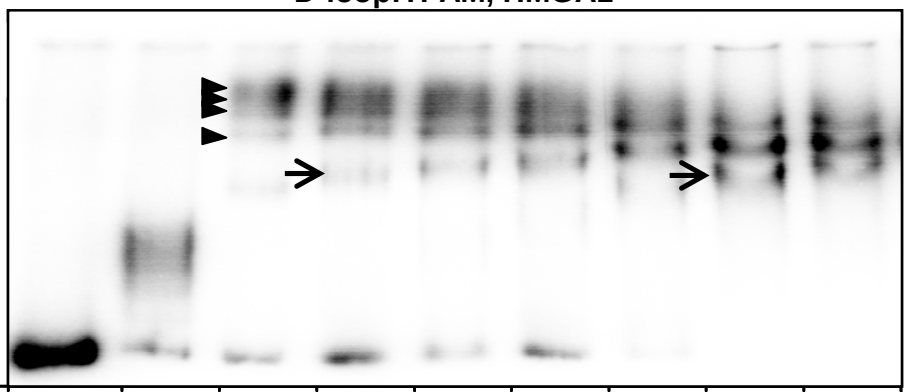
► TFAM:3WJ → TFAM/HMGA1:3WJ > HMGA1:3WJ

Figure 2.7: HMGA1 and TFAM compete for binding with B-form D-loop DNA. (**A**) TFAM protein was allowed to bind with B-form D-loop mtDNA at a protein to DNA molar ratio of 12:1. HMGA1 was then added at increasing molar ratios of 1 to 10 fold excess HMGA1 to DNA (lanes 4-9). Lanes 1-3 contained free DNA only, HMGA1 only, and TFAM only respectively. (**B**) HMGA1 was allowed to bind with the same D-loop mtDNA at 2:1 molar ratio HMGA1 to DNA prior to addition of TFAM at increasing molar ratios of 1-12 fold excess TFAM to DNA (lanes 4-10). Lanes 1-3 contained free DNA, only TFAM, and only HMGA1 respectively.

Figure 2.7

D-loop:TFAM, HMGA1

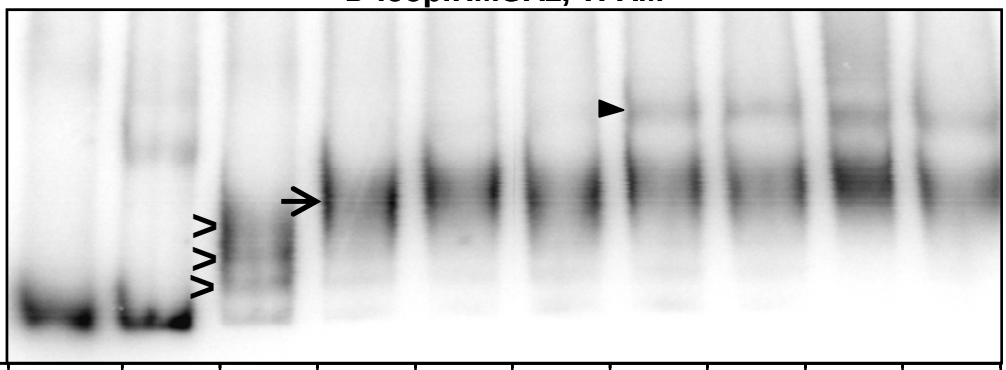
A



Lane	1	2	3	4	5	6	7	8	9
TFAM	0X	0X	12X	12X	12X	12X	12X	12X	12X
HMGA1	0X	2X	0X	1X	2X	4X	6X	8X	10X

D-loop:HMGA1, TFAM

B

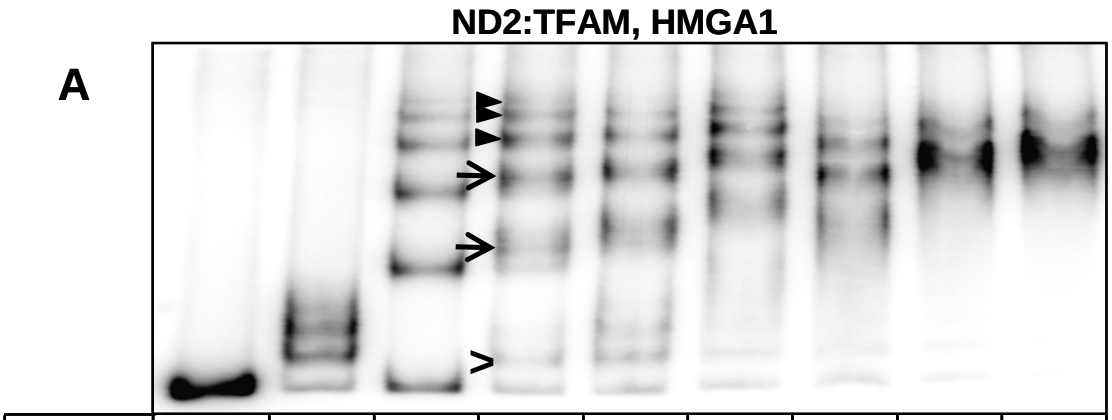


Lane	1	2	3	4	5	6	7	8	9	10
HMGA1	0X	0X	2X	2X	2X	2X	2X	2X	2X	2X
TFAM	0X	10X	0X	1X	2X	4X	6X	8X	10X	12X

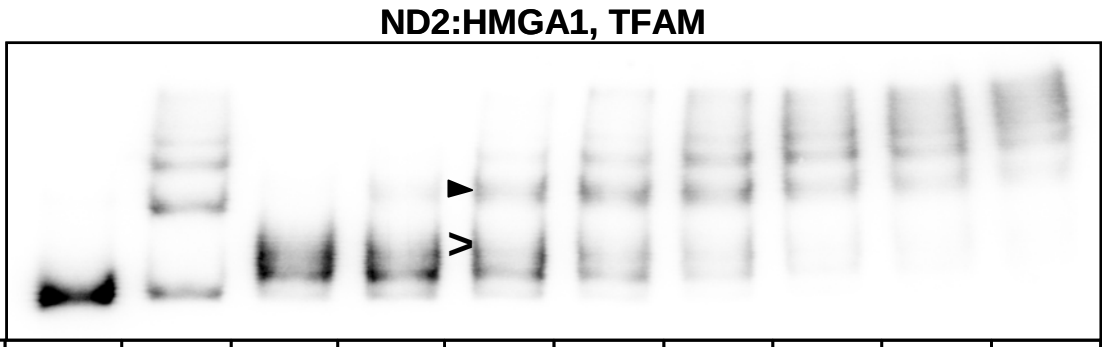
► **TFAM:D-loop** → **TFAM/HMGA1:D-loop** > **HMGA1:D-loop**

Figure 2.8: HMGA1 and TFAM compete for binding with ND2 DNA. (**A**) TFAM protein was allowed to bind with B-form ND2 mtDNA at a protein to DNA molar ratio of 8:1. After TFAM binding, HMGA1 was added at increasing molar ratios of 0.5 fold to 8 fold excess HMGA1 to DNA (lanes 4-9). Lanes 1-3 contained free DNA only, HMGA1 only, and TFAM only respectively. (**B**) HMGA1 was allowed to bind with the same ND2 mtDNA at an equal molar ratio of 1:1 prior to addition of TFAM at increasing molar ratios of 1-12 fold excess TFAM to DNA (lanes 4-10). Lanes 1-3 contained free DNA, only TFAM, and only HMGA1 respectively.

Figure 2.8



Lane	1	2	3	4	5	6	7	8	9
TFAM	0X	0X	8X	8X	8X	8X	8X	8X	8X
HMGA1	0X	2X	0X	0.5X	1X	2X	4X	6X	8X



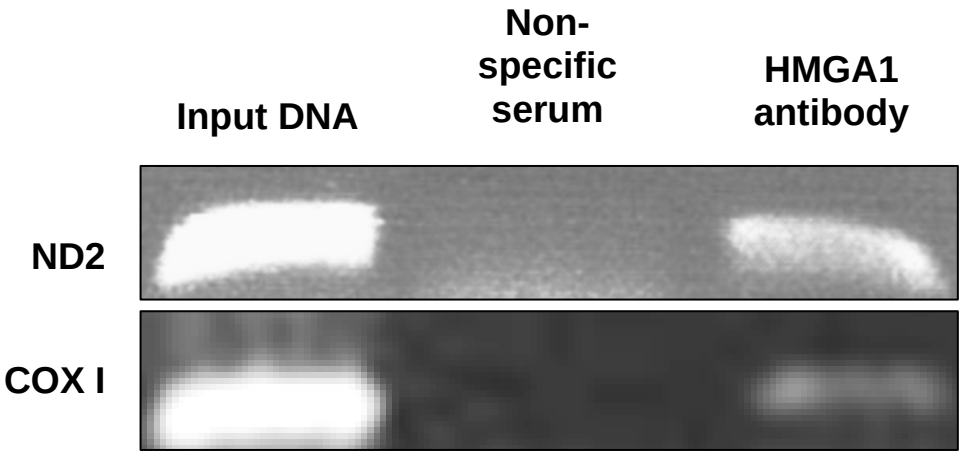
Lane	1	2	3	4	5	6	7	8	9	10
HMGA1	0X	0X	1X	1X	1X	1X	1X	1X	1X	1X
TFAM	0X	6X	0X	1X	2X	4X	6X	8X	10X	12X

► TFAM:ND2 → TFAM/HMGA1:ND2 > HMGA1:ND2

Figure 2.9: ChIP analysis demonstrates HMGA1 localizes to mtDNA *in vivo*.

7C “On” cell DNA was cross-linked and harvested for HMGA1 chromatin immunoprecipitation. Control samples were taken immediately prior to HMGA1 immunoprecipitation (Input). Non-specific rabbit serum was used in parallel with HMGA1 immunoprecipitations to verify specificity of pull-downs. Resulting DNAs were PCR amplified using primers corresponding to the ND2 and COXI mtDNA regions. Specific amplification of these two regions indicated the presence of HMGA1 at mtDNA within these primer regions.

Figure 2.9



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CHAPTER 3

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

The work presented in this thesis contains original research that improves current knowledge concerning the mitochondrial activities of HMGA1 protein. These experiments have clearly established several lines of evidence: that nuclear over-expression of HMGA1 causes the protein to accumulate at elevated levels in mitochondria; that HMGA1 interacted with multiple regions throughout the mitochondrial genome *in vitro* and *in vivo*; and that HMGA1 was able to out-compete TFAM protein for binding to both B-form and D-loop mimicking mtDNA substrates in vitro.

Previous studies have demonstrated that increased levels of HMGA1 in mitochondria have been associated with mitochondrial dysfunction [1,2], and that mtDNA binding of TFAM protein is necessary for a number of routine mitochondrial processes [3]. Given the evidence presented in this work, in conjunction with these previous findings, a potential model for HMGA1-mediated mitochondrial dysfunction has been developed in which HMGA1 and TFAM compete for functional binding to mtDNA.

This model, described in Chapter 2, maintains that in healthy mitochondria, HMGA1 is translocated in small, controlled quantities, which allows the protein to specifically bind mtDNA only at sites of highest affinity and perform a given function. TFAM, in contrast, is present in great abundance and is thus able to bind to many sites throughout mtDNA to carry out its various functions. On the other hand, in cancer cells, which routinely express elevated levels of HMGA1, the mitochondrial ratio of HMGA1 and TFAM is altered. We propose that this change in ratio is sufficient for HMGA1 to partially out-compete and displace TFAM from key sites on the mtDNA. Due to the large number of activities that TFAM carries out by means of mtDNA binding, its

displacement by HMGA1 is proposed to bring about mitochondrial dysfunction which may include altered gene expression and increased mutation caused by reactive oxygen species. Both increased mtDNA mutation and reduced mitochondrial functioning are features of cancer cells and seem to play a role disease progression. This model provides a potential premise for how HMGA1 over-expression contributes to neoplastic transformation and is associated with increasing metastatic potential of established cancers.

Future Directions

Although the work presented here has provided new insights into HMGA1 interaction with mtDNA and mitochondrial proteins, additional studies are necessary to further elucidate HMGA1 activity in mitochondria. The model derived from this evidence represents a testable hypothesis that can be evaluated through a number of additional experiments. Described below are four groups of experiments that would be the next logical steps to continue this work and yield data to either to support or refute this proposed model.

EMSAs using oxidatively damaged DNA probes

TFAM has been shown to bind preferentially to oxidatively damaged DNA and other types of lesions, potentially to facilitate recruitment of repair proteins [4]. HMGA1 has also been shown to bind to various types of DNA lesions in the nucleus, but over-expression of HMGA1 sterically hinders access of repair proteins, and thus contributes to inhibition of nucleotide excision repair [5]. Although mtDNA is mainly threatened by oxidative damage, which is generally addressed by base excision repair (BER), it is

possible that HMGA1 may carry out a similar inhibitory role in mitochondria, particularly taking into account that over-expression of HMGA1 leads to reduced mtDNA repair rates [1].

To address the question of HMGA1 and TFAM competition for binding to oxidative lesions, additional EMSA experiments should be performed using either or both of these proteins with a synthetic DNA probe containing an oxidative lesion site(s). The resulting relative affinities of TFAM and HMGA for this type of DNA probe would provide further insight into the mechanism by which HMGA1 may reduce mtDNA repair rates. If HMGA1 is found to out-compete TFAM for oxidatively damaged DNA, as it did with other mtDNA probes, it would imply that HMGA1 might block BER and contribute to accumulation of mtDNA mutations. Alternately, the lack of significant competition between HMGA1 and TFAM for binding to this probe would indicate that HMGA1 contributes to reduced mitochondrial repair by a different mechanism.

The effect of HMGA1 on mtDNA superhelicity

One of the inherent features of HMGA1 binding is that it can bend DNA substrates. In fact, HMGA1 has been found to induce positive supercoiling in A/T-rich plasmids at low molar ratios, and induce negative supercoiling at high molar ratios [6]. Considering that mtDNA is essentially a plasmid with a considerable amount of A/T rich sequence, it is likely that HMGA1 introduces topological changes in mtDNA. This idea could be evaluated by performing studies on isolated supercoiled mtDNAs to examine their topology in the presence of increasing levels HMGA1. The use of Topoisomerase I assays and 2D chloroquine gel electrophoresis would indicate the direct effect of HMGA1 over-expression on mtDNA.

If the results of these experiments were consistent with those that have been previously observed in other plasmids, low levels of HMGA1 would introduce positive supercoiling, possibly suggesting a functional role for HMGA1 in altering mtDNA structure. Additionally, if these experiments are consistent with previous studies, excess HMGA1 would cause negative supercoiling. This might potentially reflect a threshold accumulation of HMGA1 at which the protein ceases to carry out its normal function, and begins to contribute to the harmful changes in mitochondrial function that have been observed in HMGA1 over-expressing cells.

To take this idea one step further, manipulating the ratio of TFAM and HMGA1 could be used to evaluate their combined effects on mtDNA superhelicity. While HMGA1 may well be able to induce noticeable topological changes in mtDNA, TFAM may buffer, counteract, or have no effect on HMGA1's ability to change mtDNA topology. However, because TFAM is an abundant mtDNA packaging protein, it would be telling to include it in these types of studies. Whatever the result of these potential superhelical experiments, they would undoubtedly provide greater insight into the topological effects of varying levels of HMGA1 on mtDNA.

Comparative ChIP and re-ChIP of HMGA1 and TFAM

While *in vitro* experiments often provide useful answers to a given question, it is often necessary to confirm findings with an *in vivo* model. In Chapter 2, ChIP assays were presented, confirming that HMGA1 bound to two other mtDNA locations in addition to the D-loop region. While these data provide a convincing foundation that HMGA1 binds to mtDNA *in vivo*, it would be beneficial to perform further assays to evaluate HMGA1 binding to a number of regions throughout the mtDNA. Of particular interest

would be to evaluate binding of HMGA1 at locations that have been previously identified by ChIP TFAM. Ohgaki and colleagues [7] have described over 30 regions on mtDNA in HeLa cells (a cell line that over-expresses HMGA1) in which TFAM was found to immunoprecipitate, none of which have been evaluated for HMGA1. Thus, comparison of ChIP profiles for HMGA1 and TFAM generated from identical primer sets would allow a more thorough evaluation of HMGA1-TFAM competition for mtDNA binding *in vivo*. Such comparison would be particularly intriguing in multiple cells lines that express both basal and elevated levels of mitochondrial HMGA1 (i.e. normal cells and cancer cells).

In addition to single antibody ChIPs, it would be revealing to perform ChIP/re-ChIP experiments in which a second pull-down was performed on previously immunoprecipitated complexes. DNA fragments that immunoprecipitated with HMGA1 antibody, for example, would be assayed again (re-ChIP) for the presence of TFAM using a second immunoprecipitation step with TFAM antibody. Comparing this type of ChIP/re-ChIP, using both combinations of successive antibody pull-downs, would indicate if HGMA1 and TFAM are able to bind to similar locations of mtDNA *in vivo*. As a result, it would establish or challenge the notion that these two proteins are able to simultaneously bind short stretches of mtDNA *in vivo.*

In Vivo Animal Studies

After *in vitro* studies and *in vivo* cell culture studies are performed, it is valuable to confirm findings in the context of whole organisms. Although convincing evidence has been observed that over-expressed HMGA1 in transgenic or tumor cell culture aberrantly translocates to mitochondria [8], validation of these findings in mice or harvested human cancer tissue would likely strengthen these results. In fact, both of

these types of *in vivo* materials are presently available for study, given that Resar and colleagues [9] have developed a transgenic mouse line that over-expresses HMGA1, and that various types of matched set cancer samples can be made available from hospital tissue banks.

Mitochondria isolated from cancerous and healthy tissues could be used in multiple experiments that further evaluate the model proposed in Chapter 2. Western blots can be employed to evaluate the levels of HMGA and TFAM in mitochondria isolated from these tissues. In accordance with the proposed model, it would be predicted that HMGA1 levels in cancerous mitochondria would be greatly elevated as compared to HMGA1 levels in healthy mitochondria, while TFAM levels would remain unchanged. Transgenic mice and human tumor tissues could also be used to review the various ChIP experiments performed in cell culture. These potential *in vivo* animal studies would either refute or lend significant credibility to previous findings presented here and to proposed future ChIP experiments.

Performing the four types of experiments outlined above would be a valuable way to proceed with the findings presented in Chapter 2. The tools needed to conduct all of these experiments are currently available, making them a convenient place to begin future studies. In fact, work has already been initiated with matched set tumor/healthy human tissue to examine mitochondrial HMGA1 and the results thus far appear to confirm previous findings. Because there is still much to be discovered concerning the role of HMGA1 in mitochondria, and mitochondrial biology in general, it is important that this work is followed up with further investigation.

The work presented in this thesis establishes that HMGA1 binds to mtDNA *in vivo* and is able to out-compete TFAM for binding to multiple types of mtDNA structures. This evidence, in conjunction with previous findings that HMGA1 seems to undergo regulated mitochondrial translocation, suggests that HMGA1 carries out some particular function in mitochondria. However, when translocation of HMGA1 is irregular or in excess, mitochondria exhibit multiple types of dysfunction that are associated with cancer and other disease. While it is possible that this dysfunction may be related to HMGA1 binding to mtDNA, the exact activities of HMGA1 remain to be elucidated. It is also unclear as to whether HMGA1 plays a causal or contributory role in the progression of mitochondrial dysfunction and cancer. What is apparent, however, is that a greater understanding of how HMGA1 interacts with mtDNA and other mitochondrial proteins will provide significant insight into mitochondrial biology and potentially mitigation of cancer progression.

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APPENDIX A

HIGH-MOBILITY GROUP A1 PROTEINS INHIBIT EXPRESSION OF NUCLEOTIDE EXCISION REPARI FACTOR XERODERMA PIGMENTOSUM GROUP A

Summary

The work of Adair et al. entitled *High-Mobility Group A1 (HMGA1) Proteins Inhibit Expression of Nucleotide Excision Repair (NER) Factor Xeroderma Pigmentosum Group A (XPA)* was published in Cancer Research, July 1, 2007, vol. 67, pp. 6044-52. The research described in this article illustrates that HMGA1 protein is able to bind to a repressor region of the XPA gene, preventing its expression and subsequently inhibiting effective NER of cells that over-express HMGA1. Multiple distinct methods are employed to demonstrate the repressive interaction of HMGA1 with the XPA promoter. One particular method examined an A/T-rich region of the XPA promoter which appeared to be the potential HMGA1 binding site for negative regulation. Site-directed mutagenesis was used to alter the A/T-rich region of the XPA promoter to random sequence not associated with any known transcription factors. The mutated promoter and wild type promoters were then fused to luciferase reporter genes and the constructs were transfected into MCF7 and 7C ”On” cells. Figure 3 demonstrates that mutating the promoter’s A/T rich region yields increased levels of luciferase activity. This indicates that the loss of the HMGA1 binding site allow for increased gene expression.

My contribution to this work was to determine the appropriate sequence for the mutated promoter and perform the site-directed mutagenesis to form the constructs for transfection. This experiment indicated the A/T-rich region of the XPA promoter was the negative regulatory element bound by HMGA1.

Research Article

High-Mobility Group A1 Proteins Inhibit Expression of Nucleotide Excision Repair Factor Xeroderma Pigmentosum Group A

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Abstract

Cells that overexpress high-mobility group A1 (HMGA1) proteins exhibit deficient nucleotide excision repair (NER) after exposure to DNA-damaging agents, a condition ameliorated by artificially lowering intracellular levels of these nonhistone proteins. One possible mechanism for this NER inhibition is down-regulation of proteins involved in NER, such as xeroderma pigmentosum complimentation group A (XPA). Microarray and reverse transcription-PCR data indicate a 2.6-fold decrease in intracellular XPA mRNA in transgenic MCF-7 cells overexpressing HMGA1 proteins compared with non–HMGA1-expressing cells. XPA protein levels are also ~3-fold lower in HMGA1-expressing MCF-7 cells. Moreover, whereas a >2-fold induction of XPA proteins is observed in normal MCF-7 cells 30 min after UV exposure, no apparent induction of XPA protein is observed in MCF-7 cells expressing HMGA1. Mechanistically, we present both chromatin immunoprecipitation and promoter site-specific mutagenesis evidence linking HMGA1 to repression of *XPA* transcription via binding to a negative regulatory element in the endogenous *XPA* gene promoter. Phenotypically, HMGA1-expressing cells exhibit compromised removal of cyclobutane pyrimidine dimer lesions, a characteristic of cells that express low levels of XPA. Importantly, we show that restoring expression of wild-type XPA in HMGA1-expressing cells rescues UV resistance comparable with that of normal MCF-7 cells. Together, these data provide strong experimental evidence that HMGA1 proteins are involved in inhibiting XPA expression, resulting in increased UV sensitivity in cells that overexpress these proteins. Because HMGA1 proteins are overexpressed in most naturally occurring cancers, with increasing cellular concentrations correlating with increasing metastatic potential and poor patient prognosis, the current findings provide new insights into previously unsuspected mechanisms contributing to tumor progression. [Cancer Res 2007;67(13):6044–52]

Introduction

The ability to recognize and respond to DNA damage is a ubiquitous property of living organisms, from *Mycoplasma* to multicellular vertebrates. The two pathways of DNA repair thought to be responsible for a majority of day-to-day genomic maintenance are the base excision repair (BER) and nucleotide excision repair (NER) pathways. Whereas BER is known to repair small, single-base modifications, most of which arise from endogenous damage, such as oxidation (1), NER is responsible for detecting and repairing large, helix-distorting lesions, such as cyclobutane pyrimidine dimers (CPD), (6-4) photoproducts, and cisplatin adducts (2).

Deficiencies in proteins involved in NER result in severe sensitivity to DNA-damaging agents, such as UV light and cisplatin, whose lesions are normally repaired via this pathway (3). 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 xeroderma pigmentosum group A (XPA) protein display the most severe symptoms of the human disease xeroderma pigmentosum, for which eight of the NER factors are named (4).

The XPA protein is a highly conserved, 38- to 42-kDa polypeptide containing three separate protein-protein interaction domains, a nuclear localization signal, and a zinc finger domain (5). Although nuclear localization of XPA is observed under many different experimental conditions, nuclear sequestration of this protein is not required for functional participation in NER, during which it is thought to play several different roles (6). Moreover, based on the discovery of a cancer-predisposing human disease characterized by lack of functional XPA, it is apparent that this protein is essential for cellular processes of genome maintenance.

In most normal cells, the *XPA* gene is constitutively transcribed at very low levels (7, 8) and is not usually induced in response to DNA-damaging agents, such as UV light (9). Nevertheless, it has been convincingly shown that variations in the intracellular concentrations of XPA have dramatic affects on NER efficiency. For example, Cleaver et al. have shown that UV sensitivity in human cells is a linear function of intracellular XPA concentrations (10) with even modest decreases in XPA levels significantly reducing overall NER competence (10, 11).

Previous work from our laboratory has shown that transgenic MCF-7 cells induced to overexpress high-mobility group A1 (HMGA1; also known as HMG-I/Y; ref. 12) proteins, as well as cancerous cell lines that naturally overexpress these proteins, exhibit both increased UV sensitivity as well as compromised NER of CPD lesions (13). Importantly, HMGA1 proteins were directly implicated in mediating both of these cellular phenotypes because artificial reduction of the endogenous levels of HMGA1 proteins in overexpressing cells increased both their ability to repair CPD lesions and their ability to survive UV exposure (13). Additionally, recent work by Baldassarre et al. (14) has shown that embryonic stem cells overexpressing HMGA proteins are also more sensitive to treatment with cisplatin, a chemotherapeutic agent that forms bulky DNA adducts. Furthermore, it has been shown that MCF-7 cells induced to overexpress HMGA1 proteins are also more sensitive to dimethylsulfate, an alkylating agent whose base

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modifications are typically repaired via BER.[1] Finally, Boo et al. (15) have also shown that HMGA proteins potentiate genotoxic stress induced by exposure of breast cancer cells to either doxorubicin, a topoisomerase II inhibitor, or cisplatin, a DNA cross-linking agent. Given these highly suggestive data, in combination with results from our previous microarray analyses indicating decreased concentrations of XPA mRNA in cells overexpressing HMGA1 (16), we decided to experimentally address whether HMGA1 overexpression negatively regulates intracellular concentrations of XPA protein and whether XPA deficiency plays a role in the previously shown NER inefficiency displayed by cells that overexpress HMGA1 (13).

Materials and Methods

Cell culture. The human breast adenocarcinoma cell line MCF7-tet (i.e., MCF7/Tet-OFF; BD Biosciences) was cultured as described previously (13). Clonal cell line MCF7-7C-Cs is a stably transfected derivative of MCF7/Tet-OFF cells containing a tetracycline-regulated pTRE vector encoding hemagglutinin (HA)-tagged HMGA1a cDNA, maintained in the presence of 100 μg/mL hygromycin (13). This cell line expresses high levels of transgenic HA-tagged HMGA1a protein when grown in medium lacking tetracycline (these are referred to as HMGA1 "ON" cells). To prevent expression of *HMGA1* transgenes, MCF7-7C-Cs cells were cultured in medium containing 2 μg/mL tetracycline (and are referred to as HMGA1 "OFF" cells).

Cell transfections and luciferase assays. Transfections of the pGL3 Basic luciferase reporter plasmid carrying either the wild-type *XPA* promoter region (*XPA* wt-luc) or the negative regulatory mutant *XPA* promoter construct (*XPA* mut-luc) were done using Invitrogen's LipofectAMINE transfection reagent and carried out according to the manufacturer's recommendations. Briefly, 200 ng of either wild-type or mutant reporter plasmid and 50 ng of pHRGB *Renilla* plasmid (as a transfection efficiency control) were mixed with 2 μL of LipofectAMINE transfection reagent. Complexes were incubated with 70% confluent cells for 5.5 h in the absence of serum. Transfections were rescued by the addition of medium containing 2× serum directly to the transfection medium and allowed to incubate for 48 h. Twelve independent transfection experiments were done with the wild-type *XPA* wt-luc construct and at least three independent experiments with the mutant *XPA* mut-luc construct in both the HMGA1 ON and HMGA1 OFF cell lines.

Luciferase reporter assays were done with Promega's Dual-Luciferase Assay kit according to the manufacturer's recommendations and read on a Berthold Technologies Lumat LB 9507. Relative light units (RLU) were measured for both the reporter and *Renilla* plasmids and then calculated for RLUs of reporter per one RLU of *Renilla* transfection control plasmid.

Multiplex reverse transcription-PCR analysis. HMGA1 ON and HMGA1 OFF cells were cultured as described above until 90% confluent. Total RNA was isolated from cells via Trizol (Invitrogen) extraction according to the manufacturer's protocol. RNA was resuspended in RNase-free double-distilled water and quantitated via spectrophotometry. Total RNA (1 μg) from each sample was then used as template in a first-strand synthesis reaction using First-Strand cDNA Synthesis kit for reverse transcription-PCR (RT-PCR; Roche) according to the manufacturer's protocol. Single-stranded cDNA was quantitated by spectrophotometry and 50 ng were used as template in a multiplex PCR to amplify either HMGA1 and hypoxanthine phosphoribosyltransferase (HPRT) sequences or XPA and HPRT sequences. Primers used for HMGA1 amplification were GAD1 (sense, 5′-agatctatgagtgagtcgagctcgaag-3′) and GAD2 (antisense, 5′-ggatcctgctgctcctcctccg-3′); for HPRT amplification, primers were HPRT1 and HPRT2 (Clontech); and for XPA, primers were XPA mRNA S1 (sense, 5′-tactggaggcatggctaatg-3′) and XPA mRNA AS1 (antisense, 5′-ccattgtgaatgatgtggtatct-3′). PCR amplification products were resolved on 1% agarose containing ethidium bromide (0.2 μg/mL) and quantitated using Quantity One software (Bio-Rad). No-template reactions for each primer set were included as negative controls, and reactions using either a construct encoding full-length, wild-type HMGA1 or XPA as template served as positive controls for each primer set.

***In vitro* site-specific mutagenesis.** A 9-bp, suspected HMGA1-binding stretch of A/T-rich DNA (5′-tattttaaa-3′) located between nucleotides −268 and −276 in the negative regulatory region of the *XPA* gene promoter (Fig. 2*A*) was randomly changed to a nonbinding sequence (5′-gctgcgact-3′) by *in vitro* site-specific mutagenesis using the commercially available QuikChange II XL Site-Directed kit (Stratagene) following instructions provided by the supplier. The MatInspector[2] program was used to select randomly mutagenized sequences of DNA that did not contain any known transcription factor–binding sites. The 67-nucleotide-long PCR primers used for *in vitro* mutagenesis had the following sequences: 5′-gcctatactctgaatcaccaagcgagaccg*ctgcgact*ggtgaccaggtcgtgagatatgatctcag-3′ (forward) and 5′-ctgagatcatatctcacgacctggtcacc*agtcgcag*cggtctcgcttggtgattcagagtataggc-3′ (reverse).

The italicized nucleotides indicate those changed from the wild-type sequence. Both strands of the mutagenized promoter DNA were confirmed by automated sequencing.

Western blot analysis. Levels of intracellular XPA and HMGA1 proteins were determined by Western blot analysis of cell extracts prepared with Trizol according to the manufacturer's instructions. Total protein (10 μg) from either HMGA1 ON or HMGA1 OFF cells was loaded onto a 12% polyacrylamide gel in the presence of SDS. Proteins were separated electrophoretically at 100 V for 2.5 h before transferring to Immobilon-P membrane (Millipore) via tank transfer in buffer containing 25 mmol/L Tris (pH 7.5), 0.2 mol/L glycine, and 20% methanol at 100 V for 1 h. Before addition of primary antibodies, membranes were blocked in TBS containing 5% nonfat dry milk. Membranes were probed for XPA, HMGA1, and total actin (as a loading control) using either a monoclonal antibody against XPA (1:150 dilution; Santa Cruz Biotechnology), a specific polyclonal antibody against HMGA1 proteins (1:1,000 dilution; ref. 17), or polyclonal anti-actin rabbit antibody (1:5,000; Sigma). Secondary antibodies were either horseradish peroxidase (HRP)-conjugated goat anti-rabbit (1:5,000; Santa Cruz Biotechnology) or HRP-conjugated goat anti-mouse (1:10,000; Pierce). Blots were developed using SuperSignal chemiluminescent substrate (Pierce). Films were scanned and quantitated densitometrically using ImageQuant software to determine fold differences observed.

For UV induction, cells were cultured as described above until 90% to 95% confluent. Medium was removed from culture plates and cells were washed once with 1× PBS. Cells were then irradiated using low-pressure Hg lamps (model G30T8; Sylvania) at a dose of 20 J/m^2 (measured with a Spectroline DM-254N shortwave UV meter; Spectronic Corp.). Immediately after UV exposure, complete medium was replaced, and cells were incubated for 30 min before protein extraction was done as above.

For XPA overexpression, both HMGA1 ON and HMGA1 OFF cells were cultured as above until 70% confluent. Cells were either left untreated, transfected with 1 μg/mL empty pcDNA3.1 construct (Invitrogen), or transfected with 1 or 2.5 μg/mL pcDNA3.1(XPA) (a gift from Dr. Richard Wood, Hillman Cancer Center, University of Pittsburgh, Pittsburgh, Pennsylvania), containing the full-length, wild-type *XPA* gene under the control of a cytomegalovirus (CMV) promoter, using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. Total protein was isolated and analyzed via Western blot analysis as above 48 h after transfection.

Immunocytochemical analyses. HMGA1 ON and HMGA1 OFF cells were cultured on sterile glass coverslips measuring 22 mm × 22 mm under standard culture conditions as described above until 50% to 60% confluent. Medium was removed and cells were washed once with PBS. Coverslips were then irradiated at 20 J/m^2 (as described above) and either fixed immediately or replaced in culture medium and incubated to allow repair

[1] J.E. Adair and R. Reeves, unpublished data.

[2] http://www.genomatrix.de

to occur. At 6 and 24 h after irradiation, coverslips were fixed in ice-cold absolute methanol for 20 min at −20° followed by washing in PBS for 5 min before being permeabilized using PBS + 0.1% Triton X-100. Coverslips were washed thrice in PBS and subjected to 30 min in 2 mol/L HCl to depurinate and denature dsDNA. This was followed by a 5-min wash in 1 mol/L borate buffer (pH 8.0) and three washes in PBS for 5 min each. Coverslips were blocked by adding 5% nonfat dry milk in PBS and incubating at room temperature for 30 min. A primary antibody against CPD lesions (18) was added to blocking solution at a 1:500 dilution and incubated for 1 h at room temperature. Coverslips were then washed thrice with PBS + 0.1% Tween 20 before addition of anti-mouse Oregon Green–conjugated secondary antibody (for detection of CPD antibody) was added at a 1:200 dilution in blocking solution and incubated at room temperature in the dark for 1 h. Coverslips were then washed again for 5 min each in PBS + 0.1% Tween 20 before mounting on sterile glass microscope slides in mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) as a nuclear stain. Confocal microscopy was used to visualize cell nuclei via DAPI fluorescence as well as CPD lesions. Individual cells were counted for each of five random fields, and total field fluorescence was divided by the number of cells per field to obtain average per cell fluorescence intensities for each treatment and time after UV irradiation.

For colocalization analyses, micropore irradiation (19) was used as opposed to whole-cell irradiation due to the ubiquitous nuclear fluorescence of both HMGA1 proteins and CPD lesions even at low UV doses. In these experiments, HMGA1 ON cells were cultured on glass coverslips as above, medium was removed, and a 5-μm isopore polycarbonate filter (Millipore), presoaked in PBS, was overlayed before UV exposure. Filters were then removed and coverslips were treated, as above, for detection of CPD lesions, with the following exceptions. First, during primary incubation, both anti-CPD antibody (mouse origin) and an anti-HMGA1 antibody (rabbit origin) were added at 1:500 dilution. Second, both anti-mouse Oregon Green–conjugated and anti-rabbit Texas Red–conjugated secondary antibodies were added during the second incubation period. As above, confocal microscopy was used to visualize DAPI staining as well as both CPD lesions and HMGA1 proteins. Per cell fluorescence intensities for each of five random fields were calculated as above.

Chromatin immunoprecipitation analyses. To determine whether HMGA1 was associated with the endogenous *XPA* promoter, HMGA1 ON cells were cultured as indicated above until complete confluence was obtained. Approximately 4×10^6 cells were trypsinized and resuspended in 10 mL serum-free DMEM containing 1% formaldehyde and incubated for 10 min at 37°C. Glycine was added to a final concentration of 125 mmol/L to quench cross-linking, and cells were pelleted by centrifugation. Nuclei were isolated from the cell pellets and extensively sonicated, and the resulting small chromatin fragments were processed following our previously described chromatin immunoprecipitation (ChIP) techniques (20) using rabbit MR19 anti-HMGA1 antibody (17). To evaluate whether HMGA1 was endogenously associated with the *XPA* promoter, PCR of immunoprecipitated DNA was done using primers specific to a 450-bp region of the 5′-untranslated region (5′-UTR) of the *XPA* gene, which consists of one identified potential HMGA1-binding site and the only conserved regulatory element described within this promoter, a 100-bp negative regulatory region (21, 22). Primers used were XPA New Neg S1 (sense, 5′-aactgtcaagagctcagacttaacc-3′) and XPA 5′-UTR AS1 (antisense, 5′-ctccgcgggttgctctaa-3′). PCR amplification of a portion of the KIT ligand gene promoter, to which HMGA1 is bound in these cells (20), was done as a positive control to show effective immunoprecipitation. In addition, amplification of a fragment of the HPRT gene promoter, which does not bind HMGA1 *in vivo* (20), was done as a negative control for the ChIP reactions. Products of the PCRs were resolved by electrophoresis in 1% agarose in the presence of ethidium bromide.

XPA overexpression and UV survival analyses. HMGA1 ON and HMGA1 OFF cells were cultured as above until 70% confluent. Cells were then transfected with either empty pcDNA3.1 or pcDNA3.1(XPA) at a concentration of 1 μg/mL using LipofectAMINE according to the manufacturer's protocol. Medium was removed and cells were washed once with PBS 48 h after transfection. Plates were then either left untreated or irradiated at a dose of 2, 10, 25, 50, or 100 J/m^2 as above. Medium was then replaced and cells were cultured for 96 h to allow repair and/or apoptosis to occur.

To determine cell viability, cells were washed with PBS, trypsinized, and resuspended in complete medium. Cell survival after irradiation was monitored via trypan blue (0.4%) exclusion and viability counting using a hemacytometer. Briefly, cell suspension was diluted 1:1 in 100 μL trypan blue dye and incubated at room temperature for 5 min. Twenty microliters were loaded onto a Levy Ultra Plane hemacytometer, and the number of viable cells was determined. Percentage survival was calculated by comparison with non–UV-irradiated controls.

Results

XPA mRNA and protein levels are lowered in HMGA1-overexpressing cells. Our previous microarray gene expression analyses indicated that intracellular concentrations of XPA mRNA were decreased 2.6-fold in HMGA1 ON cells compared with HMGA1 OFF cells (16). To confirm this observation, multiplex RT-PCR was used (see Fig. 1*A*). In these experiments, total RNA was isolated from parental MCF-7 cells as well as from transgenic MCF-7 cells either induced (HMGA1 ON) or not induced (HMGA1 OFF) to overexpress the HMGA1 proteins. Equal amounts of total

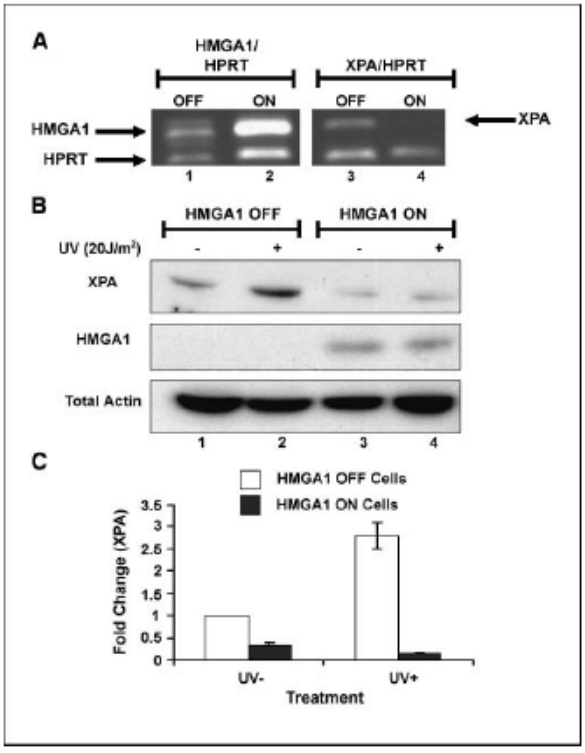

Figure 1. XPA transcript and protein are reduced in HMGA1-expressing MCF-7 cells. *A*, multiplex RT-PCR analysis of total RNA extracted from MCF-7 cells either noninduced (HMGA1 OFF; *lanes 1* and *3*) or induced (HMGA1 ON; *lanes 2* and *4*) to express HMGA1 proteins. *Lanes 1* and *2*, PCR products from reaction containing primers to both *HMGA1* and *HPRT* gene transcripts; *lanes 3* and *4*, PCR products from reactions containing primers to *XPA* and *HPRT* gene transcripts. *B*, Western blot analysis of total protein isolated from HMGA1 OFF (*lanes 1* and *2*) and HMGA1 ON (*lanes 3* and *4*) cells. *Lanes 1* and *3*, total protein from untreated cells; *lanes 2* and *4*, total proteins isolated 30 min after a 20 J/m^2 dose of UV radiation. *C*, graphical representation of Western blot densitometric analysis. *White columns*, HMGA1 OFF cell XPA protein; *dark gray columns*, HMGA1 ON cell XPA. *Columns*, mean of three independent experiments for each cell type and treatment; *bars*, SD.

RNA were used to produce cDNA, which was subsequently used as template in a multiplex PCR to amplify either HMGA1 and HPRT transcript sequences or XPA and HPRT transcript sequences. As shown in Fig. 1*A*, these analyses indicated that HMGA1 OFF cells expressed only low levels of both HMGA1 (*lane 1*) and XPA (*lane 2*) transcripts. In contrast, HMGA1 ON cells (after normalization to levels of "control" HPRT transcripts produced in the same multiplex reaction) showed ~10-fold more HMGA1 transcript (*lane 3*) and no detectable XPA transcript (*lane 4*).

As transcript levels are not always indicative of intracellular protein concentrations, Western blot analysis was used to assess intracellular concentrations of XPA in both HMGA1 OFF and HMGA1 ON cells (Fig. 1*B*). Although both cell lines showed detectable XPA levels, the amount of XPA detected in HMGA1 ON cells was significantly lower than that observed in HMGA1 OFF cells (Fig. 1*B*, compare *lanes 1* and *3*). Moreover, when total proteins were isolated from cells 30 min after a 20 J/m^2 dose of UV radiation, there was a significant increase in intracellular XPA concentrations in HMGA1 OFF cells (Fig. 1*B*, compare *lanes 1* and *2*). In contrast, XPA levels seemed unchanged in HMGA1 ON cells under the same conditions (Fig. 1*B*, compare *lanes 3* and *4*). Densitometric analyses of three independent assessments of intracellular XPA concentration revealed a basal 3-fold difference between HMGA1 OFF and HMGA1 ON cells before UV irradiation and a 6-fold difference after UV exposure (Fig. 1*C*). As indicated in Fig. 1*B*, HMGA1 protein levels were not altered in cells on UV exposure.

HMGA1 is associated with the endogenous *XPA* gene promoter in living cells. HMGA1 proteins have been shown to participate in both positive and negative regulation of transcription of a large number of mammalian genes by controlling the formation of multicomponent protein-DNA complexes on gene promoter regions (12, 20, 23, 24). Thus, ChIP assays were done to determine if HMGA1 proteins associate with the endogenous *XPA* promoter (Fig. 2). Briefly, HMGA1 ON cells were exposed to formaldehyde to cross-link proteins to proteins and proteins to DNA wherever close intracellular interactions between the two exist. Isolated chromatin was then sonicated to produce average DNA fragments of ~500 bp in size, and immunoprecipitation of protein and DNA complexes containing HMGA1 was accomplished by incubation in the presence of an antibody specific to HMGA1 proteins. Immunoprecipitated DNA was then purified and subjected to PCR analysis. In these experiments, as shown in Fig. 2, a 450-bp region of the *XPA* promoter, known to contain a negative regulatory region of ~100 bp that is conserved between mice and humans, was amplified (21, 22). Importantly, this region of the promoter also contains several potential HMGA1-binding domains, as defined by a sequence of six or more adenine or thymine bases (25), one of which is located within the negative regulatory region at nucleotides −268 to −276 (see Fig. 2*A*). As shown by the ChIP results in Fig. 2*B* (*lane 3*), preferential amplification of DNA corresponding to this region of the *XPA* promoter strongly suggests that HMGA1 proteins either are directly bound to the DNA itself or are in very close proximity to this particular genomic sequence as a result of indirect interactions with other DNA-bound proteins. As positive and negative controls for these ChIP reactions, using the same immunoprecipitation samples, PCR amplifications were also done for the promoters of genes previously shown (20) to either bind (e.g., the *KIT* ligand) or not (e.g., *HPRT*) HMGA1 proteins *in vivo* (Fig. 2*B*). Additional negative controls (Fig. 2*B*) included immunoprecipitations with either nonspecific (*lane 4*) or no (*lane 5*) antibody in the reactions.

Mutation of an A/T-rich sequence in the *XPA* promoter relieves transcriptional repression. These ChIP results provide strong support for a close association of HMGA1 with the negative regulatory region of the *XPA* promoter in living cells but do not directly show that the repressive effects of HMGA1 on *XPA* gene transcription (Fig. 1*A*) are mediated through the nine-nucleotide-long stretch of A/T-rich sequence located between nucleotides −268 and −276, the only potential binding site for the HMGA1 protein in this negative control element. To investigate this possibility, *in vitro* site-directed mutagenesis was used to randomly alter the nine nucleotides in this stretch of DNA so that they would no longer be a potential binding site for either HMGA1 (Fig. 2*A*) or any other known mammalian transcription factor (see Materials and Methods). Both the wild-type and mutant *XPA* promoter sequences were ligated into a luciferase reporter plasmid to produce expression vectors *XPA* wt-luc and *XPA* mut-luc, respectively. HMGA1 ON and HMGA1 OFF cells were individually transfected with either *XPA* wt-luc or *XPA* mut-luc, and 48 h after transfection, the cells were lysed and the amount of luciferase activity in the extracts was determined. The graph in Fig. 3 shows the results obtained when HMGA1 OFF cells were transfected with either *XPA* wt-luc (Fig. 3, *gray column*) or *XPA* mut-luc (Fig. 3, *black column*) and shows that the transcriptional activity of the *XPA* promoter significantly increases (by ~50%) when the A/T-rich stretch in the negative regulatory region is mutated to a sequence that will not bind HMGA1. Similar differential transcription trends were also observed when the *XPA* wt-luc and *XPA* mut-luc plasmids were transfected into ON cells that are overexpressing HMGA1 protein (data not shown).

HMGA1 proteins do not colocalize with CPD lesions in living cells. In accordance with phenotypes observed in human cells that have been experimentally induced to express low levels of functional XPA protein (10), defective NER of CPD lesions was observed in HMGA1 ON cells, but not in HMGA1 OFF cells or normal MCF-7 cells, both of which express low to undetectable levels of HMGA1 proteins (13). The immunocytochemical results shown in Fig. 4 qualitatively verify this phenomenon. In these experiments, a specific monoclonal antibody was used to detect *cis-syn* CPDs in nuclei of HMGA1 OFF (Fig. 4*A–D*) and ON (Fig. 4*E–G*) cells that had been exposed to UV irradiation (10 J/m^2) and then either immediately fixed (time 0; Fig. 4*B* and *F*) or allowed to undergo NER for either 6 h (Fig. 4*C* and *G*) or 24 h (Fig. 4*D* and *H*). Figure 4*A* and *E* shows control reactions in which non–UV-treated cells were reacted with the anti-CPD antibody to determine levels of background fluorescence. From these results, two main observations can be made. First, it is evident that at "time 0" the nuclei of both the OFF cells and the ON cells that have been UV irradiated fluoresce with approximately equal intensity, indicating that similar amounts of UV-induced CPDs are present in the global genomic DNA of both cell types. Thus, overexpression of HMGA1 proteins in the ON cells does not seem to enhance the overall amount of UV-induced damage formation. Second, at 6 h after irradiation, fluorescence of HMGA1 ON cell nuclei is much brighter than is the nuclear fluorescence of the OFF cells containing very low levels of HMGA1 proteins (Fig. 4*C* and *G*), and even at 24 h after irradiation, a difference in intensity between the ON and OFF cells is still apparent (Fig. 4*D* and *H*), although not to the same extent. These highly reproducible results qualitatively confirm, by an independent experimental method, our previously reported quantitative demonstration that overexpression of HMGA1 proteins inhibits global genomic NER of CPD lesions *in vivo* (13).

We have also previously shown, under *in vitro* conditions, that HMGA1 proteins exhibit a high binding affinity for non-B-form DNA (12, 24) and that they are capable of binding to, and altering the formation of, CPD lesions in a DNA fragment rich in A/T residues (13). These findings, therefore, raised the question of whether HMGA1 proteins can also selectively bind to CPD lesions in living cells, thus providing a likely explanation for the NER defects observed in HMGA1 ON cells. To investigate this possibility, immunocytochemical analyses were done using specific antibodies to determine whether HMGA1 and CPDs are colocalized in ON cells exposed to 20 J/m^2 UV irradiation. In these experiments, HMGA1-expressing cells were micropore irradiated (19) rather than whole-cell irradiated as described in Materials and Methods. Micropore analysis was used to avoid potential spurious colocalization artifacts that could arise from several sources (13, 19). In such analyses, colocalization of molecules is examined in time course experiments in only subnuclear "foci" of DNA damage corresponding to small holes in a masking micropore filter placed over the cells before UV irradiation. Figure 5 shows a representative example of such an experiment in which ON cells were replaced in culture medium following UV irradiation and allowed 30 min to equilibrate before being fixed in absolute methanol and examined. Based on kinetic data showing that HMGA1 proteins are remarkably mobile in the nuclei of living cells with rapid on/off rates at most genomic sites (26), this 30-min equilibration period would allow for HMGA1 proteins to diffuse to and/or from DNA lesions induced within micropore-irradiated nuclei. As seen in Fig. 5 (*left*), numerous sites of HMGA1 accumulation (Fig. 5, α-*HMGA1 TR, red*) are scattered throughout the nuclei of cells exposed to UV light but these localized concentrations of protein are not (for the most part) induced by the irradiation itself because similar HMGA1 distribution patterns are also observed in the nuclei of normal, nonirradiated cells (27, 28).[1] In the case of CPD lesions, direct support for this conclusion is provided by the results shown in Fig. 5 (*middle* and *right*), which show that, although localized areas of CPD lesions were readily visualized within micropore-irradiated nuclei (Fig. 5, α-*CPD OG, green*), no yellow fluorescence, indicative of HMGA1 and CPD colocalization, was observed in any merged

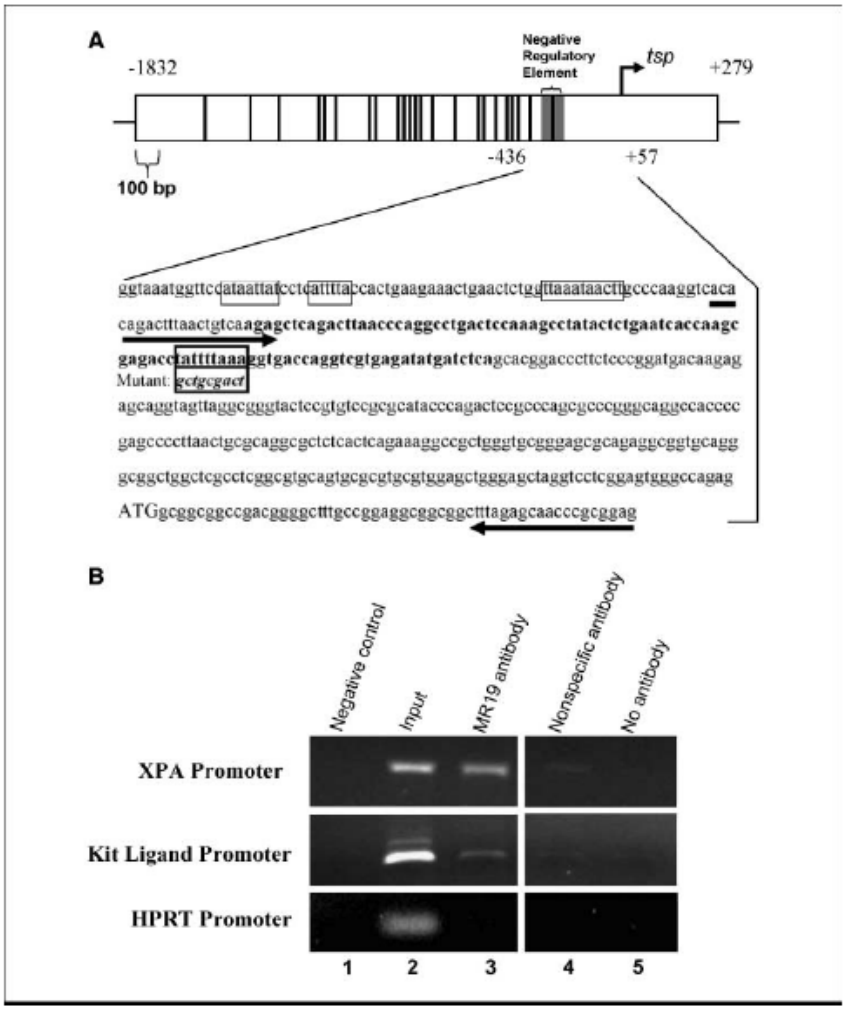

Figure 2. HMGA1 protein is associated with the endogenous *XPA* gene promoter in MCF-7 cells overexpressing HMGA1 proteins. *A, white box*, schematic diagram of the 5′-UTR of the human *XPA* gene; *gray box*, putative 100-bp negative regulatory region; *vertical black bars*, potential HMGA1-binding sites. Expanded sequence (−436 to +57) includes negative regulatory region (*bold text*), potential HMGA1-binding sites (*open boxes*), and forward and reverse PCR primer sites (*black arrows*) used to amplify this region from ChIP samples. *Bolded open box*, position of the putative HMGA1-binding site within the negative regulatory element (nucleotides −268 to −276); *underlined*, wild-type sequence. The sequence of a randomly mutated version of nucleotides −268 to −276 used in transfection experiments is shown in *italics* below the wild-type sequence. *tsp*, transcriptional start point. *B*, ethidium bromide visualization of ChIP PCR amplifications. *Top*, ChIP results obtained with a 450-bp fragment of the *XPA* promoter corresponding to nucleotides −393 to +57 with reference to the transcriptional start point; *middle*, ChIP results obtained with a known positive control, a 350-bp fragment of the *KIT* ligand gene promoter that has previously been shown to interact with HMGA1 *in vivo* (20); *bottom*, ChIP results obtained with a known negative control, a 124-bp fragment of the *HPRT* gene promoter that does not interact with HMGA1 *in vivo* (20). *Lane 2*, result of PCR amplifications in which input DNA (i.e., isolated from samples before immunoprecipitation) was used as template for each primer set; *lane 3*, results of ChIP reactions using a specific anti-HMGA1 antibody; *lanes 1, 4*, and *5*, results of additional negative controls for the ChIP reactions themselves and correspond, respectively, to no DNA template, nonspecific antibody, and no antibody reaction controls.

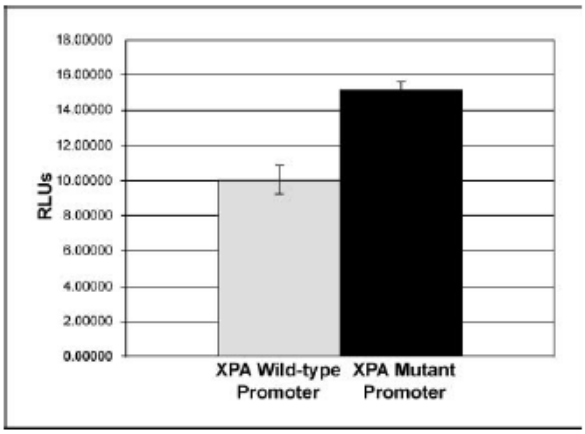

Figure 3. Site-specific mutations of the A/T-rich HMGA1-binding site in the negative regulatory region (nucleotides −268 to −276) of the *XPA* promoter relieve gene transcriptional repression. Results of luminescence assays (depicted in RLUs) obtained with HMGA1 OFF cells that were transfected with either the wild-type *XPA* wt-luc plasmid construct (*gray column*) or the mutant *XPA* mut-luc construct (*black column*). Similar differential trends were also observed when the *XPA* wt-luc and *XPA* mut-luc plasmids were transfected into HMGA1 ON cells (data not shown).

fields examined. These results indicate that HMGA1 proteins are not stably associated with sites of CPD damage in living cells soon after UV irradiation.

XPA complimentation restores UV resistance. To assess whether the observed XPA deficiencies associated with HMGA1 overexpression caused the NER defects also seen in these cells, XPA complimentation studies were done. In these experiments, a vector encoding full-length, wild-type XPA, under control of a constitutive CMV promoter, was transfected into both HMGA1 OFF and HMGA1 ON cells and cell viability after UV irradiation at a variety of UV doses was examined (Fig. 6). As shown in the Western blots in Fig. 6*A*, these transient transfections resulted in increased expression of XPA protein in both ON and OFF cells compared with control cells transfected with an empty pcDNA3.1 vector (Fig. 6*A*, compare *lanes 3–6* with *lanes 1* and *2*). Importantly, overexpression of XPA did not alter HMGA1 levels in either ON or OFF cells (Fig. 6*A*, *lanes 3–6*).

When cell survival after UV irradiation was examined in XPA-overexpressing cells, no significant difference in survival between HMGA1 OFF and HMGA1 ON cells was observed at UV doses between 2 and 100 J/m^2, indicating that XPA deficiencies are responsible for previously described UV sensitivity in these cells (Fig. 6*B*; ref. 13). This is exemplified by the significant increase in UV resistance between HMGA1 ON cells overexpressing XPA compared with HMGA1 ON cells transfected with empty pcDNA3.1 vector (Fig. 6*B*). Moreover, there were no significant increases in cell survival between HMGA1 OFF and nontransgenic parental MCF-7 cells before and after induction of XPA,[1] indicating that the endogenous concentrations of XPA in these cells are sufficient for normal levels of repair to occur. Likewise, additional control experiments showed that there was no difference between survival of parental MCF-7 and transgenic HMGA1 OFF cells that were transfected with an empty pcDNA3.1 vector (data not shown; ref. 13).

Discussion

Previous work from our laboratory showed that MCF-7 cells overexpressing HMGA1 proteins are as much as 50% less efficient in their ability to repair UV-induced CPD lesions compared with non–HMGA1-overexpressing MCF-7 cells (13). These findings, in combination with other work indicating that HMGA1-expressing cells are more sensitive to cisplatin (14), suggest that cells expressing HMGA proteins are compromised in their ability to carry out NER efficiently.

Given the diverse array of protein-protein interactions involving HMGA1, as well as the ability of HMGA proteins to bind DNA in a structure-specific manner, one possible mechanism for the observed NER deficiency in cells overexpressing these proteins would be by interference with repair factor binding. In this model, direct binding of HMGA1 proteins to CPDs could inhibit NER factor access to lesion sites as a consequence of steric hindrance. Indeed, our previously published *in vitro* analyses indicated that HMGA1 could bind to DNA fragments containing CPD lesions and, moreover, inhibit repair of DNA fragments containing CPD lesions

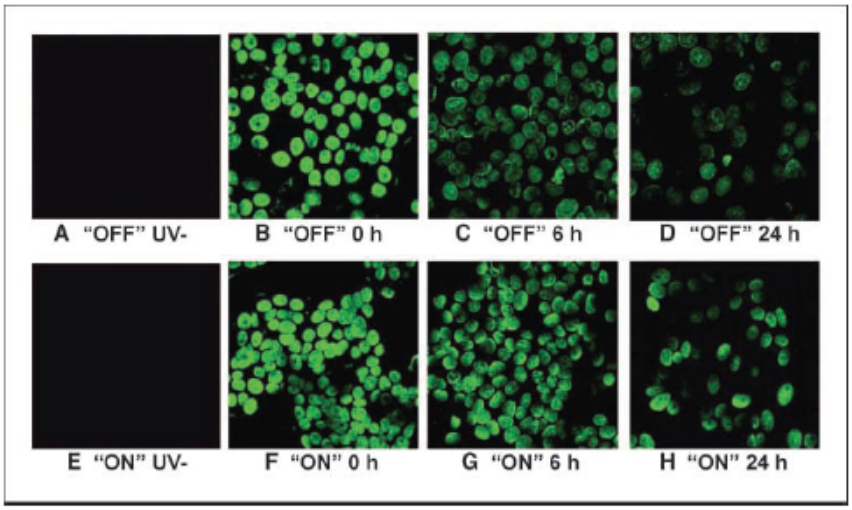

Figure 4. Global genomic NER of CPD lesions is inhibited in MCF-7 cells overexpressing HMGA1 proteins. Immunocytochemistry using anti-CPD primary antibody and Oregon Green secondary antibody in MCF-7 cells induced to either express (*bottom*) or not express (*top*) HMGA1a proteins. Cells were exposed to 10 J/m^2 and then either immediately fixed (time 0; *B* and *F*) or allowed to undergo NER for either 6 h (*C* and *G*) or 24 h (*D* and *H*) and then probed for the presence of CPDs. *A* and *E*, control reactions in which non–UV-treated cells are reacted with the anti-CPD antibody. Representative of the average total fluorescence observed in five random fields for each treatment at ×600 magnification.

by *Xenopus* oocyte nuclear extracts (13). The results of immunocytochemical studies reported here, however, do not show colocalization of HMGA1 proteins at CPD lesion sites within MCF-7 cell nuclei after UV irradiation (Fig. 5). These data suggest that *in vivo* HMGA1 proteins do not directly interfere with binding of repair factors to CPD lesions and, thereby, directly impede repair processes at the sites of damage.

Another possible mechanism for the observed NER deficiency seen in ON cells is that HMGA1 proteins limit the availability of one or more important NER factors, a likely candidate given the well-established role of the HMGA1 proteins as both positive and negative regulators of gene transcription (24). Further evidence supporting this possibility includes microarray analyses that compared basal transcriptome profiles of HMGA1 OFF and ON cells (16). In these experiments, many gene transcripts were found to be either up-regulated or down-regulated in the presence of overexpressed HMGA1 proteins. Of those transcripts that were found to be down-regulated, however, an unexpectedly large number were involved in DNA damage recognition and repair, including the XPA mRNA.

The work described here indicates that not only are XPA transcript levels decreased in MCF-7 cells overexpressing HMGA1 proteins but intracellular XPA protein levels are proportionately decreased as well in these cells (Fig. 1). Moreover, this decrease in intracellular XPA protein concentrations is exaggerated even further following exposure of cells to UV irradiation at 20 J/m^2, a dose considered to be within the physiologic range of sunlight exposure (Fig. 1*B* and *C*). These data provide convincing 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 submaximal NER observed in these cells previously (13).

There are several potential mechanisms by which overexpression of HMGA1 proteins could be negatively influencing intracellular XPA protein levels, including possible effects on transcript splicing or stability (29, 30). Nevertheless, given the extensive body of literature defining a role for HMGA1 proteins in controlling gene transcription via interaction with promoter regulatory regions (12, 24), we initially considered this to be the most likely mechanism by which HMGA1 negatively regulates *XPA* gene activity. Additional arguments for transcriptional control of the *XPA* gene include microarray and multiplex RT-PCR evidence showing that basal transcript levels are, in fact (as shown in Fig. 1), depleted in HMGA1 ON cells compared with HMGA1 OFF cells, indicating regulation at the level of either transcription or mRNA stability.

Further evidence indicating a direct role for HMGA1 in negatively regulating transcription of the *XPA* gene in overexpressing cells comes from two important experimental observations. Using ChIP analyses, we showed that in ON cells HMGA1 is associated with an ~450-bp region of the *XPA* promoter that contains a previously identified negative regulatory region with one potential HMGA1-binding site of A/T-rich nucleotide residues (Fig. 2*A*). Although these ChIP results provide firm evidence physically linking HMGA1 to the endogenous negative regulatory region, they do not, per se, show that the observed repressive effects of HMGA1 on *XPA* gene transcription are a consequence of binding of this protein to the only A/T residues within this region. Unequivocal *in vivo* evidence supporting this possibility comes, however, from experiments in which cells were transfected with expression plasmids containing a luciferase reporter gene driven either by the wild-type *XPA* promoter or by a mutant *XPA* promoter in which the nine A/T residues have been replaced by a non–HMGA1-binding stretch of random nucleotide sequence (Fig. 2*A*). The graph in Fig. 3 clearly shows that the level of transcription obtained with the mutant *XPA* promoter is significantly higher (by ~50%) than that from the wild-type promoter. Because great care was taken while designing this random replacement sequence to ensure that no binding site for other known transcription factors was inadvertently introduced into the mutant promoter, the most parsimonious interpretation of these transfection results is that binding of HMGA1 protein to nucleotides −268 to −276 of the negative regulatory region significantly contributes to repression of transcription of the XPA gene *in vivo*. Together, the results of these ChIP and promoter mutagenesis experiments provide compelling evidence that the

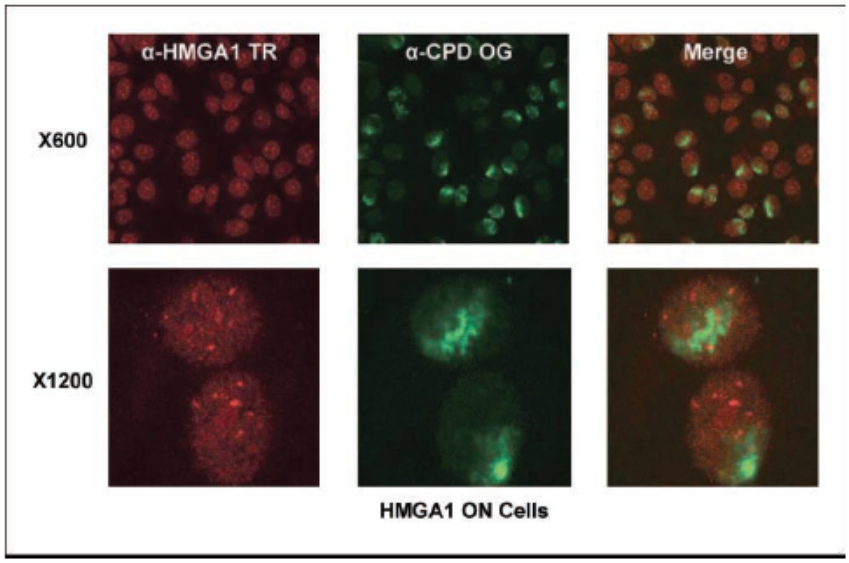

Figure 5. HMGA1 proteins do not colocalize with CPD lesions after UV exposure. Immunocytochemistry using both anti-HMGA1 and anti-CPD primary antibodies and Oregon Green (for CPD detection) and Texas Red (for HMGA1 detection) secondary antibody in MCF-7 cells induced to overexpress HMGA1a proteins. *Top*, fields observed at ×600 magnification; *bottom*, fields observed at ×1,200 magnification. *Left column*, HMGA1 staining; *middle column*, CPD staining 30 min following a 20 J/m^2 UV dose through a 5-μm pore size filter. Representative of the average total fluorescence observed in five random fields for each treatment at ×600 magnification.

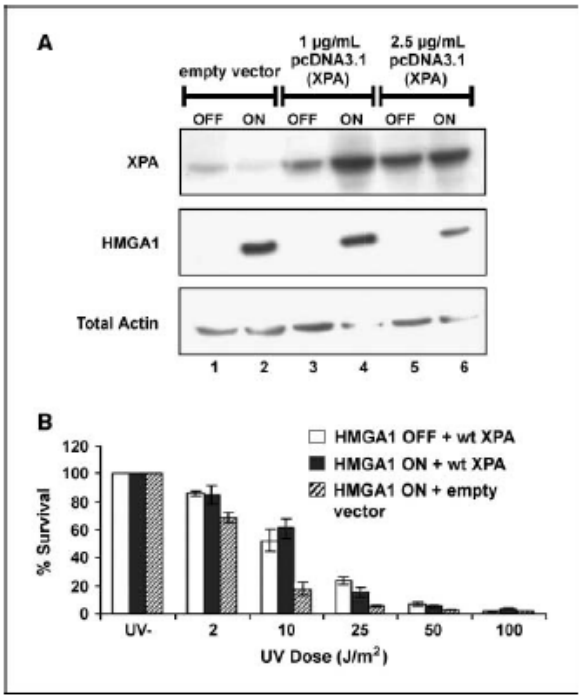

Figure 6. XPA complimentation restores UV resistance in HMGA1-expressing MCF-7 cells. *A*, Western blot analysis of total protein isolated from both nontransfected and transfected cells. *Lanes 1, 3,* and *5*, proteins isolated from HMGA1 OFF cells; *lanes 2, 4,* and *6*, proteins isolated from HMGA1 ON cells. *Lanes 1* and *2*, cells transfected with 1 μg/mL of empty vector (controls); *lanes 3* and *4*, cells transfected with 1 μg/mL pcDNA3.1(XPA); *lanes 5* and *6*, cells transfected with 2.5 μg/mL pcDNA3.1(XPA). *B*, graphical representation of survival as a function of UV dose. *White columns*, HMGA1 OFF cell survival; *dark gray columns*, HMGA1 ON cell survival; *hashed columns*, cell survival of HMGA1 ON cells transfected with empty pcDNA3.1 construct as a control. *Columns*, mean of three independent experiments for each cell line and UV dose; *bars*, SD.

promoter of the *XPA* gene is a direct cellular target for transcriptional regulation by HMGA1 proteins.

Several groups have previously reported that cells expressing low intracellular concentrations of functional XPA protein show compromised NER which, as a consequence, may only function efficiently in transcriptionally active regions of the genome (10, 11, 31). Consistent with these observations, the immunocytochemical analyses presented here not only verify deficiencies in CPD removal in HMGA1-overexpressing, XPA-depleted, cells (Fig. 4) but also show that HMGA1 proteins do not bind tightly to CPD lesions in ON cell nuclei (Fig. 5). These cellular localization studies indicate that the inhibitory effect of HMGA1 on repair of CPD lesions is indirect and most likely mediated through transcriptional down-regulation of *XPA*, a critically important gene involved in both global genomic and transcription-coupled NER (1).

Importantly, XPA deficiencies in HMGA1 ON cells seem to be directly responsible for the observed UV sensitivities displayed by these cells. The XPA complementation studies showing that overexpression of XPA completely restores UV resistance of HMGA1 ON cells to a level comparable with that of normal MCF-7 and HMGA1 OFF cells (Fig. 6*B*) strongly support this biological connection.

The gene coding for HMGA1 is a proto-oncogene whose overexpression induces neoplastic transformation of normal cells and the formation of aggressive lymphoid malignancies when overexpressed in transgenic mice (32). In many different types of human cancers (including thyroid, colorectal, prostate, breast, cervical, and lung carcinomas; neuroblastomas; and leukemias), the constitutive level of *HMGA* gene products is often exceptionally high with increasing concentrations paralleling increasing degrees of metastatic potential and malignancy (33–39). This correlation is so consistent and widespread that elevated HMGA levels are now being investigated as potential diagnostic molecular biomarkers for clinical assessment and management of patients with a variety of cancers (39–43).

Accumulation of genetic mutations (44) and increases in chromosome abnormalities and genomic instabilities (45) are also hallmarks of tumor progression in many malignant cancers, but in most cases, their underlying molecular causes remain obscure. One hypothesis, originally advanced by Loeb (46) and Loeb et al. (47), to partially explain these phenomena is that cancer cells, even in their earliest stages of development, exhibit a "mutator phenotype" that predisposes them to increased mutation rates that are essential to account for the large number of accumulated mutations observed during carcinogenesis. In this connection, HMGA1 overexpression has recently been linked to both chromosomal rearrangements in prostate cancer cells (48) and aneuploidy in colon carcinomas (49). Furthermore, the present data showing that HMGA proteins can repress transcription of the *XPA* gene in intact cells provide a plausible explanation for why NER is impaired in cells that overexpress these proteins and also suggest that such inhibition likely contributes to the accumulation of mutations and genetic instabilities commonly observed in many human cancers. Work with $XPA^{-/-}$ knockout mice, whose phenotype mimics that of humans with *XPA* mutations, supports this possibility. These NER-deficient mice have a marked predisposition for both spontaneous and genotoxin-induced gene mutations, for large genomic rearrangements, and for developing natural and carcinogen-induced tumors (reviewed in ref. 50).

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APPENDIX B

THE HIGH-MOBILITY GROUP A1a/SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION-3 AXIS: AN ACHILLES HEEL FOR HEMATOPOIETIC MALIGNANCIES?

Summary

Hillion et al. published The *High Mobility Group A1a (HMGA1a)/Signal Transducer and Activator of Transcription-3 (STAT-3) Axis: An Achilles Heel for Hematopoietic Malignancies* in Cancer Research in 2008, Vol. 68, pp. 10121-27. STAT-3 is a signaling molecule that plays a prominent role in human malignancy and has been found to be up-regulated in HMGA1 over-expressing cells. The research presented in this article evaluates the interaction of HMGA1a on STAT-3 expression and demonstrates that blocking STAT-3 function leads to apoptosis, decreased cellular motility and foci formation in HMGA1 over-expressing cells. As a result, STAT-3 is proposed as a potential target for therapeutic treatment of cancers that over-express HMGA1a.

Fig. 1 shows a series of experiments that illustrate that HMGA1a binds to the *STAT-3* promoter to drive its expression. My contribution to this work was specifically Fig. 1Ci, in which electrophoretic mobility shift assays demonstrate that HMGA1a is able to bind to a consensus site on the *STAT-3* promoter. When the consensus site it mutated, HMGA1a no longer binds, thus suggesting that this particular sequence is the target of HMGA1a regulation of *STAT-3.*

The High-Mobility Group A1a/Signal Transducer and Activator of Transcription-3 Axis: An Achilles Heel for Hematopoietic Malignancies?

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Abstract

Although *HMGA1* (*high-mobility group A1*; formerly *HMG-I/Y*) is an oncogene that is widely overexpressed in aggressive cancers, the molecular mechanisms underlying transformation by *HMGA1* are only beginning to emerge. *HMGA1* encodes the HMGA1a and HMGA1b protein isoforms, which function in regulating gene expression. To determine how *HMGA1* leads to neoplastic transformation, we looked for genes regulated by HMGA1 using gene expression profile analysis. Here, we show that the *STAT3* gene, which encodes the signaling molecule signal transducer and activator of transcription 3 (STAT3), is a critical downstream target of HMGA1a. *STAT3* mRNA and protein are up-regulated in fibroblasts overexpressing *HMGA1a* and activated STAT3 recapitulates the transforming activity of HMGA1a in fibroblasts. HMGA1a also binds directly to a conserved region of the *STAT3* promoter *in vivo* in human leukemia cells by chromatin immunoprecipitation and activates transcription of the *STAT3* promoter in transfection experiments. To determine if this pathway contributes to HMGA1-mediated transformation, we investigated *STAT3* expression in our *HMGA1a* transgenic mice, all of which developed aggressive lymphoid malignancy. *STAT3* expression was increased in the leukemia cells from our transgenics but not in control cells. Blocking STAT3 function induced apoptosis in the transgenic leukemia cells but not in controls. In primary human leukemia samples, there was a positive correlation between *HMGA1a* and *STAT3* mRNA. Moreover, blocking STAT3 function in human leukemia or lymphoma cells led to decreased cellular motility and foci formation. Our results show that the HMGA1a–STAT3 axis is a potential Achilles heel that could be exploited therapeutically in hematopoietic and other malignancies overexpressing *HMGA1a*. [Cancer Res 2008;68(24):10121–7]

Introduction

Because the *HMGA1* (formerly *HMG-I/Y*) gene is widely overexpressed in human cancer and overexpression portends a poor prognosis in some tumors, understanding the mechanisms that lead to transformation by *HMGA1* should lead to the identification of therapeutic targets for these malignancies. The *HMGA1* gene encodes the HMGA1a and HMGA1b protein isoforms, which were originally identified as abundant chromatin binding proteins (reviewed in refs. 1, 2). *HMGA1a* and *HMGA1b* also have oncogenic properties in cultured cells derived from different embryologic tissues (1–6), and inhibiting *HMGA1* expression in human cancer cell lines blocks the transformed phenotype (3, 5, 7). Transgenic mice overexpressing *HMGA1a* develop aggressive lymphoid malignancy (8, 9), uterine cancer (10), and pituitary tumors (9). These findings show that *HMGA1a* functions as an oncogene *in vivo* and suggest that it directly contributes to oncogenic transformation in human cancer.

Because these proteins function in transcription, it has been postulated that they promote malignant transformation by altering expression of specific target genes. *IFN-β* gene is the best-characterized gene target (reviewed in ref. 11), and previous studies show that HMGA1 is essential for its efficient transcription. Other candidate targets include genes that function in cell signaling, motility, and inflammation (1, 2, 5, 6, 10–13). How these genes contribute to the role of HMGA1 in malignancy is not yet clear.

To define the molecular pathways induced by HMGA1 in transformation, we looked for genes regulated by HMGA1 using gene expression profile analysis. We focused on the signal transducer and activator of transcription-3 (*STAT3*) gene because of its prominent role in human malignancy (14–17). Our studies suggest that the HMGA1a–STAT3 axis is a rational therapeutic target for hematopoietic malignancies and potentially other cancers with activation of this pathway.

Materials and Methods

Microarray analysis. Microarray analysis was done with the Clontech Atlas cDNA Rat 1.2 Array (Clontech PT3564-3) according to the manufacturer's instructions. Briefly, mRNA (1 μg) from each polyclonal cell line (Rat1a-HMGA1a, Rat1a-HMGA1b, Rat1a-pSG5 control; ref. 3) was labeled in a reverse transcriptase reaction in the presence of 35 μCi of [α-^{32}P]dATP. The hybridization signals were scanned with a PhosphorImager (Molecular Dynamics) and analyzed according to the Clontech

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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instruction manual. The intensity of signal for reference genes was adjusted so that the mean intensities for each membrane were equal as recommended in the instructions. All signals were compared with the control housekeeping genes, *β-actin* and *myosin*, according to the manufacturer's recommendations.

Quantitative reverse transcription-PCR analysis. Reverse transcription-PCR (RT-PCR) was done with SYBR Green PCR Master kit and TaqMan One-Step RT-PCR kit (Applied Biosystems) as described (10). The sequences for the forward and reverse primers that amplify STAT3α are 5′-CTGACCCAGGTAGCGCTGCCCCATACC and 5′-TCACAATGGGGGAGGTAGCGCACTCCG, respectively. Reaction conditions were as reported (8, 10).

Western blot analysis. Western blots were done with an anti-HMGA1a antibody (3) diluted 1:200, an anti-STAT3 antibody diluted 1:200 (New England Biolabs), an anti-pSTAT antibody (New England Biolabs) diluted 1:100, and an anti–topoisomerase I antibody (BD Biosciences) diluted 1:1,000.

Immunohistochemistry. The commercially available antibodies for STAT3 were used according to the manufacturer's instructions (New England Biolabs). Briefly, slides from paraffin-embedded tissues were made as described (10). Slides were heated in antigen retrieval solution (DAKO) and immunohistochemically stained for STAT3 following the manufacturer's directions (New England Biolabs, #9132).

Nuclear extract preparation. Nuclear extracts were prepared as previously described (17).

Chromatin immunoprecipitation experiments. Chromatin immunoprecipitations were done with HEL-HMGA1a or Ramos cells as described (10). Briefly, proteins cross-linked to chromatin were immunoprecipitated with the following antibodies (10): HMGA1, polymerase II, histone H3 (both polymerase II and histone H3 served as positive controls), and IgG (as a negative control). The *STAT3* promoter region with the consensus HMGA1a DNA binding site was amplified by quantitative RT-PCR from the immunoprecipitated protein-DNA complexes using the forward and reverse primers 5′-GCCAATGGGCTAGCTGGT-3′ and 5′-CTTCAGTTTCTGCGTGAGCA-3′, respectively. The control primers for histone H3 and polymerase II have been previously described (10). The *HPRT1* promoter was amplified as a negative control promoter with no HMGA1a binding sites as described (12).

Electrophoretic mobility shift assay. Electrophoretic mobility shift assays were done as previously described (12, 17). To determine if HMGA1a binds to the STAT3 promoter at the putative HMGA1a binding site, a probe containing this site was generated by annealing equimolar amounts of two 37-nucleotide complementary oligonucleotides from the *STAT3* promoter, CACTCTAGTAATTACTCTATTTCCACGTCATGTTTCC and GGAAACATGACGTGGAAATAGAGTAATTACTAGAGTG, and designated STAT3 wild-type. The probe containing the mutated site has the sequence CACTCTAGTAAggACTCTAggTCCACGTCATGTTTCC and GGAAACATGACGTGGAccTAGAGTccTTACTAGAGTG; the mutations are shown in lowercase letters. The control probe for the *Kit* ligand promoter has been described (12). The double-stranded probe was end-labeled with [γ-^{32}P]ATP and purified. Binding reactions were done as described (12).

Preparation of splenocytes. Spleens were placed in PBS supplemented with 5% fetal bovine serum (FBS) on ice and mechanically fragmented with the Stomacher 80 (Seward). Single-cell suspensions were isolated after passage through a cell strainer (70-μm pores), treated with red cell lysis buffer, and resuspended in RPMI 1640.

Plasmids, transfections, and lentiviral infections. The pSG5-STAT3-C vector was made by excision of constitutively activated STAT3 (STAT3-C) from STAT3 pcDNA3.1 vector (14) with *Pme*1 and *Bam* H1. The STAT3-C fragment was cloned into pSG5 after restriction with *Eco*R1 and Klenow treatment. The PA-HMGA1a vector was made by excision of the full-length murine *HMGA1a* cDNA from the vector pHEBoNeo-HMG-I[3] with *Hind*III, Klenow treatment, and subsequent *Not*I digestion. The HMGA1a fragment was subsequently cloned into the EF1α expression vector (18) after restriction with *Bam* HI and Klenow treatment. The *STAT* promoter vectors were described (kindly provided by K. Kohno, Department of Molecular Biology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Fukuoka, Japan; ref. 19).

Transfections were done using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions as we previously described (10). In the experiments with HEL control or HEL-HMGA1 cells, the STAT3 promoter reporter plasmid (1 μg) was cotransfected with the control pRL-TK vector (100 ng) containing Renilla luciferase (Promega) to control for transfection efficiency (3×10^5 cells/500 μL per transfection). The DNA was mixed with Lipofectamine 2000 at a ratio of 1 μg:1.5 μL (in Opti-MEM, Invitrogen) and incubated with cells for 6 h. Cells were harvested for luciferase activity 24 h after transfection and experiments were done twice in triplicate. In the titration experiment, reporter plasmid (100 ng), control Renilla luciferase plasmid (50 ng), and 0 to 150 ng of HMGA1a expression plasmid (pSG5-HMG-I) were cotransfected as described above. The pSG5 plasmid was added (0–150 ng) to keep the total DNA constant in the titration experiments (1×10^5 cells/100 μL per transfection). The DNA was mixed with Lipofectamine 2000 at a ratio of 1 μg:1.67 μL (in Opti-MEM, Invitrogen) and cells were harvested as described above.

The lentiviral construct expressing dominant-negative STAT3 (Lenti-DN-STAT3) was made from the pcDNA3.1-STAT3DN (provided by J-I. Park, the Johns Hopkins University School of Medicine, Baltimore, MD; ref. 14). The cytomegalovirus promoter was replaced by the human EF1α promoter from pEF1/Myc-His-A (Invitrogen) and the EF-DN-STAT3 cassette was cloned into the cFUGW lentivirus (provided by D. Baltimore, Division of Biology, California Institute of Technology, Pasadena, CA) at the *Pac*I site and lentiviral supernatants were made as described (20).

Cell lines and cultures. The polyclonal Rat1a-HMGA1a (HMG-I) and control cells were described (3, 4). The Rat1a-activated STAT3 cells were made by transfecting with pSG5-STAT3-C as described (3, 4). Polyclonal stable cell lines were selected in puromycin and expression of activated STAT3 was confirmed by Western blot analysis.

The parental HEL cells are human erythroleukemia cells from the American Type Culture Collection (ATCC). HEL-HMGA1a cells were made by transfection of PA-HMGA1a with DMRIE-C (Invitrogen) according to the manufacturer's instructions. Stable clones were selected in G418 (800 μg/mL). The Burkitt's cells (Ramos RA 1) were derived from a 3-y-old male Caucasian patient with Burkitt's lymphoid malignancy and grown as suggested (ATCC).

Growth curves. Cellular growth rates were determined as previously described (21) using the CellTiter Cell Proliferation Assay (Promega). Briefly, cell proliferation was evaluated at 24-h intervals by measuring the mitochondrial-dependent conversion of the tetrazolium salt, 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, to a colored formazan product using a CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer instructions. Cells were seeded in 96-well plates at a density of 1,000 per well and grown for 1 wk. A volume of 20 μL of CellTiter 96 AQueous One Solution Reagent was pipetted directly into each well of the 96-well assay plate containing cells in 100 μL of culture medium, and cells were incubated for 2 h at 37°C in a humidified 5% CO_2 atmosphere. Absorbance was read at 490 nm using a 96-well microplate reader (model 680, Bio-Rad).

Migration and soft agar transformation assays. Migration assays were done in 24-well plates containing 0.8-μm-pore cell culture inserts with polyethylene terephthalate membranes according to the manufacturer's instructions (BioCoat Cell Culture Inserts, BD Biosciences) as described (22). Briefly, filters were coated on ice with 100 μL of growth factor–reduced Matrigel at 0.5 to 0.8 mg/mL protein. The cells were seeded in 500 μL of 10% FBS-RPMI at 30,000 per well into the upper chamber. The lower chamber was filled with 750 μL of the same medium. After 48 h, migration was assessed by counting cells on the underside after fixation with 70% ethanol and staining with hematoxylin. Soft agar assays were done as described with minor modifications (3). Cells (10^3–10^4) were mixed with 0.4% agar in growth media and added as a top layer over 0.8% agar. Growth medium (1 mL) was added after 1 h at room temperature. Colonies were counted after 4 wk.

Results

STAT3 is up-regulated in cells overexpressing *HMGA1a*. To identify HMGA1 target genes, we performed gene expression profile analysis comparing RNA from Rat1a fibroblasts overexpressing HMGA1a or HMGA1b to control Rat1a cells transfected with vector alone. From a microarray (Clontech Atlas Rat 1.2 Array)

with unique cDNA fragments representing 1,176 genes, we identified genes that were differentially expressed by at least 2-fold compared with control cells after normalization to both β-actin and myosin (Supplementary Fig. S1; Supplementary Tables S1 and S2). Ninety-seven genes from the total of 1,176 genes (8.2%) were differentially regulated in cells overexpressing HMGA1a, and 103 genes (8.8%) were differentially regulated in cells overexpressing HMGA1b (Supplementary Fig. S1; Supplementary Tables S1 and S2). Fifty-five of these genes were common to both the HMGA1a and HMGA1b cells (Supplementary Fig. S1; Supplementary Tables

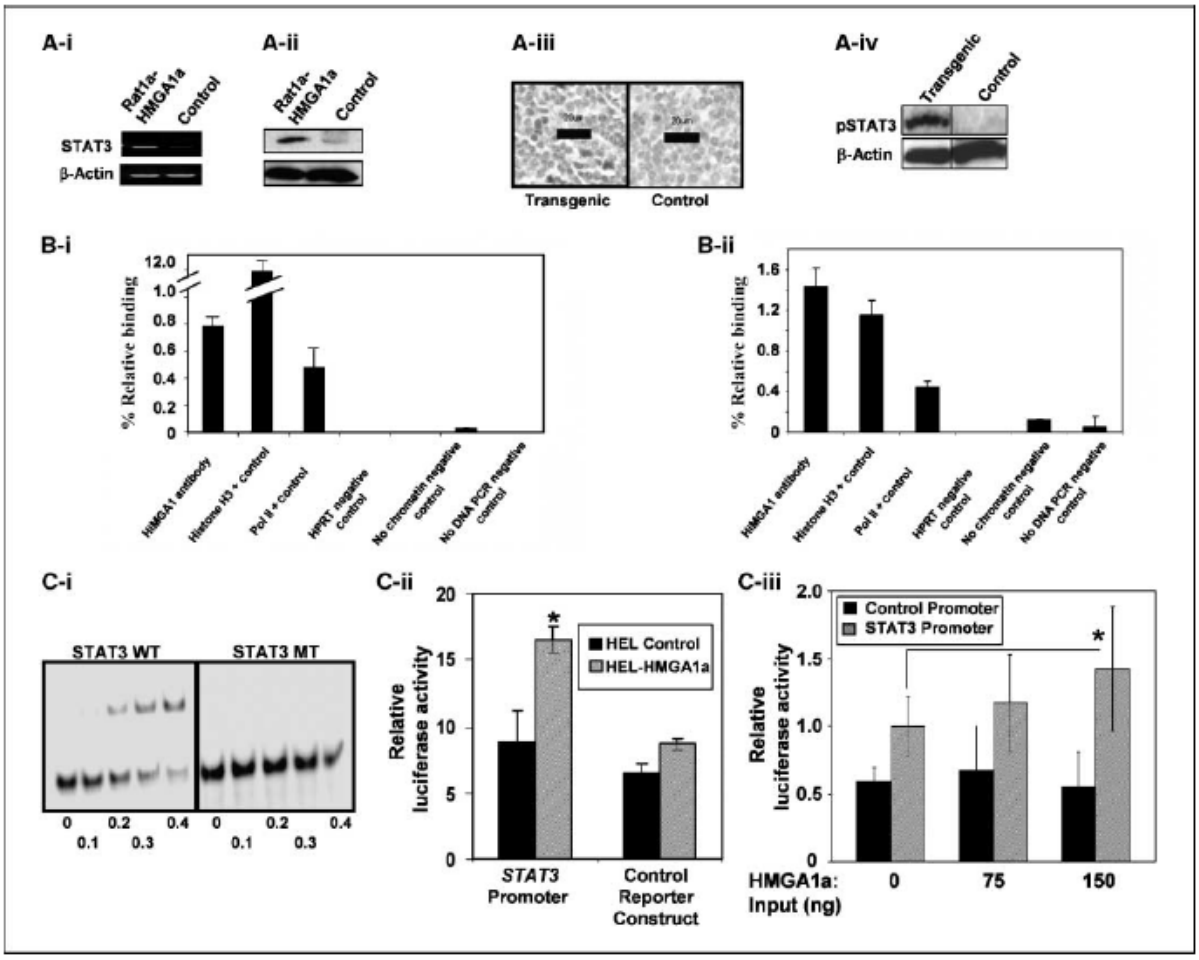

Figure 1. HMGA1a directly up-regulates *STAT3* expression. *A-i*, RT-PCR shows that Rat 1a fibroblasts overexpressing HMGA1a have increased *STAT3* mRNA compared with control Rat1a cells. *A-ii*, Western blot analysis shows increased STAT3 protein in the Rat1a-HMGA1a cells. *A-iii*, splenocytes from the *HMGA1a* transgenic mice also have increased STAT3 protein compared with splenocytes from control mice by immunohistochemical staining. *Bar*, 20 μm. *A-iv*, Western blot shows that splenocytes from the *HMGA1a* transgenics have increased phosphorylated STAT3 (*pSTAT3*) protein compared with control splenocytes. *B-i*, HMGA1a binds directly to the *STAT3* promoter region containing a conserved HMGA1a DNA binding site in HEL-HMGA1a cells. Chromatin immunoprecipitation experiments with sheared chromatin from HEL-HMGA1a cells after cross-linking proteins bound to DNA with formaldehyde (10). The bar graph shows the percent of total input DNA as assessed by quantitative RT-PCR after immunoprecipitation with HMGA1a (12) and positive control histone H3 or polymerase II (*Pol II*) antibodies (all from Upstate, excluding the HMGA1a antibody). The *HPRT* promoter sequence with the HMGA1a antibody was used as a negative control because there are no HMGA1 DNA binding sites in the region amplified and previous chromatin immunoprecipitation experiments showed no binding by HMGA1 to the amplified region (12). Additional negative controls included no chromatin and no DNA. The total input is considered to be 100% (not shown in the bar graph). Chromatin immunoprecipitation and quantitative RT-PCR experiments were done at least twice. *Columns*, mean from the repeat experiments; *bars*, SD. *B-ii*, HMGA1a also binds directly to the *STAT3* promoter in Burkitt's lymphoma cells. Chromatin immunoprecipitation experiments were done as described above in the HEL-HMGA1a cells. The graph shows the percent of total input DNA immunoprecipitated with the same antibodies from repeat quantitative RT-PCR experiments as described above. *C-i*, recombinant HMGA1a binds directly to the predicted consensus HMGA1a site in the *STAT3* promoter by electrophoretic gel shift analysis. There was no binding to the mutated site. Experiments were done with the following protein/DNA molar ratios, as indicated in the figure: 0, 0.1, 0.2, 0.3, and 0.4. For the wild-type probe, 500 nmol/L DNA was used with 0, 50, 100, 150, or 200 nmol/L protein to give the indicated protein/DNA molar ratios. For the mutated probe, 125 nmol/L DNA was used with 0, 12.5, 25, 37.5, or 50 nmol/L protein to give the indicated protein/DNA molar ratios. *C-ii*, in transfection experiments, HMGA1a activates the *STAT3* promoter containing the HMGA1 binding site (labeled *STAT3* Promoter; 1 in ref. 19) but not the control reporter vector without the *STAT3* promoter containing the HMGA1a binding site (labeled Control Reporter Construct; B2 in ref. 19). *Black columns*, luciferase reporter activity in HEL control cells; *hatched columns*, luciferase reporter activity in HEL-HMGA1a cells. Experiments were done in triplicate at least twice. There was >2-fold activation ($P = 0.0000298$, Student's t test.) *C-iii*, increasing HMGA1a plasmid results in increasing activation of the *STAT3* promoter in a dose-dependent fashion (*hatched columns*). The luciferase activity with control plasmid (0 ng input HMGA1a) was arbitrarily assigned a value of 1. There was no activation of the control plasmid lacking the HMGA1 binding site (*solid columns*). Experiments were done in triplicate at least twice. Activation at 150 ng of input HMGA1a plasmid was significant ($P = 0.028$, Student's t test).

S1 and S2). The following classes of genes involved in cell growth and transformation were highly represented in this study: (*a*) general metabolism (HMGA1a: 16 genes, HMGA1b: 23 genes, both: 11 genes); (*b*) ribosomal proteins (HMGA1a: 10, HMGA1b: 11, both: 8); (*c*) growth factors, cytokines, and chemokines (HMGA1a: 10, HMGA1b: 6, both: 4); and (*d*) oncogenes and proto-oncogenes (HMGA1a: 8, HMGA1b: 4, both: 4). Only two genes were down-regulated by at least 2-fold in this study (*CD3 γ subunit* and *γ-aminobutyric acid receptor, subunit ρ1*) and both were down-regulated by HMGA1a and HMGA1b.

From the group of 53 genes up-regulated in cells overexpressing HMGA1a and HMGA1b, the gene encoding STAT3 was studied further because of its prominent role in diverse human malignancies (14–17). Microarray analysis showed that *STAT3* mRNA was increased 2.5- to 3.1-fold in cells overexpressing HMGA1a and 2.9- to 3.0-fold in fibroblasts overexpressing HMGA1b (Supplementary Table S2). This increase was confirmed by RT-PCR from the fibroblasts overexpressing HMGA1a (Fig. 1*A-i*). STAT3 protein was also increased in the cells overexpressing HMGA1a (Fig. 1*A-ii*).

***STAT3* is up-regulated in lymphoid tumors from *HMGA1a* transgenic mice.** To determine if this pathway is dysregulated in malignancies induced by HMGA1a, we first evaluated *STAT3* expression in lymphoid tumors from our *HMGA1a* transgenic mice (8). These mice overexpress *HMGA1a* in lymphoid tissue and develop aggressive leukemia or lymphoma with complete penetrance (8). STAT3 protein is also increased in the lymphoid tumors from the transgenics compared with lymphoid cells from control mice by immunohistochemical analysis (Fig. 1*A-iii*). Because STAT3 proteins require activation by phosphorylation at Tyr^{705} for oncogenic function (14–17), we also assessed activated STAT3 protein levels by Western blot analysis and found that they are increased in the transgenic spleens compared with controls (Fig. 1*A-iv*). These studies indicate that HMGA1a up-regulates STAT3 proteins *in vivo* in the transgenic tumors.

HMGA1a binds to the *STAT3* promoter and directly up-regulates *STAT3* expression in malignant human hematopoietic cells. Next, we sought to determine if HMGA1 directly activates expression of the *STAT3* promoter. Of note, the human, mouse, and rat *STAT3* promoters contain a conserved consensus DNA binding site for HMGA1 (ATTACTCTATTTCCAC) about 650 bp upstream from the transcription start site (19). By chromatin immunoprecipitation, HMGA1 binds directly to the *STAT3* promoter sequence containing the consensus binding site in human erythroleukemia cells transfected to overexpress HMGA1a (HEL-HMGA1a cells; Fig. 1*B-i*). To expand our findings to other human hematopoietic malignancies, we also investigated Burkitt's leukemia/lymphoma cells (Ramos RA 1). In previous studies, we showed that these Burkitt's cells express high levels of the HMGA1 protein and are dependent on HMGA1 for their transformed phenotype in soft agar (3). Here, we show that HMGA1 also binds to the *STAT3* promoter *in vivo* in Burkitt's cells (Fig. 1*B-ii*).

To determine if HMGA1 is binding to the consensus DNA binding site in the *STAT3* promoter, we performed electrophoretic mobility shift assays with an oligonucleotide containing this site. We found that HMGA1a protein binds to this site in the *STAT3* promoter *in vitro*, but not to an oligonucleotide containing a mutation in the site that abrogates STAT3 DNA binding (Fig. 1*C-i*).

To determine if HMGA1 activates *STAT3* expression, we performed transfection experiments. We found that HMGA1 activates *STAT3* promoter expression by >2-fold (Fig. 1*C-ii*) in HEL-HMGA1a cells compared with HEL control cells. There was no activation of the control promoter construct lacking the HMGA1 binding site. We also showed that cotransfection of a plasmid expressing HMGA1a led to transactivation of the *STAT3* promoter in a dose-dependent fashion in HEL cells (Fig. 1*C-iii*). Specifically, there was increasing transactivation of the *STAT3* promoter with increasing quantity of HMGA1a input plasmid (Fig. 1*C-iii*). There was no activation of the control promoter construct lacking the HMGA1a binding site. Taken together, these results indicate that HMGA1 binds directly to the *STAT3* promoter to activate its transcription.

Activated STAT3 transforms like HMGA1a in rat fibroblasts. Because STAT3 has oncogenic properties and is constitutively activated in diverse human malignancies (14–17), we hypothesized that it plays a role in *HMGA1a*-mediated transformation. To compare its transforming activity to that of HMGA1a, we generated Rat1a cells overexpressing a constitutively activated STAT3 protein

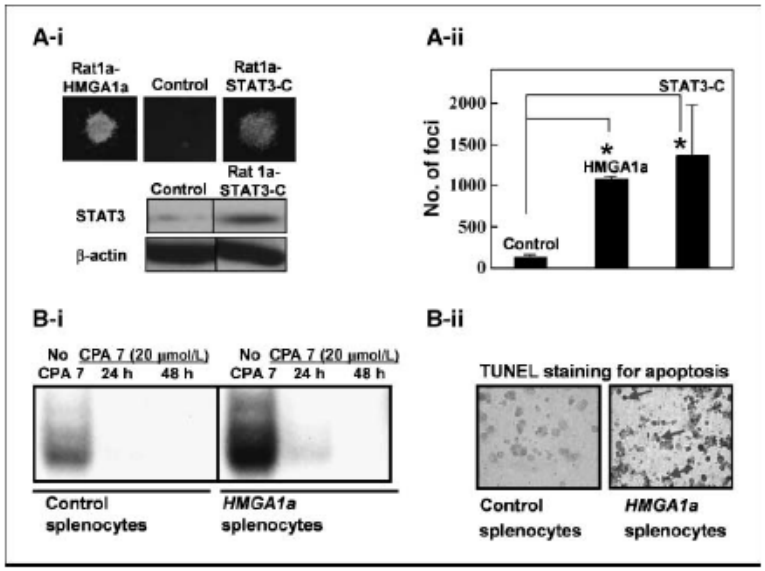

Figure 2. Activated STAT3 transforms like HMGA1a in fibroblasts and blocking STAT3 function induces apoptosis in leukemic cells from *HMGA1a* transgenic mice. *A-i*, fibroblasts overexpressing STAT3-C exhibit anchorage-independent cell growth in soft agar. *Top*, foci from cells overexpressing STAT3-C are similar in number and morphology to cells overexpressing HMGA1a. *Bottom*, Western blot analysis from the Rat fibroblasts overexpressing vector alone (control) or STAT3-C (Rat 1a STAT3C). The Rat 1a HMGA1a cells have been described and published (3). *A-ii*, foci number from two experiments done in duplicate. Rat1a cells overexpressing HMGA1a or STAT3-C exhibit significantly increased foci formation compared with controls ($P = 0.000000244$ for HMGA1a and $P = 0.038$ for STAT3-C, Student's *t* test). *B-i*, gel shift analysis shows increased STAT3 binding activity in splenocytes from *HMGA1a* mice compared with control splenocytes. The CPA-7 inhibitor blocks binding of STAT3 to its cognate binding site in both transgenic and control splenocytes. *B-ii*, blocking STAT3 induces apoptosis in leukemia cells from *HMGA1a* transgenic mice. Terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (*TUNEL*) assay shows apoptosis in the transformed splenocytes from the *HMGA1a* mice (right, see *arrows*) compared with the control splenocytes.

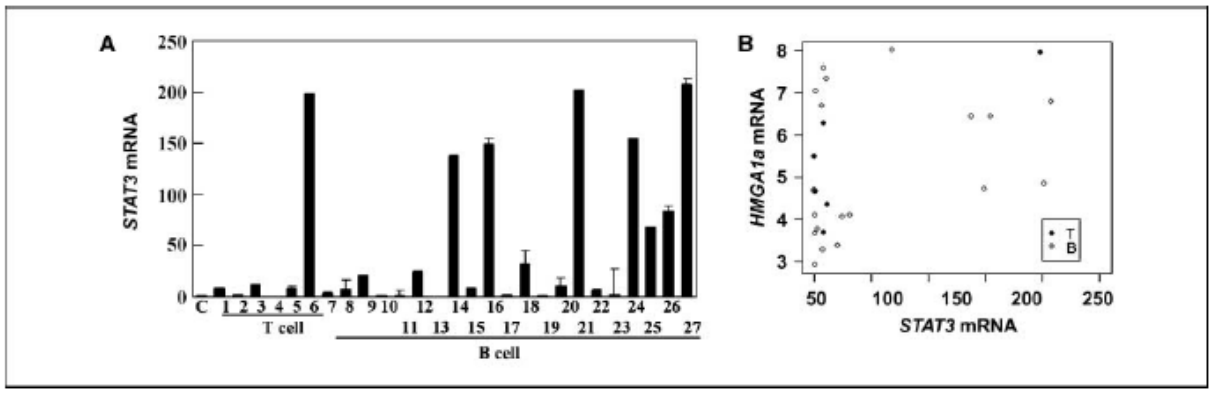

Figure 3. *STAT3* and *HMGA1a* expression are positively correlated in primary human acute lymphoblastic leukemia samples. *A*, *STAT3* mRNA is increased in most human leukemia samples (22 of 27) already shown to overexpress *HMGA1a* (8) by 2- to 200-fold compared with the control cells (*C*) by quantitative RT-PCR. Experiments were done in triplicate and performed at least twice. (*HMGA1a* mRNA levels were previously published; see ref. 8.) *B*, there is a positive correlation between *STAT3* mRNA and *HMGA1a* mRNA ($P = 0.049$, Spearman's rank correlation coefficient).

(STAT3-C) and found that STAT3-C induces foci formation like HMGA1a (Fig. 2*A-i* and *A-ii*). Fibroblasts that overexpress HMGA1a or activated STAT3 exhibit anchorage-independent cell growth in soft agar with similar colony numbers and size (Fig. 2*A-i* and *A-ii*). These results indicate that activated STAT3 is sufficient for transformation in the rat fibroblasts.

Blocking STAT3 function induces apoptosis in leukemia cells from the *HMGA1a* mice. To define the role of STAT3 in HMGA1a-mediated transformation, we blocked STAT3 function in leukemia cells from our *HMGA1a* transgenic mice with the inhibitor CPA-7 (17). CPA-7 specifically prevents activated STAT3 from dimerizing and thereby blocks binding to the target DNA (17).

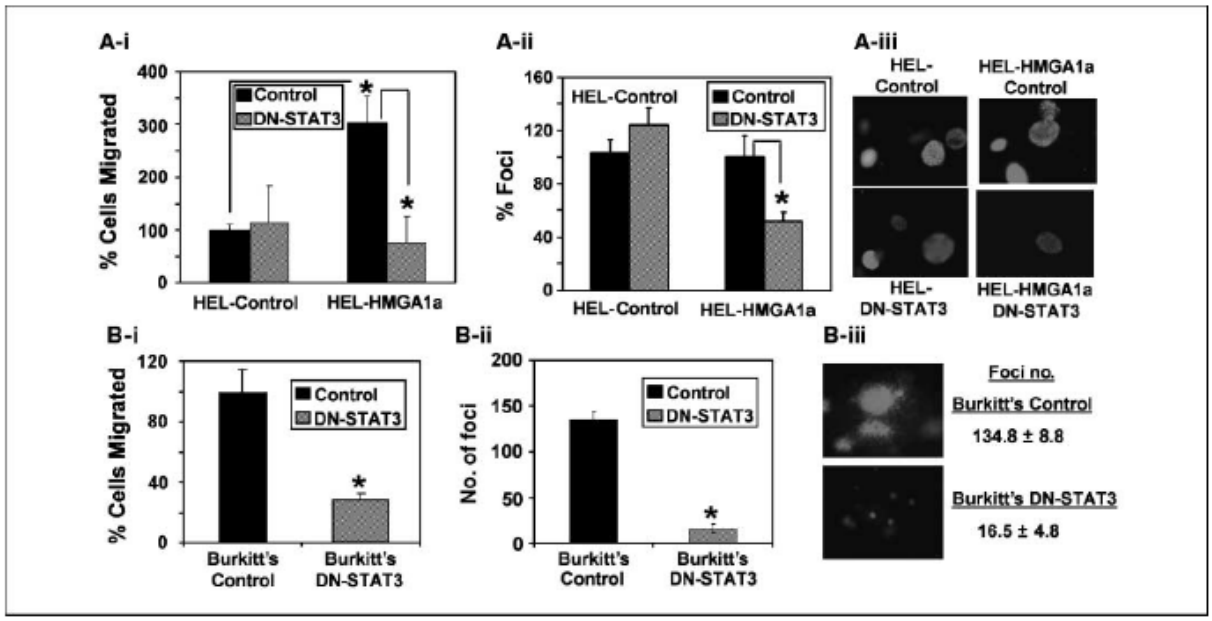

Figure 4. Blocking STAT3 function interferes with cell motility and anchorage-independent cell growth in human leukemia and lymphoma cells that overexpress *HMGA1*. *A-i*, parental human erythroleukemia HEL-Control or HEL-HMGA1a cells were transduced with a control green fluorescent protein lentivirus (*Control*) or a lentivirus expressing dominant-negative STAT3 linked to green fluorescent protein (*DN-STAT3*). DN-STAT3 significantly decreases motility in the HEL-HMGA1a cells, causing fewer cells to migrate through the membrane ($P = 0.000645$, Student's *t* test). There is no effect of DN-STAT3 on the HEL-Control cells. Experiments were done in triplicate and repeated twice. *Columns*, mean percent cells migrated from repeat experiments; *bars*, SD. HEL-Control cells transduced with the control vector were arbitrarily assigned a value of 100%. *A-ii*, DN-STAT3 decreases foci formation in HEL-HMGA1a cells ($P = 0.000248$) but has no significant effect on transformation in the HEL-Control cells. *Columns*, foci number from repeat experiments; *bars*, SD. (As above, HEL-Control transduced with control vector were assigned a value of 100%.) *A-iii*, foci from the HEL control or HEL-HMGA1a cells with or without DN-STAT3 under fluorescence microscopy. *B-i*, DN-STAT3 also decreases motility in Burkitt's lymphoma cells causing significantly fewer cells to migrate through the membrane ($P = 0.0000126$). Experiments were done in triplicate and done twice. *Columns*, mean from repeat experiments; *bars*, SD. The Burkitt's cells transduced with control vector were assigned a value of 100%. *B-ii*, DN-STAT3 also blocks foci formation in human Burkitt's leukemia cells ($P = 0.00000546$). As above, experiments were done in triplicate and performed twice. *Columns*, foci number from repeat experiments; *bars*, SD. *B-iii*, foci from Burkitt's cells with or without DN-STAT3 under fluorescence microscopy.

Because STAT3 up-regulates its own expression, CPA-7 also reduces activated STAT3 levels. In gel shift experiments, we found that STAT3 binding activity was increased in the splenocytes from the *HMGA1a* mice compared with control mice (Fig. 2*B-i*; see also Supplementary Fig. S2*A* for full gel and probe in repeat experiment). CPA-7 treatment for 48 hours effectively blocked binding of activated STAT3 to an oligonucleotide containing a consensus STAT3 binding site in splenocytes from the *HMGA1a* and control mice (Fig. 2*B-i*; Supplementary Fig. S2*A*). Moreover, blocking STAT3 activity induces apoptosis in the transformed *HMGA1a* splenocytes, but not in control splenocytes (Fig. 2*B-ii*), suggesting that STAT3 is required for viability in HMGA1a transformed lymphocytes.

***STAT3* and *HMGA1a* expression are positively correlated in primary human acute lymphoblastic leukemia samples.** To determine if *STAT3* is up-regulated in primary human leukemia, we measured *STAT3* mRNA in bone marrow samples from 27 patients with acute lymphoblastic leukemia. *STAT3* mRNA was increased in 5 of 6 T-cell leukemia samples (range, 1.7- to 198-fold) and 17 of 21 B cell leukemia samples (range, 2.0- to 207-fold) compared with control lymphocytes (Fig. 3*A*). These leukemia samples also overexpress *HMGA1a* by >2- to 8-fold above that of normal lymphocytes as we previously described (8). Spearman's rank correlation coefficient showed a significant positive correlation ($P = 0.049$) between *HMGA1a* and *STAT3* in the leukemia samples, indicating that the HMGA1a–STAT3 axis is up-regulated in these cases (Fig. 3*B*).

The HMGA1a–STAT3 axis is a potential Achilles heel in human hematopoietic malignancy. Next, we determined if STAT3 is required for the transformed phenotype in human leukemia cells. To this end, we blocked STAT3 function in HEL-HMGA1a cells and evaluated transformation phenotypes, including cellular motility and foci formation in soft agar. To block STAT3 function, we infected these cells with a lentiviral construct (20) expressing a dominant-negative STAT3 (DN-STAT3; ref. 14) linked to green fluorescent protein or with a control lentivirus linked to green fluorescent protein alone. The DN-STAT3 blocks STAT3 binding (Supplementary Fig. S2*A–C*). We found that HEL-HMGA1a cells with DN-STAT3 had decreased motility ($P = 0.000645$, Student's t test) and foci formation ($P = 0.00139$, Student's t test) in soft agar compared with controls (Fig. 4*A-i* to *A-iii*). In contrast, DN-STAT3 had a minimal effect on foci formation or motility in the control HEL cells (Fig. 4*A-i* to *A-iii*).

To further assess the role of the HMGA1a–STAT3 axis in human hematopoietic malignancy, we repeated these studies in a Burkitt's cell line (RA 1 Ramos; ATCC), derived from an aggressive B-cell childhood lymphoma/leukemia. Using an RNA interference approach, we previously showed that these cells are dependent on HMGA1a for the transformed phenotype in soft agar (3). Here, we find that the Burkitt's cells with DN-STAT3 exhibited markedly decreased motility ($P = 0.0000126$, Student's t test) and foci formation ($P = 0.00000546$, Student's t test; Fig. 4*B-i* to *B-iii*). DN-STAT3 did not affect cellular proliferation in cultured HEL-HMGA1a or Burkitt's cells (Supplementary Fig. S3*A* and *B*), showing that the DN-STAT3 vector was not toxic to cell growth in these cell lines. Taken together, these functional studies indicate that *STAT3* is a critical downstream target of HMGA1 in hematopoietic malignancy.

Discussion

We show that HMGA1 directly up-regulates *STAT3* in hematopoietic malignancy. This is the first demonstration of an oncogene (other than *STAT3*) that directly activates *STAT3* expression. Because activated STAT3 plays a pivotal role in malignant transformation (14–17), identifying the molecular pathways that lead to increases in *STAT3* expression and protein levels will be critical to better understand how cancer develops. STAT3 is a member of the STAT proteins that communicate signals from the cell membrane to the nucleus and thereby mediate diverse biological processes including cell growth, differentiation, apoptosis, fetal development, inflammation, and immune responses. Like HMGA1 (1, 2), activated STAT3 is up-regulated in human tumors arising from diverse tissues (14–17).

Our studies identify the HMGA1a–STAT3 axis as a potential Achilles heel that could be targeted therapeutically in hematopoietic malignancies. We show that the STAT3 inhibitor CPA-7 leads to apoptosis in leukemia cells from the *HMGA1a* transgenic mice but not in normal lymphoid cells. We also show that blocking STAT3 function in human leukemia or lymphoma cells overexpressing *HMGA1a* interferes with transformation by decreasing both cellular motility and foci formation. Because some cancer cells seem to be dependent on an oncogene for survival (oncogene addiction), targeting the relevant oncogenic pathways should preferentially induce cell death in these cancer cells. We found that the HMGA1a–STAT3 axis is required in some hematopoietic malignancies, and this dependence could be exploited therapeutically. The results with primary human leukemia samples also indicate that this pathway is active in both T-cell and B-cell acute lymphoblastic leukemias.

Our studies also predict a model for hematopoietic malignancy whereby overexpression of HMGA1a leads to direct induction of the *STAT3* mRNA followed by increases in activated STAT3 protein. How STAT3 becomes activated by phosphorylation in this setting is not yet clear. Because cytokines known to activate T cells also lead to STAT3 phosphorylation, T-cell activation *in vivo* should lead to STAT3 activation in these cells. Activated STAT3 protein could then contribute to neoplastic transformation. In non–T-cell leukemia, constitutive tyrosine kinase activity or alternative, undefined pathways may lead to STAT3 activation. Results from other gene expression studies with cells overexpressing *HMGA1a* also show up-regulation of cytokine receptors, cytokines, and/or their pathways that can activate STAT3 (5). Similar to other pivotal signaling molecules, there are likely to be multiple cellular pathways, including HMGA1a-dependent and HMGA1a-independent signals, that up-regulate and activate STAT3.

In summary, we show that *STAT3* is a direct HMGA1a target gene that is necessary for HMGA1a-mediated transformation in hematopoietic cells. These findings implicate the HMGA1a–STAT3 axis as a rational therapeutic target in hematopoietic malignancy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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APPENDIX C

HMGA1 DIRECTLY INFLUENCES MITOCHONDRIAL LEVELS AND

mtDNA REPAIR EFFICIENCY

SUMMARY

The manuscript by Mao et al. entitled *HMGA1 Directly Influences Mitochondrial Levels and mtDNA Repair Efficiency* has been submitted for review and is presently undergoing revisions with anticipation of its pending acceptance. The work described in this manuscript evaluates mitochondrial function in the context of HMGA1 over-expression in both transgenic and naturally occurring cancer cell lines. Multiple parameters of mitochondrial function were assessed in cells expressing normal and elevated levels of HMGA1, including mitochondrial mass, generation of reactive oxygen species, and ability to repair oxidative DNA damage. The results of these investigations have indicated that mitochondrial dysfunction tracks with levels of HMGA1, suggesting that this protein has an important effect on mitochondrial function.

While much of the work in this paper was performed in transgenic cell lines, my contribution was to confirm that these results were reflected in naturally occurring human tumor cells and their matched set normal cells (both harvested from the same individual). Figure 5 shows that the types of observations made in transgenic cells are also seen in actual tumor cells. These experiments are important because they provide additional strength and support for the transgenic evidence.

The following manuscript is formatted according to the guidelines of the journal to which it has been submitted.

HMGA1 Directly Influences Mitochondrial Levels and mtDNA Repair Efficiency

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Running Title: HMGA1 over-expression inhibits mitochondrial function

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ABSTRACT

HMGA chromatin proteins are expressed at only low levels in somatic cells but are almost universally over-expressed in cancer cells. In normal cells HMGA proteins are located primarily in the nucleus except during the late S/G2 phases of the cell cycle when a sub-population of proteins reversibly migrates to the mitochondria where they bind to mtDNA. In many cancer cells this controlled shuttling is lost and HMGA remains bound to mtDNA throughout the cell cycle. To investigate the affects of HMGA on mitochondria we employed a genetically engineered line of human MCF-7 cells in which the levels of transgenic HMGA1 protein could be reversibly controlled. Time-course "Turn ON" and "Turn OFF" experiments in which cellular HMGA1 levels were either increased or decreased demonstrated that both mitochondrial mass and mtDNA inversely parallel changing HMGA1 levels, directly implicating HMGA in the regulation of these parameters. The level of reactive oxygen species (ROS) was also demonstrated to increase, and the efficiency of repair of oxidatively damaged mtDNA to decrease, as a consequence of HMGA1 over-expression. Increased ROS levels and reduced repair efficiency in HMGA1 over-expressing cells likely contribute to the increased occurrence of mutations in mtDNA that is frequently observed in cancer cells.

INTRODUCTION

Aberrant alterations in mitochondrial function are observed in a wide range of human conditions and diseases (11,108), including cancer (14,22,50,112,123,133). Because of their prevalence, mitochondrial abnormalities in cancer cells have been extensively investigated (19,83). Even in the presence of an adequate oxygen supply, the mitochondria of cancer cells are almost universally deficient in their ability to generate ATP via oxidative phosphorylation and therefore rely heavily on glycolytic metabolism for energy production [the so-called Warburg effect; (122)]. Likewise, cancer cells often exhibit electron transport chain deficiencies (6,91,113), generate lower amounts of ATP and higher levels of reactive oxygen species [ROS; (44,63,87)], display calcium signaling defects (95,101) and frequently exhibit alterations in their mitochondrial transmembrane potential (55,56,101). Many cancers also exhibit reduced levels of both mitochondrial mass (mtMASS) and DNA (mtDNA) (31,36,79,121,130,131) as well as significantly altered levels of mtDNA transcription (49,54,92,95). It is also well documented that mtDNA in cancer cells is more susceptible to somatic mutation than is nuclear DNA (nDNA) (8,17,22,64), partially as a consequence of limited repair mechanisms in this organelle compared to those responsible for repair of nDNA (13,14,39,75,125).

The underlying molecular causes for mitochondrial dysfunction in most individual cancers are unknown, although available evidence suggests that there are many possible contributing factors that interact in complex ways. In a few instances specific mutations in either mtDNA (17,63,94), or in nDNA that codes for mitochondrial components (22,52,82,111), have been identified that lead to mitochondrial dysfunction. But not all tumors have such identifiable mtDNA or nDNA mutations and yet still exhibit a similar pleiotrophic set of abnormal organelle phenotypes, suggesting that additional, as yet unidentified, factors likely contribute to mitochondrial

dysfunction in most cancers. In this regard, we have recently demonstrated that human cells containing high levels of HMGA1 proteins exhibit many of the same mitochondrial abnormalities found in naturally occurring cancers which, almost universally, over-express these chromatin proteins (31).

The HMGA (formerly called HMG-I/Y) family of nonhistone proteins are architectural transcription factors that modulate chromatin structure and regulate expression of well over a hundred different vertebrate nuclear genes involved in normal biological processes in different species (29,81,97,99). For example, recent studies have implicated HMGA proteins in human height determination (103,124), normal heart development in amphibians (84), promotion of stem cell renewal in the young mouse brains (88), as well as in the control of cellular aging and senescence of human cells (45,124,132). HMGA proteins have also been implicated in a number of human pathological conditions. For example, members of the *HMGA* family are presently the only genes coding for *bona fide* chromatin proteins demonstrated to be proto-oncogenes whose aberrant over-expression is involved in many different aspects of both neoplastic transformation and tumor progression (33,34,114,127,129). The level of HMGA proteins is very low (often nearly undetectable (97,128)) in differentiated somatic cells but is over-expressed in most naturally occurring cancers with increasing concentrations being correlated with increasing degrees of malignancy and poor patient prognosis (46,97,115,126). This correlation is so consistent and wide-spread that elevated concentrations of HMGA proteins are being employed as diagnostic markers of both neoplastic transformation and metastatic potential for many different types of tumors (1,7,10,25,42,89,96,102,109).

Members of the HMGA1 protein subfamily (97) are almost exclusively localized in the nucleus of normal cells except for a minor fraction of these proteins that reversibly migrates,

during the late S and G2 phases of the cell cycle, out of the nucleus and into the mitochondria where they transiently bind to the regulatory D-loop of mtDNA (32). In overtly malignant cells, however, this highly regulated shuttling is frequently disrupted with HMGA1 proteins being detectable in the mitochondria bound to mtDNA throughout the cell cycle (31,32). As previously noted, the mitochondria of cancerous cells also frequently exhibit many abnormal phenotypic and metabolic characteristics but whether any of these are causally associated with the constitutive presence of HMGA1 proteins inside these organelles remains to be determined.

Previously, employing genetically engineered human MCF-7 cells, we investigated changes in a number of mitochondrial parameters observed in 'ON' cells experimentally induced to express high levels of transgenic HMGA1 protein compared to these same parameters in parental MCF-7 cells that express only very low levels of endogenous HMGA1 protein (31). Although the results from such "all-or-none" type experiments were very informative they could not, by their nature, identify which of the mitochondrial changes observed in over-expressing cells were likely to be directly attributable to the HMGA1 proteins themselves. In the present study we have again used a transgenic line of MCF-7 cells in which the intracellular concentrations of HMGA1 protein can be reversibly controlled by altering the amount of tetracycline in the culture medium. In time-course experiments we analyzed changes in mtMASS, mtDNA concentration, reactive oxygen species (ROS) levels and the efficiency of base excision repair (BER) of oxidatively damage mtDNA in cells contain varying amounts of transgenic HMGA1 protein. The results of these reversible HMGA1 protein "concentration titration" experiments with transgenic cells, combined with supporting findings from non-transgenic human cells, are discussed in terms of the likely molecular mechanisms by which high levels of HMGA1 protein

aberrantly influence mitochondrial structure/function and promote the accumulation of mutations in mtDNA in cancer cells *in vivo*.

MATERIALS AND METHODS

Cell culture and regulation of transgenic HMGA1 protein expression. Details of the creation, phenotypic characteristics and use of the genetically engineered HA7C line of human MCF-7 breast adenocarcinoma cells containing a tetracycline regulated hemagglutnin (HA)-tagged human HMGA1a cDNA transgene have been reported (2,3,80,100,119). HA7C cells grown in medium lacking tetracycline expresses very high levels (~40-fold elevated; (120)) of transgenic HA-tagged HMGA1a protein (and are referred to as 'ON' cells). In contrast, following growth of 'ON' cells for a period of days in medium containing 4 μg/ml of tetracycline, the concentration of transgenic HMGA1a protein is reduced to nearly undetectable levels (and the cells are then referred to as 'OFF' cells; see **Fig. 1**). The parental MCF-7 cell line from which HA7C was derived (i.e., M/tet-off; Clontech, Palo Alto, CA), expresses very low levels of endogenous HMGA1 proteins regardless of whether tetracycline is present in the medium. Parental MCF-7 cells exhibit few, if any, detectable phenotypic differences from transgenic 'OFF' cells (31) and in some experiments are, therefore, employed in place of 'OFF' cells as reference standards. The common growth medium for both the parental MCF-7 and transgenic 'ON' and 'OFF' cells was DMEM medium supplemented with 10% fetal bovine serum, penicillin (100 μg/ml), streptomycin (100 μg/ml), and G418 (100 μg/ml) to maintain selection of the tetracycline transactivator protein gene. In addition, the growth medium of 'ON' and 'OFF' cells was supplemented with hygromycin (100 μg/ml) to maintain constant selection for the integrated HA-HMGA1a transgene. The genetically matched set of human epithelial cell

lines Hs578Bst (ATCC catalog no.HTB-125) and Hs578T (ATCC catalog no. HTB-126), originally derived from the same individual, were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and maintained as recommended by the supplier.

Western Blot Analyses. The levels of both endogenous and transgenic HA-tagged HMGA1 proteins in all of the cells lines were routinely monitored by western blot analysis prior to, during and following all experiments using a specific anti-HMGA1 rabbit polyclonal antibody as described previously (100). Peroxidase-conjugated goat anti-rabbit IgG was employed as the secondary antibody in western blots and bound proteins detected using Super Signal West Pico Chemiluminescent Subtrate Kit (Pierce, Rockford, USA), following the manufacturer's instructions.

Isolation of purified mitochondria. Highly-purified mitochondria preparations (~95% pure), essentially free of cross-contamination with proteins or nucleic acids from other cell compartments were isolated from cells using a Pierce "Mitochondrial Isolation Kit for Mammalian Cells" (Pierce Biotechnology, Inc., Rockford, Ill.) following protocols supplied by the manufacturer.

Mitochondrial mass analyses. Fluorescence activated cell sorting (FACS; Becton Dickinson™) was used to monitor mtMASS in cells at various times points during HMGA1 "Turn ON" and "Turn OFF" time-course experiments. For these studies cells were grown in DMEM medium lacking the pH indicator phenol red which interferes with the cytometric detection method used. Mitochondrial mass was determined by staining cells with 100 nM nonylacridine orange (NAO) (Invitrogen: Molecular Probes), a metachromatic dye that binds to cardiolipin and accumulates in mitochondria regardless of organelle membrane potential or energetic state. At each experimental time point cells at ~80% confluence were incubated for

30 min in the presence of NAO, washed with PBS, trypsinized, pelleted by gentle centrifugation and then resuspended in 1 ml of PBS containing 100 nM NAO. Mitochondrial mass analysis of the stained cells was performed on a FACSCalibur instrument (Becton Dickinson: Immunocytometry Systems) with an emission wavelength of 488 nm and a 525 nm excitation detector. Data acquisition and analyses were performed as described previously (31) using Becton Dickinson Cell Quest Pro Software supplied with the instrument. In order to obtain consistent and statistically reliable results, a concentration of at least $1x10^6$ cells/ml of counting solution was required for all FACS analyses.

mtDNA analyses. Mitochondrial DNA levels were determined by semi-quantitative PCR analysis employing mtDNA specific primers that produced a 155 bp amplification product. The mtDNA primer sequences (based on the nucleotide numbering system of http://MitoMap.org) were: Sense (nt# 7773) 5'-CCG TCT GAA CTA TCC TGC CC-3' and Antisense (nt# 7928) 5'-GCC GTA GTC GGT GTA CTC GT-3'. Total cellular DNA was isolated by standard phenol/chloroform/isoamyl alcohol/EtOH (PCI/EtOH) isolation/precipitation methods (www.invitrogen.com/iprotocol). All PCR analyses were performed in triplicate with 30 ng of template DNA using a Genius Techne Thermal Cycler. PCR reaction parameters included an initial 5-min denaturation step at 94ºC follow by 20 cycles of: 94 ºC (30 second) denaturation, 59 ºC (30 second) annealing, 72 ºC (30 second) elongation followed by a final 72 ºC extension (10 min) period. Linearity of the PCR reactions were quantitatively confirmed by control 50% input template DNA reactions. PCR reaction products were electrophoresed through 1% agarose gels, exposed to a digital image screen, analyzed on a Bio-Rad Geldoc™ EQ system and quantitatively assessed using Quantity One 1-D analysis software (Bio-Rad Labs Inc., Hercules, CA).

FACS analysis of mitochondrial Reactive Oxygen Species (ROS) levels. Cells employed for the ROS time-course studies were grown as described above for mtMASS and mtDNA analyses. At each experimental time point, freshly prepared ROS indicator dye 2′7′-dicholorodihydrofluorescein diacetate (H_2DCFDA; Invitrogen:Molecular Probes) was added to the cell growth medium at a final concentration of 10 μM. The cultures were incubated (at 37° C; 5% CO_2) for 60 min, washed with PBS, trypsinized, the cells pelleted and resuspended in PBS containing 10 μM H_2DCFDA at a concentration of 1x 10^6 cells /ml. Samples were immediately analyzed on a FACSCalibur machine utilizing excitation at 488 nm and FITC filter detection parameters. Cell Quest Pro software was used for both data acquisition and analysis to produce histogram plots and mean values. 'OFF' cells exposed to H_2DCFDA (as above) and then treated with 200 μM H_2O_2 for 30 min served as positive ROS controls for the FACS analyses. Untreated 'OFF' cells, and 'OFF' cells exposed to dilute 0.1% EtOH for 60 min, served as negative ROS FACS controls.

Mitochondrial DNA oxidative damage and base excision repair (BER) analyses. Twenty hours prior to treatment with either menadione sodium bisulfate (water soluble; Sigma-Aldrich, St. Louis, MO) or hydrogen peroxide (H_2O_2; Sigma-Aldrich), exponentially growing cells at ~80% confluence were treated with hydroxyurea (HU; 4 mM final concentration) in order to lower the background level of replicative DNA synthesis and facilitate analysis of repair of oxidative damage in mtDNA. HU treated cells were washed with PBS and then warm serum-free medium was added to the culture dishes. In some experiments menadione (40-400 uM; (31,38)) was added to dishes and the cells incubated for 30-60 min at 37 °C and 5 % CO_2. In other experiments H_2O_2 at a final concentration of 750 uM was added and the cells incubated for 30 min at 37 °C and 5 % CO_2. Following exposure to either agent, cells were washed with

PBS, fresh complete medium added and the dishes returned to the incubator for various lengths of (0, 1, 2 and 4 hr, etc.) to allow for repair of oxidative lesions before being harvested by trypsinization and their DNA isolated. High molecular weight total cellular DNA was isolated from cells using a modified PCI/EtOH isolation/precipitation method. DNA concentrations were determined by use of a NanoDrop^{TM} 1000 Spectrophotometer (Thermo Fisher Scientific, Inc).

Mitochondrial DNA repair time-course experiments. Time-course analyses of the rate of repair of oxidative damage in nearly full-length (16.2 kb) mtDNA were performed using modifications of the long-range quantitative PCR (QPCR) methods developed by Van Houten and his coworkers (105,106) as previously described (80). Briefly, prior to QPCR amplification, approximately 2 ug of purified total cellular DNA was digested to completion with the restriction enzyme Xho I (4 units; 14 hr, 37° C; New England Biolabs) to linearize human mtDNA (GenBank Accession # J01415), the digested DNA isolated by PCI/EtOH precipitation and then re-dissolved in ddH_2O, pH 8.4. The Xho I digested DNA was then digested (2 units; 4 hr 37° C) with the damage-specific enzyme FPG (formamidopyrimidine [fapy]-DNA glycosylase; also known as 8-oxoguanine DNA glycosylase; New England Biolabs) which cleaves at sites of oxidized purine bases. The double-digested DNA was then accurately quantified and used as a concentrated working stock solution.

The GeneAmp XL PCR kit (Applied Biosystems) was used for QPCR amplifications following manufacturer-supplied protocols. Twenty five µl amplification reactions were performed in 0.5 ml thin-wall PCR tubes (Phenix, Hayward, CA) on a Techne Genius thermal cycler (Techne, Cambridge, UK). QPCR conditions were as follows: 40 ng total cellular DNA, 1 X Buffer XL II (Applied Biosystems), 100 ng/µL bovine serum albumin (BSA), 200 µM (each)

of the four deoxynucleotides, 400nM (each) forward and reverse mtDNA primers, 0.7 mM Mg^{+2}, and 0.5 unit r*Tth* DNA polymerase XL (Applied Biosystems). The human mtDNA primer set used for amplification was: Sense (#15149) 5'-TGA GGC CAA ATA TCA TTC TGA GGG GC-3' and Antisense (#14841) 5'-TTT CAT CAT GCG GAG ATG TTG GAT GG-3' (105). The unique Xho I cut site employed to linearize mtDNA is situated between the two primers at nt #14,955 and hence the expected QPCR amplification fragment is 16.2 kb in length (rather than full-length 16.6 kb mtDNA; GenBank REFSEQ No. AC_000021.2). Controls for the QPCR amplification reactions followed the recommendations of Santos *et al* (105). A half-template (50%) reaction was run for first set of QPCR experiments to determine the linear amplification range of template DNA. A small fragment amplification reaction was run for each set of QPCR experiments and used for the normalization of mtDNA levels. Sequences of the small (155 bp) fragment amplification primer set are given above. The 50% control reactions contained the same long-fragment (16.2 kb) and short (~155 bp) gene primer sets as above, but only half the concentration of template DNA (i.e., 5-20 ng for total cell DNA). Amplification parameters included a PCR 'hot start', in which samples were heated to 75ºC for 5 minutes prior to addition of r*Tth* DNA polymerase. DNA samples were then denatured by heating at 94ºC for 1 minute, followed by 20 cycles of denaturation at 94ºC for 30 seconds and annealing and extension at 64ºC for 12 minutes. Samples were then heated to 72ºC for 10 minutes for final elongation and stored at 4ºC until electrophoretic separation and repair analyses could be completed.

Mitochondrial DNA repair analyses. Methods for quantitative evaluation of DNA repair efficiency following initial damage using results from long-range QPCR have previously been described in detail (80,105). Briefly, 25 µL of each QPCR reaction were loaded onto a standard 0.7% agarose gel containing 2 µM ethidium bromide and amplified DNA products separated by

electrophoresis. Stained mtDNA bands resulting from QPCR reactions in the linear range of amplification (105) were visualized and digitally captured and quantified as described above. Band intensities were first corrected for gel background (average above and below each band) and then normalized to 100% control template reactions to measure variances observed between repair samples. When total cellular DNA was employed as template, a small fragment (155 bp) of mtDNA was PCR amplified in independent reactions (see above) as a measure of the actual concentration of mtDNA per sample and these results used for normalization purposes. The small mtDNA fragment normalization control was only required when whole cell DNA was used as template because previous experiments demonstrated that cells over-expressing HMGA protein have reduced levels of mtDNA compared to non-expressing control cells (31). The normalized value of each menadione- or H_2O_2-treated sample was then divided by the normalized band intensity of the non-treated control sample to give the fraction of non-damaged templates at a given dose of oxidizing agent and/or repair time (105,106). Assuming a Poisson distribution of oxidative lesions between amplified strands (e^{-S}, where S = average lesion frequency), the lesion frequency per strand was calculated using the equation $S = -\ln(A_D/A_0)$, where A_0 = intensity of the normalized full length (undamaged) mtDNA band from untreated cells and A_D = the intensity of the normalized full length band intensity from either menadione- or H_2O_2-treated samples at a given time after DNA damage. Repair efficiencies at each time point were measured from the difference in average lesion frequency of the repair time and that of the un-repaired DNA (0 hours). The mean value and standard deviation for lesion incidence and efficiency of lesion repair were calculated for at least 3 independent experiments for each gene amplification in each of the different cell lines. Best fit curve plots of the data were derived employing the PivotChart Wizard function of Microsoft Excel®.

RESULTS

HMGA1 proteins levels can be reversibly controlled in transgenic cells. The western blot (WB) results shown in **Fig. 1A** demonstrate that the intracellular concentration of HA-tagged HMGA1 protein inside transgenic cells can be reversibly controlled by addition of tetracycline to, or removal from, the cell culture medium. In "Turn OFF" experiments, addition of tetracycline to the medium of 'ON' cells that initially contain high levels of HMGA1 completely inhibits expression of this protein within about 3 days of drug exposure and thereafter the level remains at extremely low levels for extended periods of time (**Fig. 1A**, top row). The reverse is true in "Turn ON" experiments: within 24 hr of removal of the drug from the medium of non-expressing 'OFF' cells, low levels of transgenic HMGA1 protein can be detected and the intracellular concentration continues to gradually increase with time until it finally reaches the maximum level found in 'ON' cells after about 9 days (**Fig. 1A**, bottom row). Panels **1B** and **1C** graph the results of quantitative analyses of the western blot results shown in **Fig. 1A**. Two additional significant observations are evident from these WB results. First, based on its rate of disappearance in "Turn OFF" experiments (**Fig. 1B**), the half-life of the HMGA1 protein is estimated to be about 30-35 hr, a value consistent with previously reports ($T_{1/2}$ > 30 hr; (9,60)). Second, and consistent with the protein's half-life, it takes much longer (~3.7-times) to accumulate maximal levels of HMGA1 protein inside cells in "Turn ON" experiments (~11 days; **Fig. 1C**) than it does to deplete the initially high levels of protein present in 'ON' cells in "Turn OFF" experiments (~3 days). Furthermore, and of particular importance for the present study, the WB show in the lower part of **Fig. 1D** demonstrates that HMGA1 protein is present in highly purified mitochondria preparations isolated from 'ON' cells but not in mitochondria isolated from 'OFF' cells. These findings that are consistent with previous reports of

mitochondrial localized of HMGA proteins from both our laboratory (31,32) and those of others (24,70).

Mitochondria change in response to altered cellular HMGA1 protein levels. Significant changes in mitochondrial structure, function and metabolism have been demonstrated to occur in transgenic HMGA1 over-expressing 'ON' cells compared to non-transgenic parental MCF-7 cells (31). For example, mtDNA content and cellular ATP levels are substantially reduced in over-expressing 'ON' cells compared to parental MCF-7 cells. In contrast, 'ON' cells exhibit elevated ROS levels and have a more glycolytic metabolism than parental MCF-7 cells. Likewise, changes in mitochondrial membrane potential ($\Delta\Psi m$), as well as significant modulations in the levels of organelle RNA transcripts from both the heavy and light strands of mtDNA, are also observed in 'ON' cells compared to MCF-7 controls. A limitation of these earlier experiments, however, is that the results were obtained by comparing maximally HMGA1 over-expressing transgenic 'ON' cells with non-transgenic parental MCF-7 cells that only express very low levels of endogenous HMGA1 protein (31). Thus, from these findings alone, it is not possible to assess which, if any, of the observed mitochondrial changes are likely to be causally influenced by the HMGA1 protein itself. The ability to reversibly alter intracellular HMGA1 proteins levels in "Turn OFF" and "Turn ON" experiments (**Fig. 1**) allows for an alternative approach to address this important question. It is reasonable to suspect, for example, that any mitochondrial changes that clearly "track" with both increasing and decreasing intracellular concentrations of HMGA1 protein are more likely to be causally related to some direct function of the protein than are those organellar changes that don't follow such a reciprocal expression pattern.

Changes in mtMASS track with variations in HMGA1 protein levels. To experimentally test this idea, " Turn OFF" time-course experiments were performed in which tetracycline was added to the culture medium of 'ON' cells and FACS analysis was employed to monitor changes in mtMASS as a function of time (days) following transgene "turn off" and declining intracellular HMGA1 protein levels. Previous work has suggested that 'ON' cells contain less than 70% of the mtMASS of parental MCF-7 cells (31). If mtMASS is directly influenced by HMGA1 levels then the predicted results of these "Turn OFF" experiments are that, over time, mitochondrial mass will increase in cells as a consequence of dropping HMGA1 protein levels. And, as shown in **Figs. 2,** this is indeed what is observed. **Fig. 2A** shows the overlay of FACS histograms of the mtMASS distributions found in cells at various times (0, 4, 7 and 18 days) following drug-induced transgene 'turnoff'. The shift of the peak of mitochondrial mass intensity in the 'ON' cells (at day 0) toward the peak intensity level found in non-expressing MCF-7 control cells as a function of time (days) following drug addition demonstrates that mtMASS increases as the intracellular concentration of HMGA1 protein decreases. Additionally, Kolmogorov-Smirnov (K-S) test analysis (insert in **Fig. 2A**) of the FACS scans from "ON" and parental MCF-7 cells statistically confirms that the mtMASS in HMGA1 over-expressing cells is significantly reduced relative to that found in the 'OFF' cells. The graph in **Fig. 2B** shows the results "Turn OFF" experiments in which the mtMASS is monitored as a function of time following drug addition to the media of 'ON' cells and demonstrates that by 70 days the mtMASS has risen to levels comparable to those found in parental MCF-7 cells. The graph in **Fig. 2C** shows the results of a reciprocal "Turn ON" time-course experiment and demonstrates that following removal of tetracycline from the culture medium of "OFF" cells, mitochondrial mass decreases by >30% over a period of about a month and a half. Together, the

results of these complementary time-course experiments provide strong and compelling evidence that HMGA proteins are causally associated with regulating mtMASS levels *in vivo*.

Changes in mtDNA track with variations in HMGA1 protein levels. Previous work has demonstrated that 'ON' cells contain only about half the amount of mtDNA of parental MCF-7 cells (31). To determine whether changes in the levels of mtDNA also track with variations in cellular HMGA1 protein levels, time-course "Turn OFF" and "Turn ON" experiments similar to those described in **Fig. 2** were performed and changes in mtDNA levels monitored at various times using semi-quantitative PCR techniques. The graphs in **Fig. 3** plot the results of these experiments and demonstrate that mtDNA levels are also influenced by changes in the intracellular concentration of HMGA1 protein. Specifically, mtDNA levels increase in cells as a function of time during "Turn OFF" experiments (**Fig. 3A**) and, reciprocally, mtDNA levels decrease with time during "Turn ON" experiments (**Fig. 3B**). Thus, both mtMASS and mtDNA vary with changes in intracellular HMGA1 concentrations, strongly suggesting that this protein has a direct impact on both of these mitochondrial features.

Reactive Oxygen Species (ROS) concentrations track with HMGA1 protein levels. Consistent with their overtly cancerous phenotype (100), HMGA1 over-expressing 'ON' cells contain elevated levels of reactive oxygen species (31). FACS analyses were employed to monitor ROS levels in cells during "Turn OFF" experiments to determine whether they would drop as HMGA1 levels decrease, as would be expected if cellular ROS levels are directly influenced by this protein. The results of these time-course experiments are shown in **Fig. 4** and demonstrate that ROS levels do indeed decline as predicted. The results of both positive control experiments (in which H_2O_2 was added to 'OFF' cells to artificially increase ROS levels)

and negative control experiments (in which 'OFF' cells were exposed to 0.1% EtOH) validated the sensitivity of FACS analyses for detecting changes in cellular ROS levels (data not shown).

Mitochondria in native human cells are similar to those in transgenic 'ON' and 'OFF' cells . Although the level of HMGA1 protein over-expression in the transgenic 'ON' cells is well within the range of over-expression of this protein found in naturally occurring human cancers (120), a potential concern with the above findings is that they were obtained with an artificial experimental system and thus might not faithfully reflect the situation with mitochondria in non-transgenic, naturally occurring cells. If the results shown in **Figs. 1- 4** are generally applicable to non-transgenic cells, a number of biologically relevant and testable predictions can be made. Among these, for example, is the expectation that HMGA1 protein concentration in naturally occurring cancers will be significantly higher than in normal cells. Likewise, both mtMASS and mtDNA in naturally occurring cancers are predicted to be noticeably lower than that in normal cells whereas ROS levels are expected to be higher. To test these predictions we determined HMGA1protein, mtMASS, mtDNA and ROS levels in a matched set of human mammary cells (Hs578 Bst and Hs578T) derived by fresh tissue biopsies from the same individual but with markedly different tumorigenic phenotypes (53). The aneuploid Hs578T cell line originated from an epithelial carcinoma and causes aggressive, highly metastatic tumors when injected into nude mice. In contrast, the diploid Hs578Bst myoepithelial cell line is both phenotypically and biochemically normal in all respects (53,76) including undergoing natural senescence in culture (65). From the results of western blots for HMGA1 protein (**Fig. 5A**), from FACS analyses monitoring both ROS levels (**Fig. 5B**) and mtMASS (**Fig. 5D**), as well as from quantitative PCR determination of mtDNA levels (**Fig. 5C**), it is evident that all of these predictions are

confirmed: the highly malignant Hs578T cancer cells have a much higher level of HMGA1 protein, lower mtMASS and mtDNA levels and elevated amounts of ROS compared to the normal Hs578 Bst cells. Interestingly, the differences in all of these mitochondrial parameters between the Hs578 Bst and Hs578T cells are equal to, or greater than, the differences observed between the transgenic 'OFF' and 'ON' cells employed in the present studies. These results, obtained with naturally occurring cells derived from the same patient lend considerable credenc to biological relevance of the findings obtained with transgenic 'ON' and 'OFF' cells.

HMGA1 over-expression inhibits BER repair of oxidatively damaged mtDNA. We have previously demonstrated that nucleotide excision repair (NER) of bulky DNA lesions induced b ultraviolet (UV) light is severely compromised in cancer cells that over-express HMGA1 proteins (2,80). Two molecular mechanisms are believed to contribute to this NER deficiency: (i) inhibition by HMGA1 of transcription of DNA repair genes (e.g., *Xeroderma pigmentosum A XPA*) whose encoded protein products are essential for NER to occur (2); and (ii) direct binding of HMGA1 to UV-induced DNA lesions (e.g., cyclobutane pyrimidine dimers (CPDs) and 6-4 photo-products) thereby preventing access of repair enzymes to the lesions by steric hindrance (2).

Mitochondria, however, lack the NER enzymes required for repairing bulky lesions in mtDN (16,26,43). Rather, the mechanisms of repair of lesions in mtDNA are complex and highly dependent on both the type and extent of damage involved (43,71), with base excision repair (BER) playing a primary role in removing oxidatively damaged bases (35,77,85). Preliminary cell survival studies using the base alkylating agent dimethylsulfate suggested that BER of nDNA, like NER, is reduced in HMGA1 over-expressing cells (unpublished data). We

therefore decided to examine whether BER of oxidatively damaged mtDNA is also compromised in over-expressing 'ON' cells. For these experiments both "ON' and parental MCF-7 control cells were briefly treated with the vitamin K(3) analog menadione, a redox-cycler that selectively induces excess superoxide radicals in mitochondria. Following menadione treatment, removal of oxidative lesions from mtDNA was followed as a function of time employing long-range QPCR to monitor the time-course of repair in nearly full-length (16.2 kb) mtDNA. In these analyses oxidatively damaged bases interfere with elongation during the PCR amplification reaction and hence reduce the yield of the 16.2 kb product. Thus, the amount of QPCR amplified product is a quantitative measure of amount of damage/repair that has occurred in mtDNA templates as a function of time. **Figure 6A** shows the results of such an experiment in which the percentage of adjusted volume of amplified mtDNA products (i.e., a direct measure of the extent of lesion removal) is plotted as a function of time post-treatment. It is evident from this graph that HMGA1 over-expression significantly reduces the efficiency of BER as evidenced by the fact that ~60% of the lesions in mtDNA in the parental MCF-7 control cells have been removed by 2.5 hr post-damage whereas little, if any, repair has occurred in the mtDNA from over-expressing 'ON' cells. By 4 hr post-damage >80% of the mtDNA damage has been repaired in parental MCF-7 cells compared with only around 30% in the 'ON' cells. Also shown in **Fig. 6A** are the results of control QPCR experiments with undamaged mtDNA [minus (-) menadione; 100% and 50% input] templates used to verify that the PCR reactions were in the required linear range of amplification (106). **Fig. 6B** graphically depicts these results as the percentage repair and clearly shows the dramatic difference observed between 'ON' (open boxes) and parental MCF-7 control cells (closed boxes) during the first two hours following menadione treatment.

Hydrogen peroxide (H_2O_2) is frequently used as an alternative/complementary oxidative damaging agent to menadione in studies of mitochondrial DNA damage and repair in mammalian cells (23,51,86). Optimization experiments in which 'ON' and 'OFF' cells were exposed to varying concentrations of H_2O_2 (50 uM-1 mM) for different lengths of time (15-60 min) demonstrated that an exposure of 750 uM H_2O_2 for 30 min induced, on average, approximately 1 oxidative lesion/kb of mtDNA in these cells (data not shown). The results of time-course of mtDNA repair experiments following exposure of 'ON' and 'OFF' cells to this dosage of H_2O_2 are shown in **Figs. 7A** & **7D**, respectively. From these figures it apparent that immediately following H_2O_2 treatment (i.e., at 0 hr before any repair has occurred) the amount of amplified 16.2 kb product is significantly reduced (>75%) in both the 'ON' and 'OFF' cells compared to the amount of amplified product in the undamaged mtDNA controls. Following H_2O_2 treatment (i.e., at 1, 2 and 4 hrs) the amount of amplified 16.2 kb product increases with time in both the 'ON' and 'OFF' cells indicating that oxidative lesions are being progressively removed from the damaged mtDNA by *in vivo* repair processes. Importantly, however, it is obvious that at 1 hr post-damage much less mtDNA repair has occurred in the 'ON' cells (**Fig. 7A**) than in the 'OFF' cells (**Fig. 7D**). Quantitative assessments of these QPCR results in terms of both the relative number of lesions/kb removed and the percentage repair that occurs with time are shown, respectively, in **Figs. 7B** & **7C** for 'ON' cells and **Figs. 7E** & **7F** for 'OFF' cells. From these graphs it is seen that by 1 hr post-damage 'OFF' cells have repaired greater than 60% of the oxidative lesions in mtDNA whereas only around 15% of the lesions have been repaired in the 'ON" cells. The lower panels in **Figs. 7A** and **7D** also show the results of important QPCR control amplification reactions for both the 'ON' and 'OFF' cells. Namely, that at each point during the repair time-course repair experiment approximately equal amounts of a

155 bp sub-mtDNA fragment are amplified from the cells demonstrating that the observed variations in the intensity of amplified 16.2 kb product are due to variations in the amount of repair that has occurred and not the result of differences in the amount of mtDNA present in the reactions themselves (105).

Having established that the greatest observed difference in mtDNA repair between the 'ON' and 'OFF' cells occurs at 1 hr following H_2O_2 treatment, an HMGA1 "Turn OFF" time-course experiment was conducted to determine whether HMGA1 proteins levels directly influence the efficiency of mitochondrial BER. In these experiments tetracycline was added to the medium of 'ON' cells to inhibit HMGA1 protein production and on subsequent days aliquots of cells were exposed to H_2O_2, allowed to recover for 1 hr, and the amount of mtDNA repair that has occurred during this recovery period quantitatively assessed by long-range QPCR. **Figure 8** shows the results of these experiments and clearly demonstrate that as the levels of HMGA1 protein decrease in cells following transgene 'Turn-OFF' the efficiency of repair of oxidative damage in mtDNA increases as a function of time. Thus, analogous to the situation for both mtMASS and mtDNA, over-expression of HMGA1 in 'ON' cells decreases the efficiency of base excision repair of oxidatively damaged mtDNA and this inhibition is relieved once the intracellular levels of the protein drop. This, to our knowledge, is the first reported evidence demonstrating that over-expresssion of HMGA proteins influences the efficiency of BER in either mtDNA or nuclear DNA.

DISCUSSION

Previous studies have demonstrated that, in contrast to 'OFF' cells, the 'ON' cells used in this study acquire the ability for anchorage-independent growth in soft agarose and to form both primary and metastatic tumors when injected into nude mice (100). Immunohistochemical

analyses of these metastatic tumors indicate that the 'ON' cells have undergone an epithelial-mesenchymal transition *in vivo*, demonstrating that HMGA1 over-expression promotes both neoplastic transformation and malignant metastatic progression. Furthermore, and of considerable importance for the present study, these cancerous phenotypic changes have also been shown to be reversible following 'Turn OFF' of HMGA1 transgene expression(100). Additionally, stable transfection of non-transgenic human cancer cells that have naturally elevated levels of HMGA1 protein (e.g., Hs578T and HeLa cells) with either antisense or dominant-negative *HMGA1* constructs has been demonstrated to inhibit both their rate of proliferation and their ability to grow in soft agar (100). The reversible nature of many of the cancerous phenotypic changes induced in cells by HMGA over-expression has been confirmed by numerous laboratories (33,37,61,127,128) and serves as the basis for suppression of HMGA gene expression as a promising new therapeutic approach for the treatment of cancer (12,93,107,117).

A comprehensive picture of the complex and varied molecular mechanisms by which over-expression of *HMGA* genes is induced in cells, as well as how this aberrant expression promotes cancer, in now beginning to emerge (reviewed in: (29,41,46,48,99,127)). Among the most significant biological changes induce by HMGA1 over-expression are alterations in specific signal transduction pathways (28,100,120,128) that, in turn, lead to anomalous transcriptional activation/repression of a subset of cancer-related 'target genes' as a result of HMGA1 protein binding to regulatory regions in their promoters (3,27,40,58,62,116,119).

Against this background information, the present demonstration that several mitochondrial characteristics frequently altered in naturally occurring cancers (e.g., mtMASS, mtDNA and ROS levels; **Fig. 5**) reversibly change in response to controlled alterations in the cellular

concentrations of the HMGA1 (**Figs. 1-4**) strongly implies that these proteins are causally involved in mediating such phenotypic changes. The fact that not all mitochondrial parameters (e.g., membrane potential; data not shown) exhibit such a clear reciprocal pattern also emphasizes the biological importance of the affects of HMGA1 proteins on those organellar properties that do.

Additional experimental support for such a causal relationship, especially for the observed reductions of mtDNA and mtMASS in both 'ON' cells (**Figs. 2** & **3**) and naturally occurring Hs578T cells (**Fig. 5**), comes from a number of sources. For example, we have previously demonstrated by chromatin immunoprecipitation (ChIP) analysis that in 'ON' cells HMGA1 protein binds to regions of the mitochondrial D-loop referred to as conserved sequence blocks (CSBs) (32), regulatory units involved in controlling replication of the vast majority of mtDNA (18,110). Given such localized and specific D-loop binding we have speculated that HMGA1 proteins in over-expressing cells likely inhibit mitochondrial replication processes *in vivo* (32) and the findings from the present studies are entirely consistent with this suggestion. One possible mechanistic explanation for how this inhibition might occur is that HMGA1 is acting at the level of transcript cleavage during the formation of RNA primers from the light strand promoter (P_L), primers (32). These short RNA primers are required for the transition from RNA to DNA synthesis and for the initiation of mtDNA replication at the heavy strand origin (OH) by mitochondrial DNA polymerase (pol γ) (78). During asymmetric mtDNA replication transcripts from the P_L are cleaved in the vicinity of CSB sites, the first of which (CSB I) has a very high A/T content (20,21) and is a specific binding site for HMGA1 both *in vivo* (32) and *in vitro* (data not shown). Since HMGA1 binding induces bends and other structural distortions in A/T-rich DNA substrates (99), any alterations to the CSB region resulting from HMGA1 binding could

potentially hinder the transition process and thus interfere with initiation of replication and result in the observed decreased amounts of mtDNA and mtMASS in over-expressing 'ON' cells.

Another line of evidence strongly supportive of a causal relationship between HMGA1 over-expression and reduced levels of mtDNA and mtMASS derives from the fact that the processes of transcription and replication in mitochondria are so intimately coupled (18,110) that any protein involved in regulating transcription will also likely influence mtDNA replication, as has been so elegantly shown for mitochondrial transcription factor A (TFAM). TFAM is a DNA-binding protein that interacts with mitochondrial DNA pol γ and another transcription factor (either TFB1M or TFB2M) and stimulates mtDNA transcription through binding to specific D-loop sequences called upstream enhancers (5,47,69). In addition to sequence-specific promoter recognition, however, TFAM also binds nonspecifically to DNA and, due to its high abundance, plays an architectural role in the stabilization and maintenance of mtDNA and its compaction into a nucleoid structure (4,66,68). Substantial evidence indicates that TFAM is required for mtDNA replication (reviewed in: (69)) including the fact that TFAM levels are elevated in naturally occurring ragged-red muscle fibers which have high levels of mtDNA and the levels are decreased in the cells of infants that are naturally depleted of mtDNA. The most compelling evidence, however, is the experimental demonstration that the amount of mtDNA closely parallels (both upward and downward) changes in cellular TFAM levels (67). TFAM is an "HMG box-containing" protein that, like HMGA1, can bend and unwind DNA- properties linked to the biological functions of both proteins. We have previously reported (57) that HMGA1 has the ability to "out-compete" HMG-box containing proteins for binding to distorted DNA structures similar those thought to exist in the mtDNA D-loop (57,110). More recently, *in vitro* binding competition studies (Wertzler *et al*., manuscript in preparation) demonstrated that

HMGA1 can displace recombinant TFAM protein from both B-form D-loop DNA as well as from synthetic cruciform junctions designed to mimic the presumed structure of this DNA *in vivo* (90). Therefore a second likely mechanistic explanation for the decreased levels of mtDNA and mtMASS found in 'ON' cells is that HMGA1 binding to D-loop DNA prevents or displaces TFAM binding, thus interfering with mtDNA replication.

Although available evidence suggests two alternative, but not mutually exclusive, reasonable explanations for how HMGA1 over-expression leads to decreased levels of mtDNA and mtMASS in cells, a plausible mechanistic explanation that accounts for increases in ROS levels in over-expressing cells (**Figs. 4** & **5**) is not as easily discernable. One reason for this is that, based on gene expression microarray analyses (118), HMGA1 over-expression has been shown to modulate the transcription of a number of nuclear genes that code for mitochondrial proteins whose altered expression could contribute to the observed increases in ROS levels. Another caveat is that real-time PCR analyses have demonstrated that in 'ON' cells the HMGA1 protein directly influences transcription of both the heavy and light strands of mtDNA (31), alterations that could likewise influence ROS levels. Further investigations are, therefore, required in order to determine whether any of these induced nuclear and mitochondrial transcriptional changes are causally responsible for the elevated ROS levels in over-expressing cells.

Mitochondrial DNA is demonstrably more susceptible to mutation than genomic DNA and numerous somatic mutations have been observed in the mtDNA of several types of cancers (22). Although some of these mtDNA mutations may eventually turn out to be experimental artifacts, a considerable number are likely to be authentic and have a direct relationship with cancer development (63,104). The present work suggests that HMGA1 over-expression may contribute to increased mutation rates in the mtDNA of cancer cells in at least two ways: first, by inducing

increased levels of reactive oxygen species (**Figs. 4** & **5**) and, second, by decreasing the efficiency of base excision repair of oxidative lesions (**Figs. 6-8**). The mechanism(s) by which HMGA1 over-expression inhibits BER in mitochondria are unknown but, by analogy with the way these proteins inhibit NER of lesions in nuclear DNA (2,3), are likely to be a combination of: (i) binding to lesions in mtDNA and preventing access of repair enzymes; and (ii) repression of transcription of nuclear encoded BER genes that are essential for repair to occur. Although the first possibility hasn't yet been explored there is experimental evidence supporting the latter in that gene expression array analyses have shown that transcription of the nuclear gene coding for DNA ligase III is significantly down-regulated in HMGA1 over-expressing cells (98). DNA ligase III, in complex with XRCC1, is the main nick sealing enzyme involved in nuclear short-patch BER (43,59). The human DNA ligase III gene encodes a transcript containing two in-frame ATG translation initiation start sites, one used to produce an isoform of the protein with an amino-terminal mitochondrial targeting sequence and the other a shorter form of the protein involved in BER in the nucleus (72). Inside the mitochondria DNA ligase III functions independently of XRCC1 (73) and forms a complex with DNA pol γ (30) that is involved in both BER of oxidative lesions (15) and the maintenance of mtDNA integrity and stability (74). Furthermore, given that cells deficient in DNA ligase III are also defective in nuclear base excision repair (73), it is not surprising, as demonstrated here (**Figs. 6-8**), that the efficiency of repair of oxidative lesions in mtDNA is reduced in 'ON' cells. It is therefore reasonable to suspect that repression of DNA ligase III transcription in HMGA1 over-expressing contributes to the increased levels of somatic mutations observed in the mtDNA of cancer cells.

Results from this study also suggest a novel and unexpected biological role for the HMGA1 protein in nuclear-mitochondrial communication in normal, non-cancerous cells. In normal cells

a sub-population of HMGA1 proteins reversibly migrates between the nucleus and mitochondria in a cell cycle dependent manner and, while within the mitochondria, transiently binds to the regulatory D-loop (32). The direct affects of the HMGA1 protein on both mtDNA and mtMASS levels demonstrated here strongly imply that the tightly controlled shuttling of this protein between the nucleus and mitochondria in normal cells likely functions as a 'messenger' to coordinate the metabolic and replication activities of these two sub-cellular compartments.

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FIGURE LEGENDS

Fig. 1. Time-course "Turn OFF" and "Turn ON" experiments demonstrate that the concentration of HMGA1 protein in transgenic cells can be reversibly controlled by the addition/removal of tetracycline to the culture medium. (**A**) Western blot analyses of the relative levels of HMGA1 protein present in 'ON' cells as a function of time (days) following addition of tetracycline to the medium to inhibit transgene expression (upper panel) and in "OFF" cells following removal of the drug from the medium to activate transgene expression (lower panel). (**B**): Quantitative assessment of the results of "Turn OFF" experiment shown in '**A**' (upper panel). (**C**) Quantitative assessment of the results the "Turn ON" experiment shown in '**A**' (lower panel). All results shown are the average of three or more independent experiments. (**D**) Upper panel: Coomassie stained SDS-PAGE gel of total proteins from mitochondria (>95% pure) isolated from 'ON' cells (lane 1) and 'OFF' cells (lane 2). Lane 3 contains trace amounts of recombinant HMGA1 protein as a reference standard and lane 4 contains molecular weight marker proteins with the 25 kd protein labeled. Lower panel: Western

blot analysis of a portion of the gel shown in upper panel demonstrating that mitochondria isolated from 'ON' cells contain readily detectable HMGA1 protein (lane 1) whereas those isolated from 'OFF' cells (lane 2) do not. The absence of detectable HMGA1 protein in the mitochondria 'OFF' cells is consistent with the fact that in logarithmically growing populations of these cells the protein is only found in the organelle during a short, restricted phase of the cell cycle (32). Lane 3 is recombinant HMGA1 included as a positive marker. No other anti-HMGA1 antibody cross-reacting proteins were observed on the western blot.

Fig. 2. FACS analysis of mitochondrial mass (mtMASS) found in cells during "Turn OFF' and 'Turn ON' experiments as a function of time (days) following addition/removal of tetracycline from the culture medium. **(A)** Overlay of mtMASS histograms of cells on various days (0, 4, 7, 18) following drug addition to the medium in "Turn OFF' experiments. The intensity of nonylacridine orange (NAO) stained mitochondria (a measure of mtMASS) is shown on the logarithmic X axis and cell counts on the Y axis. Key to the color-coded scan lines is shown in figure. **Insert:** Kolmogorov-Smirnov (K-S) test statistical analysis of FACS results confirms that the mtMASS in "ON" cells is significantly increased up to levels follow in control 'OFF' cells following tetracycline treatment. **(B)** Quantitative plot of the results of mtMASS FACS data such as that shown in **(A)** as a function of time (up to 70 days) following addition of drug to the growth medium. **(C)** Quantitative plot of the results of "Turn ON" experiments in which mtMASS is measured as a function of time (up to 46 days) following removal of tetracycline from the medium. Differences between time points were calculated using mean peak mtMASS values as reported by Cell Quest Pro FACS analysis software (Becton-Dickenson Corp.). All results shown are the average of three or more independent experiments.

Fig. 3. Semi-quantitative PCR analyses of the levels of mtDNA found in cells during "Turn OFF' and 'Turn ON' experiments as a function of time (days) following addition/removal of tetracycline from the culture medium. (**A**) Graph showing that the relative levels of mtDNA increase in cells as a function of time during "Turn OFF" experiments. (**B**) Graph showing that the relative levels of mtDNA decrease in cells as a function of time during "Turn ON" experiments.

Fig. 4. Reactive oxygen species (ROS) levels in cells decline as HMGA1 protein levels drop in "Turn OFF" experiments. FACS analyses of ROS levels in cells as a function of time (days) during "Turn OFF" experiments.

Fig. 5. Naturally occurring human breast carcinoma cells (Hs578T) over-express HMGA1 protein (panel A), have reduced levels of both mtMASS (panel B) and mtDNA (panel C) and increased ROS levels compared to normal breast epithelial cells (Hs578 Bst). (A) Western blot with anti-HMGA1 antibody. (**B**) FACS analysis of mtMASS levels in Hs578Bst and Hs578T cells. (**C**) Semi-quantitative analysis of mtDNA in Hs578Bst and Hs578T cells.. The insert in the upper part of the figure shows the ethidium bromide-stained QPCR products amplified from Hs578T and Hs578T cells. (**D**) FACS analysis of ROS levels in Hs578Bst and Hs578T cells.

Figure 6. Time-course of repair of oxidative damage in mtDNA following treatment of HMGA1 over-expressing 'ON' and parental MCF-7 control cells with menadione (40 uM, 1hr, 37°C; average of 3 independet experiments). **Panel (A):** Bar graph of the percentage of

adjusted volume of quantified QPCR bands of mtDNA from 'ON' cells (white bars) and parental MCF-7 control cells (black bars) following drug treatment. **Panel (B):** Results of panel A plotted as percentage repair in 'ON' (open boxes) and parental MCF-7 control (black boxes) cells following drug treatment.

Fig. 7. Time-course of mtDNA repair experiments following exposure of 'ON' and 'OFF' cells to 750 uM H_2O_2 for 30 min to induced, on average, approximately 1 oxidative lesion/kb of mtDNA. The results of long-range QPCR analyses employed to follow the repair mtDNA in both 'ON' cells (**A**; upper panel) and 'OFF' cells (**D**; upper panel) as a function of time (hours) post-H_2O_2 treatment. The lower panels in (A) and (D) show the results QPCR control amplification reactions for a 155 bp sub-mtDNA fragment and demonstrate equal input loading in all of the samples. Quantitative assessments of the QPCR results shown in (**A**) and (**D**) in terms of both the relative number of lesions/kb remaining in mtDNA, and the percentage repair that has occurred, as a function of time are shown, respectively, in panels (**B**) and (**C**) for 'ON' cells and panels (**E**) and (**F**) for 'OFF' cells.

Fig. 8. "Turn OFF" time-course experiments in which the efficiency of repair of mtDNA damage (at 1 hour post-H_2O_2 treatment) is measured as a function of time (days) following addition tetracycline addition to the medium of 'ON' cells.

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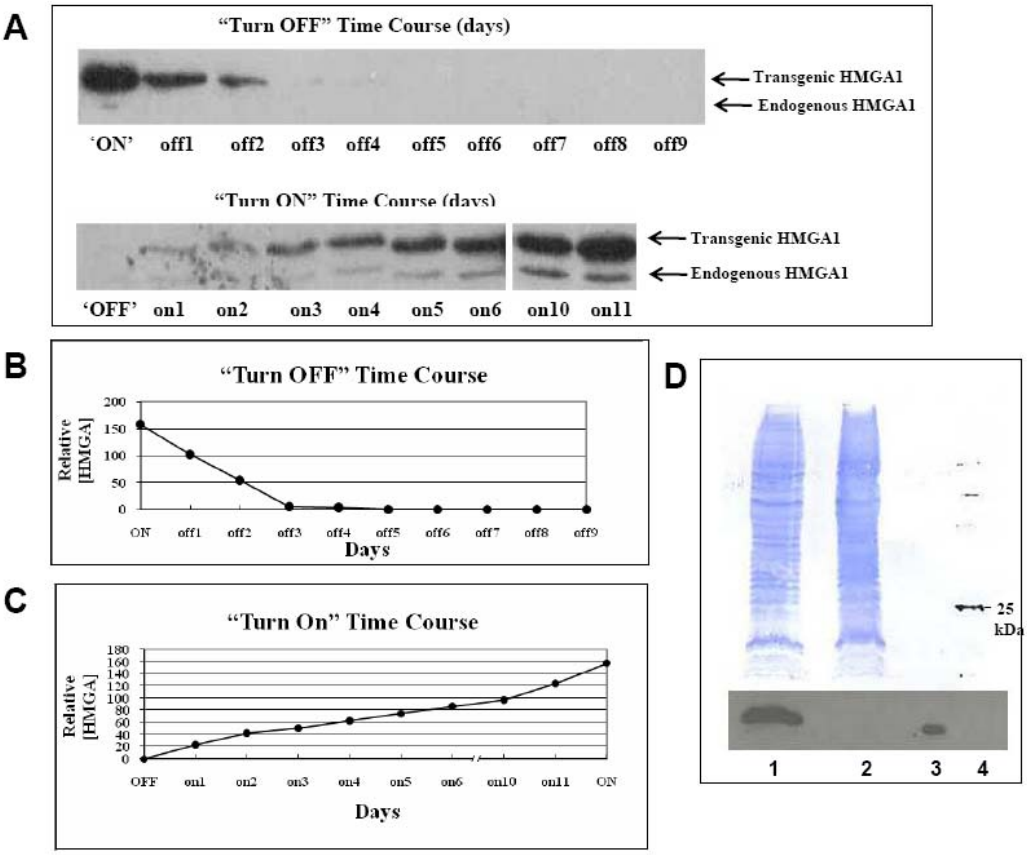
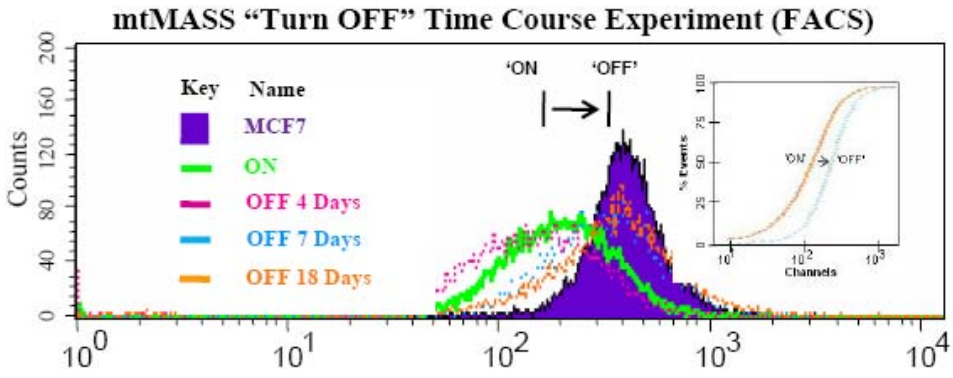
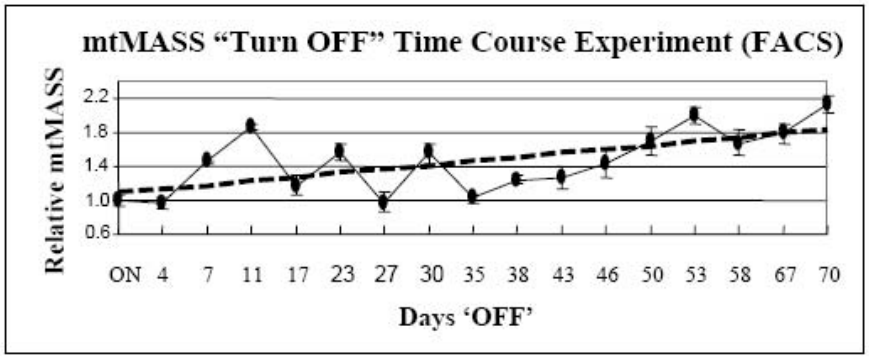

A
"Turn OFF" Time Course (days)
Transgenic HMGA1
Endogenous HMGA1
'ON' off1 off2 off3 off4 off5 off6 off7 off8 off9
"Turn ON" Time Course (days)
Transgenic HMGA1
Endogenous HMGA1
'OFF' on1 on2 on3 on4 on5 on6 on10 on11
B
"Turn OFF" Time Course
Relative [HMGA]
200
150
100
50
0
ON off1 off2 off3 off4 off5 off6 off7 off8 off9
Days
C
"Turn On" Time Course
Relative [HMGA]
180
160
140
120
100
80
60
40
20
0
OFF on1 on2 on3 on4 on5 on6 on10 on11 ON
Days
D
25 kDa
1 2 3 4

Fig. 1

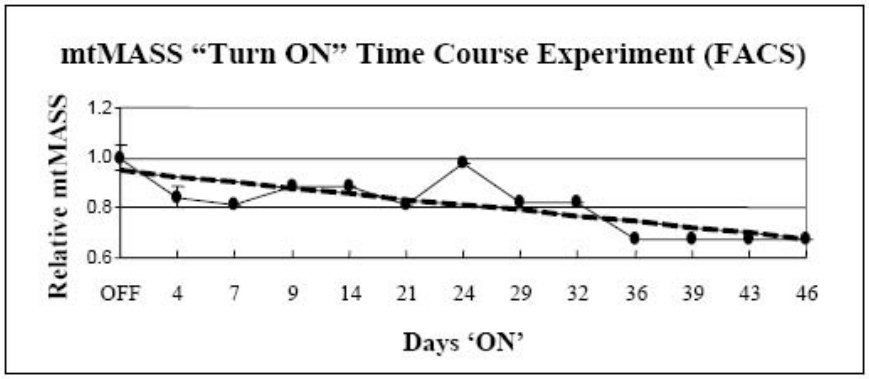
A


mtMASS "Turn OFF" Time Course Experiment (FACS)
Key
Name
MCF7
ON
OFF 4 Days
OFF 7 Days
OFF 18 Days
'ON'
'OFF'
Counts
% Events
Channels

B


mtMASS "Turn OFF" Time Course Experiment (FACS)
Relative mtMASS
ON 4 7 11 17 23 27 30 35 38 43 46 50 53 58 67 70
Days 'OFF'

C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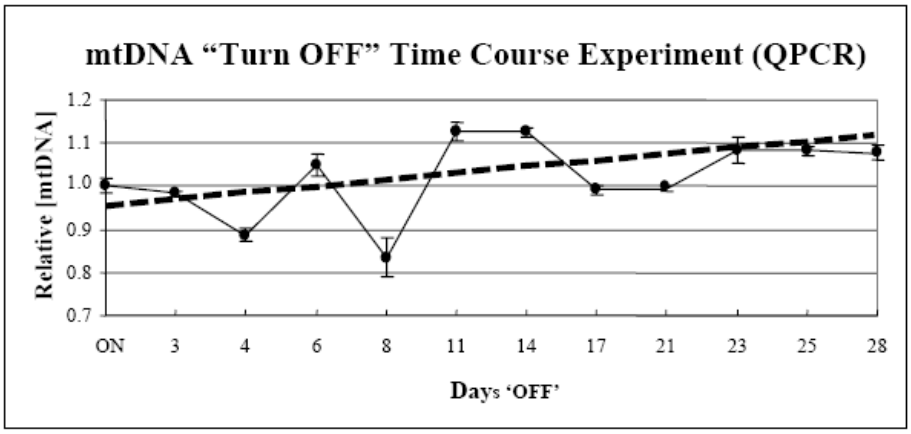
Relative mtMASS
OFF 4 7 9 14 21 24 29 32 36 39 43 46
Days 'ON'

Fig. 2

A


mtDNA "Turn OFF" Time Course Experiment (QPCR)
Relative [mtDNA]
1.2
1.1
1.0
0.9
0.8
0.7
ON 3 4 6 8 11 14 17 21 23 25 28
Days 'OFF'

B

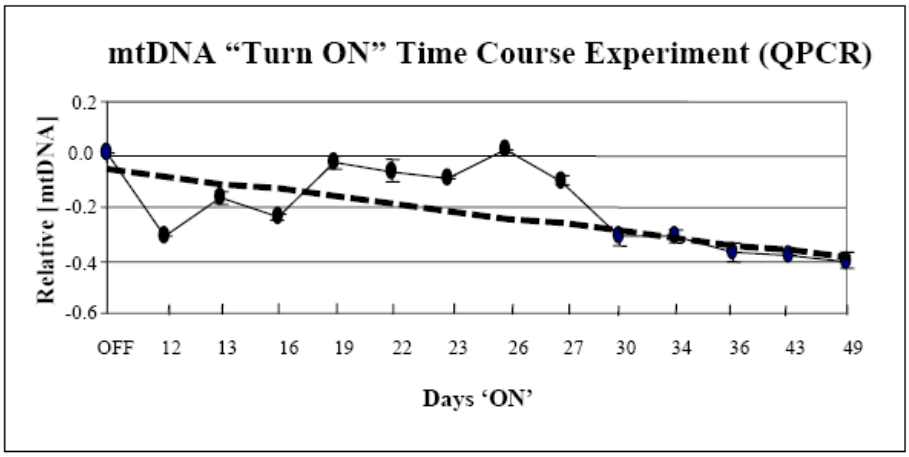

mtDNA "Turn ON" Time Course Experiment (QPCR)
Relative [mtDNA]
0.2
0.0
-0.2
-0.4
-0.6
OFF 12 13 16 19 22 23 26 27 30 34 36 43 49
Days 'ON'

Fig. 3.

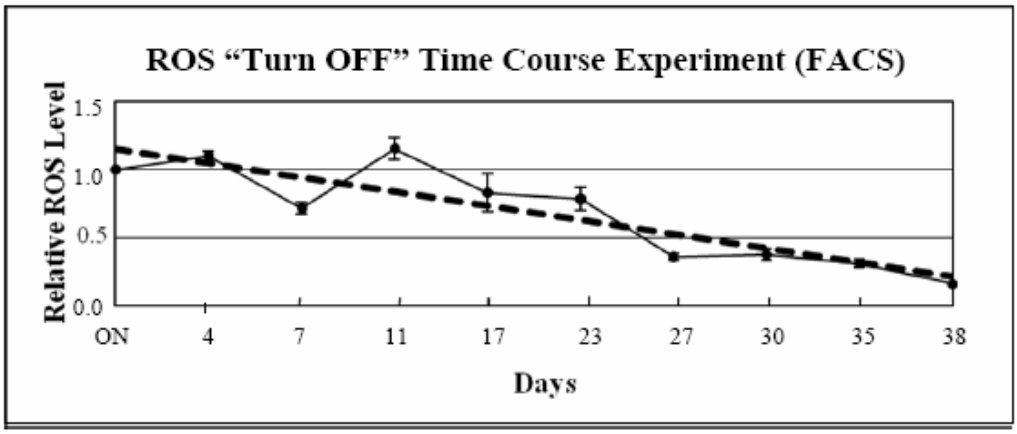

ROS "Turn OFF" Time Course Experiment (FACS)
Relative ROS Level
1.5
1.0
0.5
0.0
ON
4
7
11
17
23
27
30
35
38
Days

Fig. 4

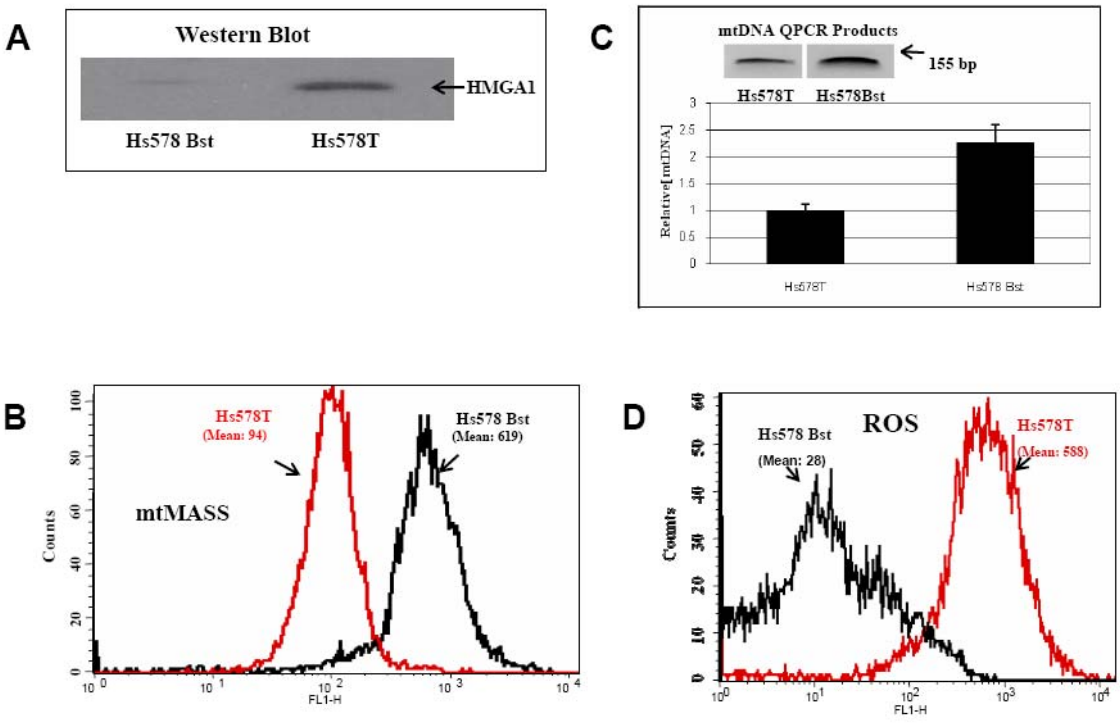

A
Western Blot
HMGA1
Hs578 Bst
Hs578T
C
mtDNA QPCR Products
155 bp
Hs578T
Hs578Bst
Relative[mtDNA]
Hs578T
Hs578 Bst
B
Hs578T
(Mean: 94)
Hs578 Bst
(Mean: 619)
mtMASS
Counts
FL1-H
D
ROS
Hs578 Bst
(Mean: 28)
Hs578T
(Mean: 588)
Counts
FL1-H

Fig. 5.

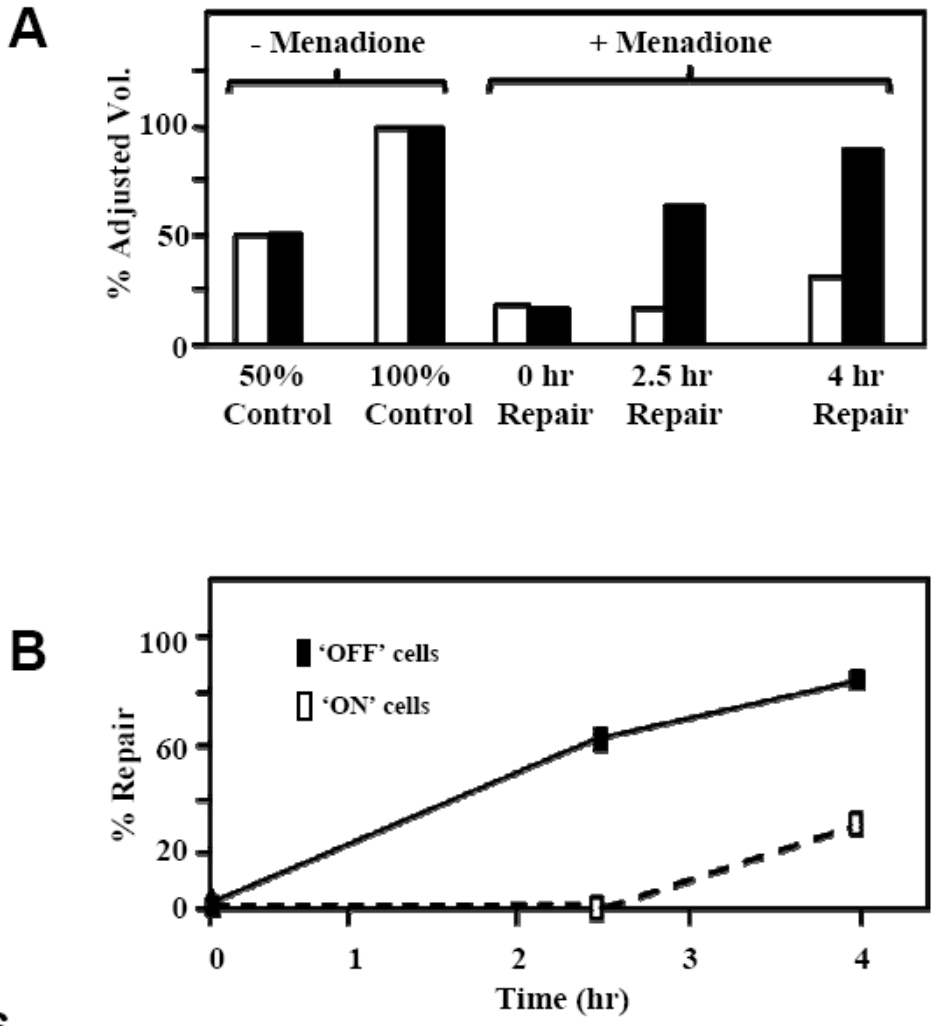

A
- Menadione
+ Menadione
% Adjusted Vol.
100
50
0
50% Control
100% Control
0 hr Repair
2.5 hr Repair
4 hr Repair
B
'OFF' cells
'ON' cells
% Repair
100
60
20
0
0
1
2
3
4
Time (hr)

Fig. 6

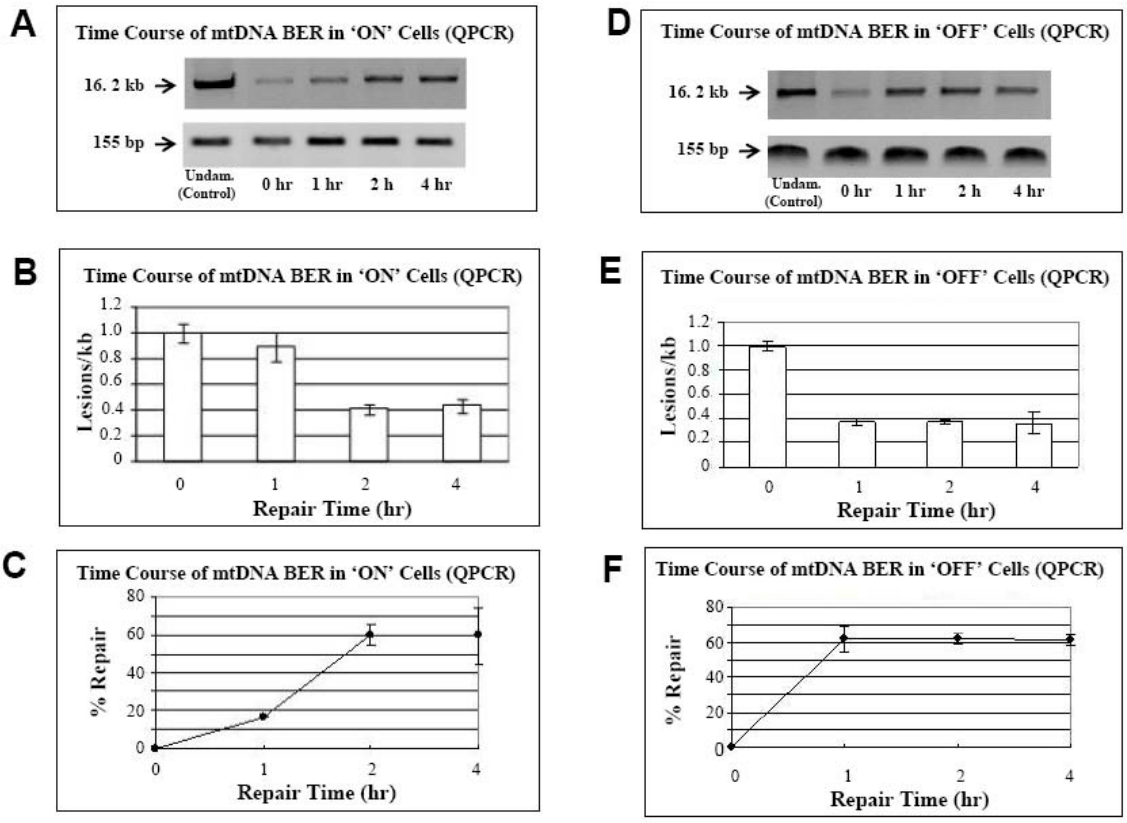

A
Time Course of mtDNA BER in 'ON' Cells (QPCR)
16. 2 kb
155 bp
Undam. (Control)
0 hr
1 hr
2 h
4 hr
D
Time Course of mtDNA BER in 'OFF' Cells (QPCR)
16. 2 kb
155 bp
Undam. (Control)
0 hr
1 hr
2 h
4 hr
B
Time Course of mtDNA BER in 'ON' Cells (QPCR)
Lesions/kb
1.2
1.0
0.8
0.6
0.4
0.2
0
0
1
2
4
Repair Time (hr)
E
Time Course of mtDNA BER in 'OFF' Cells (QPCR)
Lesions/kb
1.2
1.0
0.8
0.6
0.4
0.2
0
0
1
2
4
Repair Time (hr)
C
Time Course of mtDNA BER in 'ON' Cells (QPCR)
% Repair
80
60
40
20
0
0
1
2
4
Repair Time (hr)
F
Time Course of mtDNA BER in 'OFF' Cells (QPCR)
% Repair
80
60
40
20
0
0
1
2
4
Repair Time (hr)

Fig. 7.

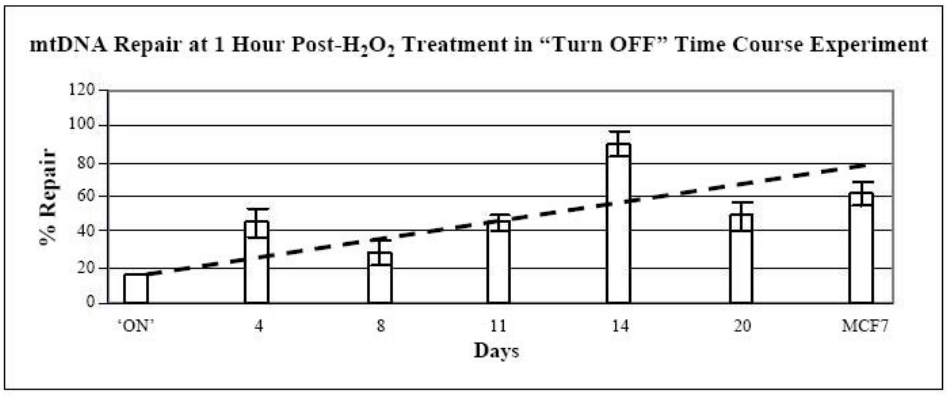

mtDNA Repair at 1 Hour Post-H_2O_2 Treatment in "Turn OFF" Time Course Experiment
% Repair
120
100
80
60
40
20
0
'ON'
4
8
11
14
20
MCF7
Days

Fig. 8.