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Peptidylarginine deiminases in citrullination, gene regulation, health and pathogenesis

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Abstract

Peptidylarginine deiminases are a family of enzymes that mediate post-translational modifications of protein arginine residues by deimination or demethyelimination to produce citrulline. *In vitro*, the activity of PADs is dependent on calcium and reductive reagents carrying a free sulfhydryl group. The discovery that PAD4 can target both arginine and methyl-arginine for citrullination about 10 years ago renewed our interest in studying this family of enzymes in gene regulation and their physiological functions. The deregulation of PADs is involved in the etiology of multiple human diseases, including cancers and autoimmune disorders. There is a growing effort to develop isoform specific PAD inhibitors for disease treatment. However, the regulation of the activity of PADs *in vivo* remains largely elusive, and we expect that much will be learned about the role of these enzymes in normal life cycle and under pathology conditions.

Keywords

peptidylarginine deiminase; histone; gene regulation; cancer; autoimmunity

1. INTRODUCTION

1.1 The early discovery of PADs as protein citrullination enzymes

Citrulline residues were detected in early 1960s from polypeptide hydrolysates of hair inner root sheath cells and medullary cells [1]. Since there is no citrulline tRNA *in vivo*, citrulline can only be produced by enzymatic modifications after protein synthesis. Subsequently, the peptidylarginine deiminases (PADs, also called PADIs) were identified, which convert protein arginine residues to citrulline in a calcium dependent manner ([2], reviewed in [3]). PADs are involved normal functions in the immune and reproduction systems as well as in the skin (see recent review in these particular areas in [4–6]). In each of the mammalian vertebrate genomes, five highly conserved PADs exist, including PAD1, 2, 3, 4 and 6. (Fig.

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1A). In the last decade or so, more research on the gene features, tissue-specific distribution and preferred substrates of PADs has been performed. Human PAD4 can bind five calcium ions (Fig. 1B). Several flexible parts of PAD4 form stable secondary structures after the binding of calcium and substrate, indicating that calcium stabilizes the conformation of PAD4 and may facilitate the formation of the active site cleft (Fig. 1C and 1D). The calcium binding sites in PAD4 are conserved in PAD1, -2, and -3 except for PAD6. Thus, calcium is an important regulator of the active PAD enzymes [7].

1.2 The five PADs

1.2.1—PAD1 is mainly expressed in epidermis and uterus [8, 9]. PAD1 deiminates keratin K1 and is involved in the cornification of epidermal tissues [10].

1.2.2—PAD2 is widely expressed in multiple tissues, including secretory glands, brain, uterus, spleen, pancreas, skeletal muscle [11–16]. The expression of PAD2 can be regulated at both mRNA splicing and protein translation levels [15, 16]. Myelin basic protein of the central nervous system and vimentin in skeletal muscle and macrophages are long known substrates of PAD2 [16, 17]. Recently, α - and β -actins were identified as PAD2 substrates in human neutrophils [18]. PAD2 is mainly a cytoplasmic protein, but a fraction of PAD2 may become nuclear in canine and human mammary epithelial cells [19, 20]. Nuclear PAD2 may citrullinate histones H3 and H4 [19, 21–23], suggesting a role of this protein in gene regulation.

1.2.3. PAD3 is localized to epidermis and hair follicles [24–26]—PAD3 is colocalized with trichohyalin, a structural protein in the inner root sheath and medulla of hair follicles [25, 26]. In addition, PAD3 colocalizes with profilaggrin and filaggrin in the epidermis [26]. PAD3 targets filaggrin, which interacts with keratin intermediate filament to regulate epidermal homeostasis in the granular layer and lower stratum corneum of human epidermis [2, 25, 26]. Deimination of filaggrin and trichohyalin *in vitro* by recombinant PAD3 further supports that PAD3 is involved in regulating epidermis functions [25, 26].

1.2.4—PAD4 (also called PADV and PADI4) is detected mainly in white blood cells including granulocytes and monocytes under normal physiological conditions [16, 27, 28]. However, in a wide range of tumors of various tissue origins, the overexpression of PAD4 was detected, suggesting that PAD4 plays a role in tumorigenesis [29, 30]. PAD4 is localized primarily in the nucleus and contains a nuclear localization signal sequence at its N-terminus. PAD4 citrullinates a range of nuclear proteins, such as histones H2A, H3 and H4, ING4, p300/CBP, nucleophosmin and nuclear lamin C, thereby playing an important role in nuclear functions [31–36].

1.2.5—PAD6 was originally identified from mouse eggs and embryos and was named ePAD (egg PAD) [37]. PAD6 regulates oocyte cytoskeletal sheet formation and female fertility [38]. Recently, it is found that PAD6 localizes to the cytoplasmic lattices and regulates the function of microtubules during early embryo development [39]. In human tissues, PAD6 is mainly restricted to ovary, testis and peripheral blood leucocytes [40]. Interestingly, unlike the other PADs, PAD6 have lost some of the conserved Ca^{2+} binding residues and the active center cysteine residue is also different from other PADs [41], suggesting that PAD6 is likely not an active deiminase.

Recent studies have revealed the role of PADs in physiological and pathological conditions. In the following sessions, we will focus on the role of PADs in gene regulation, innate immunity, cancers and autoimmune diseases. We will further discuss the potential of PADs as druggable targets for disease treatment.

2. PAD4 and PAD2 IN GENE REGULATION

2.1. PAD4 in gene regulation

In eukaryotic cells, nuclear DNA is organized with two of each histones H3, H4, H2B, and H2A to form a nucleosome core particle, which is further organized with the linker DNA and histone H1 to form the 10 nm chromatin fiber and folded to form the higher order chromatin structures. The nuclear structure and 3D organization is under continuous remodeling to adapt to the physiological and environmental changes that the cells are exposed to. Because of this structural organization, histone modifications, including methylation, acetylation, phosphorylation and citrullination, work as a signaling network to provide the on the off signal for gene expression and/or a landing platform for effector protein binding [42–44].

Histone modifying enzymes with opposite activities counteract each other's effect, such as histone acetyltransferases (HATs) and histone deacetylases (HDACs), kinases and phosphatases [45, 46]. Until the Lys demethylases, such as LSD1 and JmjC domain-containing dioxygenase, as well as the Arg deiminase PAD4 were identified [47, 48], histone methylation on Arg and Lys residues was considered as static rather than dynamic because of the low turnover rate of the methyl groups [49]. PAD4 antagonizes CARM1 (also called PRMT4) and PRMT1 mediated histone H3 and H4 Arg methylation through a reaction dubbed as demethylimination in reflecting the removal of the methyl-imine group from monomethyl-arginine residues [33]. The activity of PAD4 on asymmetrical dimethyl-arginine residues is very low. We have observed that PAD4 prefers methyl-Arg in histone proteins over methyl-Arg in short peptides as substrates, suggesting the substrate has an allosteric effect on PAD4 [33]. CARM1 and PRMT1 function as transcription coactivators by catalyzing histone Arg monomethylation and asymmetrical dimethylation [42, 50–53]. By antagonizing Arg methylation, PAD4 functions as a transcription corepressor. In the case of ER target genes in the breast cancer MCF-7 cells, PAD4 regulates histone Arg methylation via its citrullination activity on the gene promoters [33, 54]. Interestingly, the modification of histone Arg residues on the ER (estrogen receptor) target promoters fluctuates over time after estradiol treatment, whereby the increase in histone citrullination correlates with the decrease in histone Arg methylation, indicating that opposite enzymes are alternatively working on the ER target gene promoters [55].

In addition to the ER target genes, our group has found that PAD4 interacts with the tumor suppressor and transcription factor p53 and functions as a corepressor to regulate the expression of multiple p53 target genes [56, 57]. Before DNA damage, a high level of histone citrullination and PAD4 was detected on the promoter of p53 target genes, such as p21/CIP1/WAF1, GADD45 and PUMA [56, 57]. After DNA damage, PAD4 association and histone citrullination decreases on these gene promoters with a concomitant increase in histone Arg methylation, suggesting that citrullination and arginine methylation counteract each other's function to regulate gene expression.

The role of histone acetylation in gene activation has been long established. A landmark 2004 paper with rich *in vitro* biochemical analyses showed that protein Arg methyltransferases and histone acetyltransferase p300/CBP cooperatively activate the p53-mediated transcription [42]. Reversely, we found that PAD4 and HDAC2 associate with p53 in a dynamic fashion [56]. Before DNA damage, both PAD4 and HDAC2 are associated with p53 target gene promoters, while they dissociate from the gene promoters after DNA damage allowing the activation of the p53 target genes, such as p21, GADD45 and PUMA [56]. Taken together, we propose that histone Arg modification in concert with histone Lys acetylation forms a molecular switch on the p53 target gene promoters for gene regulation (Fig. 2).

Although the corepressor function of PAD4 has been well established, it may also play a coactivator role in a promoter context dependent manner. A genome wide ChIP-chip study of PAD4 promoter association in MCF-7 cells found that PAD4 is enriched on the promoter regions of actively transcribed genes [58]. Motif analyses found that many of the PAD4 bound genes contain potential binding sites for Elk-1, a member of the ETS oncogene family [58]. It was proposed that PAD4 interacts with and citrullinates Elk-1 thereby facilitating Elk-1 phosphorylation to activate transcription [58]. Additionally, PAD4 can target histone H3 Arg8 for citrullination and subsequently affects the binding of HP1 (heterochromatin protein 1) to the nearby H3 Lys9 methylation site [59, 60]. The dissociation of HP1 from its binding cognate sites after citrullination activates transcription in multiple sclerosis patients [60]. In addition, the dissociation of HP1 likely regulates chromatin decondensation during the formation of neutrophil extracellular traps (NETs) [59].

The hypothesis of the “histone code” predicts that histone modifications may function in a combinatorial manner to regulate chromatin biology [61]. Two adjacent modification sites were proposed to form a binary code to antagonize or synergize the function of each other [62]. The effect of histone H3 Ser10 phosphorylation on the function of H3 Lys9 methylation and HP1 in cell cycle and gene regulation was well studied [63, 64]. The new finding of the effect of H3 Arg8 citrullination on HP1 binding to H3 Lys9 methylation greatly enriched the binary code concept and highlights the dense cell signaling information that a cluster of histone residues carries (Fig. 3).

2.2. PAD2 in gene regulation

That PAD2 can citrullinate histone H4 Arg3 *in vitro* raises a role of PAD2-mediated histone citrullination in transcriptional regulation [21]. The expression of PAD2 is regulated by estrogen in vertebrate uterus and pituitary gland [65–67]. Recent data from the Coonrod's group support that PAD2 responds to cellular signals to regulate transcription via histone citrullination. Only during the diestrus phase of the reproduction cycle, PAD2 in mammary gland epithelial cells was found to citrullinate the histone H3 N-terminus [19]. Moreover, PAD2 is detected in human breast luminal epithelial cells, and associates with target gene promoters in the ER⁺ positive breast cancer MCF-7 cells to regulate histone H3 Arg26 citrullination and transcription [20]. Estradiol stimulates the recruitment of PAD2 to the estrogen-response element of estrogen receptor alpha (ER⁺) target gene promoters. Since PAD2 does not have a nuclear localization signal, its association with ER⁺ may facilitate its translocation from the cytosolic to the nuclear compartment [20]. As such, PAD2 likely mediates chromatin decondensation and activation of target gene transcription during cellular response to estrogen stimulation [23]. These studies indicate that PAD2 functions as an epigenetic regulator of gene activity and plays a potential role in breast cancer progression.

3. PAD4 and NETs

3.1. NETs, a highly decondensed form of chromatin

Peripheral blood neutrophils serve as the first line of defense against microbial infection [68–70]. Although neutrophils are well known for their antimicrobial function via phagocytosis, it was reported in 2004 that a distinct mechanism of killing bacteria in the extracellular space using a meshwork of highly decondensed chromatin in association with antimicrobial proteins (e.g., myeloperoxidase and neutrophil elastase) [68]. Upon encountering bacteria, a certain percentage of neutrophils release their chromatin into the extracellular space, forming chromatin-based structures called neutrophil extracellular traps (NETs) (reviewed by [71]). NET formation can be induced by IL-8, PMA, LPS and diverse microbes, such as bacteria and fungi, as well as protozoan parasites [68, 72]. As a

mechanism of immune defense, NETs could mediate the microbial death and/or limit pathogen spread in host [68, 73, 74]. Although the role of NETs in innate immunity and human disease has gained much attention, the mechanism regulating the NET formation is less clear. The activation of the intracellular signaling pathways, such as the MAPK pathway and the ROS (reactive oxygen species) signaling pathways, has been implicated in the formation of NETs [75–77]. Since NET formation directly leads to cell death (dubbed netosis) and the release of nuclear and cytoplasmic components into the extracellular space has harmful consequences to the surrounding normal tissues, this process needs deliberate regulation.

3.2. Histone hypercitrullination in NET formation

Chromatin in NETs is extremely decondensed, with a diameter ranging between 10–30 nm [68]. This degree of chromatin decondensation indicates that the neutrophil chromatin can be unfolded extensively to form the polymers of nucleosomes, i.e., the 10 nm chromatin fibers. A proteomic study of the NET protein components has found that even linker histone H1 is diminished from NET chromatin [78]. However, the chromatin decondensation mechanisms underlying NET formation remain further explored.

Retrospectively, our finding that PAD4 and histone hypercitrullination are involved in the formation of neutrophil extracellular traps is a serendipitous story. In the early months of 2004, we found that prolonged treatment of HL-60 granulocytes with calcium ionophore produced sticky chromatin that glues many of cells in a cluster. The sticky chromatin was stained with the DNA dye and very strongly stained with the histone H4Cit3 antibody, suggesting that there is a link between the high-degree of histone citrullination and the formation of NETs. The link of PAD4 and NET formation was eventually published in 2009 [79]. Around the time we were preparing to publish our report, the Radic group published immunostaining images to show that NET chromatin can be stained by a histone citrullination antibody [80]. It is now clear that one of the normal physiological functions of PAD4 in peripheral blood neutrophils is to regulate the formation of NETs.

Histone hypercitrullination is an important intermediate step for the formation of NETs. In HL-60 granulocytic cells, the inhibition of PAD4 and histone hypercitrullination decreases chromatin decondensation and the formation of NET-like structures [79]. Importantly, citrullination of a 12-mer nucleosome array decreased the chromatin folding by the linker histone H1 [79]. The role of PAD4 in NET formation is further supported by genetic studies in mice. The PAD4 knockout mice can survive to adulthood and are fertile with a normal number of neutrophil counts [81]. In PAD4 null neutrophils, the basal level of histone citrullination is undetectable. After stimulation with LPS, H₂O₂, PMA and bacteria, histone citrullination is greatly increased in wild type neutrophils but remains undetectable in the null cells [81]. The lack of histone citrullination is correlated with a lack of NET formation in the null cells, indicating that PAD4 is required for NET formation under these experimental conditions [81]. Furthermore, elevated PAD4 activity is sufficient for the chromatin decondensation to form NET-like structures. First, treatment of detergent permeated cells with GST-PAD4 but not its catalytic inactive mutant GST-PAD4 C645S induced the release of chromatin from HL-60 cells to form decondensed chromatin structures [79]. Surprisingly, forced overexpression of PAD4 in osteosarcoma U2OS cells was also sufficient for the formation of NET-like chromatin structures [59]. These results indicate that PAD4 activity is required and sufficient for the formation of decondensed chromatin, a process that is essential for the formation of NETs.

How does citrullination mechanistically unfold chromatin? First, the degree of histone citrullination associated with NET formation is very high. Based on Edman degradation experiments to analyze histone citrullination, we estimate that over 50% of histone H3 Arg8

can be converted to citrulline [33]. Since citrullination eliminates the positive charge of histones, this modification may weaken the interaction of histones and DNA thereby decreasing chromatin fiber compaction to facilitate NET formation. Second, PAD4 mediated histone hypercitrullination could exclude HP1 from binding to chromatin, offering a new mechanism for PAD4 mediated NET formation [59]. Third, citrullinated nucleosome arrays are not folded properly by the linker histone H1. Moreover, many other proteins can be targeted by PAD4 for citrullination. These other PAD4 substrates could play a modulatory role for the process of NET formation.

3.3.3 PAD4 in NET-mediated killing of bacteria and many inflammatory conditions—Evolution is a driving force behind the adaption of pathogenic bacteria to the host's immune defense system, while the host constantly creates mechanisms to eliminate invading microbes. Indeed, many pathogenic bacteria that are infectious to humans produce extracellular DNase to facilitate them to evade killing by NETs. For example, the *EndA* gene in *Streptococcus pneumoniae* encodes a membrane bound and extracellular faced endonuclease and the *Nuc* gene of *Streptococcus aureus* encodes the secreted micrococcal nuclease (MNase) [82]. Moreover, certain serotypes of the group A *Streptococcus pyogenes* secrete a DNase called Sda1, which facilitates the bacteria to generate a local skin infection called fasciitis [73]. Consistent with the role of NETs in bacteria killing, the PAD4 knockout mice are more susceptible to bacterial infections in the experimental fasciitis model, and neutrophils from the PAD4 deficient mice showed significantly decreased bacteria killing activity *in vitro* [81].

Like many other immune defense mechanisms in the human body, NETs serve as an antimicrobial defense weapon as well as fan the fire for inflammation, leading to tissue damages. NETs are implicated in many pathological conditions, such as appendicitis, cystic fibrosis, tuberculosis, sepsis, lupus and nephritis [4, 68, 83–86]. A common theme is that neutrophils recruited to the inflammation sites will die by netosis to form NETs and release their nuclear and cytoplasmic components into the extracellular space. The PAD4 protein released from neutrophils may citrullinate proteins and produce autoimmune antigens to cause chronic autoimmune diseases, such as rheumatoid arthritis (see below). Furthermore, NETs may have a quick impact on thrombosis by contributing the chromatin threads as a building material for the formation of thrombus [87]. Conversely, the lack of NET formation in the PAD4 null mice decreases the formation and maintenance of thrombi in the blood veins [6].

4. PAD IN HUMAN DISEASES

In addition to the above-mentioned pathological conditions associated with NETs, PADs and its citrullinated products have been associated with many human diseases. In this section, we will discuss the potential involvement of PAD4 in rheumatoid arthritis (RA) and PAD2 in multiple sclerosis (MS). We will then discuss the involvement of PAD4 in cancers.

4.1 Rheumatoid Arthritis

RA is a chronic autoimmune disease featured with inflammatory synovium and infiltration of activated macrophages. The anti-citrullinated protein antibodies are the most specific autoantibodies present in the RA sera [88–90]. Most of these autoantibodies can be detected in the early stage of the disease [91, 92], which makes them useful diagnostic markers of RA [93–95]. Those citrullinated proteins, including fibrin, fibrinogen and vimentin, were produced in synovial fluid of the inflammatory joints [96–101]. The presence of citrulline residues in these proteins sends the immune cells a false alarm and initiates immune responses to generate anticitrulline antibodies against these proteins [102, 103]. Many PAD2 and PAD4 expressing leucocytes infiltrate into the inflammatory synovial tissues in RA

patients and release large amount of PAD2 and PAD4 in the synovial fluid, which in turn produce high levels of citrullinated proteins [104, 105]. Since the calcium concentration in the extracellular space is at the millimolar levels, PAD4 released from the dying neutrophils during the NET formation process can be super-activated to citrullinate joint proteins [16]. In addition to these pathology links, the involvement of PAD4 in rheumatoid arthritis is also supported by human genetic studies, in particular in the oriental ethnic groups [106–108].

4.2 Multiple Sclerosis and Alzheimer's

MS is mainly a PAD2 related inflammatory disorder in the central nervous system associated with excess citrullination of myelin basic protein (MBP), resulting in the demyelination of the myelin sheath and affecting the nerve signal transduction [109, 110]. About 18% of MBP protein is citrullinated at 6 out of its 19 Arg residues in healthy individuals, while 45% or more of MBP is citrullinated in MS patients [111]. Hypercitrullination of MBP weakens its interactions with phospholipid and disrupts the formation of normal multilayer myelin sheath structures. In addition, hypercitrullinated MBP is more susceptible to protein degradation by the protease cathepsin D [112–114]. Elevated PAD2 in the myelin sheath is related to an increase in MBP citrullination prior to the onset of MS symptoms [109, 115]. PAD2 overexpression in transgenic mice increases the amount of citrullinated MBP and accelerates the development of demyelination [116–118]. Moreover, upregulation of PAD2 and the presence of the inflammatory signals at the affected axon regions might further increase PAD4 locally to exacerbate the inflammatory disease [115, 119]. It is surprising that knockout of PAD2 did not produce a significant phenotype in the nervous system given the importance of MBP citrullination in the axon electrical signal transmission [120]. Whether other PAD enzymes, such as PAD4 play a redundant role with PAD2 in the central nervous system remain to be solved.

In addition to multiple sclerosis, PAD2 and PAD4 are also expressed in neurons [121]. Protein citrullination in the central nervous system may cause protein denaturation and precipitation, which in turn lead to the Alzheimer's disease [121–123]. These studies highlight the possibility of PAD2 as a drug target for the treatment of nervous system diseases.

4.3 Cancer

Epigenetic modification plays an important role in tumorigenesis [124, 125]. Two major epigenetic alterations in cancer cells are histone modifications and DNA methylation. In this session we will focus on the effect of histone modifications on aberrant tumor suppressor gene silencing during tumorigenesis [126, 127]. Tumor suppressor p53 is one key transcription factor in maintaining cellular homeostasis. In response to diverse upstream signals, such as starvation, DNA damage and various stress signals, p53 regulates its downstream genes to cope with stress and control the cell fate [128–131]. Many of the p53 target genes are tumor suppressor genes that regulate the cell cycle arrest, programmed cell death, and autophagy.

Under normal conditions, PAD4 is mainly expressed in the peripheral blood leukocytes. In pathology studies using a large cohort of human patient samples, PAD4 is overexpressed in a majority of tumor tissues, including osteosarcoma, colon adenocarcinoma, esophagus adenocarcinoma, ovary adenocarcinoma, pancreas adenocarcinoma, and stomach adenocarcinoma [29, 30]. Given that PAD4 functions as a corepressor of p53 to regulate its downstream tumor suppressor genes, the overexpression of PAD4 in tumor tissues suggests that it might be involved in tumorigenesis. Interestingly, the expression of PAD4 is directly regulated by p53, suggesting that PAD4 may form a negative feedback loop to regulate p53 [132]. As we have discussed in the previous section, PAD4 catalyzed histone citrullination

coupled with HDAC2 catalyzed deacetylation represses p53 target gene expression [56]. Since HDACs are targets for cancer drug development, we recently devoted a solid effort to test if PAD4 is a druggable target for cancer treatment (see below).

5. PAD proteins as druggable targets for disease treatment

A benzoyl-arginine-derived compound Cl-amidine has been reported as a pan PAD inhibitor *in vitro* by forming covalent bond with the active center Cys of the enzyme thereby blocking the binding of natural substrates to the active site [133–135]. However, Cl-amidine inhibits cancer cell growth with an IC₅₀ of ~150–200 μ . [56, 57], which limits its clinical value in cancer treatment. Recently, we performed medicinal modification of Cl-amidine and developed novel PAD inhibitors with low micromolar IC₅₀ for PAD4 enzymatic activity inhibition and cancer cell growth inhibition [22]. Compared with Cl-amidine, the novel inhibitors show increased efficacy in PAD4 inhibition and more importantly increased membrane penetration [22]. The lead compound YW3–56 alters the expression of a cohort of genes, including many p53 target genes, to control the cell cycle and cell death [22]. Treatment of human cancer cells with YW3–56 induces the expression of SESN2, a recently identified p53 target gene that serves as an upstream inhibitor of the mTORC1-signaling pathway [22, 136, 137]. YW3–56 disturbs the autophagy flux and leads to cell death [22]. YW3–56 has demonstrated anti-tumor activity in mouse xenograft studies with no overt adverse effect to vital organs, whereas a combination of YW35-6 with HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) further improved the tumor growth inhibition [22]. Future studies to explore the spectrum of cancers that can be targeted and the molecular mechanisms underlying the anticancer effect of YW3–56 will be key to evaluate its potential as an anticancer drug.

In addition to the chloroacetamidine-based inhibitors, other types of PAD inhibitors have been developed [113, 138–142]. The efficacy and specificity of these inhibitors will need to be further improved. Interestingly, the mitotic inhibitor paclitaxel used in cancer chemotherapy can inhibit PAD2 mediated MBP citrullination *in vitro* [143]. Paclitaxel attenuated demyelination symptoms and induced the remyelination of the damaged myelin sheaths [144], suggesting that paclitaxel is a possible anti-MS compound. Recently, fluorescence-based PAD4 activity assay approaches were reported thus opening the door for future large-scale screening of PAD4 specific inhibitors [145, 146]. Furthermore, given that a combination of DNA methyltransferase inhibitor and HDAC inhibitor like SAHA can synergistically activate tumor suppressors in cancer cells and animal tumor models [147–150], the combined usage of PAD4 and HDAC inhibitors might be a promising strategy in future cancer treatment.

6. Conclusions and future perspectives

The citrullination of structural protein in the epidermis and neuronal system by peptidylarginine deiminase likely plays important roles in regulating the structure and function of these tissues. In addition, histone citrullination by PAD4 and PAD2 may influence the expression of many genes that are important for physiological functions. Deregulated citrullination has profound impact on human disease etiology. The role of histone and protein citrullination in cancers is emerging in recent years. Tumorigenesis is featured with many spontaneous driver and standby mutations. Targeting individual mutations one at a time may not be efficient. Therefore, it is still an attractive idea to reverse the tumorigenesis by altering the gene expression profile of cancer cells to that of the normal cells. One view of the tumorigenesis is the gradual silencing of the tumor suppressor genes. Many chromatin DNA and histone modifying enzymes are involved in this process, such as the DNA methyltransferases, histone deacetylases, and peptidylarginine deiminases (Fig. 4).

Others and us have shown that inhibition of PAD4 by small molecules can turn on the expression of the tumor suppressor genes in cancer cells, raising a possibility that PAD4 is druggable target for cancer treatment. In the field of cancer epigenetics, inhibitors for both DNA methyltransferases and histone deacetylases have been successfully used for cancer treatment. Only time will tell if inhibitors of PAD4 will eventually be effective in clinical tests.

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Highlights

- PADs are a family of enzymes that citrullinate protein Arg residues.
- PADs are involved in regulating p53 and nuclear receptor target genes.
- PAD4 plays a role in the formation of NETs and antibacterial innate immunity.
- Human cancers and autoimmune disorders involve deregulation in protein citrullination.
- PADs are promising drug targets for disease treatment.

Fig 1. The PAD family of proteins and the effect of calcium on the PAD4 structure
 (A) Phylogenetic analyses of 26 PAD proteins from several vertebrate species. Sequences of mammalian PADs were derived from databases for Human (*Hs: Homo sapiens*), mouse (*Mm: Mus musculus*), cow (*Bt: Bos taurus*), dog (*Cf: Canis lupis familiaris*). Also included are PADs from other species, such as chicken (*Gg: Gallus gallus*), trout (*Om: Oncorhynchus mykiss*) and frog (*Xl: Xenopus laevis*; *Xt: Xenopus Silurana tropicalis*). The unrooted phylogenetic tree was generated using ClustalW2 on EMBL-EBI website and visualized by MEGA 5.2. Scale bar represents 0.05 amino acid substitutions per site. Accession number for PADs in alignment are as follows: HsPAD1 (NP_037490.2), HsPAD2 (NP_031391.2),

HsPAD3 (NP_057317.2), HsPAD4 (NP_036519.2), HsPAD6 (NP_997304.3), MmPAD1 (NP_035189.1), MmPAD2 (NP_032838.2), MmPAD3 (NP_035190.3), MmPAD4 (NP_035191.2), MmPAD6 (NP_694746.2), BtPAD1 (NP_001094742.1), BtPAD2 (NP_001098922.1), BtPAD3 (XP_003585850.1), BtPAD4 (NP_001179102.1), BtPAD6 (XP_002685843.1), CfPAD1 (XP_851932.1), CfPAD2 (XP_544539.2), CfPAD3 (XP_535391.2), CfPAD4 (XP_848494.1), CfPAD6 (NP_001091016), GgPAD1 (XP_425729.3), GgPAD2 (XP_425730.3), GgPAD3 (NP_990374), OmPAD1 (NP_001153973.1), XlPAD2 (NP_001080369), XtPAD2 (NP_001096490.1).

(B) Ribbon presentation of superimposed structures of Ca^{2+} and substrate bound PAD4 (green, 1WDA) and free PAD4 (Red, 1WD8). Five Ca^{2+} ions bound to PAD4 are shown as purple spheres. Substrate BAA (benzoyl-L-arginine amide) is in a stick model.

(C) Superimposed structure of Ca^{2+} and substrate bound PAD4 (green) and free PAD4 (red) in boxed region 1 (N-terminal amino acids 126–300). The arrow denotes the region with major conformational changes after calcium binding.

(D) Superimposed structure of Ca^{2+} and substrate bound PAD4 (green) and free PAD4 (red) in boxed region 2 (C-terminal amino acids 301–420 and 624–663). Arrows denote the regions with major conformational changes.

Fig 2. A molecular switch operated on histone Arg and Lys residues of p53 target promoters for gene regulation

This molecular switch model predicts that p53 target genes can be activated through PAD4 and HDAC2 inhibition.

(A) Before p53 target gene activation, PAD4 and HDAC2 function at the promoters to citrullinate Arg residues and deacetylate Lys residues on histone proteins to suppress gene expression. RNA Pol II is not recruited to the promoter or paused at the promoter under these conditions.

(B) Upon activation signal such as DNA damage is sensed by p53, it switches its interaction partners from PAD4 and HDAC2 to PRMT1/4 and p300/CBP. These later two histonemodifying enzymes generate histone Arg methylation and Lys acetylation at target gene promoters, such as p21, GADD45 and PUMA to facilitate gene activation.

Fig 3. Cit-methyl and methyl-phos binary codes regulate the function of HP1

(A) The trimethylation of histone H3 Lys9 (K9me3) by the Suv39 histone methyltransferase leads to the binding of heterochromatin protein 1 (HP1) to regulate heterochromatin formation and/or to repress gene expression.

(B) The citrullination of H3 Arg8 by PAD4 produces a Cit-methyl binary code to decrease the binding of HP1 to K9me3.

(C) The phosphorylation of H3 Ser10 by aurora B or JIL-1 produces a methyl-phos binary code to decrease the binding of HP1 to K9me3.

Fig 4. An epigenetic view of tumorigenesis and cancer treatment

During the process of cancer initiation and progression, DNA methylation catalyzed by the DNA methyltransferases (DNMTs), histone deacetylation catalyzed by histone deacetylases (HDACs), and histone citrullination catalyzed by PAD4 work singularly or synergistically to epigenetically silence tumor suppressor genes thereby leading to untamed growth of cancer cells and tissues. Based on this theory, cancer treatment can be achieved with inhibitors targeting these epigenetic modifiers to restore histone modification patterns that are favorable for tumor suppressor gene expression thereby promoting cell cycle arrest and cell death.

Table 1

Tissue distribution, substrates, biological functions and related diseases of PADs.

	Protein distribution	Substrates	Biological functions	Diseases
PAD1	Epidermis; Uterus	Keratin K1; Filaggrin [26]	Cornification of epidermal tissues	Psoriasis [151]
PAD2	Broadly expressed: Pituitary gland; Brain; Uterus; Spleen; Spinal cord; Skeletal muscle	MBP Vimentin and -actins Histones H3 and H4	Plasticity of the CNS; Transcription regulation; Innate immune defense [152]; Female reproduction [153]	Multiple sclerosis; Rheumatoid arthritis; Alzheimer's disease [118, 119, 154, 155]; Prion disease [122]
PAD3	Epidermis; Hair follicles	Filaggrin; Trichohyalin	Regulation of epidermal functions	Unknown
PAD4	Neutrophils; Monocytes; Macrophages; Mammary gland Epithelial cells; Tumors;	Histones H2A, H3 and H4; ING4; p300/CBP; Nucleophosmin; Nuclear lamin C	Chromatin decondensation; transcription regulation; Tumorigenesis; Innate immunity; NET formation	Rheumatoid arthritis; Multiple sclerosis [156]; Cancers
PAD6	Eggs; Ovary; Early embryo;	Unknown	Oocyte cytoskeletal sheet formation and female fertility; early embryo development (mouse)	Unknown