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Nuclear HMGA1 Nonhistone Chromatin Proteins Directly Influence Mitochondrial Transcription, Maintenance, and Function

Gregory A. Dement, Scott C. Maloney, and Raymond Reeves*

School of Molecular Biosciences, Washington State University, Pullman, WA, 99164-4660

Abstract

We have previously demonstrated that HMGA1 proteins translocate from the nucleus to mitochondria and bind to mitochondrial DNA (mtDNA) at the D-loop control region [11]. To elucidate possible physiological roles for such binding, we employed methods to analyze mtDNA transcription, mitochondrial maintenance and other organelle functions in transgenic human MCF-7 cells (HA7C) induced to over-express an HA-tagged HMGA1 protein and control (parental) MCF-7 cells. Quantitative real-time (RT) PCR analyses demonstrated that mtDNA levels were reduced approximately 2-fold in HMGA1 over-expressing HA7C cells and flow cytometric analyses further revealed that mitochondrial mass was significantly reduced in these cells. Cellular ATP levels were also reduced in HA7C cells and survival studies showed an increased sensitivity to killing by 2-deoxy-D-glucose, a glycolysis-specific inhibitor. Flow cytometric analyses revealed additional mitochondrial abnormalities in HA7C cells that are consistent with a cancerous phenotype: namely, increased reactive oxygen species (ROS) and increased mitochondrial membrane potential ($\Delta\psi_m$). Additional RT-PCR analyses demonstrated that gene transcripts from both the heavy (ND2, COXI, ATP6) and light (ND6) strands of mtDNA were up-regulated approximately 3-fold in HA7C cells. Together, these mitochondrial changes are consistent with many previous reports and reveal several possible mechanisms by which HMGA1 over-expression, a common feature of naturally occurring cancers, may affect tumor progression.

Keywords

High Mobility Group; MCF-7; Mitochondria; D-loop; transcription factor; NADH dehydrogenase; cytochrome c oxidase; glycolysis; oxidative phosphorylation; reactive oxygen species; ATP; membrane potential

Introduction

The gene encoding for mammalian high mobility group A1 (HMGA1) proteins is a proto-oncogene whose over-expression is one of the most consistent biochemical markers of cancer cells, with increasing intracellular levels correlated with increased metastatic potential and malignancy [1]. HMGA1 proteins act as dynamic regulators of transcription within the nucleus where they alter global and local chromatin structure to induce or inhibit gene expression in response to multiple external and internal cell stimuli [1]. Such stimuli affect HMGA1 activity, in part, by altering their pattern of post-translational modifications which results in changes to

*To whom correspondence should be addressed: Rm. 639, Fulmer Hall; Ph: 509-335-1948; FAX: 509-335-9688; Email: reevesr@mail.wsu.edu

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the proteins ability to interact with different DNA and protein substrates [2,3,4]. Because of their small size (10.6-12 kDa) [5], inherent flexibility [6], and high levels of post-translational modifications, the proteins are uniquely equipped to adapt and function in a highly regulated manner. Additionally, HMGA1 proteins are unified by their possession of a versatile DNA binding motif called the AT-hook [7]. Each isoform resulting from alternative splicing of the *HMGA1* gene [8,9] contains three separate AT-hooks that allow the proteins to bind in a structurally specific manner within the minor groove of AT-rich B-form DNA [7,10]. This characteristic non-sequence specific DNA binding is a major factor contributing to the proteins ability to bind to multiple different genomic substrates. Finally, the ability of HMGA1 to mediate protein-protein interactions adds a level of regulated diversity to their activities displayed by their numerous protein partners and multiple specific gene substrates [4]. As a consequence of these unique characteristics, the number and types of demonstrated molecular and cellular functions for the HMGA1 proteins are still continuously expanding. Recent studies from our laboratory have revealed previously unrecognized rapid translocation of HMGA1 from the nucleus to the cytoplasm where it accumulates in the mitochondria and is able to bind to AT-rich DNA within the control D-loop region of the circular mitochondrial genome [11]. Evidence indicates that this nucleocytoplasmic movement is very dynamic, is cell-cycle dependent, and is both directional and reversible, with the protein moving from the nucleus to the cytoplasm and then back again. Earlier studies, interestingly, also demonstrated specific post-translational phosphorylation of the HMGA1 proteins by cdc2 kinase during the late G2 stage of the cell cycle, the precise stage at which the nucleocytoplasmic translocation was shown to occur [12]. This phosphorylation was further shown to reduce the capacity of the proteins to bind to DNA by 20-fold, indicating mobilization of the protein and a possible mechanism of translocation initiation. Regardless of translocation mechanism, the mitochondrial localization of the HMGA1 proteins opened new areas of research regarding possible organelle-specific functions for the proteins in respect to normal and abnormal cellular functions, including cancer.

Specifically, the mitochondrial genome is quite AT-rich [13] and because HMGA1 was found associated with the control D-loop region in living cells, the proteins likely play a role in expression and potentially replication of the mtDNA as these processes are intimately coupled [14]. Furthermore, because HMGA1 proteins are major contributors to the process of carcinogenesis, the many abnormalities being discovered in mitochondrial genome stability and overall organelle and organelle-related function associated with cancer phenotypes are intriguing. Particularly, it has been shown that mtDNA levels in multiple different cancer types are significantly altered as are the mitochondrial gene expression profiles. For example, rat fibroblast cells immortalized by viral and cellular oncogenes displayed increased transcription of the mitochondrial genes for COXI, COXII, and 16S rRNA [15]. Activation of genes coding for ATP6, ND5, cytochrome b, COXI, and COXII was also observed in papillary thyroid carcinomas as compared to normal thyroid tissue [16]. In fact, a general theme in the literature is the up-regulation of mitochondrial gene expression in the context of a cancer phenotype, while both increases and decreases in the levels of tumor cell mtDNA have been reported [17]. Nevertheless, there are also a few exceptions to this generality since some studies do show reduction in gene activity in certain cancer types such as glioblastomas [18]. Many of these changes in mtDNA expression and replication are thought to reflect mitochondrial genomic adaptation to perturbations in cellular energy requirements [19]. However, changes to mitochondrial genomic expression and replication may precede overall cellular metabolic abnormalities associated with cancer.

In fact, the role of mitochondria in carcinogenesis was first considered following observation, in 1956, that rodent tumors exhibited reduced respiration-coupled oxidative metabolism and increased glycolysis [20]. To date, these observations have not been adequately or fully explained but the above studies suggest that changes to mitochondrial gene expression profiles

observed in cancer cells may be involved in this Warburg phenotype. That is, deficiencies in the ability of a cell to perform oxidative phosphorylation, resulting in a switch to a glycolytic phenotype, may be a result of abnormalities at the mitochondrial genome level. Furthermore, such changes may also result from changes in expression levels of oncoproteins like HMGA1 that are consistently over-expressed in cancers and likely act as transcriptional regulators within the mitochondria.

In the current study, we explore the various possible functions that HMGA1 proteins might influence in the mitochondria. It was hypothesized that the proteins affect transcription/maintenance of the mtDNA and subsequently contribute to alterations in the overall cellular mitochondrial content and metabolic function. As reported below, this suggestion has proven to be correct and the present work reveals new roles for HMGA1 proteins in the process of normal cell function and carcinogenesis as it relates to mitochondrial activity.

Materials and Methods

Cell Culture

The tetracycline-regulated M/tet (referred to as parental or MCF-7) and HMGA1 transgene containing M/tet/HA-I (referred to as transgenic or HA7C) human breast epithelial MCF-7 cells were developed as described previously [21]. Briefly, the parental M/tet (MCF-7/tet-off) cell line was purchased from Clontech, Palo Alto, CA. This cell line was stably transfected with a plasmid vector (Clontech) containing the tetracycline response element (pTRE) driving the expression of a hemagglutinin (HA)-tagged HMGA1a transgene to generate the M/tet/HA-I (HA7C) line. All MCF-7 cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin (100 ug/ml), streptomycin (100 ug/ml), and G418 (100 ug/ml) to maintain selection of the tetracycline transactivator protein gene. HA7C cells were maintained and selected in the same media as for the MCF-7, and supplemented with hygromycin (100 ug/ml) for selection of clones containing the HA-HMGA1a expression vector. Western blot analyses of endogenous and transgenic HA-tagged HMGA1 protein levels in all cells lines were continuously monitored using a specific anti-HMGA1 rabbit polyclonal antibody as previously described [11].

Quantitative real-time RT-PCR analysis

Real-time PCR was performed using Custom TaqMan® Gene Expression Assays for mitochondrial and beta-globin DNA sequences and mitochondrial genes: cytochrome c oxidase subunit I (COXI), ATP Synthase 6 (ATP6), and NADH dehydrogenase subunit 6 (ND6) (Applied Biosystems, Foster City, CA, USA). Assays-on-Demand were utilized for human target genes, NADH Dehydrogenase subunit 2 (ND2) (assay ID: Hs00846374_s1 MTND2) and HPRT1 (assay ID: Hs99999909_ml HPRT1) (both assay kits were supplied by Applied Biosystems). TRIzol and phenol chloroform extracted total cellular DNA was isolated from MCF-7 and HA7C cells and 100 ng was used as template for real-time PCR for analysis of mitochondrial DNA levels using the beta globin gene as an endogenous control. Assays were performed in triplicate and repeated utilizing DNA isolation with Genomic Tip/20 Columns (QIAGEN). For the analysis of the mitochondrial ND2 gene using HPRT as the endogenous control, TRIzol extracted total RNA was purified using an RNeasy Kit (QIAGEN) and reverse transcribed using 2 µg in a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche) following the recommendations of the supplier. Following the reverse transcriptase reaction, 3 µl of the resulting cDNA was used as template for the RT-PCR using TaqMan Universal PCR Master Mix, No AmpErase (Applied Biosystems) as with the mitochondrial DNA assays. All reactions were performed in triplicate. All RT-PCR applications were performed on an ABI PRISM® 7000 Sequence Detection System. Amplification plots were generated from delta Rn values exported from the system software. Fold change was determined by the

comparative Ct method as described in User Bulletin #2: Relative Quantitation of Gene Expression (P/N 4303859) (Applied Biosystems).

FACS Analysis for Mitochondrial Mass

Wild-type MCF-7 and HMGA1 over-expressing transgenic HA7C cells were grown in DMEM without phenol red and supplemented as described above. Removal of phenol red was done in order to prevent detection interference during flow cytometry. Cells were stained with 100 nM nonyl acridine orange (NAO) (Invitrogen: Molecular Probes), a metachromatic dye that accumulates in mitochondria regardless of the mitochondrial membrane potential. Specifically, the dye binds to cardiolipin in all mitochondria, regardless of their energetic state. Cells were grown to 70-80% confluence and incubated at 37°C in 5% CO₂ for 30 min. in the presence of 100 nM NAO. Cells were then washed with PBS, trypsinized, spun at 1000 rpm on a clinical centrifuge for 10 min. and resuspended in 1 ml of either pre-warmed PBS or media. Fluorescence activated cell sorting (FACS) analysis was performed on a FACScalibur (Becton Dickinson: Immunocytometry Systems) utilizing an emission wavelength of 488 nm and a 525 nm excitation detector. Data acquisition and analysis was performed using Cell Quest Pro Software.

ATP Bioluminescence Assay

Cells were grown to approximately 90% confluence under normal growth conditions and harvested by trypsinization. Following a single wash in 1X PBS, cells were resuspended in 10 ml of 1X PBS and the cell resuspension for each cell type counted three separate times using nine grids of a hemacytometer and 2X Trypan Blue stain. Cells were then diluted or spun down and resuspended to 5×10^5 cells/ml and subjected to eight 1:2 serial dilutions in PBS. Serial dilutions were prepared to ensure accurate determination of ATP utilizing the highly sensitive ATP Bioluminescence Assay Kit (Sigma Corp., St. Louis, MO). Accuracy was checked by comparing theoretical cell counts, based on serial dilutions, to final ATP luminescence values from each dilution and plotting the reduction in signal versus cell number. Additionally, reproducibility was accomplished by a tightly controlled regimen of sample analysis involving careful 3 min. incubations between each sample read following addition of the ATP Detection Reagent to the assay tube. Utilizing this method, data was highly reproducible and displayed minimal decreases in apparent ATP levels overtime. It was determined via the production of slope plots that although ATP levels for both cell types decreased overtime, the rate of decrease was equal for both cell types (data not shown).

2-Deoxy-D-Glucose Sensitivity Assay

MCF-7 and HA7C cells were seeded (200 μ l) into a 96-well tissue culture plate from a resuspension at a concentration of 1×10^4 cells/ml. Following 24 hrs to allow adherence and growth, media was replaced with complete growth media containing 0 (untreated), 1, 1.5, 2, 2.5, 3, 3.5, 4, and 4.5 mM 2-deoxy-D-glucose (Sigma), a specific inhibitor of glycolysis. Treatments were performed in triplicate within individual plates. The cells were then incubated at 37°C in 5% CO₂ for 96 hrs without changes to the media. Following drug treatment, DNA content was determined by a Hoechst 33258 (Sigma) staining method. Briefly, cells were washed in serum free media once and fixed by adding 100 μ l of 100% ethanol per well and letting sit at room temperature for 10 min. Following a second wash with serum free media, 100 μ l of TNE buffer (10 mM Tris, 1 mM EDTA, 2M NaCl, pH 7.4) was added to the cells. Hoechst 33258 stain was then diluted to 20 μ g/ml in TNE buffer and 100 μ l was added to each well. Cells were incubated at 37°C for 30 min. before analysis. Relative DNA content was determined by reading fluorescence intensities with a 360 nm excitation filter and a 460 nm emission filter on a 96-well plate reader (Wallac Victor-2). Arbitrary fluorescence units were

used to plot relative DNA content which was considered as relative cell number using Microsoft Excel.

FACS Analysis of Reactive Oxygen Species (ROS) Levels

Cells were seeded into phenol red (PR) free DMEM medium supplemented with 10% fetal bovine serum, penicillin (100 ug/ml), streptomycin and cell type specific selection reagents as in complete growth media described above. At approximately 90% confluence, freshly reconstituted cell permeant nonfluorescent 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) (Molecular Probes - Invitrogen) at a stock concentration of 10 mM (5 mg/ml in DMSO) was loaded directly into the growth plate to equal a final concentration of 10 uM (10 ul stock per 10 ml of PR (-) DMEM). The cultures were then incubated at 37°C and 5% CO₂ for 30 min. Following this incubation, positive control plates were treated with either 200 uM H₂O₂ or 400 uM menadione sodium bisulfite (water soluble), an inducer of ROS (Sigma) and incubated for an additional 30 min. Cells were then washed with PBS, trypsinized, centrifuged, and resuspended in 1 ml of PBS containing 10 uM H₂DCFDA excluding 'no stain' control samples which were resuspended in PBS alone. Samples were then analyzed immediately on a FACSCalibur utilizing excitation at 488nm and FITC filter detection parameters. Cell Quest Pro software was used for both data acquisition and analysis to produce histogram plots and median peak values.

FACS Analysis of Mitochondrial Membrane Potential ($\Delta\psi_m$)

Cells were grown in complete DMEM to an approximate confluence of 90%. Following harvest by trypsinization, cells were counted, spun down and resuspended in complete growth media to a concentration of 0.5×10^6 cells/ml. Cells were then distributed in 1 ml aliquots to eppendorf tubes and DiOC₆ (Molecular Probes – Invitrogen) (stock was at 40 uM in EtOH) was added to a final concentration of 20 nM (0.5 ul to 1 ml sample to keep EtOH concentration below 0.1%) in samples labeled for staining. This was followed by incubation in a 37°C water bath for 30 min. As a control for reduced $\Delta\psi_m$ the uncoupling agent, CCCP, was added to certain samples to a final concentration of 10 uM for an additional 30 min. at 37°C. Stock CCCP (20 mM) was in EtOH and was thus added at low volumes (0.5 ul) to keep EtOH concentrations below 0.1% total in the treated samples. Samples were then analyzed immediately on a FACSCalibur utilizing excitation at 488nm and FITC filter detection parameters. Cell Quest Pro software was used for both data acquisition and analysis to produce histogram plots and median peak values.

Results

HMGA1 Proteins Alter Mitochondrial DNA Transcription and Replication

Previous work in our laboratory, utilizing a modified “chromatin” immunoprecipitation (ChIP) assay, revealed that HMGA1 proteins associate with the D-loop region of the mitochondrial DNA, in living cells [11]. Binding of HMGA1 in these studies was precisely shown to occur at a specific region upstream of the mtDNA heavy strand promoter (HSP) transcription start site and downstream of the light strand promoter (LSP) transcription start site (see Fig. 8). Due to the proximity of this region to important DNA elements involved in regulation of both mitochondrial DNA replication and transcription, we hypothesized that HMGA1 proteins may affect both of these processes directly. To test these ideas, HMGA1 proteins were over-expressed in a genetically engineered line of human mammary epithelial cells (MCF-7) and the affects of over-expression, regarding mitochondrial function, investigated.

In order to determine the effect of HMGA1 over-expression on the levels of mitochondrial DNA within MCF-7 breast cancer epithelial cells, a transgenic cell line (HA7C), expressing an HA-tagged HMGA1 fusion protein, under the control of a tetracycline-regulated promoter

[21], was used in comparison to parental (control) MCF-7 cells. Western blot results (Fig. 1) demonstrate that the total concentration of HMGA1 protein in the HA7C cells (as determined by densitometry) was ~ 39-fold greater than in the control MCF-7 cells, a level consistent with previous reports [21,45]. Highly purified DNA was isolated from both the HA7C and MCF-7 cell lines and used as template to perform quantitative real-time (RT) PCR. Triplicate data from individually isolated DNA sample sets revealed an overall down-regulation (-1.7 ± 0.5 fold) in HA7C mtDNA relative to MCF-7 cells, using beta globin as an endogenous DNA control (Fig. 2). The data indicated that over-expression of HMGA1 proteins results in a reduction in mtDNA copy number in HA7C cells. This suggested that HMGA1 over-expression might also reduce transcript levels of mitochondrially transcribed genes in these cells. In order to study this, the levels of four distinct mitochondrial gene transcripts (three from the heavy and one from the light mtDNA strands) were measured in the two cell types, again, using RT-PCR.

RNA harvested from the same TRIzol samples used for the isolation of DNA collected for mtDNA quantification was reverse transcribed to yield total cellular cDNA. This cDNA was subsequently used as template in RT-PCR reactions as above, using primers and probes specific to NADH Dehydrogenase subunit 2 (ND2), cytochrome c oxidase subunit I (COXI), ATP Synthase 6 (ATP6), NADH dehydrogenase subunit 6 (ND6), and the nuclear gene HPRT1 promoter as an endogenous control. Again, in triplicate, results indicated an overall up-regulation of ND2 (1.7 ± 0.3 fold), COXI (1.6 ± 0.2 fold), ATP6 (1.8 ± 0.2 fold), and ND6 (1.3 ± 0.2 fold) gene transcript levels in HA7C cells relative to the wild-type MCF-7 cells. However, because the mitochondrial DNA levels in these same cells was shown to be reduced by approximately two fold, the total fold change for the ND2, COXI, ATP6, and ND6 genes were normalized to mtDNA levels. After normalization, the total up-regulation of these genes is approximately 3-fold in HA7C cells over-expressing HMGA1 protein compared to control MCF-7 cells (Fig. 2).

HMGA1 Over-expression Alters Mitochondrial Mass and Activity

Because mitochondrial DNA levels were shown to be reduced in HMGA1 over-expressing cells, it was assumed that other mitochondrial functions or characteristics would also be altered. Though the link between mitochondrial genome levels and organelle mass within cells is poorly understood, it was hypothesized that reduction in mtDNA levels would translate into a reduced level of total mitochondria in the cell. Using fluorescence activated cell sorting (FACS) analysis, transgenic HA7C and wild-type MCF-7 cells were comparatively studied by staining with the metachromatic dye, nonyl acridine orange (NAO), which stains mitochondria independently of mitochondrial membrane potential. Data analysis showed that a majority of the HMGA1 over-expressing cells had significantly reduced mitochondrial mass to control MCF-7 cells (Fig. 3A,B).

Because both mtDNA and overall mitochondrial mass in cells over-expressing HMGA1 was reduced relative to wild-type cells, experiments to determine potential corresponding alterations to cellular ATP production were performed. In order to ensure accurate cell counts and ATP detection, analysis was performed following stringent and repetitive cell counts as well as serial dilutions of cell re-suspensions. As expected, HMGA1 over-expressing cells displayed significantly reduced levels of overall cellular ATP production when compared to wild-type MCF-7 cells, as displayed by reduced luminescence detection utilizing a luciferase based ATP assay kit (Fig 4.). This reduction in the ATP levels of HMGA1 over-expressing cells was observed regardless of the cell number over a wide range of dilutions. We thus conclude that HMGA1 over-expression is associated with deficiencies in oxidative phosphorylation and may contribute to the glycolytic phenotype that is often observed in cancer cells which, almost universally, over-express HMGA proteins [1,4].

Having established that HMGA1 over-expression induces reproducible alterations in a number of mitochondrial parameters, studies were undertaken to determine the biological ramifications of such changes. Due to observations that tumor cells have a substantially greater capacity for aerobic glycolysis consistent with a reduction in oxidative phosphorylation [22], it was hypothesized that HMGA1 might contribute to deficient mitochondrial oxidative metabolism. If correct, treatment with 2-deoxy-D-glucose (2-DG), a specific inhibitor of glycolysis, would be expected to preferentially kill HA7C cells over-expressing HMGA1 in comparison to control, non-over-expressing MCF-7 cells. To test this prediction, exponentially growing populations of both cell types were treated for 96 hr with varying concentrations of 2-DG. The treated cells were then fixed, stained Hoechst 33258, and the DNA content in each population determined and used as an indicator of cell number or survival. The results of these cell growth assays (Fig. 5) showed that the number of phenotypically normal MCF-7 cells was largely unaffected by drug treatment up to between 4-5 mM concentrations. In contrast, the number of HA7C cells, which are overtly malignant [21], was noticeably reduced by as little as 1.5 mM 2-DG. At 3 mM 2-DG, HA7C cells were maximally sensitive in comparison to MCF-7 cells. These results are consistent with the suggestion that cellular reductions in mtDNA and mitochondrial mass caused by HMGA1 over-expression result in increased dependency on glycolysis for the production of ATP.

HMGA1 Contributes to Cancer Related Mitochondrial Abnormalities

In addition to ATP, oxidative phosphorylation in the mitochondria produces reactive oxygen species that can damage mtDNA and negatively affect protein function, leading to enhanced cancerous transformation and increasing oxidative stress [19]. Due to its effects on cellular activities described above, it was hypothesized that HMGA1 over-expression may indirectly lead to changes in total cellular ROS via inefficiencies in the electron transport system of individual mitochondria. To investigate this possibility, H₂DCFDA, which is cleaved within cells and fluoresces in response to oxidation, was used as an indicator of oxidative stress in MCF-7 versus HA7C cells. Analysis of cell staining using flow cytometry revealed an approximately 40% increase in relative ROS levels in the majority of cells over-expressing HMGA1 (Fig 6). Control studies were done to ensure proper staining with H₂DCFDA and actual detection of oxidative stress. These controls included treatment of both cell types with either H₂O₂ or menadione (an ROS generator) and the results showed increased fluorescence detection indicative of increased levels of ROS as a result of both treatments (Fig. 6). Furthermore, unstained cells, as expected, showed only very low levels of autofluorescence in both cell types (Fig. 6). Increases in ROS levels have been associated with a cancerous phenotype and are thought to play a major role in cancer related increases in mtDNA mutations and alterations to electron transport efficiency [19]. Therefore, it was hypothesized that mitochondria from HMGA1 over-expressing cells may display downstream effects of these changes, such as altered membrane potential.

Mitochondrial membrane potential ($\Delta\psi_m$) was assessed in MCF-7 versus HA7C cells utilizing flow cytometry and the indicator DiOC₆, a probe that fluoresces in response to increasing $\Delta\psi_m$. Interestingly, results showed that HA7C cells had a significantly higher $\Delta\psi_m$ than MCF-7 cells (Fig. 7) indicating that HMGA1 may play a role in altering this fundamental characteristic of mitochondrial function. To verify that membrane potential in this assay was being accurately measured, the uncoupling agent CCCP was added to cells and, as expected, reduced $\Delta\psi_m$ (Fig. 7). Importantly, unstained cells showed a lack of significant interfering autofluorescence (Fig. 7).

Discussion

Here, we have demonstrated that HMGA1 proteins are not restricted to nuclear function, but rather influence essential mitochondrial DNA maintenance and organelle function as well. This

occurs through the ability of the proteins to associate with the regulatory D-loop of the mitochondria [11] and affect mtDNA levels as well as mitochondrial gene transcript levels (Fig. 2). To our knowledge, the results regarding the involvement of HMGA1 in the regulation of both mtDNA transcription and mitochondrial maintenance represent the only known incidence of a *bona fide* nuclear transcription factor being exported from the nucleus and functioning in a similar regulatory capacity within the matrix of the mitochondria. The downstream effects of these novel activities, particularly in the context of a cancerous phenotype in which the HMGA1 proteins are naturally over-expressed, are alterations to mitochondrial function and thus overall cellular metabolism (Figs 3-7). Such results strongly suggest that the HMGA1 proteins play a role in many of the mitochondrial abnormalities observed in cancer cells and may shed light on both normal and disease related mitochondrial function.

Localization of the HMGA1 proteins within mitochondria is a phenomenon that holds multiple new and exciting potential areas of research in the field. Based on the data presented here, it is clear that HMGA1 is playing a mitochondrial role that results in changes to the characteristics of the entire cell. Regarding non-disease related mitochondrial activity, there appears to be a normal function for HMGA1 proteins within the organelle, as association of the proteins with mtDNA occurs in both normal and highly-metastatic cells [11]. Furthermore, reversible movement of HMGA1 between the nucleus and mitochondria appears to be cell cycle dependent, indicating a potential role for HMGA1 in regulating mitochondrial biogenesis during the process of mitotic cell division. Thus, although the focus of the research presented in this manuscript has been on the exploration of a potential joint role for HMGA1 and mitochondria during carcinogenesis, the data may shed light on normal processes mediated by HMGA1 as well as processes related to other mitochondrial diseases.

Given the results shown in Figure 2 demonstrating that mtDNA levels are approximately 2-fold lower in over-expressing cells compared to controls, it seems likely that HMGA1 is playing a significant role in causing this reduction. Although we did not investigate mtDNA replication directly in the present study, several lines of evidence, nevertheless, suggest that HMGA1 proteins might inhibit this process *in vivo*. Perhaps the strongest support for this idea comes from our previous demonstration that in living cells HMGA1 protein binds to regions of the D-loop referred to as conserved sequence blocks (CSBs) [11], regulatory units involved in controlling replication of the vast majority of mtDNAs [46,47] (Fig. 8). Given such localized and specific D-loop binding, we suggest that if HMGA1 is indeed involved in inhibition of mtDNA replication *in vivo* it most likely is acting at the level of transcriptional termination during the formation of RNA primers from the LSP. During asymmetric mtDNA replication transcripts from the LSP are terminated at CSB sites, the first of which (CSB I) is quite high in A/T content [23], a preferred DNA-binding context for HMGA1 proteins [1]. The CSB blocks are essential for proper termination and primer formation that allows for a transition from RNA to DNA synthesis for the initiation of replication at the heavy strand origin (O_H) by mitochondrial DNA polymerase (pol γ) [24]. Since HMGA1 binding bends and induces other structural distortions in DNA substrates [4,6], any alterations to this D-loop region resulting from its binding could serve to inhibit the transition process and disallow initiation of replication. Because the processes of transcription and replication in mitochondria are so intimately coupled [46,47], any protein involved in altering or regulating transcription will also likely influence replication, as has been so elegantly shown for Tfam, an HMG box-containing mitochondrial transcription factor [48,49]. Thus, the demonstration (Fig. 2) that the level of ND6 message (derived from LSP transcripts- see below) is up-regulated in HA7C cells provides additional support for possible involvement of HMGA1 in replication since these cells also exhibit decreased levels of mtDNA. Although these findings are suggestive, further studies are certainly necessary to determine whether there is any direct involvement of HMGA1 in the inhibition of mtDNA replication.

In contrast to reduced mtDNA levels, we observed increased expression of ND2, COXI, ATP6, and ND6 mitochondrial gene transcripts by approximately 3-fold (Fig. 2) in HA7C cells over-expressing HMGA1. The messages coding for ND2, COXI and ATP6 are derived from a polycistronic transcript originating from the mtDNA heavy chain whose initiation is controlled by the HSP while the message coding for ND6 originates from a light chain transcript whose expression is controlled by the LSP (Fig. 8). The up-regulation of gene transcripts coded for by both mitochondrial DNA strands in over-expressing cells is of particular importance given that HMGA1 binds to a site in the D-loop that is located in close proximity to both the LSP and HSP promoters. This site could thus easily function as an enhancer element for both promoters and, following HMGA1 binding, increase transcription from both the heavy and light DNA strands, consistent with the present observations. Interestingly, in this connection, there are many examples of up-regulated heavy chain gene expression in different cancer types [15,16,25] and ND2 is especially significant since it has been reported to be specifically activated in malignant tissue as compared to normal tissue taken from the same colorectal cancer patients [26]. Taken together, the current data and published results support the possibility that HMGA1 proteins may serve a dual organelle function whereby binding to a single site in the D-loop influences the regulation of not only transcription but also replication of mtDNA (Fig. 8).

The consequence of this dual regulation may be the alteration of overall mitochondrial number, morphology, and ultimately function as suggested by the observed reduction in organelle mass within the HMGA1 over-expressing cells (Fig. 3). However, because the exact relationship between mitochondrial mass, mRNA, and mtDNA copy number have yet to be determined, it remains to be proven that the various changes in mitochondrial function reported here are a direct result of low mtDNA levels [19]. Nevertheless, while some cancer types show an increase in mtDNA levels, many also display an approximately 50% reduction in mitochondrial mass [27], a number consistent with our current findings presented here. Downstream of reductions in mitochondrial mass is the potential for obvious alterations in cellular metabolic activity.

Observed reductions in overall cellular ATP levels within HA7C cells versus MCF-7 cells (Fig. 4) are consistent with lower mitochondrial mass and furthermore suggest possible inefficiencies in oxidative phosphorylation within individual mitochondria. Reduced ATP levels are likely an indirect result of HMGA1 mediated reductions in mtDNA, as a loss of mitochondrially generated ATP has been shown within EtBr treated cells displaying a large percent reduction in mtDNA [28].

Further metabolic studies utilizing the MCF-7 cell model revealed that HMGA1 proteins may play a causative role in well characterized mitochondrially related cancer phenotypes. As demonstrated by Warburg in 1956, and confirmed here by results showing the reduced capacity of the highly metastatic HA7C cell line to survive in the presence of a glycolytic inhibitor, 2-DG (Fig. 5), cancer cells have abnormal metabolism. That is, cancer cells display a substantially greater capacity for aerobic glycolysis and an apparent decreased level of oxidative phosphorylation [20]. The role of the HMGA1 proteins in this phenotype is difficult to interpret but data suggest that the alterations to metabolic activity may be a consequence of HMGA1 activity at mtDNA elements of HSP and LSP. However, it has been suggested that increased expression of mtDNA-encoded respiratory chain complexes observed in solid tumors may reflect mitochondrial gene adaptations to perturbations in cellular energy requirements [19]. In order to sort these possibilities out, depletion studies are currently being performed in the laboratory utilizing tetracycline treatment to inhibit the expression of the HMGA1 transgene that is under the control of a tetracycline responsive promoter. Initial data suggests that the sensitivity to 2-DG treatment is reversed following down-regulation of the HMGA1 transgene (data not shown), indicating that HMGA1 may play a causative role.

Our results show that mitochondrially related cellular function is clearly altered by HMGA1 mediated reduction in mtDNA and subsequent changes to oxidative phosphorylation. Such changes may likely be mediated, or at least exacerbated, by the observed increase in ROS levels within HMGA1 over-expressing cells (Fig. 6). ROS levels are likely increased in this situation due to inefficiencies in electron transport resulting from reductions in mtDNA and subsequent alterations in the production of essential mitochondrially encoded oxidative phosphorylation enzyme complexes. Under these conditions, and to a lesser extent in normal functioning mitochondria, electrons may escape or leak from transport complexes I and III and react with molecular oxygen to form ROS [29,30]. The work contained here provides evidence that these alterations to ROS levels within the context of a cancer phenotype may, in part, be a product of aberrant HMGA1 expression.

Finally, in apparent contradiction to reductions in ATP production and inhibited mitochondrial function, HMGA1 over-expression within the MCF-7 cell model revealed a significant increase in $\Delta\psi_m$ (Fig. 7). Within normal cells, decreased ATP production is often accompanied by decreased $\Delta\psi_m$, however, this is not the case in transformed cells. In fact, many carcinoma cells have been shown to display increased $\Delta\psi_m$ [31], including human colonic carcinoma cells in which an increased intrinsic $\Delta\psi_m$ was associated with an increased probability of tumor progression [32]. Given the clearly defined glycolytic phenotype of cancer cells and their common characteristic of increased $\Delta\psi_m$, our results which show both a decrease in ATP production and an increase in $\Delta\psi_m$ within HMGA1 over-expressing cells are consistent with previous findings. This leaves many unanswered questions regarding how mitochondria, within the context of cancer, maintain $\Delta\psi_m$ and yet display reduced capacity for oxidative phosphorylation. Regardless of direct causation, increased $\Delta\psi_m$, potentially mediated indirectly by HMGA1 activity within the mitochondria, has been shown to be associated with resistance to apoptosis [33], again consistent with a cancerous phenotype and HMGA1 over-expression.

Historically, aberrant mitochondrial activity has been recognized as a consistent occurrence associated with the process of carcinogenesis and metastatic progression [34-42]. Additionally, mitochondria display abnormal genotypic and phenotypic characteristics within the context of multiple non-cancer diseases and aging [43]. Increased mutations, abnormal organelle morphology and membrane potential, changes in mtDNA expression and replication, and altered cellular metabolism are distinct changes to mitochondria and mitochondrial related processes observed in association with multiple cancer types and diseases [28,20,44]. Our results provide evidence that altered mitochondrial function within the context of a cancer phenotype may, in part, be a product of aberrant HMGA1 expression. Data suggests that such effects represent abnormalities in the normal mitochondrial role for HMGA1, which may be to regulate mitochondrial activity or biogenesis during the process of cell division. These findings regarding the relationship between HMGA1 proteins and mitochondrial function are compelling and certainly warrant further investigation.

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Figure 1.

Western blot using a specific anti-HMGA1 polyclonal antibody demonstrating that HA7C cells over-express transgenic HA-tagged HMGA1 protein ~39-fold above the level of endogenous protein found in parental, control MCF-7 cells. Lanes: (1) Recombinant human (rh) HMGA1 protein reference standard; (2) empty; (3) Extract of parental, non-transgenic control MCF-7 cells (12.5 ug of total protein isolated from $\sim 2 \times 10^6$ cells); (4) Extract of induced, transgenic MCF-7 cells (12.5 ug of total protein isolated from $\sim 2 \times 10^6$ cells) over-expressing HA-tagged HMGA1 protein. The positions of the endogenous, non-transgenic HMGA1 proteins, plus the HA-tagged transgenic HMGA1 protein in the gels are indicated by the arrows at the right-hand side of the figure.

Figure 2.

Fold change data for mtDNA levels and mitochondrial gene transcript NADH Dehydrogenase subunit 2 (ND2), cytochrome c oxidase subunit I (COXI), ATP Synthase 6 (ATP6), and NADH dehydrogenase subunit 6 (ND6) levels as determined by real-time PCR. Results demonstrate an approximately 1.5-2-fold down regulation in the levels of mitochondrial DNA in HA7C cells relative to MCF-7 cells. In contrast, there is an approximately 3-fold increase in the level of the analyzed mitochondrial gene transcripts when adjusted mathematically for the observed decrease in mtDNA in the HA7C cells. Fold change relative to an endogenous control was determined using the comparative Ct method (see Methods and Materials).

Figure 3.

Mitochondrial mass within HA7C cells is reduced relative to MCF-7 cells. Histograms show nonyl acridine orange (NAO) staining intensity on the logarithmic X axis and cell counts on the Y axis. A total of 10,000 events were analyzed by FACS analysis. **Panel A:** In the NAO (-) samples, autofluorescence was minimal. The shift in the HA7C peak to a lower staining intensity in the NAO(+) column represents an approximately 20% lower level of mitochondria in the majority of these HMGA1 over-expressing cells as compared to MCF-7 cells. Percent difference was calculated using median peak values as reported by Cell Quest Pro FACS analysis software. **Panel B:** Overlay histogram in which sample detection was reduced to center the peaks and provide a visual representation of the differences represented by the different median values.

Figure 4.

Total cellular ATP levels were reduced in HA7C cells as compared to MCF-7 cells. Analysis of ATP levels using a luciferase based bioluminescence assay kit required accurate cell counts and serial dilutions for accurate readings. Thus, data is represented as cell counts (x-axis) determined by serial dilutions of original cell resuspensions versus luminescence (y-axis) which is proportional to cellular ATP levels. Data shows a consistently lower level of ATP in HA7C across multiple serial dilutions and repetitions.

Figure 5.

Increased sensitivity of HA7C transgenic cells to treatment with the inhibitor of glycolysis, 2-Deoxyglucose (2-DG), as compared to wild-type MCF-7 cells. Relative fluorescence units represent Hoechst 33258 staining of 2-DG treated samples (1-4mM) relative to untreated samples. A value of 1 represents cell numbers equal to that of the non-treated samples.

Decreasing values represent a decrease in the number of cells as compared to the cell type specific non-treated control. The greatest difference between the HA7C and MCF-7 cells lines exists at 3 mM 2-DG.

Figure 6.

Transgenic HA7C cells display increased levels of reactive oxygen species (ROS) when compared to wild-type MCF-7 cells. Histograms show H₂DCFDA staining intensity on the logarithmic X axis and cell counts on the Y axis. A total of 10,000 events were analyzed by FACS analysis. Cells that were not stained (H₂DCFDA(-)) showed a lack of significant autofluorescence. The shift in the HA7C peak to a higher staining intensity in the H₂DCFDA (+) column represents an increased level of ROS production in the majority of these HMGA1 over-expressing cells. Treatment with H₂O₂ or menadione sodium bisulfite was used as a positive control and showed expected increased in ROS detection. Median peak values were those reported using Cell Quest Pro FACS analysis software.

Figure 7.

Mitochondrial membrane potential is increased in HA7C cells as compared to wild-type MCF-7 cells. Histograms show DiOC₆ staining intensity on the logarithmic X axis and cell counts on the Y axis. A total of 10,000 events were analyzed by FACS analysis. CCCP treatments showed reduced membrane potential as expected and verifying the efficacy of the staining method. Median peak values were reported by Cell Quest Pro FACS analysis software.

Figure 8.

Postulated dual function for HMGA1 binding at a single D-loop site. As adapted from [23], the schematic displays several important regulatory features of the D-loop region of mtDNA including the heavy and light strand promoter start site (HSP and LSP, respectively) and the conserved sequence blocks (CSB's). HMGA1, as determined in living cells by ChIP analysis, associates with the D-loop DNA close to CSB I [11] which is an essential region for proper termination of LSP transcription, a process required for the production of RNA

'primer' transcripts involved in the initiation of mtDNA replication [23,24]. Due to the ability of HMGA1 to bend DNA upon binding [1], this close proximity of HMGA1 association to CSB I potentially inhibits termination of LSP transcription and thus, as a consequence, prevents replication initiation. This possibility is supported by the observations that LSP regulated transcription of the ND6 gene (coded for by mtDNA light strand) is up-regulated ~3-fold in HA7C cells. Similarly, binding of HMGA1 to the same D-loop site, which is upstream of the HSP start site, could potentially also enhance transcription of the mtDNA heavy strand and explain the increase observed in ND2, COXI and ATP6 gene transcripts in over-expressing cells.