Tumor and Stem Cell Biology

PAD2 Overexpression in Transgenic Mice Promotes Spontaneous Skin Neoplasia


Abstract

Peptidylarginine deiminase 2 (PAD2/PAD2) has been implicated in various inflammatory diseases and, more recently, cancer. The goal of this study was to test the hypothesis that PAD2 promotes oncogenesis using a transgenic mouse model. We found that about 37% of transgenic mice overexpressing human FLAG-PAD2 downstream of the MMTV-LTR promoter develop spontaneous neoplastic skin lesions. Molecular and histopathologic analyses of the resulting lesions find that they contain increased levels of markers for invasion, inflammation, and epithelial-to-mesenchymal transition (EMT) and that a subset of the lesions progress to invasive squamous cell carcinoma (SCC). We then stably overexpressed FLAG-PAD2 in the human SCC cell line, A431, and found that the PAD2-overexpressing cells were more tumorigenic in vitro and also contained elevated levels of markers for inflammation and EMT. Collectively, these studies provide the first genetic evidence that PAD2 functions as an oncogene and suggest that PAD2 may promote tumor progression by enhancing inflammation within the tumor microenvironment. Cancer Res; 74(21); 6306–17. ©2014 AACR

Introduction

The peptidylarginine deiminase (PADI or more commonly referred to as PAD) family of posttranslational modification enzymes converts positively charged arginine residues on substrate proteins to neutrally charged citrulline. This activity, alternatively called deimination or citrullination, has been shown to have wide-ranging effects on target protein structure, function, and protein–protein interactions. Increasingly, the dysregulation of PAD activity is associated with a range of diseases, including rheumatoid arthritis, multiple sclerosis, ulcerative colitis, neural degeneration, chronic obstructive pulmonary disorder (COPD), and cancer (1–3). While the presumptive function of PAD activity in most diseases is linked to inflammation, the role that PADS play in cancer progression is still under investigation (4–7). Our recent studies suggest a role for PAD2 in the oncogenic progression of breast cancer (8–11) and provide preclinical evidence showing that the PAD inhibitor, C1-amidine, could be used as a therapeutic agent for the treatment of tumors in vivo (11). To further investigate the involvement of PAD2 in the oncogenesis of epithelial tumors, we generated FVB/N mice expressing FLAG-PAD2 under the control of the mammary tumor virus (MMTV) promoter. As with previous studies (12, 13), we found that the MMTV promoter drives transgene expression in a range of tissues, including the mammary gland, salivary gland, ovaries, and skin. Results from our spontaneous tumor study show that about 37% of the PAD2 transgenic mice developed skin lesions within a period of 4 to 12 months after birth. These tumors expressed high levels of transgenic human PAD2 and display markers of increased invasiveness and epithelial-to-mesenchymal transition (EMT). Furthermore, a subset of these tumors displays the hallmarks of malignant progression to highly invasive squamous cell carcinomas (SCC). The findings identify a novel genetic mouse model of skin neoplasia and define a role for PAD2 in cancer progression.

Materials and Methods

Generation of MMTV-FLAG-PAD2 mice

To generate the MMTV-FLAG-PAD2 construct, human PAD2 cDNA was subcloned from pcDNA3.1-FLAG-PAD2 (14) into the EcoRI sites of the MMTV-SV40-Bssk plasmid (Addgene plasmid #1824), originally generated in the laboratory of Dr. Philip Leder at Harvard Medical School (Boston, MA; ref. 15). The linear MMTV-FLAG-PAD2 construct was purified and microinjected into the pronuclei of fertilized embryos from superovulated FVB/N (FVB/NJ, JAX) mice, and 2-cell stage embryos were transferred to pseudopregnant mothers. The microinjection and embryo transfer were performed by the Stem Cell and Transgenics Core at Cornell University’s College of Veterinary Medicine (Ithaca, NY). Mice were genotyped for...
the presence of an integrated human PAD2 transgene by PCR with the primers hPAD2-cds-F/R, and mouse Pad4 was used as genomic control (see Supplementary Table S1). All mouse experiments were reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) at Cornell University.

**Stable FLAG-PAD2 expression in A431 cells**

The human SCC A431 cell line was obtained from ATCC (CRL-1555) in 2013 and cultured according to manufacturer’s directions for 2 weeks before the generation of stable lines. Authentication was performed by ATCC using short tandem repeat (STR) DNA fingerprinting (16). To generate A431 cells overexpressing FLAG-tagged PAD2, two separate plasmids were generated by subcloning FLAG-PAD2 into the pIREs2-EGFP vector (Clontech) and pLenti-PGK-GFP-Puro plasmid (Addgene #19070), followed by transfection and transduction, respectively (see Supplementary Methods).

**Immunohistochemistry and immunofluorescence**

Immunohistochemistry (IHC) and immunofluorescence experiments were carried out using a standard protocol as previously described (9). Primary antibodies are as follows: anti-PAD2 (12110-1-AP, ProteinTech), anti-FLAG-M2 (F1804, Sigma), anti-pan-Citrusline (ab66464, Abcam), anti-Ki67 (ab15580, Abcam), and anti-IL6 (ab6672, Abcam). Negative controls were either normal rabbit or mouse IgG.

**Western blotting**

Western blotting was carried out as previously described (9). Primary antibodies against PAD2 (12110-1-AP, ProteinTech) and FLAG-M2 (F1804, Sigma) were incubated overnight at 4°C. To confirm equal protein loading, membranes were stripped and reprobed with anti-β-actin (ab8227, Abcam).

**RNA isolation, semiquantitative, and quantitative real-time PCR**

RNA was purified using the Qiagen RNeasy Kit, including on-column DNase treatment, and reverse-transcribed using the Applied Biosystems High Capacity RNA-to-cDNA kit according to the manufacturer’s protocol. See Supplementary Methods for a more detailed description of semiquantitative and quantitative real-time PCR (qPCR) and Supplementary Tables S1–S3 for primer sequences.

**Assay for cellular malignancy and invasion**

Collagen-coated inserts for 24-well plate wells (Falcon BD Fluoroblok; #351152) were used to conduct Transwell migration assays as previously described (17) and further detailed in the Supplementary Methods. Focus formation assays were carried out as previously described (18).

**Statistical analysis**

All experiments were independently repeated at least three times unless otherwise indicated. Values were expressed as the mean ± SD and analyzed using Student t test or ANOVA (with Tukey post hoc analysis) where three or more groups were compared with one another using continuous data. Incidence of spontaneous lesions in MMTV-FLAG-PAD2 mice was measured 4 to 12 months post-birth, along with age-matched FVB/N mice from the corresponding founder line. Data were analyzed using a χ² test with Yates correction for continuity. Significance was set at an α of 0.05.

**Results**

**Generation of MMTV-FLAG-PAD2 transgenic mice**

To assess the potential role of PAD2 in the oncogenesis of epithelial tissue, we generated a mouse model in which the human PAD2 gene is overexpressed under the control of the hormone-responsive MMTV-LTR promoter. The transgenic construct consists of an MMTV-LTR promoter placed upstream of the human FLAG-tagged PAD2 cDNA, followed by an SV40 splice/polyadenylation site (Fig. 1A). MMTV-FLAG-PAD2 mice were generated, and seven potential founders were tested for the presence of transgene by PCR. Four founders (4807, 4853, 4680, and 4863) were identified (indicated in red, Fig. 1B) that carried germline transmission of the FLAG-PAD2 transgene. We note that 4807 was not included in subsequent analyses due to poor breeding. Using semiquantitative RT-PCR, we confirmed the presence of FLAG-PAD2 transcript in the skin, salivary gland, mammary gland, and ovary of the transgenic mice (Fig. 1C). These four tissues were chosen for analysis because they have previously been shown to express high levels of MMTV-LTR-driven transgenes (13). qPCR analysis of the same tissues demonstrated that while PAD2 expression was highest in salivary and mammary glands, significant levels of transgenic PAD2 were also found in the skin of all three founders (P < 0.01; Fig. 1D). We note here that while transgenic expression of PAD2 was observed in the mammary glands of both virgin (Supplementary Fig. S1A) and multiparous mice (Supplementary Fig. S1B), we did not detect any gross abnormalities or any observable phenotype in the mammary glands of these mice. The potential mechanisms that may be blocking PAD2-mediated oncogenesis in the mammary gland are currently being investigated.

**PAD2 transgenic mice develop skin lesions with the potential to advance to invasive SCCs**

Analysis of spontaneous neoplastic growth in experimental mice found that about 37% of the mice across all founder lines developed skin lesions between 4 and 12 months of age (Table 1). When compared with age-matched wild-type (WT) FVB/N mice, the increased rate of neoplastic growth in FLAG-PAD2 transgenic mice was highly significant (χ² = 30.65; P < 0.001), suggesting that the PAD2 transgene is likely promoting oncogenesis. The percentage of males developing tumors was about 44%, whereas females developed tumors at a rate of about 56%, which was not significantly different across genders. These lesions occurred on both the dorsum and ventrum of transgenic mice and are characterized by gross abnormalities, such as alopecia, multifocal epidermal ulceration often covered in serocellular crust, dysplasia, and thickening of the adjacent epidermis (Fig. 2A).

Histologic evaluation of skin lesions from PAD2-overexpressing mice by hematoxylin and eosin reveals that these sites
contain highly neoplastic tissue, having features consistent with invasive SCC. We found nests of neoplastic cells that appeared to originate from the epidermis and to invade into the dermis and subcutis (Fig. 2B, i and ii); moreover, the epidermis overlying the neoplasm was extensively ulcerated and frequently adjacent to hyperplastic epidermal layers (Fig. 2B, i). In addition, we observed that concentric layers of keratin surrounded tumor cells forming keratin pearls, which are characteristic of SCC (Fig. 2B, ii). We found that tumor borders were poorly defined, often with highly anaplastic tumor cells invading the deeper dermis and subcutis, and that neoplastic cells frequently infiltrated and separated skeletal myofibers (Fig. 2B, iii). The nests and islands of neoplastic cells within the subcutis showed a high degree of anisokaryosis and anisocytosis, with the nuclei often containing one to two prominent nucleoli (Fig. 2B, iv). Clusters of neoplastic cells were found to be surrounded by loose collagenous stroma with a marked loss of adnexal structures, consistent with desmoplastic response (Fig. 2B, ii–iv). Finally, we observed that these lesions often contained carcinoma cells budding from the primary neoplasm (Fig. 2B, iv), again indicating an invasive component to these tumors.

Figure 1. MMTV-FLAG-PAD2 transgenic construct. A, schematic of the linearized MMTV-FLAG-HPAD2 transgene, containing FLAG-tagged human PAD2 cDNA cloned between the EcoRI sites of the MMTV-SV40-Bssk plasmid (15). The construct used for generation of FLAG-HPAD2 transgenic mice (herein FLAG-PAD2) consists of FLAG-PAD2 under the control of the hormone-responsive MMTV-LTR promoter enhancer with an SV40 splice-polyadenylation signal (SV40pA). B, PCR screening of DNA extracted from mouse tails for the presence of integrated human PAD2 transgene (TG; HPAD2). Four founders were identified (red); primers for mouse Pad4 were used as a WT (mPad4) control for amplification. Primer details can be found in Supplementary Table S1. C, semiquantitative RT-PCR was performed on tissues known to have high expression in MMTV-LTR transgenic mice: skin, salivary gland (SG), mammary gland (MG), and ovary. Relative mRNA levels were measured for the transgenic human PAD2, along with endogenous mouse Pad2. Mouse Gapdh was used as the loading control. D, qPCR for the human PAD2 transgene was performed across the same tissues, using WT (FVB/N) skin as the reference, with mouse Gapdh normalization (*, P < 0.01).
Recombinant PAD2 is expressed in the skin and tumor tissue of PAD2 transgenic mice

To test whether the observed skin neoplasia in the transgenic mice was driven by overexpression of PAD2, we next investigated PAD2 expression levels in these mice by IHC. We probed these tissues with an anti-human PAD2 antibody and found that PAD2 protein was observed in the hyperplastic epidermis, neoplastic islands, and in the hair follicular epithelium (Fig. 2C, i). In addition, we observed high PAD2 expression in the neoplastic epithelium surrounding keratin pearls (Fig. 2C, ii and iii). PAD2 was also expressed in the invasive, budding nests of carcinoma cells, which appeared to invade stromal tissue adjacent to the primary neoplasm (Fig. 2C, iv).

To test whether PAD2 overexpression correlated with increased deimination activity, we next probed sections from WT skin and transgenic PAD2 lesions with a pan-citrulline antibody that has been previously shown to specifically detect citrullinated proteins (19, 20). Results show that the skin lesions from MMTV-FLAG-PAD2 mice appear to have increased pan-citrulline staining when compared with WT skin (Fig. 2D), with the most intense levels of citrullination appearing to occur in the hyperplastic regions. In addition, we found strong nuclear anti-pan-citrulline staining in these lesions (Fig. 2D, inset bottom image), which is in line with our previous studies showing that PAD2-mediated histone deimination regulates gene expression (9, 10, 14, 21). Because the anti-human PAD2 antibody likely cross-reacts with endogenous mouse PAD2, we also stained these tissues with an anti-FLAG antibody to specifically detect the PAD2 fusion protein. We observed a similar expression pattern within the proliferating basal layers of hyperplastic/neoplastic epidermis, suggesting that much of the PAD2 signal originates from over-expression of the transgene (data not shown). We confirmed this prediction using indirect immunofluorescence to co-stain for FLAG and PAD2 expression in the skin lesions of transgenic mice (Fig. 3). Both PAD2 and FLAG were seen to colocalize to the neoplastic epithelial cells (Fig. 3A, i–iv). While PAD2 shows strong staining in multiple layers of the epidermis, we found that FLAG-PAD2 expression is slightly more restricted to the basal layer, which is known to be more stem cell-like and highly proliferative (22). Interestingly, we also see an apparent slight increase in the proliferative marker, Ki67, within the FLAG staining section of the SCC lesion (Fig. 3B, ii–iv). This observation supports the hypothesis that PAD2 overexpression in the skin promotes epidermal proliferation, which is further supported by the identification of a subset of lesions that are highly proliferative and characteristic of invasive SCC. We also note that control staining with rabbit IgG was negative (Supplementary Fig. S2) and that the anti-FLAG antibody did not stain epidermal tissue from WT mice (Fig. 3C, i–vi) or adjacent normal skin from transgenic mice (data not shown).

Enhanced inflammation marker expression is observed in the skin of transgenic mice when compared with WT skin and with the mammary gland of transgenic animals

Previous reports have implicated PAD2 in a wide range of inflammation-associated diseases (23–25), including our recent work where we reported that PAD2 appears to promote cancer progression in vitro and in vivo using a xenograft model of breast cancer (11). Therefore, in an effort to identify potential pathways involved in the progression of MMTV-FLAG-PAD2 skin lesions, we next investigated the expression of a subset of inflammation and invasion-associated genes in the transgenic PAD2 lesions. Interestingly, the lesions containing high levels of transgenic PAD2 (Fig. 4A–C, lesions 2 and 4) also expressed high levels of Ki67, Il6, and the functional murine orthologs for human Lta and Cxcl1 (KC; ref. 26), Cxcl2 (MIP-2; ref. 27), and Cxcl5 (LIX; ref. 28; P < 0.001; Fig. 4D). These lesions also expressed high levels of the inflammatory mediator, Cox2 (Fig. 4E), which has previously been shown to induce Il6 expression (29). In addition, these tumors also displayed elevated levels of the EMT markers, vimentin (Vim) and the E-cadherin repressor, Snail (Snai1, or more commonly referred to as Snail), along with decreased levels of E-cadherin (Cdh1, or hereafter Ecad; P < 0.01) and Gata3 (Fig. 4E), which has been
previously shown to be downregulated in SCCs of the tongue (30) and mouse models of skin tumorigenesis (31). 

Our finding that PAD2 overexpression in the skin, but not the mammary gland, leads to neoplastic growth was somewhat surprising, and we speculated that the lack of tumor growth in the mammary gland may have been due to a reduced ability of FLAG-PAD2 to promote inflammatory and/or EMT marker expression in this tissue. To test this hypothesis, we next examined inflammatory/EMT marker expression in skin and mammary gland from WT and transgenic mice. Results show that, in large part, while PAD2 overexpression enhanced inflammatory marker expression in the skin, similar levels of
induction were not observed in the mammary gland (Supplementary Fig. S3). These observations supported the hypothesis that PAD2 overexpression selectively promotes inflammatory marker expression and tumorigenesis in the skin but not the mammary gland. To test this hypothesis, we probed tissue sections with a well-validated anti-IL6 antibody. Results show that protein levels of Il6 appeared to be higher in the transgenic skin lesions than in the transgenic mammary gland and in WT skin (Fig. 4F). Taken together, these findings suggest that, in the skin, PAD2 overexpression may enhance tumor progression by selectively promoting inflammation and by altering the invasive properties of epithelial cells. We also evaluated the effect of PAD2 overexpression on inflammatory and EMT marker expression in the salivary gland and found that EMT marker expression appeared to be dampened, whereas inflammatory marker gene expression was not noticeably affected when compared with skin (Supplementary Fig. S3). Interestingly, there does not appear to be a direct PAD2 gene dosing effect with respect to inflammatory mediator/EMT expression in the different tumors and we predict that this discrepancy may be due to opposing and/or synergistic interactions between PAD2 and other PAD family members within individual tumors. In support of this prediction, we found that expression levels of PAD1–4 varied dramatically between tumors (Supplementary Fig. S4B and S4C). While speculative, it is possible that the ratio of PAD isozyme expression levels could modulate inflammatory/EMT expression levels and tumor growth and/or invasiveness to varying degrees.

Overexpression of PAD2 in human SCC A431 cells increases invasiveness and malignancy

Outcomes from our transgenic MMTV-FLAG-PAD2 mouse study show that ectopic expression of PAD2 is sufficient to drive tumorigenesis in epithelial cells and that this expression correlates with an increase in the expression of markers of inflammation and invasion. To test whether PAD2 might play a similar role in humans, we next investigated whether PAD2 overexpression in the human skin cancer cell line, A431, might lead to a similar phenotype. This cell line functions as a model system for human SCC and was derived from a vulvular epidermoid carcinoma (32). To investigate the oncogenic
Figure 4. Transgene expression in the skin lesions of MMTV-FLAG-PAD2 mice. A, semiquantitative RT-PCR of four representative skin lesions from transgenic mice (lesions 1–4). Relative mRNA levels were measured for the transgenic human PAD2, along with endogenous mouse Pad2. Mouse Gapdh was used as the loading control. (Continued on the following page.)
potential of PAD2 in these cells, we first transiently expressed FLAG-PAD2 using mammalian vectors (pcDNA3.1 and pIRES2) and found that these cell lines expressed high levels of the FLAG-PAD2 protein (Fig. 5A and B). In addition, we found that PAD2 overexpression suppressed E-cadherin and increased vimentin protein expression when compared with A431 cells that had been transfected with an empty vector (Fig. 5A). We next tested whether PAD2 overexpression altered the expression of inflammation and EMT markers and found that, similar to what was seen in transgenic mice, IL6 and IL8 gene levels were significantly increased in the PAD2-overexpressing cells, along with the inflammatory mediators COX2 and NFκB/RELA. Moreover, in addition to increasing the proliferation marker Ki67, we found that transient PAD2 overexpression also increased SNAIL and SLUG levels while suppressing E-cadherin levels (P < 0.05; Fig. 5C). Finally, we tested the effects of PAD2 overexpression on the expression of two proliferative oncogenes H-Ras (33, 34) and c-Myc (35), which are known to be upregulated in skin carcinomas. Results show that levels of these oncogenes were unchanged in the PAD2-overexpressing cells, suggesting that the observed effects of PAD2 on oncogenesis are more direct in nature.

Given the data from our transiently transfected A431 cells, along with our data from the transgenic mice, we decided to investigate whether stable overexpression of FLAG-PAD2 might have an effect on the cellular malignancy and/or invasiveness of A431 cells. Using both lentiviral transduction of FLAG-PAD2 and traditional transfection, we created two stable PAD2-overexpressing lines: (i) pLenti-FLAG-PAD2 (pLenti-FP2), which expresses low levels of FLAG-PAD2 and (ii) pIRES2-FLAG-PAD2 (pIRES-FP2), which expresses high levels of FLAG-PAD2 (Fig. 6A and B). Next, we tested the invasive properties of these cell lines by measuring their ability to migrate through a collagen matrix. For both cell lines, we see...
a significant increase in cellular migration after 24 hours ($P < 0.01$), with the pRES2-FP2 cell line also showing a significant increase in migration after only 4 hours ($P < 0.01$; Fig. 6C). These results suggest that PAD2 dosage might correlate with invasion. Finally, we wanted to examine the stable A431-pRES2-FP2 cells for any increase in cellular malignancy. Assaying for focus formation, we show a significant increase in the FLAG-PAD2–overexpressing A431 cells compared with the empty vector control ($P < 0.001$; Fig. 6D). In addition, we find that the morphology of these cells also displays an elongated, fibroblast-like shape, indicative of cells that have undergone EMT (Fig. 6E).

Discussion

In the present study, we demonstrate that transgenic overexpression of PAD2 in the epidermis promotes carcinoma formation in the skin. In addition, we found that these lesions have the potential to advance to invasive SCC. These in vivo findings were supported by our in vitro studies, which showed that stable overexpression of PAD2 in the human SCC line, A431, resulted in increased cellular invasiveness and malignancy. At a more mechanistic level, we also demonstrate that PAD2 may promote carcinogenesis by enhancing the production of factors that promote inflammation and an EMT phenotype.
A number of mouse models, including models of advanced SCC, have documented a role for inflammation in cancer progression (36). In humans, the links between inflammation and cancer are now so strong that inflammation is considered to be the seventh hallmark of cancer (36, 37). Two key mediators of inflammation-driven cancers are the cytokines, IL6 and IL8, which promote cancer progression by stimulating tumor growth and angiogenesis (38–48). Interestingly, a number of previous studies have documented a role for PAD2-mediated deimination in regulating inflammatory activities during disease progression (23, 24). In addition, PAD activity has also been found to regulate tissue inflammation via direct modification of IL8 (25). Given these observations, we hypothesized that PAD2 may promote tumorigenesis by regulating the expression of IL6 and IL8. We tested this hypothesis and found that IL6 and IL8 ortholog expression is, in fact, increased in the skin lesions of PAD2-overexpressing mice and that IL6 and IL8 expression is elevated in PAD2-overexpressing cells.

In this study, we also show that PAD2 overexpression in transgenic mice, in addition to human cell lines, increased markers of invasiveness and EMT. An early critical molecular feature of EMT is the downregulation of E-cadherin (Cdh1), a cell adhesion molecule present in the plasma membrane of most normal epithelial cells. As cells progresses from an epithelial-like to mesenchymal-like state, E-cadherin downregulation is accompanied by the increased expression of Snail (Snai1) and the intermediate filament vimentin (Vim) (reviewed by Kaluri and colleagues; ref. 49). In our study, we found that PAD2 overexpression in mice induced an EMT-like phenotype in the resulting tumor cells as evidenced by the reduction in E-cadherin expression, along with an increase in Snail and vimentin expression. This molecular characterization of an EMT transition matches well with the invasive histology of the PAD2-overexpressing lesions.

A number of previous studies have shown that inflammatory cytokines, such as IL8, appear to promote cancer progression by inducing an EMT in tumor cells (41). Thus, it is possible that PAD2 overexpression promotes cell invasiveness and an EMT phenotype by upregulating cytokine production in tumor cells. Interestingly, however, we recently reported that another PAD, PAD4, plays an important role in regulating an EMT in breast cancer cells via regulation of GSK3β–TGFβ signaling (50). In that study, we found that PAD4 targets GSK3β for citrullination and that PAD4 depletion dramatically reduces levels of nuclear GSK3β, promotes EMT, and increases tumor invasiveness. In the current study, we found that overexpression of PAD2 suppressed Gsk3β expression, promoted an EMT phenotype and tumor growth. Together, these results raise the possibility that PAD2 and PAD4 may oppose or counterbalance each other in the maintenance of epithelial identity and in oncogenesis.

A surprising outcome of our project was that neoplastic growth appeared to be limited to the skin of PAD2 transgenic mice and was not observed in the mammary glands. While the ultimate explanation for this outcome is likely manifold, we found that the PAD2-mediated induction of inflammatory mediator expression was suppressed in the mammary glands of transgenic mice when compared with both the normal skin and the hyperplastic lesions from transgenic mice. These results suggest that, as opposed to the skin, a dampening mechanism may exist within the mammary gland that prevents FLAG-PAD2 from inducing inflammatory cytokine expression, thereby suppressing tumorigenesis. A potential explanation for this dampening effect may be derived from our finding that FLAG-PAD2 overexpression in vivo appears to significantly modulate the expression of other PAD family members in both normal and tumor tissue (Supplementary Fig. S4). Therefore, while purely speculative, it is possible that the relatively low levels of FLAG-PAD2 that we observed in the skin promotes the expression of a specific ratio of PAD family members that is conducive to inflammation and tumorigenesis, whereas the relatively higher level of FLAG-PAD2 in the mammary gland alters this ratio, leading to reduced inflammatory mediator expression and an absence of neoplastic growth. Regarding the potential mechanisms by which PAD2 overexpression regulates gene activity, we have recently found that PAD2 functions as a transcription factor co-factor and modulates target gene expression via histone deimination at transcription factor–binding sites (9, 10, 14, 21). Therefore, we are currently testing the hypothesis that PAD2 regulates the expression of target genes via similar mechanisms.

In conclusion, this study demonstrates that overexpression of human PAD2 in mice and in human cell lines promotes carcinogenesis. Furthermore, we show that a subset of tumors in mice display hallmarks of malignant progression from skin lesions to highly invasive SCCs. In addition, we demonstrate that PAD2 overexpression in vivo and in vitro enhances the expression of inflammatory cytokines and mediators of the EMT. Collectively, these studies provide functional and mechanistic evidence establishing PAD2 as a potential novel oncogene in the initiation and progression of epidermal carcinomas.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J.L. McElwee, S. Mohanan, S. Horibata, S.A. Coonrod
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.L. McElwee, S. Mohanan, S. Horibata, K.L. Sams, L.J. Angush, D. McLean, S.A. Coonrod
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.L. McElwee, S. Mohanan, S. Horibata, L.J. Angush, D. McLean, J.J. Waksbarg, S.A. Coonrod
Writing, review, and/or revision of the manuscript: J.L. McElwee, S. Mohanan, J.J. Waksbarg, S.A. Coonrod
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Horibata, K.L. Sams
Study supervision: J.L. McElwee
Other (technical and veterinary assistance in vivo and in vitro experiments): I. Cvitaš

Grant Support
This work was supported in part by funding through the DOD Era of Hope Award W81XWH-07-1-0372 to S.A. Coonrod and through an NIH Graduate Fellowship (grant T32HD057854) to J.L. McElwee.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 14, 2014; revised July 23, 2014; accepted August 19, 2014; published OnlineFirst September 11, 2014.
References


PAD2 Overexpression in Transgenic Mice Promotes Spontaneous Skin Neoplasia

John L. McElwee, Sunish Mohanan, Sachi Horibata, et al.