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THE ROLE OF PAD4 IN CHROMATIN DECONDENSATION

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ABSTRACT

NETosis, the process where neutrophils release highly decondensed chromatin, better known as neutrophil extracellular traps (NETs), has gained much attention as an efficient means of killing bacteria. In vivo, NETs are induced by bacteria and proinflammatory cytokines. Our lab has shown that peptidyl arginine deiminase 4 (PAD4), an enzyme that converts Arg or monomethyl-Arg to citrulline in histones, is essential for NET formation. We found that areas of extensive chromatin decondensation, along NETs, are rich in histone citrullination. Although the mediators involved in NET formation are known, the overall mechanism underlying higher-order chromatin structure changes has yet to be elucidated. Upon investigating the effect of global citrullination in vivo, we have discovered that PAD4 overexpression in U2OS cells is sufficient to cause extensive chromatin decondensation independent of apoptosis. The highly decondensed chromatin is released to the extracellular space and stains strongly by a histone citrulline-specific antibody. The structure of the decondensed chromatin is reminiscent of NETs but is unique in that it occurs devoid of stimulation of cells with proinflammatory cytokines, bacteria or calcium ionophore treatment. By using this PAD4 overexpression model system in U2OS cells, we have begun to unveil the process of PAD4 mediated chromatin decondensation and show that intracellular concentrations of calcium, the activity of PAD4, and changes in higher-order chromatin regulators (i.e. NPM1) are essential for the decondensation process.
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Chapter 1  
Introduction

1.1 - The Complex Relationship of DNA, Histones and their Modifications:

The intricate physiological processes and events that occur in a single cell at any given moment stem from a chromatin network, ubiquitous to all eukaryotic genomes. Chromatin, the dynamic form of DNA, is defined as such due to its association with histones—globular proteins except for their amino- and carboxyl-terminal “tails” (1). To be exact, 147 bp of negatively charged DNA tightly associates with the positively charged octamer of four histones (H3, H4, H2A, H2B) to form a nucleosome that represents a single “bead on a string” that chromatin is organized into (2). Because the cell must contain over 2 meters of DNA in a nucleus that is merely 5 to 10µM in diameter (3), the compaction of DNA into nucleosomes is only one of the methods the cell has evolved that physically allows the nucleus to contain all of its information. After formation of the individual nucleosome, association with linker histone H1 and linker DNA promotes the formation of higher-order chromatin structure. This higher-order chromatin can interact with nuclear proteins such as the nuclear envelope and the nucleolus for further organization (3). The association with sub-nuclear proteins further condenses the immense quantity of genomic DNA and possibly indicates that changes in the structure of chromatin may involve and rely on a dynamic interplay between histones and larger structural proteins in the nucleus. In order to further understand how this “beads on a string” of chromatin mediates a plethora of seemingly diverse physiological events, an investigation of histone modifications is necessary.

Histones constantly undergo posttranslational chemical alterations most extensively at their amino-terminal “tails.” These modifications, including acetylation, phosphorylation,
methylation, citrullination, etc., add immense complexity to our genomes and dictate cellular processes such as transcription, translation, DNA damage repair and chromatin condensation/decondensation. These histone modifications, occur in response to differential intracellular signaling cascades due to an external stimulus. A simple, well-accepted model for gene transcription mediated by histone modifications, is that histone alterations affect higher-order chromatin by altering the contact of the adjacent nucleosomes with one another as well as the interaction of DNA with histones. This makes sense given that in order to engage transcription of certain target genes, polymerases need to have access to DNA that is normally inaccessible due to the strong association of DNA with histones. For example, acetylation of histones is associated with gene transcription due to the neutralization of the negative charge of lysine, which partially allows the DNA to unwind from the histones because of a loss of ionic interaction; polymerases thus have more access to the DNA. The fact that our histones are constantly modified to achieve cellular responses indicates that site- and modification-specific histone-modifying enzymes must exist (1,3).

Methylation is one of the most noted histone modifications. Lys methylation is associated with DNA damage and repair, and Arg methylation commonly regulates gene transcription. Like any dynamic process, there must be a means by which a given modification is reversible to provide a way for regulation. Lys residues are actively methylated and demethylated by histone Lys methyltransferases (HKMTs) and histone-Lys-specific demethylase (LSD) respectively. On the other hand, Arg methyltransferases (PRMTs) methylate Arg residues. But until recently no known Arg demethylating enzyme has been discovered (1,3).
1.2 - Peptidyl Arginine Deiminase 4 (PAD4) a Novel Histone Modifying Enzyme:

Of the many post-translational modifying enzymes, the PAD protein family (PADs I, II, III, IV and V) is under immense scrutiny for two reasons. Not only can they convert protein Arg to citrulline (Cit) in a calcium and dithiothreitol (DTT)-dependent reaction, but this modification is also implicated in many human diseases (4). To begin, citrullination neutralizes the positive charge of protein Arg residues thus affecting the overall quaternary structure and charge of the protein. Citrulline is so unique because it is not one of the twenty standard amino acids synthesized and incorporated into our proteins.

The tissue specific localization of PADs corresponds with the diseases they cause. For example, PAD1, which is localized in the skin, has been implicated in psoriasis due to a lack keratin citrullination. Additionally, some patients with rheumatoid arthritis (RA) have increased levels of PAD4 in their synovial joints. Citrullination of joint proteins most likely contributes to an autoimmune response where the body recognizes the citrullinated proteins as “non-self” and thus attack the joints (4,5). From various genetic studies and observed diseases, the PADs have been shown to citrullinate proteins in many different tissues in the human body. But until recently the role of PAD4 from a gene regulatory standpoint had yet to be elucidated until discovery of a novel sub-cellular substrate of PAD4.

PAD4, the only known nuclear isoform of PADs, was discovered in HL-60 granulocytes by observing the proteins cellular via fluorescent tags. Interestingly, PAD4 was shown to act on the nuclear substrates histones H2A, H3 and H4 following treatment with calcium ionophore. This discovery further expanded the cellular structures in which PAD4 interacts with and indicated that calcium signaling is essential for PAD4 activation (6). Given that there is no known histone Arg demethylating enzyme in existence, Wang et al. tested whether PAD4 could reverse histone Arg methylation on the basis of histone citrullination. Western blot analysis showed a marked decrease in H3Arg17 and H4Arg3 methylation following an in vitro reaction using
purified GST-PAD4 on histone H3 or H4 in the presence of calcium and DTT. This result was attributed to the loss of the H3 Arg17 or H4Arg3 methylation epitope normally recognized by the corresponding antibody. Additionally, a later experiment illustrated that methylamine was released following the PAD4 reaction. Detection of the released product, methylamine and the reduction of H3Arg17 and H4Arg3 methylation specific antibody staining indicated that PAD4 demethyliminates histones, thus providing a mechanism for reversing histone Arg methylation and illustrating the means by which active gene transcription is regulated (7).

The recent knowledge of the various activities of PAD4 has truly expanded the field of epigenetics and further questions the genetic, cellular and physiological role of histone citrullination. Current understanding and recent findings favor the notion that histone citrullination affects higher order chromatin structure because the overall positive charge of histone Arg residues is lost by citrullination. Interestingly, this hypothesis has been partially validated by studying a unique cell type of the innate immune response—neutrophils.
1.3 - PAD4, Neutrophil Extracellular Traps and Higher-Order Chromatin Structure:

Every day, the human body is unnoticeably under attack by foreign pathogens that are unable to cause disease because of the body’s innate and adaptive immune responses, i.e., two different branches distinguished by the cell types they contain and their specificity for recognizing pathogens. B-lymphocytes and T-lymphocytes comprise the majority of the body’s adaptive immunity and are able to differentiate individual chemical moieties of pathogens; whereas, the innate immune response contains mainly granulocytes (eosinophils, mast cells, basophils and neutrophils), that recognize less specific features of pathogens. In understanding the relationship of PAD4, higher-order chromatin structure and the immune system, a newly recognized antimicrobial mechanism often elicited by neutrophils chromatin is of great interest for exploration (8).

Neutrophils are the first line of defense against invading pathogens and upon release into the blood circulation migrate to infected tissues due to a chemotactic gradient produced at the site of infection. Once a neutrophil recognizes the pathogen and firmly attaches to the infectious agent, the process of phagocytosis commences. First, actin reorganization under the neutrophil membrane, brings the pathogen into a phagosome where subunits of the membranous NADPH oxidase system assemble to facilitate formation of reactive oxygen species (ROS). These cytotoxic ROS attack the pathogen following formation of the phagolysosome—an intracellular structure where the phagosome fuses with numerous antimicrobial peptide-containing granules. Together, the ROS and the antibmicorbial peptides effectively destroy the pathogen (9). Phagocytosis has and still is the most accepted method of “neutrophil killing.” However, a more extreme antimicrobial mechanism has been discovered that has new implications in understanding disease dynamics and the role of histone modifications in innate immunity.

In a novel series of experiments, neutrophils were observed to generate extracellular
chromatin fibers called neutrophil extracellular traps (NETs) upon activation with interleukin-8 (IL-8), phorbol myristate acetate (PMA), or lipopolysaccharide (LPS). When neutrophils were primed with Staphylococcus pyogens, the causative agent of necrotizing fasciitis, NET formation and association of NETs with the microbes was observed. Additionally, these fibers, which are mainly composed of DNA, were also shown to contain histones H2A, H2B, H3 and H4, neutrophil elastase and myeloperoxidase—all documented antimicrobial agents (10). It was also shown that patients with chronic granulomatous disease (CGD), a condition that causes severe immunodeficiency due to mutations in the phagocytic NADPH oxidase, lack NETs in vivo. When neutrophils from these patients were treated with glucose oxidase, a drug that produces hydrogen peroxide exogenously, NET formation was restored; therefore, illustrating that ROS is a key regulator of the process (9). “NETosis,” which is the process of chromatin decondensation, ultimately kills the neutrophil due to a loss of nuclear integrity. Interestingly, this innate immune response appears to be distinct from apoptosis due to a lack of DNA fragmentation, no exposure of phosphatidylserine upon DNA release and no apparent involvement of caspases (9). These results indicate an additional ROS dependent anti-bactericidal mechanism that neutrophils employ to restrict pathogens to a site of infection. However, another essential regulator of NET formation was missing, which helps explain why DNA and histones are major components of NETs.

In understanding that the neutralization of positive charges on histones by PAD4 may have important implications on higher-order chromatin structure, a study was performed to test the affects of PAD4 on NET formation. After treatment of HL-60-derived granulocytes with calcium ionophore, extensive chromatin decondensation was observed extracellularly. Additionally, histone H4Arg3 methylation was decreased and histone H4Cit3 was increased at regions of highly decondensed chromatin revealing that chromatin decondensation is associated with NET formation (11). In light that activated neutrophils undergo NETosis, isolated blood
neutrophils were treated with TNF-α, a pro-inflammatory cytokine. After treatment, the isolated neutrophils also showed a marked increase in histone citrullination in areas of greatest chromatin decondensation. More importantly, PAD4 activity was shown to be essential in NET-like chromatin structure formation and for bacterial mediated chromatin decondensation in two independent experiments using Cl-amidine—a potent PAD4 inhibitor. In the first experiment, HL-60 granulocytes, treated with Cl-amidine prior to calcium ionophore treatment, showed a significant decrease in NET-like structures. In a second experiment, diminished histone citrullination was also observed following pre-treatment of DMSO-differentiated HL-60 with Cl-amidine followed by subsequent administration of IL-8 and the bacteria Shigella flexneri (11). These studies illustrated that in addition to its gene regulatory role, PAD4-catalyzed histone hypercitrulination is essential for the formation of highly decondensed extracellular chromatin structures in neutrophils and like ROS may be a central mediator in initiating NETosis.

Despite the fact that neutrophils release their chromatin in response to pro-inflammatory cytokines and pathogens and that recent data strongly favor an epigenetic basis for the phenomenon, one vital question still needs to be answered. What is the mechanism for NET-mediated bacterial killing? While doing some preliminary work, we unexpectedly observed that mere PAD4 overexpression in osteocarcinoma U2OS cells yields extracellular fibers analogous to those observed in neutrophils after treatment of those cells with calcium ionophore and pro-inflammatory cytokines. The fact that PAD4 can lead to chromatin decondensation in cells other than neutrophils allowed us to probe the process of “NET” formation from a solely PAD4 based model. Not only do we hypothesize that PAD4 is a significant mediator of chromatin decondensation but that intracellular calcium release must be attained for the process to occur. Additionally, because chromatin, in an interphase neutrophil nucleus, is highly compacted and interwoven with nuclear proteins, we also propose that chromatin, nuclear envelope and nucleolar structures need to be drastically remodeled to form NETs. These questions were analyzed by
various PAD4-overexpression experiments and by biochemical and cell biological techniques following PAD4 overexpression.
Chapter 2

Materials and Methods

2.1 - Plasmid Constructions:

A construct capable of expressing RFP-nucleophosmin and histone H3-GFP from the same bicistronic mRNA was engineered in the pMIGR1 retroviral expression vector by a two-step cloning procedure. First, an insert containing the fusion histone H3-GFP flanked 5’→3’ by NcoI and SalI restriction sites was generated from previously purified histone H3-DNA and pMIGR1-DNA by a set of oligonucleotides. The insert was cloned into the pMIGR1 expression vector dual digested with NcoI and SalI (New England BioLabs). The construct was confirmed by DNA sequencing in the Nucleic Acid Facility located at Pennsylvania State University. This pMIGR1-H3-GFP construct was then utilized as the vector for the second cloning procedure to produce the dual protein expressing construct. The second fusion insert, Flag-mCherry-nucleophosmin flanked 5’→3’ by EcoRI and XhoI restriction sites was amplified from H2B-mCherry purchased from AddGene and purified U2OS cDNA respectively. This insert was then cloned into the previously generated pMIGR1-H3-GFP vector dual digested with EcoRI and XhoI (New England BioLabs). Final confirmation of the engineered plasmid was assessed by DNA sequencing at the Nucleic Acid Facility.

A second construct, Nucleophosmin R197Q was generated from the nucleophosmin PMIGR1 construct. Primers flanking Arginine-197 with the arginine to glutamine mutation were used in a mutagenesis PCR. Successful mutagenesis was confirmed by DNA sequencing.
**2.2 - Cell Culture and Transient Transfection:**

U2OS and 293T cells were cultured in DMEM medium supplemented with 10% FBS and 1% Penicillin-Streptomycin in a 5% CO2 37 °C incubator. Transient transfection of U2OS and 293T cells was performed by plating 2-3x10^5 cells in a 6-well plate. The cells were changed to fresh medium without antibiotics early in the morning on the day of transfection. When cells reached ~70-90% confluency, 4µg of DNA (PSG5-PAD4, NPMR197Q, mCherry-NPM-H3-GFP) was combined with OPTI-MEM to 250µL and 10µL of Lipofectamine 2000 (Invitrogen) was combined with 240µL OPTI-MEM and incubated for 5 min. at RT. The DNA/OPTI-MEM and Lipofectamine 2000/OPTI-MEM was combined and incubated for 20 minutes at RT. 500 µl of plasmid/lipofectamine complex was added to the 6-well plate and then placed in a 5% CO2, 37 °C incubator. After 12 hours, the transfection medium was replaced with fresh complete medium.

**2.3 - Generation of a Stable Cell Line:**

293T and U2OS cells (cells to be transduced) at 7.5x10^5/well were each seeded in two wells of a 6 well plate. When cells reached ~60% confluency, the 293T cells were co-transfected with 2µg packaging plasmid and 2µg of the RFP-NPM-H3-GFP construct and combined with OPTI-MEM to 250µL. 10µL of Lipofectamine 2000 (Invitrogen) was combined with 240 µl OPTI-MEM and incubated for 5 min. at RT. DNA/OPTI-MEM and Lipofectamine 2000/OPTI-MEM were mixed and incubated for 20 minutes at RT. 500 µl of plasmid/lipofectamine complex was then added to the 6-well plate and then placed in a 5% CO2, 37 °C incubator. After 12 hours, the transfection medium was replaced with 1mL of fresh complete medium. After 12 hours, ~750 µl of the filtered retroviral supernatant (µStarLB filters, 0.45 µm, Costar #8112)
supplemented with 8µg/ml polybrene was overlayed onto the U2OS cells. The plate was then centrifuged at 1400 rpm for 45 min at RT for spinoculation of cells. Following spinoculation, the plate was left in a 37 °C incubator until the next round of infection. Two additional rounds of infection were performed using the above procedure. Following subsequent infections, generation of the stable cell line was confirmed using fluorescence microscopy using the green and red wavelengths to excite the newly expressed proteins. The cells were then sorted by FACS at the Pennsylvania State University Huck Institutes of the Life Sciences core research facility.

2.4 - Immunostaining and Microscopy:

Immunostaining was performed using a previously established protocol. After fixation of samples with 3.7% paraformaldehyde in PBS supplemented with 1% Triton X-100 and 2% NP-40, cells were washed with PBST three times for 10 min each. Following the third wash, cells were blocked in 2% BSA in PBST for at least 2 hr at RT. Primary antibodies were diluted in PBST supplemented with 2% BSA and 5% normal goat serum as follows: 1:200 of NPM, 1:20 PAD4 A.P., 1:200 HA, 1:200 H3Cit, 1:200 Flag. Cellular staining was performed in a humid chamber overnight at 4°C. After application of the primary antibodies, the cells were washed with PBST three times for 10 min each. Cells were then stained with the appropriate secondary antibodies conjugated with Cy3 or Alexa488 at a 1:500 dilution in a humid chamber for at least 2 hr at RT. After washing three times for 10 min each with PBST, cells were stained with 1µg/µL Hoeschst for 15 seconds followed by a final wash with H2O. Slides were then mounted and imaged with a fluorescent microscope (Axioscope 40; Carl Zeiss, Inc.) using an A-plan 20x NA .45 (Carl Zeiss Inc.). Fluorescent images were captured via a camera (AxioCam MRM; Carl Zeiss, Inc.) and the Axiovision AC software (Carl Zeiss, Inc.) at RT. Fluorescence was also
2.5 - Protein Extraction and Western Blotting:

Western blotting was performed following protein extraction from target cells. Cells were harvested by re-suspension in an appropriate volume of cold IP buffer (10mM EDTA, 2mM Tris-HCl pH 8.0, 150mM NaCl, 0.2% Triton X-100, 0.2% NP-40) supplemented with protease inhibitors (10µg/µL PMSF, 1µg/µL Leupeptin, 1µg/µL Aproptinin). Mixture was then sonicated for ~5 min at 4 °C followed by SDS denaturation. The appropriate volume of denatured protein was loaded into a 12% SDS-Acrylamide Gel (2.15mL ddH2O, 4.6mL Acrylamide, 4.1mL 1M Tris-HCl pH 8.8, 100µL 10% SDS, 100µL 10% APS, 3.8µL TEMED) and run at 210V. Protein was then transferred to Nitrocellulose membrane, using a Semi-Dry Transferring system for 1 hr. Following Ponceau S staining, the membrane was blocked in 5% Milk TBST for ~30 min. at RT to which the following primary antibodies were added: α-PAD4 A.P., α-H3Cit (AB5103; AbCam), α-HA (H9658, Sigma), α-histone H3 (Ab1791, AbCam), α-Flag, α-, at suitable dilutions. Following overnight incubation at 4°C, membranes were washed three times in TBST for 10 min. each and were then incubated for a minimum of 2 hrs. at 4°C with the proper horseradish peroxidase-conjugated secondary antibody. Signals were detected using the Lumilight PLUS Western blotting substrate (Roche).
2.6 - Cellular Chromatin Decondensation Assay and Decondensation Assay with BAPTA-AM and BL1-07 Drug Treatments:

To assess cellular chromatin decondensation as a result of PAD4 overexpression, U2OS cells were transiently transfected with 4µg of PSG5-PAD4, a construct that allows detection of exogenous protein because of an engineered HA-Tag on the C-terminus of the PAD4 insert. After 12 hours of transfection, the medium was removed and replaced with fresh DMEM with antibiotics. At this point, 5µM and 10µM BAPTA-AM (intracellular calcium-chelator) purchased from Sigma Aldrich stock dissolved in DMSO and 5µM and 10µM BL1-07 (PAD4 inhibitor) stock dissolved in DMSO, was first diluted in complete medium and then added to the transfected cells. The concentrations of drug were used based off of experiments testing the maximum amount of drug with minimal toxicity to cells. After an additional 24 hrs., the point where PAD4 expression is greatest as determined by a previous western-blot, the medium was aspirated and the cells were immunostained with α-HA and α-H3Cit and viewed with fluorescence microscopy to observe increased PAD4 expression and chromatin decondensation.

2.7 – PAD4 Assay (GST-PAD4 treatments):

To analyze the effects of PAD4 on chromatin decondensation and citrullination of various proteins, ~5x10^6 HL-60 nuclei were added to two separate PAD4 assay reactions. The reactions contained 20µL of GST-PAD4 or the GST-PAD4(C645S) mutant in 200µL PAD4 assay buffer (50mM Tris-HCl pH 7.6, 4mM CaCl2, 4mM DTT and 1mM PMSF). The experiment was performed in a 37°C incubator for 6-8hrs. after which the incubated nuclei were prepared for Western blotting.
2.8 – Sucrose Gradient:

Sucrose gradient was prepared with the SG30 Gradient Maker (GE, 80-6197-80) following the manufacturer’s instructions. Sucrose gradient was made in Ultra-Clear Centrifuge Tubes (Beckman) with different volume. The sample was loaded on top of the sucrose gradient followed by centrifugation at the desired speed in Beckman Ultra Centrifuge Machine. Individual fractions were carefully removed.

2.9 - Scanning Electron Microscopy (SEM):

SEM was performed using a previously established protocol from the SEM facility at the Pennsylvania State University.
Chapter 3

Results

3.1 – PAD4 is sufficient for chromatin decondensation in non-granulocytic cells:

To assess cellular higher-order chromatin structure following abundant citrullination, U2OS cells were transiently transfected for PAD4 overexpression. To our surprise, PAD4 overexpression for 36 hr in U2OS cells induced the cells to rupture and release extensive web-like fibers into the extracellular space. To assess the similarity between these extracellular fibers and the NETs formed following calcium ionophore treatment of HL-60 cells (11), the U2OS cells were stained with α-H3Cit, α-PAD4 and Hoechst following PAD4 overexpression (Figure 1A, B). Not only was there increased H3Cit and DNA staining along the areas of highly decondensed chromatin, but cells positive for H3Cit and PAD4 were found to have an abnormal nuclear morphology similar to the NET producing HL-60 cells. These results indicated that PAD4 overexpression was sufficient for the observed chromatin decondensation in non-granulocytic cells (Figure 1A, B). The fact that mere transient transfection of PAD4 in U2OS cells could cause chromatin decondensation and extracellular fiber release was unexpected for two reasons. Not only was the phenomenon occurring in non-granulocytic cells, but the chromatin release was occurring devoid of cellular treatment with calcium ionophore or pro-inflammatory cytokines. A previous study hinted that PAD4 overexpression induces apoptosis due to Cytochrome c release and activated caspases (12). To further confirm that the release of chromatin was due to abundant intracellular citrullination and not just a downstream event of the apoptosis cascade, western-blot analysis was performed. Cellular lysate was obtained after U2OS
cells were transfected to express PAD4. Western-blot revealed increased H3Cit staining and no activation of Caspase-3 in cells overexpressing PAD4 as compared to the control (Figure 1C). This result further verified that PAD4 activity alone is sufficient for extensive chromatin decondensation. Additionally, this system offered us a model to analyze the molecular mechanism underlying the process of PAD4 mediated NETosis.

To obtain a better understanding of the cellular morphology and dynamics of PAD4-mediated chromatin decondensation, scanning electron microscopy (SEM) was performed of U2OS cells transfected with PAD4 as previously described. As with all transfection experiments, 100% transfection efficiency has never been achieved and thus the experiment provided a built in control of cells that received the PAD4 plasmid and those that did not. U2OS cells that did not overexpress PAD4 were flat, appeared firmly attached to the coverslip, had clearly visible nuclei and did not contain extracellular fibers (Figure 2A). However, those cells appearing to overexpress PAD4 became irregularly shaped cellular masses with little to no attachment to the substratum and no clear nuclear morphology (Figure 2B). Upon closer examination, transfected cells made prominent extracellular structures analogous to NETs (10). These structures assumed thin and thick stretches that literally extruded from the cells. In some instances this fibril network would encompass the entire cell it originated from as well as entrap other overexpressed cells (Figure 2C). Interestingly, high magnification SEM showed vesicular domains of varying lengths interdispersed within the larger stretches (Figure 2D). These results indicate that PAD4 overexpressing cells assume abnormal cellular morphology and do release intracellular structures into the extracellular space as initial immunostaining results documented.
3.2 – The extent of PAD4 mediated chromatin decondensation depends on intracellular calcium concentrations:

Literature has shown that in vivo citrullination is only observed after high intracellular calcium concentrations are achieved in cells treated with calcium ionophores (4,13). Interestingly, after 36 hrs of PAD4 transfection in U2OS cells, there was abundant citrullination along areas of highly decondensed chromatin even without ionophore treatment. This observation led us to hypothesize that the cell senses increased PAD4 levels and upon doing so releases its intracellular calcium stores into the cytosol leading to histone “hypercitrullination” and eventual chromatin decondensation. To test our hypothesis, we employed the intracellular calcium chelator, 1,2-Bis(2aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester), better known as BAPTA-AM (14) at increasing non-toxic concentrations. U2OS cells were first seeded in a 6-well plate and transiently transfected with PAD4. After 12 hrs of transfection, the media was removed and replaced with 0 µM, 5 µM and 10 µM BAPTA-AM dissolved in DMSO and brought to a final volume of 2 ml with DMEM supplemented with P/S. PAD4 was then allowed to express for an additional 24 hr. Then cells were fixed and stained with α-PAD4, α-H3Cit and Hoechst and observed under fluorescence microscopy. A similar experiment was run in parallel so that protein could be extracted for western blot analysis. After increasing BAPTA-AM concentrations, there was an obvious decrease in the extent of cells undergoing chromatin decondensation as evidenced by H3Cit staining (Figure 3A). To assess our initial observations, Western Blot (WB) analysis was performed. WB confirmed the reduced levels of H3Cit at increasing concentrations of BAPTA-AM, but more importantly showed equal levels of PAD4 expression (Figure 3B). If BAPTA-AM is truly preventing intracellular calcium from binding to PAD4 then the activity and not production of PAD4 is inhibited. Our immunostaining and WB experiments then promoted us to
quantify the effect of BAPTA-AM on PAD4 mediated chromatin released. Because PAD4 expression was unaltered by BAPTA-AM and only those cells overexpressing PAD4 should release chromatin; the total number of cells that were citrullination positive and citrullination/decondensation positive were tabulated as a percentage of PAD4 positive cells (Figure 3C). Analysis revealed that at increasing concentrations of BAPTA-AM, the percentage of cells that were solely Cit positive increased while the percentage of cells that were Cit/decondensation positive decreased. These results reveal that sufficient intracellular calcium concentrations must be attained for PAD4 mediated chromatin decondensation.
3.3 – The extent of PAD4 mediated chromatin decondensation depends on the activity of PAD4:

To further verify that the activity of PAD4 is essential for PAD4 mediated chromatin decondensation, which would support our previous finding of the role of intracellular calcium on the overall process, we employed the synthetically derived PAD4 inhibitor, BL1-07 (our unpublished new reagent). Using the same approach for studying the role of calcium on chromatin decondensation, PAD4 was transfected into U2OS cells which were then treated with 0µM, 5 µM and 10 µM BL1-07, fixed and then immunostained with α-PAD4, α-H3Cit and Hoechst and viewed under fluorescence microscopy. A duplicate experiment was run in parallel for Western-blot analysis. Consistent with our results of the role of calcium in PAD4 mediated chromatin decondensation, increasing concentrations of BL1-07 significantly reduced the amount of cells undergoing chromatin decondensation with no decrease in PAD4 expression, which was further confirmed by Western-Blot analysis (Figure 4A, B). To quantify our observations, the total number of cells that were citrullination positive and citrullination/decondensation positive were tabulated as a percentage of PAD4 positive cells (Fig. 4C). Tabulation indicated that at increasing concentrations of BL1-07, the percentage of cells that were solely Cit positive increased while the percentage of cells that were Cit/decondensation positive decreased. Overall, these results are consistent with those obtained by employing BAPTA-AM on PAD4 over expressed U2OS cells and further support the notion of PAD4 activity as a crucial determinant for cellular chromatin decondensation.
3.4 – Citrullination at NPM arginine 197th residue causes structural changes in NPM1:

To begin elucidating the mechanism of chromatin decondensation and eventual extracellular release due to PAD4, we began by examining a protein found to be an integral component of higher-order chromatin structure—nucleophosmin/B23 (NPM1). NPM1 is an ubiquitously expressed phosphoprotein located in the nucleolus that has been shown to play an important role in cell proliferation, processing of ribosomal RNA, as well as centrosome duplication (15). In addition, it has been reported that NPM1 is citrullinated following calcium ionophore treatment of HL-60 cells at the arginine 197 residue (6,16). More intriguingly, PAD4 overexpression was shown to result in NPM1 translocation from the nucleoli to the nucleoplasm, which may hint at the role of NPM1 in the process of DNA release (16).

To test this finding, a plasmid expressing a mutant NPM1 (R197Q) was engineered (Figure 8A). Because citrulline (Cit) is a nonstandard amino acid, we chose Glutmine (Q) to replace Arg (R) because it most closely, physically and structurally resembles Cit. If PAD4 truly citrullinates NPM1 at Arg197 and leads to its diffusion into the nucleoplasm, then similar results should be observed when this mutant construct is introduced into cells. U2OS cells were transiently transfected with the pMIGR1-NPM1(R197Q) construct for ~36 hr. The cells were then fixed and immunostained with α-NPM. Although NPM1 was not found to completely diffuse into the nucleoplasm like previous work has shown (16), we did observe enlarged and more lobular nucleoli than compared to the control, with normal DNA staining (Figure 5). The fact that there was not a significant dynamic alteration in NPM1 after introducing the mutant plasmid suggests that global citrullination and not just Arg197 citrullination is needed to cause NPM1 to translocate from the nucleoli.
3.5 – Global citrullination has a significant affect on NPM1 structure in vivo and in vitro:

Our previous result of the sufficiency of PAD4 to cause chromatin decondensation allowed us to further assess the role of global citrullination on NPM1 in vivo. Following PAD4 overexpression in U2OS cells, the cells were fixed, stained with H3Cit and NPM1 antibodies and viewed under fluorescence microscopy. NPM appeared to lose all nucleolar integrity and was observed to completely diffuse into the nucleoplasm as compared to the control (Figure 6B, A). Additionally, NPM1 seemed to colocalize with PAD4. This result correlates the effect of global citrullination on NPM1 higher-order genome structure that may occur during chromatin decondensation.

To further explore our above observation, we decided to perform an in vitro biochemical experiment that would assess the role of global citrullination on the oligomeric structure of NPM1. As previously mentioned, NPM is apart of higher-order chromatin structure, is found to exist in multiple oligomeric forms in the cell and is a substrate of PAD4 (15), (6), (16). We postulated that citrullination of NPM would cause the protein to lose structural integrity due to a decrease in the protein's positive charge and due to initial observation that NPM-R197Q transfected cells have more lobate and diffuse nucleoli. Purified His6-NPM was treated with wild-type GST-PAD4 fusion protein and the catalytically inactive GST-PAD4 (C645S) mutant in the PAD assay buffer. Both samples were then subjected to sucrose gradient analysis followed by western-blotting. α-NPM staining revealed that citrullinated NPM treated with GST-PAD4 had a greater sedimentation coefficient (Figure 7, Fraction 8 ) than the NPM treated with the mutant PAD4 (Figure 7, Fraction 5). This result indicates that globally citrullinated PAD4 may have a substantial affect on the overall 3D-structure of NPM1.
Chapter 4

Discussion

In this study, we showed that 1) PAD4 overexpressed cells release highly decondensed chromatin rich in histone citrullination which is dependent upon the activity of PAD4 and 2) nucleophosmin (NPM1), a protein involved in higher order chromatin undergoes structural changes during chromatin release. My study provides a novel model for assessing the biological role of PAD4 in chromatin decondensation and for the first time hints at the effects of alterations in higher-order chromatin structure in the process. More importantly, these experiments provide a link to further delineating the mechanism of NET formation, in primary neutrophils, which is now recognized as an important innate immune response.

It has previously been shown that not only is PAD4 responsible for histone “hypercitrullination” found along NETs (11) but that PAD4 is also required for bacterial killing mediated by these chromatin “webs” (17). These findings provide a role for histones and their epigenetic modifications in a physiological process that was originally never fully recognized. Here we show that PAD4 overexpression in non-granulocytic cells devoid of pro-inflammatory cytokines or calcium ionophore treatment is sufficient to induce extensive chromatin decondensation. Interestingly, the chromatin stained positive for histone citrullination, similar to NETs (11,17) and upon further analysis occurred devoid of Caspase-3 cleavage. Other work has shown that NET formation results in neutrophil death independent from apoptosis because NETotic cells do not display “eat-me signals” such as phosphatidyl-serine (18) and no caspase activity is detectable (18). The process of NET formation is thus truly unique to other cell suicidal programs. Our results suggest that PAD4 alone is capable of catalyzing chromatin decondensation and that this observed process may be mechanistically similar to what is observed
in vivo. Therefore, it is valid to study the mechanism of chromatin decondensation as a model for NETosis via overexpressing PAD4 in non-granulocytic cells.

As previously stated, PAD4 seems to be crucial for mediating chromatin decondensation as indicated by our immunofluorescence studies, but to further solidify our findings and begin to analyze the cellular effects of chromatin decondensation, it was necessary to microscopically examine the “NET-like” structures formed by PAD4 overexpressed cells. Some of the primary literature examining NETs provides electron microscopy studies illustrating that neutrophils undergoing NETosis will flatten, will form extensive membrane protrusions and the released DNA forms a meshwork or fibers (10). Our SEM analysis revealed similar results, in that PAD4 overexpressed cells eminated both long and dense stretches of chromatin. The denser areas of fibrillar matrix were marked by what appears to be vesicular structures probably the result of the chromatin interacting with the cell membrane before extracellular release. In contrast, we show that PAD4 overexpressed cells loose contact with their substratum and round-up instead of flattening-out. This dissimilarity is perhaps a consequence of the wild type morphology of the cells under study prior to chromatin decondensation, nuclear swelling and then subsequent chromatin release. Neutrophils are oval and non-adherent cells and U2OS cells are flat and are adherent in vivo. Therefore, the morphology of the two different cells would appear unique upon extracellular chromatin release. Although there exists a difference in cell morphology upon chromatin release, it is not significant enough to devalue the notion that PAD4-mediated chromatin decondensation and NET formation are mechanistically similar.

Although PAD4 is shown to be a crucial mediator of NETosis (17), how PAD4 fits into the overall mechanistic model for NET formation is poorly understood. In vivo studies of the role of PAD4 and NETosis require that primary neutrophils or differentiated HL-60 cells be pre-treated with calcium ionophore because PAD4 requires calcium for catalytic activity (11,13). Inhibition of PAD4 with Cl-amidine followed by calcium ionophore treatment of
differentiated HL-60 cells results in a significant reduction of histone citrulline positive decondensed chromatin (11). Intriguingly, our experiments showed that chromatin decondensation can be induced in cells without prior treatment with calcium ionophore. Moreover, chelating intracellular calcium stores via BAPTA-AM during PAD4 overexpression in U2OS cells results in a marked decrease in decondensed chromatin and H3Cit positive decondensed chromatin. Our results thus highlight a possible calcium regulated mechanism leading to PAD4-mediated chromatin decondensation. Under physiological conditions, the cell must sequester its calcium in specific organelles, most notably in the endoplasmic reticulum, and when needed be released to serve as a second-messenger in signaling processes. From the perspective of PAD4, the intracellular calcium concentration is 10^{-8}-10^{-6} M and thus PAD4 is normally inactive (13).

From our work, we can postulate that increased expression of PAD4 leads to the release of Ca^{2+} from one of its sub-cellular compartments, which then binds PAD4 elevating PAD4's activity to a sufficient level required for extensive "hypercitrullination" of histones. Biochemically, the ample conversion of positively charged arginine to neutral citrulline on histones could potentially promote weaker interactions with DNA forcing the nucleosomes to significantly "relax," leading to chromatin decondensation and eventual release into the extracellular space. The notion that the activity of PAD4 is needed for chromatin decondensation is supported from our study employing the PAD4 inhibitor, BL1-07 following PAD4 overexpression (Figure 4).

Excessive citrullination of histones due to PAD4 leads to chromatin decondensation and this gene regulatory event is one of the final steps just prior to nuclear envelope disintegration and breaking of the cell membrane to form NETs (9,11). But to limit to the mechanism of NET formation to this model is not sufficient given that the nuclear genome is more complex than just DNA wrapped around histones in a confined nuclear space--the role of
global citrullination and proteins involved in higher-order chromatin structure must be considered. Previous work has shown that NPM1, a protein involved in higher-order chromatin is another substrate of PAD4 (19). Additionally, citrullination of NPM1 at the Arg 197th reside can cause its translocation from the nucleolus to the nucleus (16). Using the NPMR197Q to mimic citrullination at this site, we have shown that citrullination here is not necessary to cause complete diffusion of NPM1 to the nucleoplasm. However, citrullination at Arg 197 does seem to cause a change in the structure of NPM1 from a more compact, confined nucleolar localization to a more lobate and irregular one. This result supports the notion that global citrullination may be necessary to observe complete structural deformity of NPM1 in vivo. This effect of NPM1 global citrullination in vivo was further confirmed in PAD4 overexpressed cells stained with PAD4 and NPM1 antibodies (Figure 6). However, although NPM1 is normally localized in discrete bundles in the nucleous, it can possess many shapes and sizes due to the dynamic nature of the protein, thus posing a limitation to our results.

In addition, our sucrose gradient analysis of purified NPM1 reacted with purified PAD4 via an in vitro assay also supports the idea of an overall change in NPM1 structure followed by global citrullination. NPM1 exists in many oligomeric forms (15) and our work would suggest that during elevated expression and activity of PAD4, global deimination of NPM1 would result in a more compact monmeric form of NPM1 instead of a more complex 3D-structure prior to citrullination. Although our sucrose gradient work would support this idea, the only limitation is that this assay occurred devoid of chromatin-bound NPM1 so that the true in vivo 3D conformation of NPM as a part of higher-order chromatin structure is missing. However, this deformation of NPM1 along with increased "relaxing" of histones may facilitate PAD4-mediated chromatin decondensation and for the first time hints at the role of genome organization in the formation of a physiological response.
In order to fully observe the changes in higher-order chromatin that occur during PAD4 overexpression in vivo, it is best to employ live-cell imaging of the process. Our construct that allows expression of, RFP-NPM1 and H3-GFP from the same polycistronic mRNA (Figure 8B) would allow the changes in NPM1 to be observed during PAD4 overexpression. It is postulated that upon elevated levels of PAD4, the nuclei of the cells will swell (visualized with H3-GFP), and NPM1 will begin to diffuse into the nucleoplasm and eventually completely disappear (visualized with RFP-NPM) as the cells release their chromatin. Unfortunately, due to experimental constraints such as timing the exact moment when PAD4 expression is elevated in U2OS following transfection, this goal was unable to be accomplished. However, given that the stable cell line exists allows for the investigation of this process and will serve as an essential tool for analyzing and further understanding the changes in higher-order chromatin during NET formation.

The fact that our results prove a sufficiency for PAD4 mediated chromatin decondensation in non-granulocytic cells truly elevates the significance of this epigenetic protein in vivo and solidifies its role in eliciting NET formation in primary neutrophils. In the formation of NETs, PAD4 is one of the final downstream effectors that enters the nucleus mediating extensive histone “hypercitrullination.” Together with nuclear membrane disruption, release of the nuclear chromatin into the cytoplasmic space and eventually extracellularly marks NETosis (18). Although this mechanism intuitively makes sense, there still needs to be further investigation into the regulation leading up to activation of PAD4. Despite a requirement for sufficient intracellular calcium concentrations, perhaps PAD4 also gets phosphorylated, via a downstream kinase cascade, further promoting activity and nuclear localization, as some recent studies would suggest (20). Additionally, delineating the interplay of higher-order chromatin structure and PAD4 that leads to chromatin decondensation will give new insight into the role the genome plays in mediating physiological responses.
Figure 1: PAD4 overexpression in U2OS cells is sufficient for chromatin decondensation. A and B, U2OS cells transiently transfected with HA-tagged PAD4, fixed and stained with α–H3Cit, α–HA and Hoechst.

(A) H3Cit and PAD4 staining are along areas of highly decondensed chromatin. (B) H3Cit and Hoechst staining showing highly decondensed structures colocalize DNA indicating the released structures are chromatin. (C) Western blot analyses of H3Cit and Caspase-3 in PAD4 overexpressed cells.
Figure 2: PAD4 overexpression in U2OS cells followed by SEM analysis. (A) Untransfected U2OS cells have normal nuclear morphology and attach firmly to slide. (B) Transfected U2OS cells show de-attachment from slide, a non-distinguishable nucleus and an extensive fibrillar network that branches from one cell to the next. (C) Higher magnification of panel B showing branching of fibers. (D) Higher magnification of panel B showing fibrillar network with vesicular domains dispersed throughout. Arrows (Panel B) denoting regions of increased magnification and globular domains (Panel C).
Figure 3: Sufficient intracellular calcium concentrations are required for PAD4 mediated chromatin decondensation.
(A) U2OS transiently transfected with HA-tagged PAD4 with addition of increasing concentration of BAPAT-AM (calcium chelator) followed by fixation and staining with α-H3Cit, α-HA and Hoechst shows a marked decrease in chromatin decondensation. (B) Western blot analyses of PAD4 and H3Cit levels following PAD4 over expression and BAPTA-AM treatment. (C) Quantification of total citrullination+ and citrulline+/decondensation+ cells as a percentage of total PAD4+ cells reveals the need for suitable intracellular calcium levels for chromatin decondensation to occur.
Figure 4: The relative activity of PAD4 is essential to induce chromatin decondensation.
(A) U2OS cells transiently transfected with HA-tagged PAD4 with addition of increasing concentrations of BL1-07 (PAD4 inhibitor) followed by fixation and staining with α-H3Cit, α-HA and Hoechst shows a marked decrease in chromatin decondensation. (B) Western blot analyses of PAD4 and H3Cit levels following PAD4 overexpression and BL1-07 treatment. (C) Quantification of total citrulliune+ and citrulline+/decondensation+ cell as a percentage of total PAD4+ cells, shows that the sufficient enzymatic activity of PAD4 must be attained to induce chromatin decondensation.
Figure 5: Citrullination of NPM1 at ARg197th residue results in abnormal NPM1 morphology. (A) Transfected U2OS cells with NPM shows NPM1 in discrete bundles within the nucleolus. (B) Transfected U2OS cells with NPMR197Q depicts more lobate irregular NPM1 morphology within the nucleolus.
Figure 6: Global citrullination, via PAD4 overexpression causes a structural change in the higher-order chromatin protein (NPM1). (A) Untransfected U2OS cells stained with α-NPM, α-PAD4 and Hoechst show that PAD4 is localized in the nucleus, NPM1 is located in discrete bundles within the nucleolus and the nucleus has normal morphology. (B) U2OS cells transfected with HA-tagged PAD4 followed by staining with α-NPM, α-HA and Hoechst shows abundant PAD4 expression, diffusion of NPM1 from the nucleolus to the nucleoplasm and irregular nuclear morphology.
Figure 7: NPM changes oligomeric form upon global citrullination. His6-NPM reacted with WT-PAD4 and PAD4(C645S) in PAD4 assay buffer for 4hrs. at 37°C followed by sucrose gradient analysis of the fractions. Fraction 8-11 show that His6-NPM reacted with WT-PAD4 have a higher sedimentation coefficient than compared to Fractions 5-7.
Figure 8: Molecular construct design. (A) NPMR197Q mutant construct for analyzing role of citrullination at Arg 197th residue on NPM1 structure and localization. (B) RFP-NPM-H3-GFP construct for in vivo live-cell imaging following PAD4 over expression to assess changes in higher-order chromatin during extensive citrullination.
References

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