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THE ENZYMATIC PROPERTIES OF PEPTIDYLARGININE DEIMINASE 4 AND ITS NEGATIVE REGULATORY EFFECT UPON NSAID ACTIVATED GENE-1

A Thesis in

Biochemistry, Microbiology and Molecular Biology

by

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ABSTRACT

The repressive structure of chromatin is subject to a diverse collection of post-translational histone modifications which control transcription by regulating access to the underlying DNA. Combinations of epigenetic modifications affect gene expression through the 'histone code' in a synergistic or antagonistic fashion. These modifications are dynamically regulated allowing chromatin to transition between transcriptionally active or silent states. Thus, epigenetic marks arranged on chromatin function as a fundamental regulatory mechanism to control eukaryotic transcription.

Numerous residues located on histones are covalently modified by regulatory enzymes. Specifically, arginine residues located on core histone N-terminal tails may be mono-methylated, asymmetrically or symmetrically di-methylated. These arginine isoforms affect gene expression through two possible consequences: creation of possible binding sites for proteins and disruption of potential hydrogen bonding. Thus, methylated arginine residues function as crucial epigenetic marks.

Throughout this study, the histone modifying enzyme peptidylarginine deiminase 4 (PAD4), which catalyzes the deimination or demethylimination of arginine and mono-methyl arginine, was examined. PAD4 dynamically regulates arginine residues by producing citrulline, an unconventional amino acid. Although PAD4 modifies both arginine and mono-methyl arginine, there has been no evidence supporting that PAD4 regulates the di-methyl form. My studies present data indicating PAD4 may catalyze the 'demethylation' of di-methyl arginine through a novel regulatory mechanism.

Furthermore, two compounds were used to study the repressive effects of histone citrullination on gene expression. The first compound, Cl-amidine, irreversibly inhibits PAD4 activity thus preventing the dynamic regulation of arginine residues. The second compound,

resveratrol, increases expression of p53 target genes. MCF-7 cells treated with either compound displayed elevated expression of the TGF-β family member NSAID activated gene-1 (NAG-1). Chromatin immunoprecipitation following treatment with resveratrol revealed reduced levels of histone citrullination and increased levels of histone arginine methylation. Together, these studies indicate that PAD4 negatively regulates the transcription of NAG-1 through citrullination of the promoter nucleosomes.

MCF-7 cells treated with either compound showed reduced levels of growth, a possible downstream effect of the up-regulated tumor suppressor NAG-1. Following translation and secretion, this protein has been postulated to function through membrane bound receptors. Immunostaining against NAG-1 showed increased translocation to the cellular membranes after resveratrol treatment, indicating the protein may be inducing apoptosis. Thus, the inhibition of proliferating MCF-7 cells by resveratrol or Cl-amidine may be, in part, due to the increased expression of NAG-1.

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Chapter 1

INTRODUCTION

Chromatin: the Physiological Template of Eukaryotic Genetic Information

Within the eukaryotic nucleus resides the genetic coding material deoxyribonucleic acid (DNA), which is packaged around histones forming multiple nucleosome complexes (1). This heritable genetic material encodes genes that may be transcribed and ultimately translated. Although there are approximately three billion base pairs of DNA within the human genome, only a small fraction of the genetic material encodes genes or regulatory regions. The incredible length of DNA must be condensed to properly fit into the eukaryotic nucleus. DNA interacts with an octamer composed of four histone proteins: H2A, H2B, H3 and H4. The core histone octamer is composed of two dimers of H2A and H2B that interact with a tetramer of H3 and H4 (2). When combined, these proteins form a complex that allows 146 base pairs of DNA to be wrapped 1.7 times around the nucleosome (3). Each histone contains an extended N-terminal tail which protrudes from the octamer. These N-terminal tails retain chromatin structural stability, as certain histone tails interact with neighboring nucleosomes (4). For example, a cluster of basic residues on the N-terminal tail of H4 interacts with an acidic surface located on the H2A-H2B dimer of an adjacent nucleosome (4). Therefore, the core octamer maintains a repressive structure whereby DNA is condensed and obscured by histone proteins. Further compaction of genetic material is mediated by the linker histone H1. This protein interacts with the core histone octamer to allow further compression of DNA within the nucleus. The combined interactions between histones and DNA form chromatin, the physiological template of all eukaryotic genetic information.

The Histone Code and Cross-Talk

Post-translational histone modifications have been identified which regulate the transcription of genes by altering access to the enclosed DNA (5). Specifically, the core histone N-terminal tails may be modified by a plethora of enzymes that target specific amino acid residues. Histone modifications such as methylation, acetylation, phosphorylation, ubiquitination and citrullination have been shown to activate or repress transcription by different mechanisms. It has been proposed that these post-translational modifications work in either a synergistic or antagonistic fashion to regulate the transcription of genes, better known as the 'histone code' (5). For example, N-terminal tails of core histones are abundant in lysine and arginine residues. These amino acids may be post-translationally modified by specific enzymes to alter certain properties of the nucleosome complex. Lysines may be mono-, di- , or tri-methylated, acetylated or ubiquitinated, whereas arginine residues may be mono- or di-methylated. These modifications alter access to the underlying DNA individually or collectively. For example, it is generally accepted that hyperacetylated histones are associated with activated genomic regions (6). In contrast, deacetylated regions mainly result in repression and silencing (7). Thus, specific modifications upon the histone tails alleviate the repressive conditions created by nucleosomes.

Furthermore, certain epigenetic marks affect the placement of other modifications, which forms the basis of histone cross-talk (8). For example, a recent study has revealed a novel mode of histone cross-talk, in which H2A ubiquitylation controls the di- and tri-methylation of histone H3 lysine 4. Once histone H2A is ubiquitylated, histone H3 lysine 4 cannot be di- or tri-methylated, thus repressing transcription of target genes (9). Another study has shown that the di-methylation of histone H3 arginine 2 antagonizes histone H3 lysine 4 tri-methylation. Here, the di-methylated arginine residue represses transcription by inhibiting the tri-methylation of

histone H3 lysine 4 (10). Thus, specific modifications of histone tail residues can influence surrounding epigenetic marks. The histone code asserts that covalent histone modifications are not randomly arranged on chromatin, but rather specific epigenetic marks are found at precise locations in the genome and are therefore associated with different functional states of transcription.

Histone Arginine Modifications: Methylation and Its Counterparts

Protein arginine methyl-transferases (PRMTs) catalyze the methylation of epsilon nitrogen atoms of guanidinium side chains of arginine amino acids (11). These proteins transfer methyl groups from S-adenosyl-L-methionine (SAM) to targeted residues (12). Although certain PRMT isoforms reside in the cytosol such as PRMT5, particular proteins have been shown to reside in the nucleus (13). These nuclear isoforms epigenetically modify histone N-terminal tails by methylating specific arginine residues. Methylation is hypothesized to have two main consequences: first, it creates possible binding sites for proteins and second, it disrupts potential hydrogen bonding (14). Thus, these proteins function as a part of the histone code by affecting expression levels of targeted genes. There are currently two groups of PRMTs composed of eleven different isoforms (13). The first group, type I PRMTs, is composed of PRMTs 1, 2, 3, 4, 6 and 8, and is known to produce mono-methyl or asymmetric di-methyl arginine (figure 1). The second group, or type II PRMTs, is composed of PRMTs 5, 7 and 9, and produces either mono-methyl or symmetric di-methyl arginine (figure 1). Currently there is no known function for PRMTs 9, 10 and 11 (13). Both groups of PRMTs have been shown to alter the transcription rate of genes (12). Mono-methyl and asymmetric di-methyl arginine are associated with transcriptional activation, whereas symmetric di-methyl arginine is associated with transcriptional repression. Although these epigenetic marks correlate with specific gene

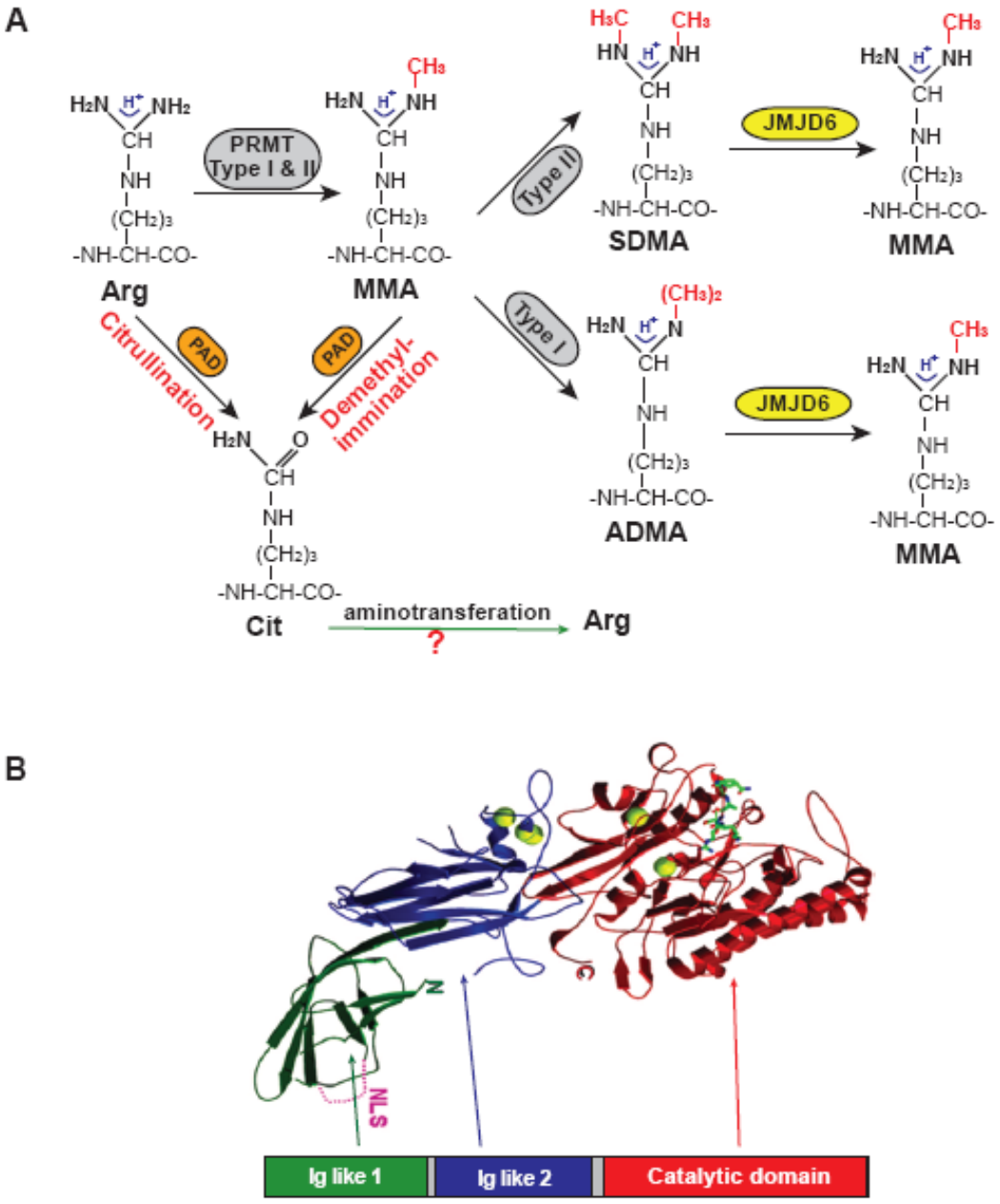

Figure 1: Known modifications of arginine residues. (A) Arginine residues are modified by PRMTs to produce either mono- or di-methyl isoforms. Although both type I and type II PRMTs produce mono-methyl arginine, they produce different di-methyl isoforms. Type I PRMTs produce asymmetric di-methyl arginine whereas type II PRMTs produce symmetric di-methyl arginine. Once di-methylated, JMJD6 removes a single methyl group from either guanidinium side chain. Unmodified or mono-methyl arginine residues are citrullinated by PAD4. Citrulline may be modified by an unknown aminotransferase allowing the reversion to the original arginine residue. (B) The crystal structure of PAD4 bound to five Ca^{2+} ions. The citrullination and demethylimination activity of PAD4 relies on the presence of DTT and Ca^{2+}. The crystal structure of PAD4 is altered by the presence of the Ca^{2+} ions.

expression levels, they cannot be exclusively used to determine transcriptional conditions. Arginine methyl marks work in conjunction with different histone modifications to synergistically or antagonistically regulate gene expression. Thus, modifications by PRMTs upon arginine residues function as a crucial part of the histone code by modifying many histone N-terminal arginine residues (figure 2).

Previous research alleged that methylated histone arginine residues were irreversibly modified by PRMTs (15). Although this is possible, it would require the removal of modified histones from nucleosome complexes to allow the reversion of arginine to the original form. Thus, the dynamics of the histone code would be distorted. For example, actively transcribed genes are associated with mono-methyl arginine residues (16). Upon inactivation, this epigenetic mark would need to be removed. This would require the substitution of an entire histone and the replacement of the epigenetic marks. This would disturb the dynamic nature of the histone code. However, a family of enzymes termed protein arginine deiminases (PADs) were known to produce citrulline (50), an unconventional amino acid created from arginine. Initially, these PAD isoforms were thought to produce citrulline from unmodified arginine residues. However, a protein containing a nuclear localization sequence termed peptidylarginine deiminase 4 (PAD4) was found to actively modify mono-methyl arginine (17). This led to the hypothesis that modified arginine histones could be dynamically regulated and incorporated into the histone code.

PAD4 was determined to citrullinate specific histone arginine and mono-methyl arginine residues (17). Citrullination removes the positive charge of the arginine residue, thus altering certain properties of the amino acid. This modification has been associated with repressed transcription of target genes (16). Thus, increased transcription by methylated arginine produced from PRMTs was reversed by citrullination. Although PAD4 has been shown to

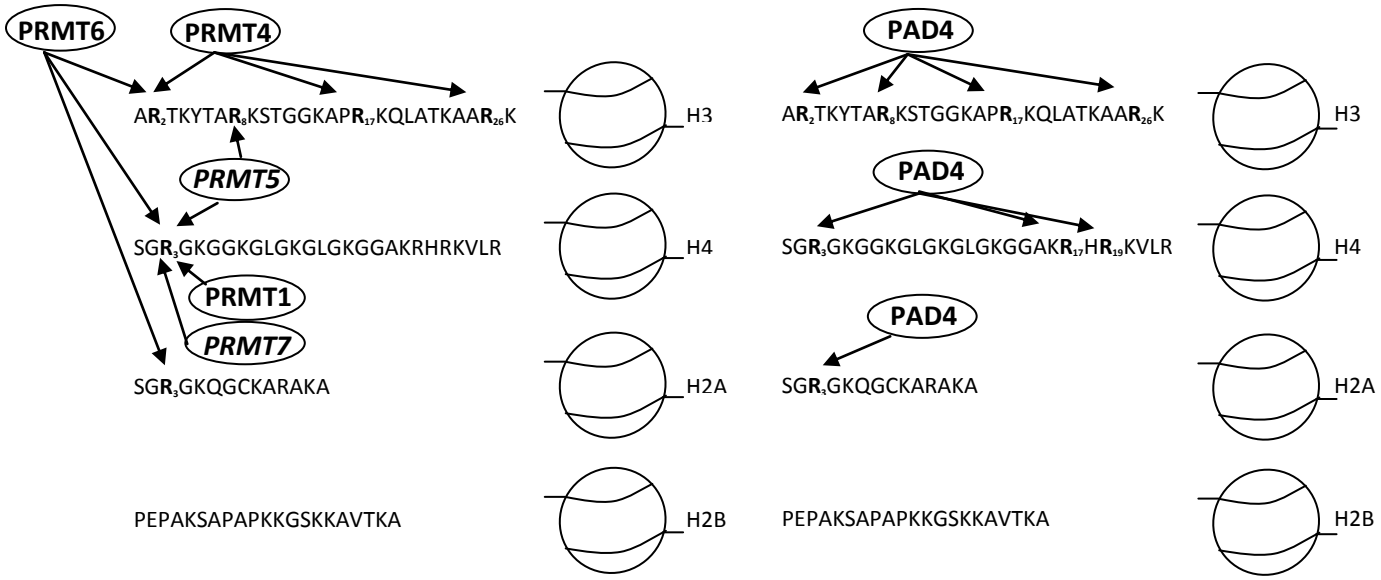

Figure 2: Histone N-terminal arginine residues associated with the histone code. The type I PRMTs 1, 4 and 6 produce mono- or asymmetric di-methyl arginine on histones H2A, H3 and H4. Specifically, these enzymes modify histone H3 arginine 2, 8, 17, 26, and histone H2A/H4 arginine 3. Type II PRMT5 produces mono- or symmetric di-methyl residues upon histones H3 and H4. In particular, PRMT5 modifies histone H3 arginine 8 and histone H4 arginine 3. PAD4 produces citrulline on numerous residues upon histones H2A, H3 and H4. Histone H2B remains unaffected by all enzymes.

'reverse' the specific epigenetic marks by PRMTs (figure 2), the complete reversal has been unresolved, as citrulline has been shown as an intermediate residue during this 'demethylation' process (figure 1). Once modified, there may be an unknown aminotransferase that converts citrulline to an unmodified arginine residue (16). Although arginine and mono-methyl arginine residues can be modified by PAD4, it was unclear whether both di-methyl forms of arginine could be altered. These findings left researchers questioning the existence of a dynamic regulatory enzyme that could 'reverse' the effects of di-methylated arginine.

Recently, a protein named JMJD6 was determined to reverse the effects of asymmetric and symmetric di-methylated arginine by removing a single methyl group from the terminal nitrogen atom (figure 1) (18). The discovery of JMJD6 proved that di-methylated arginine residues could be dynamically regulated by the reversion to the mono-methyl form. However, the activity of this protein to demethylate the mono-methylated arginine isoform is very poor. Nevertheless, following the reversion to the mono-methyl form, PAD4 could citrullinate the modified residue. Thus, PAD4, functions as an essential component in the regulation of histone arginine residues.

Data gathered from my studies indicate PAD4 functions as a larger part of the histone code than previous postulated. Past research has shown that arginine and mono-methyl arginine residues were targeted by PAD4 (17). However, my recent data has shown di-methylated residues may be modified by PAD4. Following an incubation of purified nucleosomes with PAD4, there were reduced levels of di-methyl arginine shown by Western blot analyses. This supports a hypothesis that PAD4 modifies di-methyl arginine residues. Although further research must be completed, these studies uncovered a possible novel regulatory mechanism by PAD4.

The Transcription of NSAID Activated Gene-1 is Negatively Regulated by PAD4

Previously, a PAD4 inhibitor termed Cl-amidine was produced which irreversibly inactivated the enzyme (19). Cl-amidine inhibited PAD4 activity by covalently modifying an active cysteine residue within the protein. The addition of Cl-amidine *in vitro* or *in vivo* inhibited PAD4 in a dose dependant manner (figure 3). By using this compound, the downstream effects of arginine regulation may be studied. Theoretically, the inhibition of PAD4 would subsequently reduce or eliminate the dynamic regulation of histone arginine residues by preventing the citrullination of arginine and mono-methyl arginine. Therefore, Cl-amidine should affect the expression of genes regulated by PAD4. Microarray results on MCF-7 breast cancer cells treated with Cl-amidine indicated the inactivation of PAD4 increased the expression of numerous genes (Table 1). Particularly, a gene up-regulated following Cl-amidine treatment was non-steroidal anti-inflammatory drug activated gene-1 (NAG-1). The induction of this tumor suppressor gene has been associated with decreased proliferation of a multitude of cancer cell lines (20). Thus, the repressive effect of citrullination by PAD4 upon the promoter region of NAG-1 was studied in greater detail.

Resveratrol, a polyphenolic compound isolated from grape skin which exhibits many anti-oxidant and anti-tumorgenic properties has been shown to increase NAG-1 expression levels (21). This compound has undergone extensive investigation because of its potential as a chemo-preventative agent. Recent studies have shown numerous cell lines treated with resveratrol undergo either cell cycle arrest or apoptosis (21). Microarray analysis of prostate cancer cells treated with resveratrol showed increased expression of numerous genes associated with apoptosis, including NAG-1 (22). Furthermore, this compound has been shown to inhibit the expression of cyclooxygenase-2 (COX-2), a protein associated with tumor formation (22). However, many tissue types have reduced levels of this protein (24).

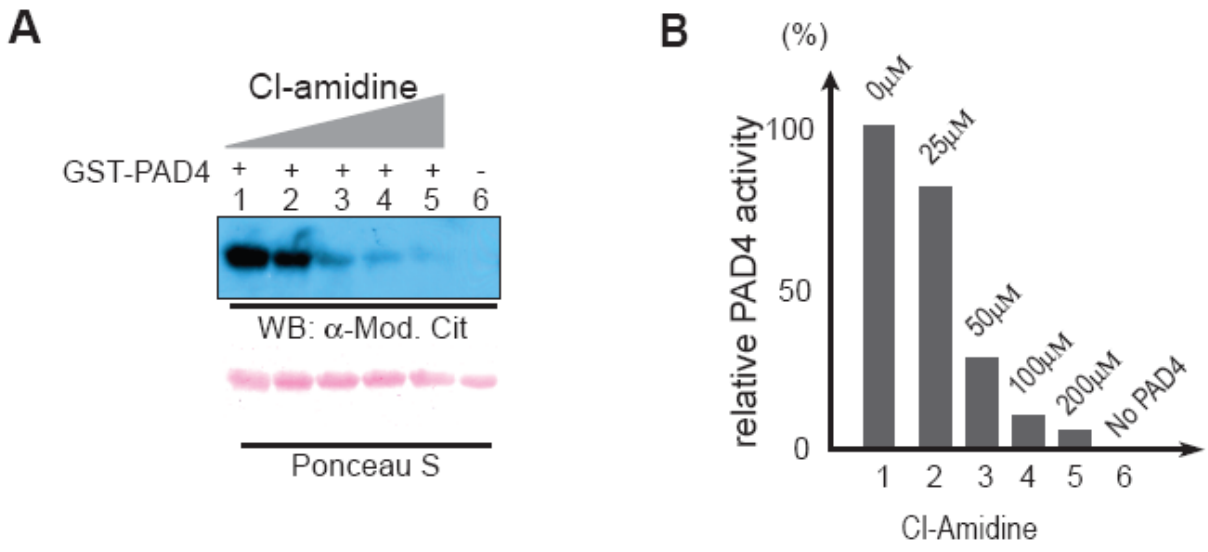

Figure 3: Cl-amidine inhibits the activity of PAD4 in a dose dependant manner. Various concentrations of Cl-amidine were incubated with 0.5 μg GST-PAD4 for 10 minutes in PAD assay buffer (10 mM Tris-HCl pH 7.6, 4 mM DTT, 4 mM $CaCl_2$, 1 mM PMSF) prior to incubation with 2 μg purified histone H3. An antibody against modified citrulline residues (α-mod-cit) determined the citrullination levels following PAD4 incubation. (A) Western analysis indicated Cl-amidine reduced the ability of PAD4 to citrullinate purified histone H3. Increased concentrations of Cl-amidine reduced the effectiveness of PAD4 by inhibiting the functionality of the enzyme. (B) Reduced levels of citrullination by PAD4 after treatment with Cl-amidine were quantified by the NIH Image J program. A near complete inhibition of PAD4 activity is found at the 200 μM concentration.

For the apoptotic effect of this protein to commence, it must be expressed in higher quantities. For example, previous studies indicated that resveratrol induced NAG-1 expression through increased p53 levels, leading to cellular apoptosis (23). Therefore, by using resveratrol, I determined the specific co-factors and the underlying histone modifications upon promoter nucleosomes which regulate the expression of NAG-1.

NAG-1 is a member of the transforming growth factor-beta (TGF-β) family cytokines that regulate many physiological processes such as cell proliferation, differentiation, motility and apoptosis (25). TGF-β family members exert their biological effects by signaling through membrane-bound receptors. Following translation, these proteins undergo di-sulfide dimerization within the endoplasmic reticulum and are secreted. Secreted proteins are known to interact with type II receptors (TβRII) located on the membrane of neighboring cells. The interaction with TβRII will cause the formation of a heterodimeric cell surface receptor complex with a type I receptor (TβRI) (26). Subsequently, the receptors initiate a phosphorylation cascade whereby the SMAD proteins are activated by phosphorylation within the cytosol. Once activated, the SMAD proteins translocate into the nucleus and initiate transcription of target genes (26). This pathway has been well studied and has been shown to initiate apoptosis in numerous cell lines (26).

The cellular processes of NAG-1 are similar to other TGF-β family members. Initially, NAG-1 is synthesized as an inactive protein precursor that undergoes di-sulfide-linked dimerization in the endoplasmic reticulum before being transported to the Golgi apparatus (27). The dimeric precursor is then cleaved at an RXXR site that separates the proprotein from the mature domain (27). The di-sulfide linked protein is then released and may interact with the membrane of neighboring cells. However, NAG-1 displays slightly different properties from other TGF-β family members. Mainly, the NAG-1 mature peptide can be correctly folded and

secreted without a propeptide (27). Nevertheless, overexpression of this gene increases apoptosis in a multitude of cell lines (20).

Previous reports indicated elevated levels of PAD4 in many tumor tissues, especially various types of adenocarcinoma (39). This indicates the inhibition of PAD4 in specific cancer types may subsequently reduce cell proliferation. Microarray analysis of MCF-7 cells treated with Cl-amidine determined possible genes negatively regulated by PAD4. Treated MCF-7 cells show numerous up-regulated p53 target genes, including OKL38 and NAG-1. This suggests that PAD4 may regulate the transcription of a subset of p53 target genes. Data gathered from my studies indicate that PAD4 negatively regulates NAG-1, a gene targeted by p53. This TGF-β family member has been shown as a potent inhibitor of cell proliferation, allowing cells to engage in apoptosis (20). My study addresses the transcriptional mechanism of NAG-1 initiation and demonstrates that PAD4 reduces NAG-1 expression through citrullination of the promoter nucleosomes. Therefore, inhibiting the negative regulatory enzyme PAD4 may reduce the abhorrent growth of cancerous cells, in part, through the activation of the tumor suppressor NAG-1.

Chapter 2

METHODS AND MATERIALS

Cell Culture

The cell lines, MCF-7, H1299 $p53^{-/-}$, HeLa S3, and U2OS, were cultured with DMEM (Gibco) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a 5% CO_2 incubator kept at 37°C. When sub-cultured, cells were washed with PBS and digested with trypsin for 3 minutes. Cells were then centrifuged at 1000 rpm for 5 minutes, resuspended in warm DMEM and transferred to new dishes. Cell number was determined using a hemocytometer.

Cell Proliferation Assay

MCF-7 cells were seeded for 24 hours in 10 cm dishes prior to treatment with either 50 μM resveratrol or 100 μM Cl-amidine in 6 ml DMEM for 12, 24 or 48 hours. Following treatment, cells were harvested with trypsin, and the cell number was determined using a hemocytometer. Shown data is representative of three independent experiments. An untreated culture was used as a control.

Microarray Analysis

Microarray analyses were performed on oligo DNA microarray chips carrying the majority of the human genes (Operon Biotech. Inc., https://www.operon.com/arrays/omad.php). Briefly, MCF-7 cells were treated with 200 μM Cl-amidine for 24 hours and total RNAs were extracted from treated and untreated groups. Differences in the gene

expression profiles were analyzed by hybridizing Cy3- or Cy5-labeled probes to the DNA chips. Results from the microarray were confirmed by RT-PCR.

RT-PCR

RNA was prepared from MCF-7 and U2OS cells in 10 cm diameter dishes with 6 ml DMEM. Briefly, cells were treated with 50 μM resveratrol or 100 μM Cl-amidine for 24, 48 or 72 hour periods. Cells were harvested by removing DMEM, washed with PBS and digested with trypsin. After centrifugation at 1000 rpm for 5 minutes, cells were collected and washed again with PBS. Using the RNAeasy kit (Qiagen), RNA was extracted following the manufacturers' protocol. The RNA was quantified by a UV spectrometer and normalized using the 18s RNA. Subsequently, RT-PCR (Invitrogen Superscript) was completed with the following primers; GAPDH: forward 5' TGAACGGGAAGCTCACTGG, reverse 5' TCCACCACCCTGTTGCTGTA, NAG-1: forward 5' CGCTCCGCGCGTCGCTGGAAG, reverse 5' GGAGCGACTCCCCGGTGTCGG, p53: forward 5' CCCACCATGAGCGTTGCT, reverse 5' CCACCCGGATAAGATGTTGG, PAD4: forward 5' GCTGTCTTGGAACACCAC, reverse 5' GGACTGCGAGGATGATG. PCR signals were detected between 26-30 cycles on agarose gels. Gel images were quantified using the NIH image J program.

Western Blots

MCF-7 cells were seeded for 24 hours in 10 cm dishes at 37°C with a 5% CO_2 incubator prior to treatment with either 50 μM resveratrol or 100 μM Cl-amidine in 6 ml DMEM for 12, 24 or 48 hours. Following treatment, cells were harvested by digestion, washed with PBS, and collected by centrifugation at 1000 rpm for 5 minutes. Subsequently, cells were resuspended in IP buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.2% Triton X-100, 0.2% NP-40,

supplemented with protease inhibitors), and sonicated (Diagenode Biodisruptor) for 5 minutes at high power. The lysate was centrifuged at 13000 rpm for 30 minutes and the supernatant was collected. Protein extract was run on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was stained with Ponceau S, imaged, and blocked with 5% non-fat milk for 30 minutes. Primary antibodies were added at the proper dilution and the membrane was rotated at 4°C overnight. Antibodies used throughout these assays were as follows; NAG-1 antibody 1:1000 (a generous gift from Dr. Thomas Eling), p53 antibody 1:3000 (Clone BP53-12, Sigma), β-actin antibody 1:10000 (Ab3280, Abcam), PRMT5 antibody 1:1500 (Ab50933, Abcam) and PAD4 antibody 1:1000 (rabbit polyclonal antibody against GST-PAD4). The following day, the membrane was washed extensively in tris buffered saline with 0.1% Tween 20 (TBST) at room temperature and incubated with a 1:1000 dilution of HRP-conjugated anti-rabbit or anti-mouse secondary antibody. Subsequently, the membrane was placed in a rotor at 4°C for 2.5 hours followed by three 10 minutes washes with TBST. The labeled proteins were detected using enhanced chemiluminescence reagents (Roche).

Chromatin immunoprecipitation (ChIP) Assay

MCF-7 cells were grown to 65-70% confluency in 14 cm diameter plates, followed by treatment with 50 μM resveratrol or 100 μM Cl-amidine in DMEM for 12 hours. Following treatment, cells were incubated with 1% formaldehyde for 10 minutes at room temperature. 2 M glycine was added to a final concentration of 0.125 M and further incubated for 5 minutes. Cells were then washed with cold PBS, scraped, collected and centrifuged at 1500 rpm for 5 minutes at 4°C. The supernatant was removed and cells were resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 5 mM EDTA, 0.5% SDS, NaCl 50 mM, supplemented with protease inhibitors). The solution was then centrifuged for 6 minutes at 1500 rpm at 4°C. Cells were re-

suspended in 3 ml ChIP buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5% Triton-x100, supplemented with protease inhibitors), sonicated for 3 minutes (Diagenode Biodisruptor), and centrifuged for 30 minutes at 13200 rpm at 4°C. Chromatin was run on a 1.25% agarose gel to monitor sizes of the fragments. Subsequently, 160 μL of the sonicated chromatin was diluted with 340 μL IP buffer and the solution was pre-cleared with 25 μL protein A beads at 4°C while rotated. After 2 hours, the beads were centrifuged at 2000 rpm for 3 minutes, removed, and the collected supernatant was centrifuged at 13200 rpm for 15 minutes. The desired antibodies were added and rotated overnight at 4°C. The following antibodies were used: p53: 6 μl (Clone BP53-12, Sigma), PAD4: 3 μl (GST produced), H3: 3 μl (07-690, Upstate Inc), H3Cit: 3 μl (Abcam, ab5103), H4Cit3: 5 μl (07-596, Upstate Inc), H3R17Me2: 5 μl (Abcam, ab8284), H4R3Me: 5 μl (07-213, Upstate Inc), Pol II Phospho Ser2: 3 μl (Abcam, ab5092), Pol II: 3 μl (Abcam, ab27459).

The following day, the mixtures were collected and centrifuged at 13200 rpm for 30 minutes. Subsequently, 20 μL protein A beads were added and the solution rotated at 4°C. After two hours incubation, the beads were collected by sedimentation for 10 minutes and then a total of nine washes were completed. Three washes were completed with Mixed Micelle buffer, two washes with Buffer 500, two washes with LiCl Detergent Buffer and 2 washes with EDTA Buffer. The beads were washed with 1 ml solution for 5 minutes by rotation at 4°C, followed by centrifugation at 2100 rpm for 1.5 minutes. Following the washes, the DNA was eluted by two successive washes with 100 μL elution buffer (1% SDS, 0.1M $NaHCO_3$) for 15 minutes. The collected samples were combined and then incubated at 65°C overnight to reverse the crosslinks. The following day, the solutions were collected, and treated with RNase A and proteinase K for a period of 3 hours at 37°C. The DNA was then collected by a PCR purification Kit (Quiagen) following the manufactures' protocol. PCR products were visualized

between 29-34 cycles of PCR reactions. Real time PCR (qPCR) reactions were performed using the SYBR-Green reagents (4367659, Applied Biosystems). QPCR primers used in these assays were located 254 base pairs upstream of the NAG-1 promoter, or +2976 downstream: -254 upstream: forward 5' AGACAGCCACCTAAACTCTTGTG, reverse 5' GCTCCTAGTAAAGCTAGGGCACAT. +2976 upstream: forward: 5' GGGAAGCTGACCTTGAGAAGATC, reverse 5' AGCCCACAGGTGTCGTAAGC.

Immunostaining

Untreated, 50 μM resveratrol or Cl-amidine treated MCF-7 cells were incubated in 6-well plates on a glass slide in 2 ml DMEM for 0, 24 and 48 hours at 50% confluency. Following 24 or 48 hours, the cells were fixed for 15 minutes in Solution I (5% paraformaldehyde, 0.1% Triton X-100, 0.2% NP40 in PBS) washed and then blocked with 2% BSA supplemented with Phosphate Buffered Saline with 0.1% Tween 20 (PBST) for 30 minutes. Subsequently, cells were extensively washed with PBST. Following the washes, cells were incubated overnight at 4°C with NAG-1 antibody diluted (1:500) in 2% BSA supplemented with PBS and 5% normal goat serum. The following day, MCF-7 cells were then washed extensively with PBST and incubated in a 1:1000 diluted Cy3 red-conjugated donkey anti-rabbit secondary antibody for 2 hours at room temperature. Cells were then washed extensively with PBST in the dark. Subsequently, cells were briefly stained with 1μg/ml Hochst, washed, and mounted on slides using glycerol-slow fade mounting media. The slides were viewed under a Zeiss microscope equipped with 40x objective lens.

PAD4 Activity Assay

For the PAD assay, 0.5 – 2.0 μg of GST-PAD4 or inactive GST-PAD4 C645S mutant was incubated with 1 μg nucleosomes for 1 hour at 37°C in PAD buffer containing 50 mM Tris-HCl pH 7.6, 4 mM $CaCl_2$ and 4 mM dithiothreitol. HeLa nucleosomes were a gracious gift from Dr. Joe Reese. Following 1 hour treatment, SDS loading buffer was added and the solution was heated to 95°C for 5 minutes to quench the reaction. The proteins were loaded on a 17% SDS PAGE gel, resolved and transferred onto a nitrocellulose membrane. The blot was then stained with Ponceau S and imaged. Western analysis was completed as previously described. Throughout the assay, the following antibodies were used: H3 R17 asymmetric di-methyl arginine: 1:1500 (Abcam, ab8284), H3 Cit: 1:3000 (Abcam, ab5103), H4/H2A Cit 3: 1:1500 (07-596, Upstate Inc), H4/H2A R3 symmetric di-methyl arginine: 1:1500 (Abcam, ab5823), H4/H2A R3 asymmetric di-methyl arginine: 1:1500 (Abcam, ab23574).

For the detection of citrullinated proteins, slight alterations were used within the procedure. Also, a modified citrulline antibody (Upstate, 17-341) was used. The assays were preformed as previously described with slight alteration. Following the transfer, the membrane was placed in 4% formaldehyde solution for 15 minutes. The membrane was then treated with two reagents that alter citrullinated arginine residues for a period of 3 hours at 37°C. Subsequently, the Western analysis was completed as previously described. Briefly, membranes were blocked with 5% non-fat milk with TBST and incubated overnight at 4°C with α-Mod-Cit (1:5000). The blots were then washed, incubated with a 1:5000 dilution of HRP-conjugated anti-rabbit secondary antibody and the labeled proteins were detected using enhanced chemiluminescence reagents (Roche).

Purification of PRMT5

Flag-tagged pcDNA3.1 PRMT5 plasmid was received from Dr. Xinyong Zhao. The plasmid was transformed into DH5α *E.coli* by heat shock. Briefly, 0.1 ng plasmid was added in a tube containing DH5α *E.coli*, left on ice for 30 minutes, heated at 42°C for 2 minutes, and placed on ice for 5 minutes. Subsequently, 1 ml 2x YT (16 g Bacto Tryptone, 10 g Bacto Yeast Extract, 5 g NaCl, per 1 L H_2O) was added to the test tube, and the *E.coli* were allowed to recover in a shaker at 37°C for 45 minutes. The *E.coli* was streak plated and individual colonies were grown overnight at 37°C. The following day, 6 tubes containing 3 ml YT and ampicillin were incubated in the rotor at 37°C overnight. The next day, PRMT5 plasmid was extracted using a plasmid extraction kit (Qiagen).

H1299 $p53^{-/-}$ cells were seeded in 10 cm dishes with DMEM 12 hours prior to the transfection at 85% confluency. Per plate, 60 μL 0.5 M HEPES was mixed with 60 μL 2 M $CaCl_2$ and 10 μg PRMT5 plasmid. Subsequently, 200 μl 2x HBS was added and the solution was incubated at room temperature for 20 minutes. The mixture was added to H1299 $p53^{-/-}$ cells. After 12 hours incubation, the media was replaced and cells were incubated further for 24 hours. Cells were then harvested and resuspended in IP buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.2% Triton X-100, 0.2% NP-40, supplemented with protease inhibitors). Cells were sonicated (Diagenode Biodisruptor) for 5 minutes and were then centrifuged at 13200 rpm for 30 minutes. 50 μL M2 agarose beads were added to the supernatant and the solution rotated overnight at 4°C. The beads were extensively washed with IP buffer the next day, followed by elution using Flag peptide. The elution process was repeated twice. The purified protein was analyzed by Ponceau S staining, Coomassie blue staining, Western blotting and silver staining to analyze the presence of PRMT5.

Silver Staining

Silver staining Kit (Invitrogen) was used following the manufactures' protocol. Sequentially, the gel was washed with water, placed in 20 ml fixative for 20 minutes, 30% ethanol for 10 minutes, 20 ml sensitizing solution for 10 minutes, 30% ethanol for 10 minutes, 20 ml of water for 10 minutes, 30 ml of staining solution for 15 minutes, 20 ml water for 40 seconds followed by the addition of 20 ml of developing solution for 8 minutes. The 3 ml stopper solution was added once the desired intensity was reached and the gel was imaged.

Methylation Assay

The methylation assay was performed using acid extracted HeLa core histones with Flag-tagged PRMT5 in a 25 μL reaction mixture containing 15 mM HEPES pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 20% glycerol, 1 mM EDTA, 0.25 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 2.75 μCi of *S*-[^{3}H]-adenosyl-L-methionine (Amersham Pharmacia Biotech., Inc.). After 1.5 hours incubation at 30°C, HeLa core histones were treated with SDS buffer, heated to 85°C for 4 minutes, and separated on a 17% acrylamide gel. The gel was fixed, treated with the enhancing solution NAMP100 (Amersham Pharmacia Biotech., Inc.), dried and exposed to x-ray film for 1 week at -80°C.

Nuclear Extract Preparation

Nuclear extracts were prepared following a protocol from Dr. Roeder's laboratory (Rockefeller University) with slight modifications. Briefly, about $1x10^8$ cells were collected by scrapping to detach the cells from the dishes. Cells were washed twice with PBS and once with hypotonic solution (10 mM Tris-HCl pH 7.3, 10 mM KCl, 1.5 mM $MgCl_2$ supplemented with protease inhibitors). Cells were then swelled in 5 ml of hypotonic solution for 10 minutes, and

homogenized in a Dounce homogenizer 15 times with a tight pestle. Cell lysis was checked under a microscope. Nuclei were collected by centrifugation at 3000 rpm for 10 minutes at 4°C, and then extracted with high salt buffer (20 mM Tris-HCl pH 7.3, 600 mM KCl, 1.5 $MgCl_2$, 0.2 mM EDTA, 25% glycerol, supplemented with protease inhibitors). The extracted nuclear proteins were dialyzed against a medium salt buffer (20 mM Tris-HCl pH 7.3, 300 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, with 1 mM PMSF).

Chapter 3

RESULTS

PAD4 Citrullinates Histone H2A, H3 and H4

Previously, PAD4 had been shown to produce citrulline from both arginine and mono-methyl arginine residues upon histone N-terminal tails (17). To determine the specific histones targeted for deimination or demethylimination, GST-PAD4 or inactive mutant GST-PAD4 C645S was incubated with purified HeLa nucleosomes followed by Western analysis. Initially, an antibody against modified citrulline residues was used to identify the precise histone proteins targeted by GST-PAD4. Figure 4A shows that histones H2A, H3 and H4 were citrullinated, whereas H2B and H1 remain unmodified following PAD4 treatment. Thus, three histones within the nucleosome structure were modified by GST-PAD4. Although these results indicate only GST-PAD4 treated nucleosomes contain citrulline, low levels of this unconventional amino acid occur naturally. An extended exposure showed low levels of citrulline upon GST-PAD4 C645S treated nucleosomes (figure 4D). Furthermore, Ponceau S staining throughout these assays showed a mobility shift of histones. This may be a downstream effect of the increased citrulline levels and consequent decreased positive charge upon arginine residues. Nevertheless, these results indicate PAD4 citrullinates histones H2A, H3 and H4, whereas histones H2B and H1 remain unmodified.

PAD4 Affects Unmodified, Mono- and Di-Methylated Arginine Residues

PAD4 has been shown to modify both arginine and mono-methyl arginine residues upon purified histone proteins (17). However, the di-methylated isoforms were unaltered by GST-PAD4 incubation (15). Previous assays incubated PAD4 with purified histone proteins or peptides, rather than entire nucleosome complexes. Thus, to determine if the nucleosome

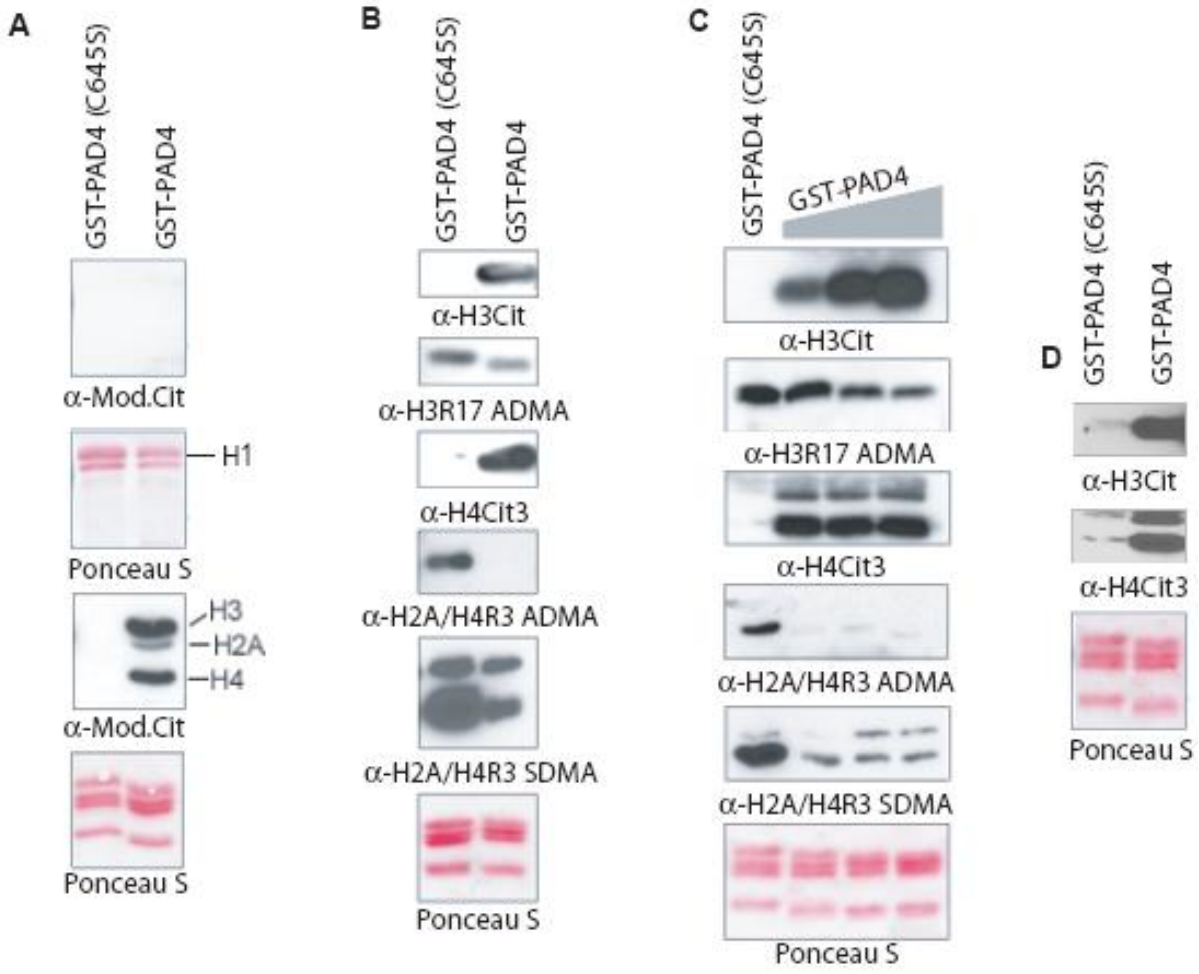

Figure 4: PAD4 increases histone citrullination and reduces histone arginine methylation. Purified HeLa nucleosomes or linker H1 histones were incubated with PAD4 in PAD assay buffer for an hour. Following treatment, Western analyses were completed using the specified antibodies. (A) The α-Mod-Cit antibody determined PAD4 affected histones H2A, H3 and H4, but not histones H2B or H1. (B and C) Nucleosomes treated with PAD4 show increased citrulline levels at histone H3 arginine 2, 8, 17 and histone H4 arginine 3. Asymmetric di-methyl arginine levels were reduced following PAD4 treatment at histone H3 arginine 17 and histone H4 arginine 3. Symmetric di-methyl arginine levels were reduced at histone H4 arginine 3. (D) Extended exposure showing natural levels of citrulline upon untreated nucleosomes.

structure affected the activity of GST-PAD4, purified nucleosomes were treated with the enzyme. Following GST-PAD4 treatment, Western analyses were completed against specific modifications upon histone N-terminal tails. Initially, an antibody against histone H3 citrulline 2, 8 and 17 showed increased levels of citrullination following GST-PAD4 incubation (figure 4B, H3Cit). Figure 4C showed increased concentrations of enzyme subsequently produced elevated amounts of citrulline upon histone H3. Although treatment with GST-PAD4 C645S indicated no citrulline expression, an extended exposure revealed the presence of the unconventional amino acid (figure 4D). Western analysis against histone H3 arginine 17 asymmetric di-methylation showed reduced levels of the di-methyl isoform following PAD4 incubation (figure 4B). Increased concentration of PAD4 incubation consequently reduced levels of the di-methylated isoform (figure 4C). However, the moderate reduction of histone H3 arginine 17 asymmetric di-methylation may be attributed to the unspecific ability of the antibody to detect the mono-methyl form (figure 5). Expectedly, PAD4 incubation increased histones H4 citrulline 3 levels (figure 4B). However, increased concentrations of PAD4 did not produce higher levels of citrullination (figure 4C). This indicates a saturation effect may have occurred whereby the majority of histone H4 arginine 3 residues were citrullinated with the lower concentration of PAD4. Another explanation may be that PAD4 preferentially affects histone H4 arginine prior to the modification of other residues. Histone H4 arginine 3 asymmetric di-methyl arginine levels reduced following PAD4 incubation. Increased treatment levels of PAD4 reduced the modified arginine levels to a negligible amount. However, this may also be attributed to the unspecific ability of the antibody to detect the mono-methyl form (figure 5).

PAD4 incubation with purified nucleosomes showed reduced levels of histone H4 arginine 3 symmetric di-methyl arginine (figure 4C). However, increased concentrations of PAD4 did not reduce the amount of the di-methylated arginine isoform. Once again, a saturation

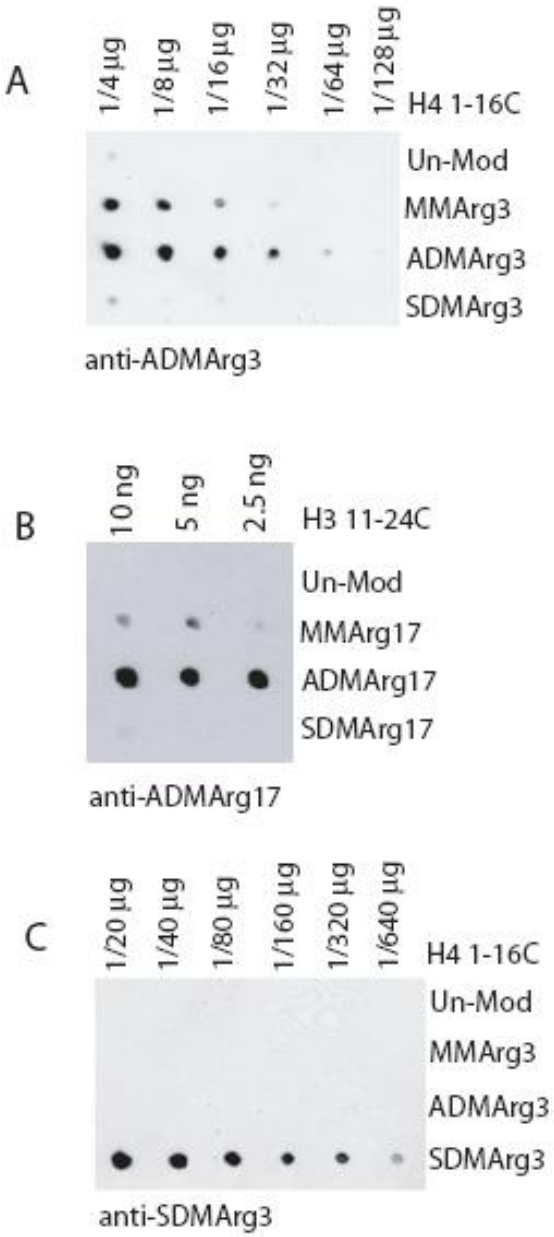

Figure 5: Dot blot analysis of histone methylation antibodies. The specificity of the antibodies used during the PAD4 modification assays upon purified HeLa nucleosomes was determined. (A and B) Both the asymmetric di-methyl arginine histone H3 arginine 17 and Histone H4 arginine 3 detect low levels of the mono-methyl form. (C) The symmetric di-methyl arginine antibody is very specific for histone H4 Arginine 3 symmetric di-methyl form. Dot blot analysis for the asymmetric di-methyl arginine histone H3 arginine 17 was completed by Upstate Biotech. The asymmetric di-methyl arginine Histone H4 arginine 3 was completed by Abcam.

effect may have occurred during the reaction. Dot blot analysis of the antibody showed a strong specificity for the symmetric di-methylated form. Therefore, these results indicate that PAD4 may affect symmetric di-methyl arginine residues within nucleosome complexes through a novel mechanism.

Purification of PRMT5

Previous results indicated PAD4 modified histone H4 arginine 3 symmetric di-methyl arginine residues. To confirm the demethylation ability of PAD4 upon di-methylated arginine, the type II methyl transferase PRMT5 was purified. Although primarily cytosolic, this protein has a nuclear localization sequence and has been determined to negatively regulate transcription (13). For example, tumor suppressor proteins non metastatic 23 (NM23) and suppressor of tumorigenicity 7 (ST7) are regulated by PRMT5 (40). Thus, symmetric di-methyl arginine residues function as an important epigenetic regulatory mark. Furthermore, PRMT5 requires post-translational processing to become active, thus requiring purification by cell culture (27). Therefore, Flag-PRMT5 was purified in H1299 $p53^{-/-}$cells. Figure 6A shows the purified PRMT5 protein from the H1299 $p53^{-/-}$cells. Subsequently, a methylation assay was completed to determine if the purified enzyme remained active. Figure 6C shows the enzyme actively methylated histone H3. Further experimentation using active PRMT5 upon purified nucleosomes needs to be completed to confirm the demethylation ability of PAD4 upon symmetric di-methyl arginine residues.

Microarray Analysis of Cl-amidine Treated MCF-7 Cells Show Increased Expression of NAG-1

PAD4 has been determined to negatively regulate gene transcription through the process of citrullination (17). By using an inhibitory compound to inactivate the enzyme, the

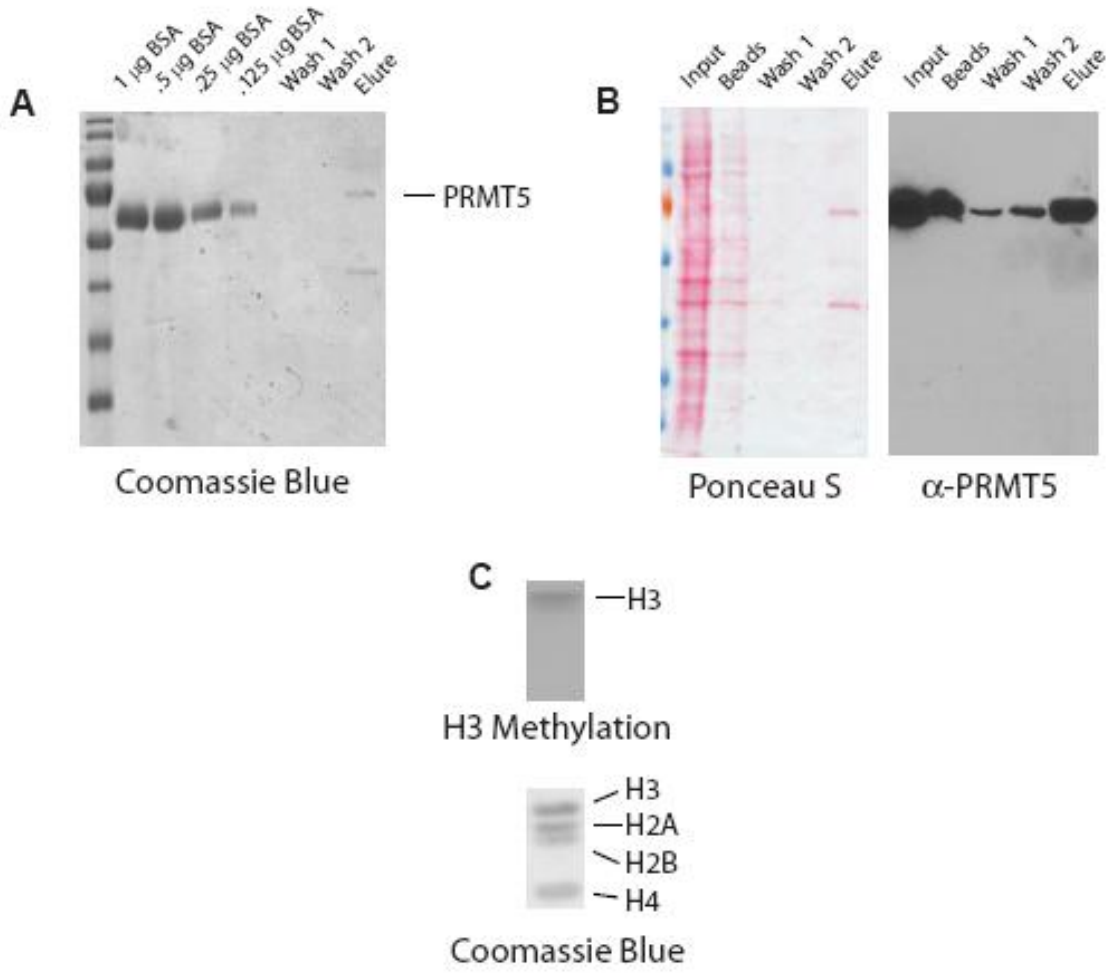

Figure 5: Purified Flag-PRMT5 methylates HeLa histones. Calcium phosphate co-precipitation was used to transfect the pcDNA3.1 PRMT5 plasmid into $p53^{-/-}$ H1299 cells. Following transfection, PRMT5 was purified from the protein extracts and confirmed by (A) Coomassie blue and (B) Ponceau S staining. Western analysis confirmed PRMT5 presence by anti-Flag and anti-PRMT5 antibodies. (C) Flag-PRMT5 was incubated with 2.5 μg HeLa histones in PRMT5 buffer. Histone H3 methylation was detected by fluorography.

negative regulatory effects of PAD4 upon transcription should be prevented. Therefore, MCF-7 breast cancer cells were incubated with the PAD4 inhibitor Cl-amidine for 24 hours. Microarray analysis of MCF-7 cells treated with 200 μM of the inhibitor showed doubled expression of 36 genes (table 1). In contrast, 3 genes had their expression rates reduced by a factor of two. Since Cl-amidine irreversibly inactivates PAD4, increased expression of genes in this microarray assay may indicate a dynamic regulatory action by citrullination. To confirm these results, a single gene from the microarray was studied in greater detail. Specifically, NSAID Activated Gene-1 (NAG-1) was studied to verify the negative regulation by PAD4.

Resveratrol and Cl-amidine Impede MCF-7 Cell Proliferation

Previous results indicated that resveratrol decreased or inhibited cell proliferation in a multitude of cell lines (22, 23, and 28). Recent microarray analysis of CD-18 cells treated with resveratrol indicated NAG-1 was up-regulated by 5.8 fold, nearly two fold higher than any other gene (22). Furthermore, resveratrol-induced growth inhibition was abolished in CD-18 cells transfected with NAG-1 siRNA (22). These results indicated NAG-1 acts as a potent anti-tumorgenic protein which causes apoptosis when overexpressed. Therefore, MCF-7 cells were treated with either resveratrol or Cl-amidine to determine if either compound reduced proliferation.

When MCF-7 cells were treated with resveratrol, a significant inhibition of cell proliferation was observed. Figure 7A shows an extensive inhibition of cell growth following treatment over 48 hours. At 12 hours post treatment, there was a reduction of cell density when compared to the initial count. Subsequently, the 24 hour and 48 hour growth rates showed a near complete inhibition of proliferation. Similar results were gathered when resveratrol was incubated with HCT-116 cells over a period of 48 hours (23). Therefore, these

Table 1. Gene expression increased by Cl-amidine in MCF-7 cells

Top increased genes after inhibitor treatment (36 genes increase >2fold)				
ID	Name	Log Ratio (Red/Green)	Log Ratio (Green/Red)	AVERAGE
H300004073	aldo-keto reductase family 1, member C1	3.76	3.14	3.45
H300010096	aldo-keto reductase family 1, member C2	3.51	3.24	3.38
H200014949	heme oxygenase (decycling) 1	3.59	3.08	3.33
H300016159	aldo-keto reductase family 1, member C3	3.19	2.96	3.07
H300021913	aldo-keto reductase family 1, member C3	2.97	2.99	2.98
H200000662	glutathione peroxidase 2 (gastrointestin	2.87	2.48	2.67
H200015716	similar to tripartite motif-containing 1 **(EBBP)**	2.52	2.14	2.33
H300007284	pregnancy-induced growth inhibitor **(OKL38)**	1.72	2.25	1.99
H300018984	leukotriene B4 12-hydroxydehydrogenase	1.96	1.97	1.96
H200002223	thioredoxin reductase 1	1.57	2.25	1.91
H300022279	UDP glycosyltransferase 1 family, polype	2.21	1.42	1.81
H300010767	tumor protein D53 **(TPD52L1)**	1.41	2.02	1.71
H300005394	ferritin, light polypeptide-like 1	1.63	1.67	1.65
H200009720	growth differentiation factor 15 **(NAG-1)**	1.77	1.52	1.64 ←
H300015102	leukotriene B4 12-hydroxydehydrogenase	1.68	1.55	1.61
H300003912	NADP-dependent malic enzyme	1.71	1.27	1.49
H200000006	ferrochelatase (protoporphyria)	1.15	1.73	1.44
H300005395	ferritin, light polypeptide	1.66	1.16	1.41
H200010382	glutamate-cysteine ligase, modifier subu	1.46	1.30	1.38
H200011541	ATP-binding cassette, sub-family B (MDR/	1.55	1.09	1.32
H200008571	sequestosome 1	1.58	1.02	1.30
H300014864	ankyrin repeat domain 24	1.28	1.27	1.27
H300002854	aldo-keto reductase family 1, member C4	1.14	1.30	1.22
H300016518	NAD(P)H:quinone oxireductase gene	1.54	0.86	1.20
H200013975	carbonyl reductase 3	1.08	1.30	1.19
H200010725	Ras-like without CAAX 1	1.18	1.09	1.13
H300007143	RAS, dexamethasone-induced 1	0.98	1.27	1.13

Table 2. Gene expression decreased by Cl-amidine in MCF-7 cells

Top decreased genes after inhibitor treatment (3 genes decrease>2fold)				
ID	Name	Log Ratio (Red/Green)	Log Ratio (Green/Red)	AVERAGE
H300015674	cytosolic sialic acid 9-O-acetylesterase	-1.41	-1.13	-1.27
H200006265	EGF-containing fibulin-like extracellula	-1.37	-0.77	-1.07
H300008416	selenium binding protein 1	-1.22	-0.83	-1.03

Table 1: Microarray analysis of MCF-7 cells treated with Cl-amidine. (A) MCF-7 cells were treated with 200 μM Cl-amidine for 24 hours to inhibit the dynamic regulation of histone arginine residues by PAD4. The transcription rate of a total of 36 genes doubled following microarray analysis. Conversely, the transcription rates of 3 genes decreased by a factor of two following PAD4 inhibition. These results correlate with the concept that citrulline residues are associated with repressed levels of transcription. The tumor suppressor NAG-1 expression levels increased over 3 fold following Cl-amidine treatment.

results are consistent with previous reports indicating that resveratrol inhibits growth and induces apoptosis.

Upon the addition of Cl-amidine to the culture, a reduction in cell proliferation was observed over a period of 48 hours. Figure 7A shows reduced cell proliferation following 48 hours treatment. An obvious reduction in cell growth of the Cl-amidine treated cells was displayed when compared to the untreated sample. These results suggest that PAD4 inhibition is responsible for impeding cell proliferation. This growth inhibition may be a result of the increased transcription of the tumor suppressor NAG-1, as seen in the microarray analysis.

Resveratrol and Cl-amidine Increase the Expression of NAG-1

Previous reports indicated that resveratrol increased NAG-1 expression in HCT-116 and CD-18 cell lines over 48 hours (22, 23). The elevated expression was correlated with increased levels of apoptosis within both cell lines. Therefore, further studies were completed to determine a possible relation between reduced MCF-7 growth rates and NAG-1 expression. RT-PCR and Western analyses were completed in MCF-7 cells treated with 50 μM resveratrol or 100 μM Cl-amidine to determine NAG-1 expression levels.

Initially, MCF-7 cells were treated with resveratrol or Cl-amidine for 24 or 48 hours. Following 24 hour resveratrol treatment, RT-PCR displayed elevated mRNA levels of NAG-1 and p53 as shown in figure 7B. The increased p53 levels correlate with increased NAG-1 expression, since there are two p53 binding motifs located near the NAG-1 promoter (29). Similarly, Cl-amidine treated MCF-7 cells showed increased NAG-1 expression; however, p53 levels remained comparable to the untreated group. This suggests the increased NAG-1 levels after Cl-amidine treatment are not related to p53 expression. Similar results were gathered following 48 hours

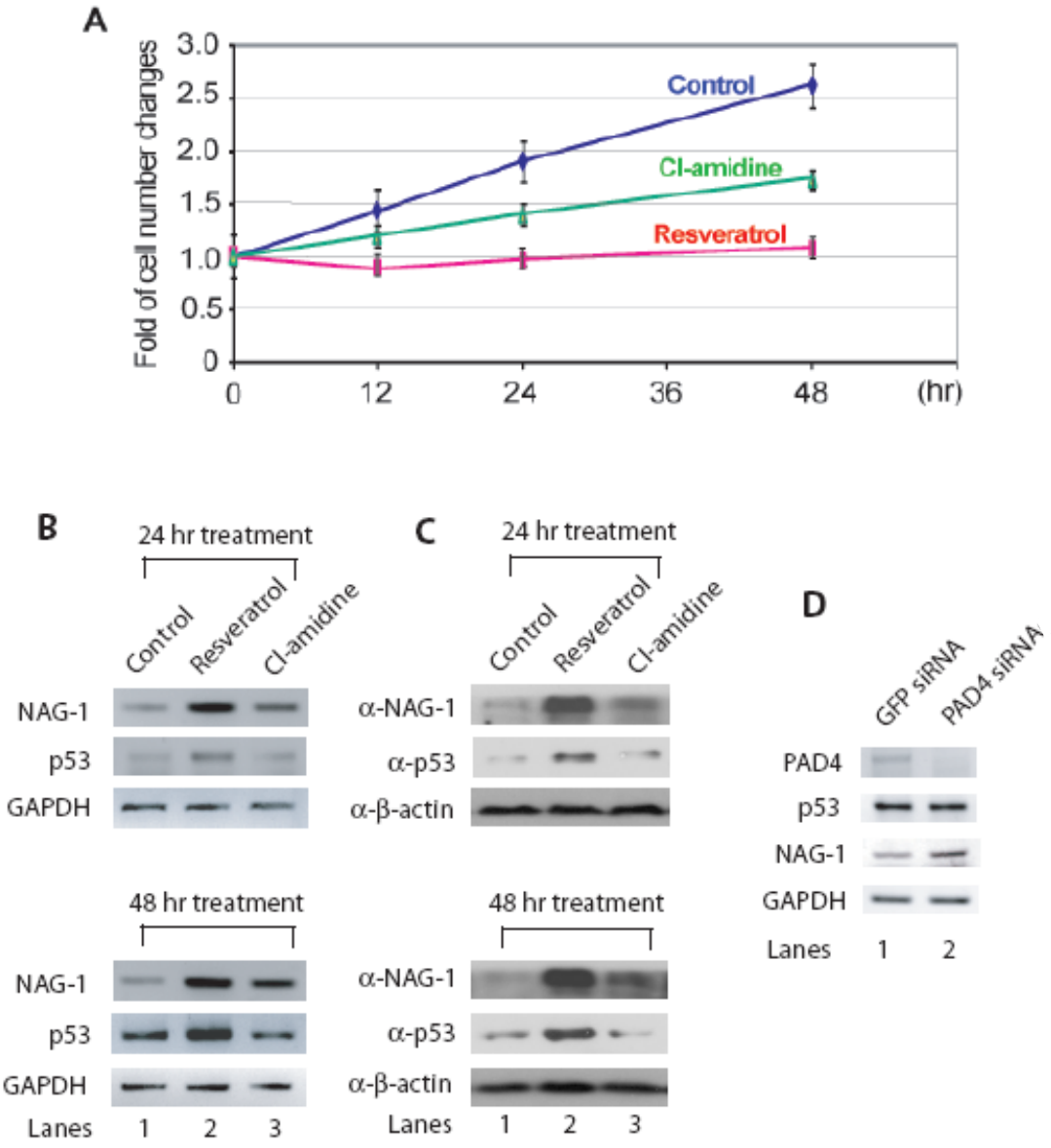

Figure 7: Resveratrol and Cl-amidine decrease MCF-7 cell proliferation and increase NAG-1 expression. (A) MCF-7 cells treated with 50 mM resveratrol or 100 mM Cl-amidine show reduced growth rates over 48 hours. Cell proliferation was determined using a hematocytometer. (B) RT-PCR show increased NAG-1 expression following treatment with either compound. Resveratrol treatment increased p53 expression; however, Cl-amidine did not affect p53 levels. (C) The increased NAG-1 expression was also detected by Western analyses. Similarly, p53 levels increased following resveratrol treatment, whereas Cl-amidine p53 levels remain unaffected. (D) U2OS cells treated with PAD4-siRNA for a period of 72 hours show increased levels of NAG-1 mRNA.

treatment. Resveratrol induced a dramatic increase of both NAG-1 and p53 levels, whereas Cl-amidine treated cells showed an increase of only NAG-1. Thus, resveratrol and Cl-amidine induce expression of NAG-1 through two possible different mechanisms.

Western analyses of resveratrol or Cl-amidine treated MCF-7 cells showed similar results when compared to the RT-PCR. Resveratrol treated cells showed increased NAG-1 and p53 expression within 24 hours. Cl-amidine treatment increased NAG-1 protein levels, but did not affect p53 expression. Similar results were gathered from MCF-7 cells treated for 48 hours. Resveratrol treatment increased both NAG-1 and p53 protein levels, whereas Cl-amidine treated cells showed only increased NAG-1 levels. These studies suggest resveratrol treated cells induce NAG-1 expression, at least partially through increased p53 levels, whereas Cl-amidine treated cells induce NAG-1 expression through the inhibition of PAD4.

To confirm that the increased NAG-1 expression was due to inactivation of PAD4 rather than a downstream effect of Cl-amidine, U2OS cells were transfected with PAD4 siRNA. Treated cells showed a notable PAD4 mRNA reduction following siRNA treatment. Concomitantly, reduced levels of PAD4 increased NAG-1 expression. The RT-PCR results within figure 7D is representative of 3 individual experiments. These studies suggest the inactivation of PAD4 through Cl-amidine or si-RNA increase expression of NAG-1. Thus, the negative regulatory effect of PAD4 upon arginine residues affects transcription levels of NAG-1.

Resveratrol Increases p53 and Reduces PAD4 Association with the NAG-1 Promoter

Resveratrol treated MCF-7 cells display elevated expression levels of NAG-1 and p53. Previous experiments showed resveratrol-mediated apoptosis was directed through increased p53 levels (22). Given that NAG-1 has two p53 binding motifs located 140 base pairs upstream or 50 base pairs downstream of the promoter (29), chromatin immunoprecipitation experiments

were completed to confirm increased p53 association to this region. Also, Cl-amidine treated MCF-7 cells display increased levels of NAG-1, indicting PAD4 negatively affects transcript levels. Thus, similar experiments were completed to determine if PAD4 was located at the promoter region of NAG-1. Using primers located -254 base pairs upstream of the promoter, p53 and PAD4 levels were determined before and after NAG-1 induction. Initially, low levels of p53 were detected upstream of the gene, whereas PAD4 levels were elevated prior to resveratrol treatment (figure 8A and 8B). These results correlate with a reduced transcriptional state. However, 12 hours following treatment, p53 levels at the promoter region increased considerably. In contrast, PAD4 levels were reduced to a fraction of their initial quantity. Together, these results indicate resveratrol increases NAG-1 expression through increased translocation of the p53 protein to the promoter region. Consequently, PAD4 levels were decreased, which is consistent with previous reports indicating this enzyme negatively regulates NAG-1 expression.

NAG-1 Transcription Increases Histone Arginine Methylation and Reduces Citrullination

The increased p53 and reduced PAD4 levels located at the promoter region of NAG-1 indicate that the gene may be transitioning to an active state. To confirm negative regulation by PAD4 upon NAG-1, chromatin immunoprecipitation experiments were completed to determine the patterns of citrullination or arginine methylation at the promoter region. Since the negative regulatory enzyme PAD4 increases citrulline levels (17), transcription activation through resveratrol should remove these epigenetic marks. MCF-7 cells were treated with resveratrol for 12 hours, and real-time (qPCR) analysis was completed to measure the varying histone epigenetic marks during transcription. Figure 8C shows prior to resveratrol treatment, histone H3 citrulline levels were high at the promoter region whereas histone H3 arginine 17 levels were

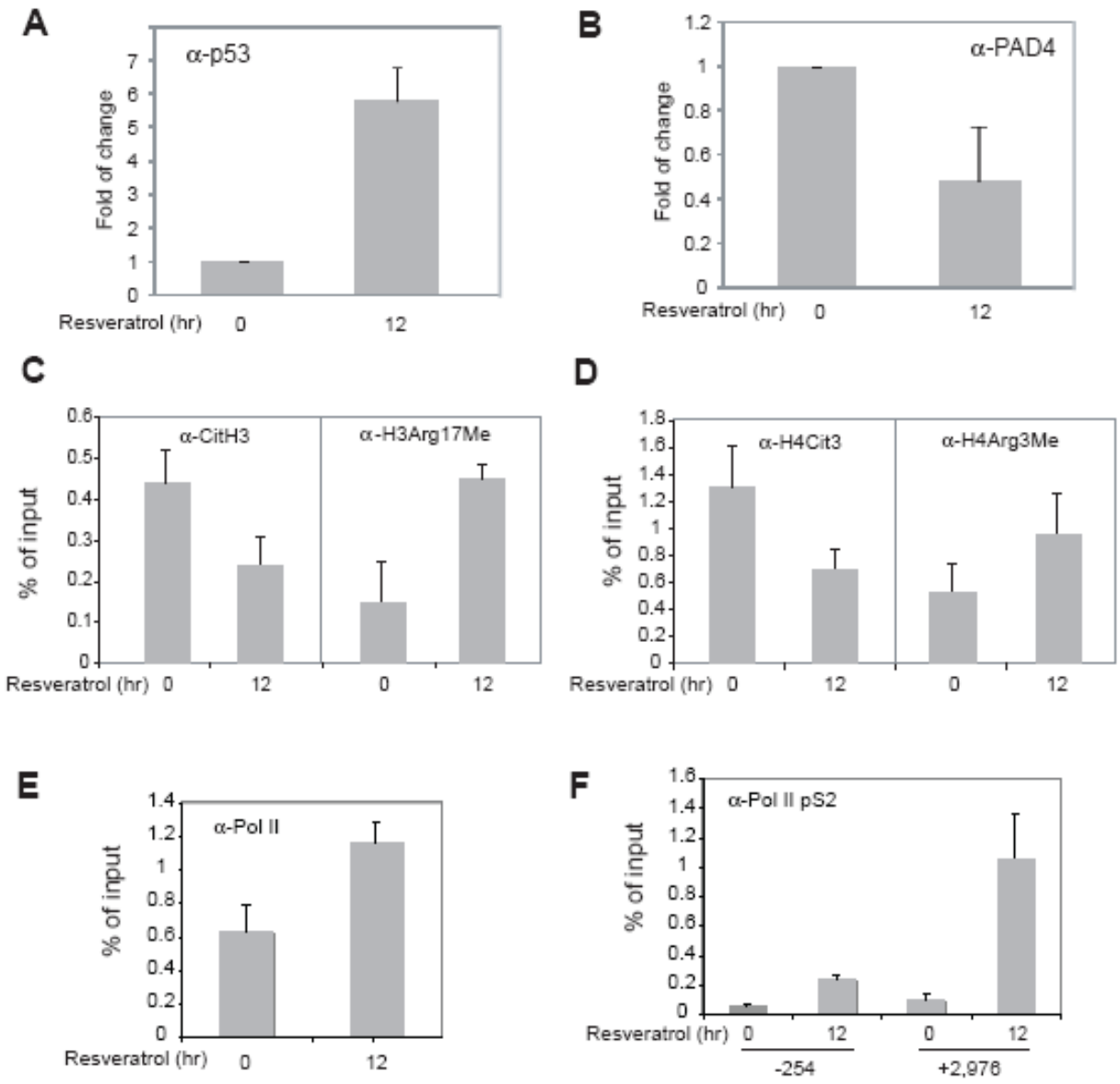

Figure 8: Chromatin immunoprecipitation assays indicate resveratrol induces NAG-1 Transcription. (A) p53 levels at the promoter region increased following NAG-1 induction. (B) Accordingly, PAD4 levels were reduced. (C) Initially, histone H3 citrulline levels were elevated whereas histone H3 arginine 17 methylation levels were reduced. Following 12 hours treatment, histone H3 citrulline levels decreased whereas histone H3 arginine 17 methylation levels increased. (D) Similarly, histone H4 citrulline 3 levels decreased while histone H4 arginine 3 methylation levels increased following resveratrol treatment. (E) RNA polymerase II levels increased following treatment. (F) RNA polymerase II serine 2 phosphorylation levels were increased downstream of the promoter region. All assays were normalized to histone H3 levels.

low. These epigenetic modification levels correlate with a repressed transcriptional state. However, following resveratrol treatment, the levels of these epigenetic marks varies inversely. Citrulline levels decreased at histone H3 citrulline 2, 8, 17, whereas methylation levels at histone H3 arginine 17 increased. These epigenetic marks correspond to an actively transcribed gene. Similar results were gathered upon histone H4. Prior to resveratrol treatment, histone H4 citrulline 3 levels were high whereas histone H4 arginine 3 levels were low (figure 8D). Following treatment, the epigenetic modification levels varied inversely. Citrulline levels decreased whereas arginine methylation increased. Thus, before transcription, a repressive chromatin state is maintained through increased citrulline and reduced methylation levels. Following treatment, the epigenetic modifications invert, showing decreased citrulline and increased methylation levels. These studies suggest PAD4 negatively regulates NAG-1 transcription through citrullination of the promoter nucleosomes. Once activated, the arginine epigenetic marks located at the promoter region undergo methylation, whereas the citrulline levels decrease.

Unmodified RNA Polymerase II and the Phosphorylated Serine II Isoform Levels Increase Following Resveratrol Treatment

Resveratrol treated MCF-7 cells increased NAG-1 expression considerably, as seen by the RT-PCR data. Furthermore, the histone modifications upstream of the promoter following treatment suggested chromatin near NAG-1 was conformed for active transcription. To confirm this, two monoclonal antibodies against RNA polymerase II were used to determine the levels of the transcribing enzyme throughout the gene. The C-terminal domain of RNA polymerase II has a repeated sequence (YSPTPSP) which remains unmodified during the pre-initiation complex formation and becomes phosphorylated at serine 2 during elongation (48). Initially, unmodified

RNA polymerase II levels were determined at the promoter region 254 base pairs upstream of the transcription state site. Similarly, the active elongating RNA polymerase II serine 2 phosphorylation levels were established through chromatin immunoprecipitation 254 base pairs upstream and 2974 base pairs downstream of the transcription start site.

Prior to treatment, unmodified RNA polymerase II was found at the upstream region of the NAG-1 promoter. These RNA polymerase II levels suggest a pre-initiation complex has been formed at the promoter prior to transcription. However, resveratrol treated MCF-7 cells showed increased levels of RNA polymerase II (figure 8E). Together, these results suggest the increased levels of the enzyme indicate NAG-1 may be undergoing pre-initiation complex formation.

The serine 2 phosphorylated isoform has been associated with transcriptional elongation. Therefore, there should be elevated levels of RNA polymerase II serine 2 phosphorylation downstream of the promoter during active transcription. Before treatment, the RNA polymerase II serine 2 phosphorylation levels were relatively low at the upstream region of NAG-1 in both groups (figure 8F). The resveratrol treated sample showed slightly increased levels of the enzyme over the untreated sample at this region. However, the untreated sample also showed low levels of RNA polymerase II serine 2 phosphorylation downstream of the transcription start site, which indicated the MCF-7 cells were undergoing low levels of NAG-1 transcription. However, resveratrol treated cells showed dramatically increased levels of RNA polymerase II serine 2 phosphorylation downstream of the promoter. These increased RNA polymerase II levels suggest that resveratrol induces active transcriptional elongation of NAG-1.

NAG-1 Localizes to MCF-7 Cell Membranes

NAG-1 has been classified as a member of the TGF-β super family due to a characteristic consensus RXXR cleavage signal for processing the immature form to the active secreted protein (30). Once secreted, it has been shown to bind to TGF-β receptors on the membrane of adjacent cells (31). However, under stable conditions it has been shown that translated NAG-1 undergoes constant proteolysis (26), thus meaning certain cellular stresses are required for secretion of the protein. Therefore, experiments were completed to determine if increased NAG-1 expression by resveratrol correlated with enhanced cellular membrane localization. Figure 9A shows untreated cells having a relatively low abundance of NAG-1 protein located on the cell membrane. However, following 24 hour treatment with resveratrol, an increased amount of NAG-1 protein was localized to this area (figure 9B). Similar results were gathered following treatment of cells for a period of 48 hours. Furthermore, figure 9A shows abundant nuclear staining, indicating that NAG-1 may localize to the nucleus. However, this is due to the unspecific binding of the antibody, as shown by Western analysis of total cellular extract alongside nuclear extract (figure 9D). Thus, resveratrol increases expression of NAG-1 in MCF-7 cells where it is translocated to the cell membrane and may then act through the TGF-β signaling pathway (31).

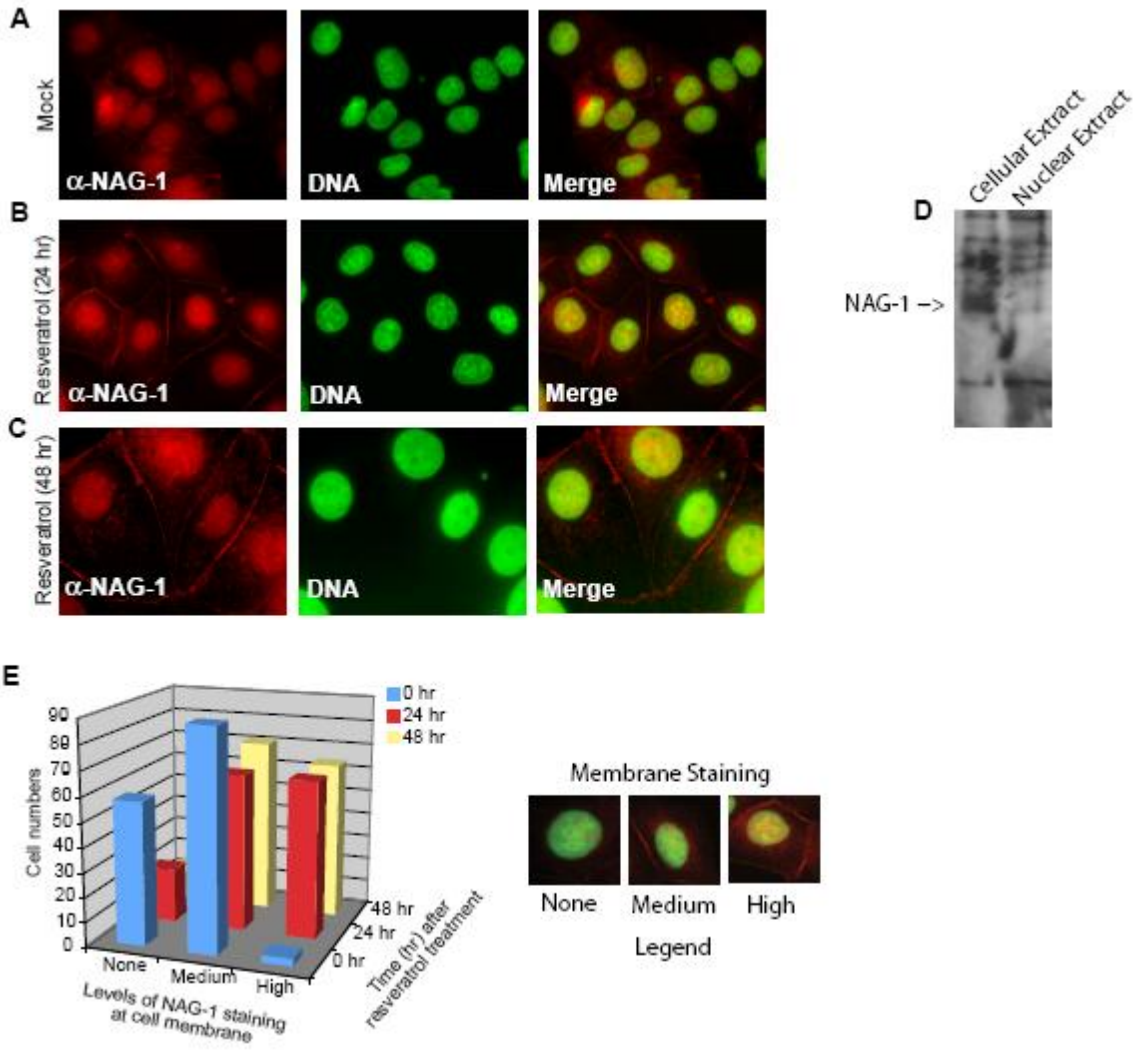

Figure 9: Resveratrol induces NAG-1 localization to MCF-7 cell membranes. Following resveratrol treatment, an antibody against NAG-1 was used to determine the localization of the protein. (A) Untreated cells showed reduced levels of membrane staining. (B and C) Treatment with resveratrol induced NAG-1 localization at MCF-7 cellular membranes. (D) Western analysis of nuclear extract against total cellular extract. (E) Counting of 150 cells was performed by an unbiased individual. Cell membranes were assigned levels of staining correlating to NAG-1 staining intensity. Legend for cell staining assay: Cells lacking any NAG-1 staining were given the classification of "none". Cells with 10-50% NAG-1 membrane localization were given the classification of "medium" intensity. Cells containing greater than 50% staining were given the classification of "high" intensity.

DISCUSSION

PAD4 Regulates Histone Arginine Residues

Histones are subjected to numerous post-translational modifications that have been closely linked to gene expression. Although chromatin is generally viewed as a repressive structure, epigenetic modifications to histone proteins have been shown to regulate the suppressive nature of nucleosome complexes (4). Within the past decade, epigenetic modifications to histone proteins have been correlated with altering gene expression levels (5). Until recently, there have been no true arginine demethylases. PAD4 was the first enzyme shown to antagonize histone arginine methylation by producing citrulline. However, recent findings have shown that JMJD6 preferentially modifies di-methyl arginine to the mono-methyl form (18). Thus, the dynamic regulatory mechanism of histone arginine residues remains in its infancy and further research must be completed to expand upon the current knowledge.

Modified histone arginine residues have been determined to alter gene expression levels singularly or in conjunction with surrounding epigenetic modifications. Chromatin immunoprecipitation analyses have showed that histone H3 arginine 17 methylation levels are increased when the estrogen receptor regulated pS2 gene is activated (41). When repressed, PAD4 and histone H3 citrulline levels increase upon the pS2 promoter, whereas RNA polymerase II levels drop following transcription initiation (42). Thus, modified arginine residues have been associated with specific levels of transcription. Furthermore, recent findings have shown modified arginine residues regulate surrounding epigenetic modifications. For example, PRMT6 mediated di-methylation of histone H3 arginine 2 has been determined to negatively regulate histone H3 lysine 4 tri-methylation (10). Thus, the combinatorial epigenetic marks upon nucleosomes correlate with specific states of transcription.

The type II PRMT5 mediated symmetric di-methyl mark upon histone N-terminal arginine residues have been correlated with epigenetic gene silencing (28). Previous research has shown that PRMT5 mediated methylation of histone H3 arginine 8 negatively regulates expression of suppressor of tumorigenicity 7 and non-metastatic 23 tumor suppressor genes (40). Furthermore, an anti-sense PRMT5 cell line showed increased expression of 227 genes, whereas 46 genes were down regulated (40). This further suggests PRMT5 mediated arginine methylation maintains an inhibitory effect upon gene regulation. Interestingly, homozygous PRMT5 knockout embryos immediately die, indicating the importance of PRMT5 during growth (28). Thus, PRMT5 functions as an essential protein required for cell growth, differentiation and gene regulation.

My studies show a potential novel regulatory mechanism upon histone N-terminal di-methylated arginine residues. To date, arginine and modified arginine residues are regulated by small set of proteins. PAD4 and JMJD6 have been shown to regulate the demethylation of arginine residues through two different mechanisms (17 and 18). PAD4 mediated regulation is important due to the numerous arginine residues modified by citrullination (34). Any residue mono-methylated by PRMTs may be targeted *in vitro* by PAD4. However, these results herein may alter the previously held perception that di-methyl arginine was resistant to deimination. Western analysis showed a convincing reduction in the amounts of asymmetric and symmetric di-methyl arginine upon purified HeLa nucleosomes incubated with PAD4. These results lead to the hypothesis that PAD4 impacts a larger part of the histone code than previously postulated.

Although more research is required to support these findings, this study suggests a novel method of gene regulation upon di-methylated arginine residues. PAD4 completes demethylimination of mono-methyl arginine by a hydrolation reaction that produces equal amounts of citrulline and methylamine (17). Thus, it is possible to imagine a mechanism by

which PAD4 modifies asymmetric di-methyl arginine by releasing di-methylamine. The removal of this byproduct would produce the unconventional amino acid citrulline. However, the reduced levels of asymmetric di-methyl arginine may be attributed to the unspecific binding ability of the antibody (figure 5). Both asymmetric di-methyl arginine antibodies interact with mono-methyl form. This is also supported by the hypothesis that the active pocket of PAD4 cannot accommodate asymmetric di-methyl arginine due to steric hindrance (51). Thus, it may be improbable that PAD4 modifies asymmetric di-methyl arginine. However, PAD4 treatment convincingly lowered the levels of symmetric di-methylation upon histone H4 arginine 3. These results are further confirmed by the specificity of the antibody. However, the mechanism for demethylation upon symmetric di-methylated arginine remains unclear. Should methylamine be released from the modified arginine, the epigenetic mark methyl-citrulline would remain. From here, it is possible to envision an aminotransferase acting to restore the residue to the mono-methyl form. However, this data requires further research to confirm the 'demethylation' reaction of PAD4 upon di-methylated arginine residues.

Summarily, my studies indicate that PAD4 modifies histone H2A, H3 and H4 within the nucleosome complex. Histones H2B and H1 remain unmodified following PAD4 incubation. PAD4 increased citrulline levels upon histone proteins, thus subsequently reducing unmodified, mono-methyl, or di-methyl arginine levels. Asymmetric di-methyl arginine levels appear to be reduced following PAD4 treatment; however, it may be attributed to the unspecific antibody detecting the mono-methyl form. On the other hand, the symmetric di-methyl arginine antibody has a strong specificity for targeted residues (figure 5). The antibody specifically detects the symmetric di-methyl form, and does not recognize other modified arginine isoforms. Thus, the reduced levels of symmetric di-methyl arginine following PAD4 incubation merits

further research. Therefore, my studies indicate that PAD4 may modify symmetric di-methyl arginine residues produced by the type II methyl transferase PRMT5.

NAG-1 Acts as a Potent Tumor Suppressor

PAD4 has been studied as a protein associated with rheumatoid arthritis (35), cancer (39), and gene regulation (17). The PAD4 inhibitor Cl-amidine was initially produced as a compound that would alleviate pain associated with rheumatoid arthritis (35), as patients with this disease displayed elevated levels of citrullinated proteins (36). The increased citrulline levels eventually caused the immune system to attack its own tissues through anti-citrulline antibodies. Interestingly, recent studies have also associated elevated PAD4 levels with a multitude of cancerous cell lines (39). PAD4 medicated citrullination of cytokeratin, a protein associated with tumor formation, appears to interfere with the cell death. Upon citrullination, cytokeratin is rapidly degraded which interferes with cellular apoptosis and induces cancer formation (39). Furthermore, PAD4 has been shown to regulate transcription through citrullination of promoter nucleosomes (17). The enzyme was established at the promoter region of the pS2 gene during low expression levels (42). To further study the downstream effects of PAD4, Cl-amidine could be used to deplete dynamic regulation of arginine residues. In theory, genes affected by citrullination would have their expression levels increased through PAD4 inactivation. Therefore, my study utilized Cl-amidine to study PAD4 function upon the tumor suppressor NAG-1 and the subsequent effects caused by histone citrullination.

Multiple studies have demonstrated the ability of naturally occurring substances to prevent experimental carcinogenesis. Diets having an abundance of fruits and vegetables have been shown to prevent the onset of cancer. Resveratrol, a compound isolated from red wine and grape skin, has been determined to have antitumorigenic effects in multiple cancers (21).

As a chemoprevention agent, resveratrol has been shown to inhibit tumor initiation, promotion, and progression (21). Furthermore, the 'French paradox' whereby the French population has low rates of cancer and obesity has been related to the ingestion of resveratrol (43). Numerous studies have been completed to determine the mechanism by which resveratrol maintains its chemopreventative properties. Microarray analyses of multiple cell lines treated with resveratrol showed increased levels of numerous proteins associated with tumor suppression (44). Most notably, NAG-1 expression increased dramatically over untreated cells (22). Therefore, the chemopreventative properties of resveratrol appear to be linked to the increased expression of NAG-1.

Microarray analyses of cells treated with Cl-amidine or resveratrol demonstrate a specific commonality. Particularly, the expression of NAG-1 increased considerably following treatment with either compound. In MCF-7 cells, Cl-amidine increased NAG-1 expression whereas resveratrol treated CD-18 cells increased expression six fold (22). Although both compounds work through different mechanisms, each treatment increased the expression of the tumor suppressor gene NAG-1.

The NAG-1 protein has been associated with tumor suppression and cancer prevention (20). Initially, NAG-1 was described as a protein which strongly disrupted cell growth and tumor formation (31). For instance, transgenic mice overexpressing the human NAG-1 protein showed considerably less tumor formation than mice lacking the transgenic expression of NAG-1 (20). Also, overexpression of NAG-1 by a retrovirus system inhibited cell growth of MCF-7 human breast cancer cells (20). In contrast, evidence has also been presented to support a role for NAG-1 in the progression of tumors. Microarray studies comparing matched samples from normal and primary prostate tumors found increased NAG-1 expression in 21 out of 24 cancers (45). However, NAG-1 expression was down-regulated in 8 out of 10 primary prostate cancers

(46). These observations provide evidence for a dual role for NAG-1 in cancer progression: suppression of tumorigenesis at the early stages of cancer and promotion of cancerous survival at more advanced stages of disease.

PAD4 Negatively Regulates NAG-1 by Histone Citrullination

Treatment of MCF-7 breast cancer cells with either resveratrol or Cl-amidine decreased cell proliferation over a period of 48 hours. Previous reports showed resveratrol-mediated apoptosis within CD-18 cells have been related to the increased NAG-1 expression (22). When cells were incubated with NAG-1 siRNA, the apoptotic effect of resveratrol was diminished. The authors suggested resveratrol mediated apoptosis within CD-18 cells may rely, in part, upon the up-regulation of NAG-1 (22). My study of resveratrol treated MCF-7 cells showed similar levels of NAG-1 induction through RT-PCR and Western analysis when compared to resveratrol treated CD-18 cells. Thus, the mechanism of apoptosis through resveratrol may remain similar to CD-18 cells. Cl-amidine treated MCF-7 cells also showed reduced levels of proliferation. This may also be related to the increased NAG-1 expression levels. It may also be reliant upon increased tumor suppressor expression as seen in the microarray results. Also, the inactivation of PAD4 could affect cell proliferation through other pathways. For example, upon citrullination, cytokeratin is rapidly degraded which interferes with cellular apoptosis (39). Inactivation of PAD4 may interfere with this process. Thus, MCF-7 cells treated with either compound show reduced levels of growth; a possible downstream effect of the up-regulated tumor suppressor NAG-1.

Chromatin immunoprecipitation experiments showed resveratrol mediated NAG-1 induction relies upon specific co-factors, histone modifications and RNA polymerase II levels upstream or downstream of the transcription start site. Previous studies have shown that p53 is

required for NAG-1 expression following resveratrol treatment (23). This was confirmed by the increased levels of p53 translocated to the promoter region of NAG-1 following resveratrol treatment. However, the co-factor PAD4 has not been shown to negatively regulate NAG-1 through citrullination of histone arginine residues. Resveratrol treated MCF-7 cells showed reduced levels of PAD4 at the promoter region, accompanied by subsequently decreased histone citrulline levels. Conversely, the histone arginine methylation levels increased. The reduced citrulline and increased methylation levels indicate that resveratrol initiated the transformation of repressed chromatin to the active chromatin configuration. These results correlate with previous studies showing citrullinated histone residues correlate with decreased gene expression levels (17). Similarly, the increased methylation patterns correlate to increased transcription levels (41). Also, the induced NAG-1 expression showed increased RNA polymerase II levels located at the promoter region, indicating RNA polymerase II is recruited constantly. The increased levels may also result in the formation of the pre-initiation complex (47). When phosphorylated at serine 2, RNA polymerase II has been shown to undergo transcription elongation (48). Active transcription of the 2900 base pair NAG-1 transcript (23) should show increased RNA polymerase II serine 2 phosphorylation levels downstream of the promoter. RNA polymerase II serine 2 phosphorylation levels downstream of the promoter clearly show active transcription of NAG-1. These Chromatin immunoprecipitation experiments allowed the proposal of a resveratrol-stimulated NAG-1 expression model, as seen in figure 10. Thus, these assays indicated that p53 and PAD4 play conflicting roles at the NAG-1 promoter. PAD4 negative regulation is reduced following NAG-1 induction, thus reducing histone citrullination and increasing arginine methylation. RNA polymerase II and the active RNA polymerase II levels increase upstream and downstream of the promoter region, thus indicating the NAG-1 is being actively transcribed.

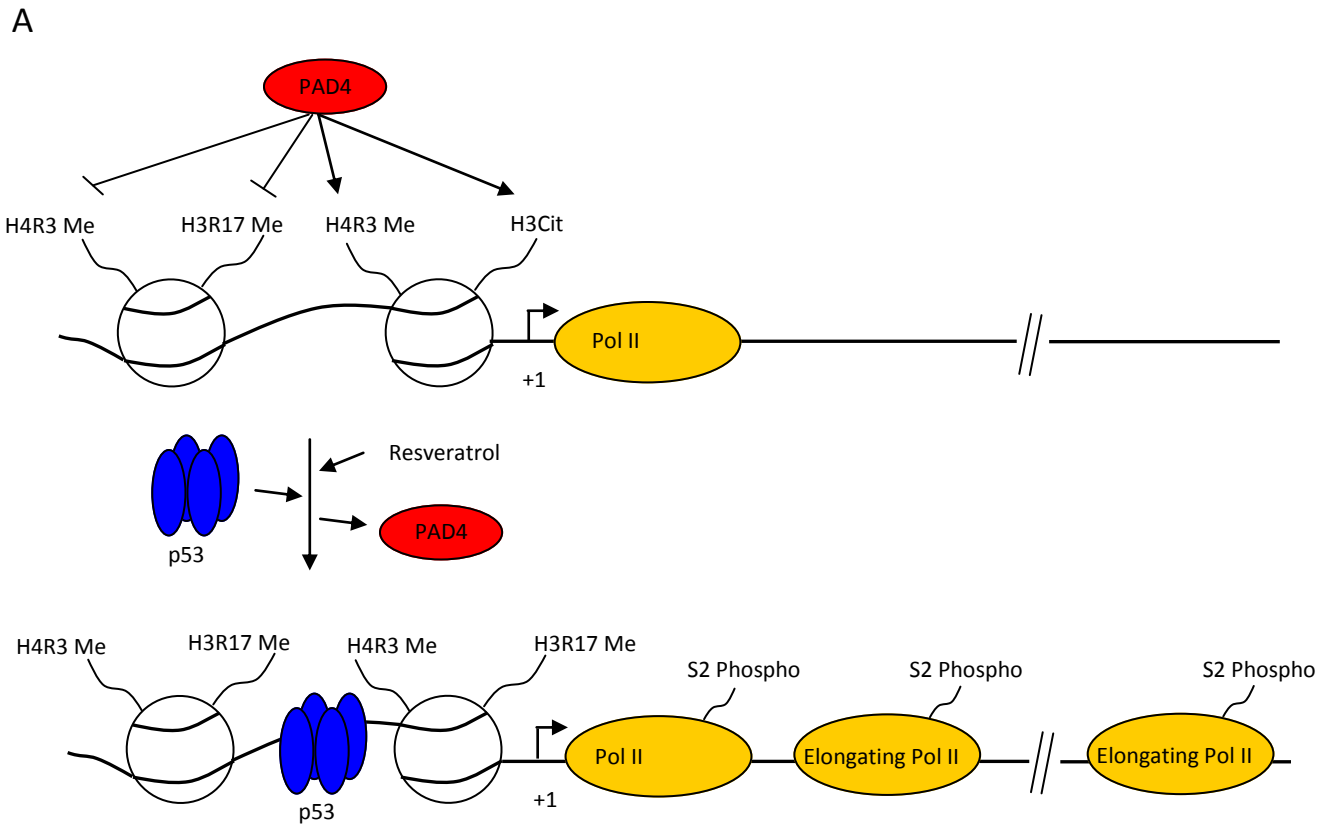

Figure 10: Proposed model for resveratrol-stimulated NAG-1 expression. Prior to resveratrol treatment, PAD4 and histone citrullination maintain histone arginine methylation at a basal level. RNA polymerase is bound to the promoter region; however, it is not transcribing the gene. Resveratrol treatment induces p53 translocation to the promoter region of NAG-1, followed by a concurrent decrease of PAD4 levels from the same region. Histone citrulline levels decrease followed by an increase in the histone arginine methylation levels. Polymerase II serine 2 phosphorylation levels are increased upstream and downstream of the promoter region indicating NAG-1 is being actively transcribed.

Following translation and secretion, NAG-1 has been postulated to function through membrane bound receptors. Recent studies have shown that NAG-1 co-localized with TGF-β I receptors at cell membranes. This induced an altered expression of SMAD protein localization within the cells, suggesting NAG-1 functions through the TGF-β/SMAD pathway (32). Resveratrol or untreated MCF-7 cells showed different patterns of NAG-1 localization. Untreated MCF-7 cells displayed low levels of NAG-1 membrane localization. This may be a byproduct of the constitutively expressed TGF-β/SMAD pathway associated with MCF-7 cells (49). Resveratrol treated MCF-7 cells displayed considerably increased levels of NAG-1 translocation to MCF-7 cellular membranes. This indicates that resveratrol induced apoptosis may, in part, be related to the increased NAG-1 protein levels. Overexpression of NAG-1 has been determined to induce apoptosis in cells with actively expressed TGF-β pathways (20). Thus, these studies suggest NAG-1 may be a suitable target for tumor prevention through the inactivation of PAD4.

Furthermore, PAD4 overexpression has been associated with various types of cancer (39). Previous reports indicated elevated levels of PAD4 in many tumor tissues, especially various types of adenocarcinoma (39). This indicates that inhibiting the effects of PAD4 in specific cancers types may subsequently inhibit cell proliferation. In this study, the PAD4 inhibitor Cl-amidine decreased MCF-7 breast cancer cell proliferation over a 48 hour period. Microarray analysis of cells treated with Cl-amidine showed increased expression of genes associated with tumor suppression. Specifically, OKL38 and NAG-1 expression levels were increased. NAG-1 induction showed reduced levels of PAD4 from the promoter region, followed by the subsequent reduction of histone citrullination. The increased RNA polymerase II serine 2 phosphorylation levels downstream of the promoter region indicated the gene became transcriptionally active. Following transcription, NAG-1 was determined to localize to MCF-7

cellular membranes, where it interacts with TGF-β membrane bound receptors to induce apoptosis. Thus, these studies indicate that PAD4 negatively regulates NAG-1, a protein associated with reduced cellular growth. Since PAD4 may be overexpressed in numerous types of carcinomas (39), it may be a target for future chemotherapy treatment. Thus, these studies suggest PAD4 inhibition may reduce cancerous cell proliferation, in part, by the increased expression of NAG-1.

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