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Osteopontin Facilitates Ultraviolet B-induced Squamous Cell Carcinoma Development

Pi-Ling Chang, Ph.D.^{1,2,5}, Yu-Hua Hsieh, Ph.D.¹, Chao-Cheng Wang, Ph.D.², M. Margaret Juliana, V.M.D., Ph.D.³, Yuko Tsuruta, M.D.², Laura Timares, Ph.D.², Craig Elmetts, M.D.^{2,5}, and Kang-Jey Ho, M.D., Ph.D.⁴

¹Department of Nutrition Sciences, University of Alabama at Birmingham, Birmingham, Alabama USA

²Department of Dermatology, University of Alabama at Birmingham, Birmingham, Alabama USA

³Department of Genetics, University of Alabama at Birmingham, Birmingham, Alabama USA

⁴Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama USA

⁵Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, Alabama USA

Abstract

Background—Osteopontin (OPN) is a matricellular glycoprotein that is markedly expressed in cutaneous squamous cell carcinomas (cSCCs) and in actinic keratoses implicating its role in photocarcinogenesis.

Objective—To determine whether OPN facilitates the development of cSCC and its function.

Methods—cSCCs development was compared between wild-type (WT) and OPN-null mice subjected to UVB irradiation for 43 weeks. UVB-induced OPN expression was determined by Western blot, immunoprecipitation, ELISA, and semi-quantitative RT-PCR. Epidermal layer and TUNEL analyses assessed if OPN mediates UVB-induced epidermal hyperplasia or suppresses UVB-induced apoptosis of basal keratinocytes, respectively. *In vitro* experiments determined whether OPN enhances cell survival of UVB-induced apoptosis and its potential mechanisms. Immunohistochemical analyses of epidermis assessed the expression of CD44 and focal adhesion kinase (FAK), molecules that mediate OPN survival function.

Results—Compared to female WT mice, OPN-null mice did not develop cSCCs. UVB irradiation stimulated OPN protein expression in the dorsal skin by 11 h and remains high at 24 to 48h. OPN did not mediate UVB-induced epidermal hyperplasia; instead, it protected basal keratinocytes from undergoing apoptosis upon UVB exposure. Likewise, the addition of OPN

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Correspondence: Pi-Ling Chang, 404 Webb Building, Department of Nutrition Sciences, University of Alabama at Birmingham, 1720 2nd Avenue South, Birmingham, AL 35294 USA. Tel: 205-975-6624; Fax: 205-934-7049; plchang@uab.edu.

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suppressed UVB-induced OPN-null cSCC cell apoptosis, the activation of caspase-9 activity, and increased phosphorylation of FAK at Y397. Furthermore, the expression of CD44 and FAK in WT mice epidermis was greater than that of OPN-null mice prior to and during early acute UVB exposure.

Conclusion—These data support the hypothesis that chronic UVB-induced OPN expression protects the survival of initiated basal keratinocytes and, consequently, facilitates cSCC develop.

1. Introduction

Ultraviolet B (UVB) irradiation contributes to the integrity of skin and bone through the production of vitamin D. Paradoxically, it is also a major risk factor for the development of nonmelanoma skin cancer. Most nonmelanoma skin cancers develop late in life, implicating the requirement of chronic exposure to UVB. Nonmelanoma skin cancers consist of basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs). These skin cancers are the most common malignancy in the United States with more than 2 million new cases per year [1]. Nonmelanoma skin cancer is also expected to increase because depletion of the ozone layer permits more UVB radiation to reach the earth [2].

UVB irradiation acts as both an initiator and a promoter. As an initiator, it has been shown to cause permanent genetic alterations resulting in the generation of initiated cells. The most common UVB-induced mutations include tumor suppressor genes, *p53* and *PATCH*, and the proto-oncogene, *RAS* [3-5]. As a promoter, it triggers cell proliferation and transformation of initiated cells to malignant tumors. The mechanism by which UVB acts as a promoter, however, is still not well understood. The tumor promotion stage is the major rate-limiting step as initiated cells can remain dormant for an extended period prior to acquiring their ability to proliferate and transform to malignancy [6]. The fate of an initiated cell to survive long-term is likely dependent on its intrinsic and extrinsic factors, such as those in the microenvironment.

Alterations in the microenvironment of initiated and tumor cells include changes in the expression of matrix proteins and their interaction with their cell surface receptors. These changes have been shown to play a critical role in regulating tumorigenesis and metastasis [7-9]. We have shown that lack of induced expression of a matricellular protein, osteopontin (OPN), reduces the rate of papilloma development in the model for two-stage skin carcinogenesis supporting its role in facilitating tumor promotion [9]. Whether OPN is an important factor in driving UVB-induced photocarcinogenesis is not known.

OPN is mainly a secreted, adhesive glycoprotein that interacts with integrins and the hyaluronic acid receptor, standard CD44 (CD44s) or its variant isoforms. It is normally expressed in selective tissues, but can be stimulated in various cell types by hormones and growth factors [10]. OPN contains three conserved functional motifs: the 8-10 conserved Asp that binds calcium or hydroxyapatite, the Arg-Gly-Asp sequence that binds to integrin receptors and the Asp-Arg-Tyr-Leu-Lys-Phe-Arg-Ile sequence that binds to the hyaluronic acid receptor CD44. Thus far, one of the major physiological roles of OPN in the context of its cell-binding and signaling ability has been to enhance cell survival [9-12].

OPN is expressed in several types of cancer [13-16] and in premalignant tumors [17-19]. It is highly expressed in cutaneous squamous cell carcinoma (cSCCs) and in actinic keratoses, which are precursors of cSCCs [20]. Further, we have shown that OPN expression in the human adult epidermis is elevated in skin frequently exposed to sunlight as oppose to non-exposed regions [20], supporting the likelihood of UVB inducing OPN expression [20].

UVB may induce epidermal OPN expression in adult human/mouse skin by indirect mechanisms. Human epidermis produces the active form of vitamin D₃, 1 α , 25-dihydroxyvitamin D₃ (calcitriol), upon UVB exposure [21]. Calcitriol can transcriptionally regulate OPN expression as its promoter consists of functional vitamin D response element [22]. We have previously shown that calcitriol stimulates the synthesis and secretion of OPN in mouse epidermal-like cells [23, 24]. Additionally, human OPN promoter consists of RAS-activated enhancer and AP-1 response element both of which can be activated by the RAS and AP-1 protein, which are stimulated upon UVB exposure [20]. Collectively, these findings suggest that UVB-induced OPN expression may play a role in photocarcinogenesis. The present study was undertaken to determine if OPN facilitates the development of UVB-induced cSCCs and to elucidate its potential function in this process.

We found that UVB-induced OPN is a facilitator of photocarcinogenesis; female mice lacking OPN did not develop cSCCs. OPN did not stimulate epidermal hyperplasia; instead, it enhanced the survival of UVB exposed basal keratinocytes *in vivo*. Additional *in vitro* studies with OPN-null cSCC (ONSC) cells [25] showed that OPN suppressed cell apoptosis is mediated, in part, through suppressing caspase-9 activation and increasing the activation of focal adhesion kinase (FAK) phosphorylation at Y397 in UVB-induced apoptosis assay. Additionally, the expression of CD44 and FAK, molecules that mediate OPN survival function, was lower in OPN-null epidermis than that of WT mice prior to and during early acute UVB exposure, suggesting potential direct or indirect regulation of these molecules by OPN. In summary, we postulate that the lack of UVB-induced expression of OPN decreases the survival of initiated basal keratinocytes and consequently, causes a reduction of UVB-induced cSCCs.

2. Materials and Methods

2. 1. Mouse model

All animal experiments were approved and conducted according to Institutional Animal Care and Use Committee guidelines for the University of Alabama at Birmingham. WT in 129S6/SvEv (Taconic, German Town, NY) and OPN-null mice in the same background were bred in-house [9].

2. 2. UVB-induced tumor development

Female mice at 10 wks of age were shaved and exposed dorsally to broad band UVB range from 290-320 nm (Daavlin, Bryan, OH) three times per week. At the first, second, and third week, mice were exposed to 100, 150, and 150 mJ/cm² of UVB, respectively, followed by 200 mJ/cm² for the next 40 weeks. Tumor growth and body weights were monitored weekly. Tumor volume was calculated using the formula: $V=ab^2/2$, where V is mm³; a and

b are the maximum and minimum tumor diameter, respectively. Mice were euthanized 4 days after the last UVB exposure at 43 weeks, the time at which 80% of WT mice developed tumor.

2. 3. Cell lines and primary keratinocytes

JB6 Cl41.5a cells, a murine preneoplastic epidermal cell line (from Dr. Nancy Colburn, Fredrick, Bethesda, MD [26], were maintained in Minimum Essential Medium (MEM) eagles supplemented with 5% heat inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT) as previously described [27]. Cells were used as a positive control for elevated OPN expression when treated with the tumor promoter, 12-*O*-tetradecanoyl-13-phorbol acetate (TPA). HaCaT cells (adult immortalized human keratinocytes) [28] were obtained from Dr. Andrzej T. Slominski (University of Tennessee Health Science Center) and grown in DMEM with 5% FBS. Osteopontin-null squamous cell carcinoma cell line (ONSC) was previously established from cSCC of OPN-null female mouse [25]. These cells were maintained in 10% FBS in Dulbecco MEM without Ca and Mg. Primary fetal keratinocytes were isolated according to previously described protocol [29]. Cells were maintained in 0.045 mM of CaCl₂ in keratinocyte-SFM medium (Invitrogen, Grand Island, NY) with 1% antibiotics (Cellgro, Manassas, VA).

2. 4. UVB-induced ONSC apoptosis

ONSC cells were seeded at equal density in 60 mm dishes. At near confluent state, they were exposed to 0, 25, and 50 mJ/cm² of UVB (quadruplicate dishes/dose of exposure) to induce cell apoptosis, prior to the addition of 1% BSA or non-degraded rat recombinant OPN (rOPN) [30, 31] at a single concentration. Similar experiments were performed where cells were exposed to 50 mJ/cm² of UVB and incubated with various concentrations of rOPN or 1% BSA as control. After 24 h, cells were trypsinized, stained with trypan blue, and live cells were counted by hemocytometer.

2.5 Caspase-9 activity

ONSC cells were treated with 50 mJ/cm² with UVB to induce apoptosis followed by the addition of rOPN at 1, 3 or 5 µg/ml or 1% BSA as control. After 6 h of incubation, cells were lysed in lysis buffer (0.1 M Hepes, pH 7.4, containing 2 mM dithiothreitol, and 0.1% Chaps) and protein concentration was determined by bicinchoninic acid method. Protein lysates were incubated with 50 µl of 50 mM fluorogenic caspase-9 peptide substrates, Ac-LEHD-AMC (7-amino-4-methoxy coumarin) from Bachem, Torrance, CA. The release of AFC was measured with excitation at 400 nm and emission at 505 nm using a fluorescence spectrophotometer. Data are presented as fold-increase of caspase activity relative to that of BSA-treated cells as control.

2. 6. Histopathological and immunohistochemical analyses

Skin biopsies and tumors were fixed in 10% buffered formalin and paraffin embedded. The tissue sections were either stained with hematoxylin and eosin (H&E) for histopathological analyses or used for immunohistochemical analyses of OPN and other proteins as previously described [9, 32] using anti-mouse OPN (Akm2A1, Santa Cruz Biotechnology Inc., Santa

Cruz, CA) and its isotype control mouse IgG_{1,κ} (BD Biosciences, San Jose, CA). Kidneys from WT and OPN-null mice were used as positive and negative controls for OPN, respectively. For detection of CD44 and CD44v6 expression, tissues were antigen-retrieved, blocked, and incubated with antibodies to CD44 (clone IM7, BioLegend, San Diego, CA) or CD44v6 (Calbiochem, EMD Biosciences, Inc. La Jolla, CA) and their respective controls, rat IgG_{2b,κ} (BD Biosciences) and purified rabbit IgG (Southern Biotechnology Associates, Inc., Birmingham, AL). For detection of focal adhesion kinase (FAK), tissues were treated as above and incubated with anti-total FAK antibody (Santa Cruz Biotechnology Inc.) or the control rabbit IgG. To detect phosphorylated FAK, parallel sections were incubated with anti-phospho (Y397) FAK from three different sources (EMD Millipore, Billerica, MA; Invitrogen; and GenScript, Piscataway, NY) or their corresponding isotype controls (rabbit IgG, Southern Biotechnology Associates, Inc.). Photographs of sections were made with a digital Nikon camera (Coolpix 4500) attached to Olympus CX31 microscope.

2. 7. Western blot analyses, immunoprecipitation and ELISA

Epidermal protein was obtained by scraping the dorsal epidermis with a razor blade and processed as described [9]. Equal amounts of protein were pre-cleared with antibody to albumin, separated on 10% SDS-polyacrylamide gels, and transferred onto polyvinylidene fluoride membranes (EMD Millipore) prior to incubation with monoclonal antibody to mouse OPN. Epidermal proteins or ONSC cells were also immunoprecipitated with antibody to OPN or total FAK prior to Western blot analyses. In the latter experiment, blots were probed first with anti-pY397 FAK (GenScript) followed by anti-FAK antibody. Secondary antibodies to mouse monoclonal or rabbit polyclonal were HRP-conjugated. The substrates used were ECL Amersham (GE Healthcare Bio-Sciences, Piscataway, NJ) or Luminata Forte (EMD Millipore). All blots were exposed to x-ray films except for Figure 7D, which was detected using the Bio-Rad ChemiDoc™ MP imaging System.

To quantify the amount of OPN induced by UVB in the epidermis, total epidermis protein was analyzed by ELISA (Duo-set, R& D Systems, Minneapolis, MN). The values of OPN concentration were normalized to mg protein.

2. 8. Semi-quantitative reverse transcription-PCR

Total RNA extracted from the epidermis of mice treated with UVB at 200 mJ/cm² was transcribed to cDNA by reverse transcriptase using oligo(dT). Primer pairs specific for mouse *Spp1* and *Gapdh* were as described in [9]. To determine expression of OPN mRNA in cSCCs and sarcomas of WT mice, these regions were isolated from paraffin-embedded tumor samples by a surgical blade, and total RNA was extracted and processed as described above.

2. 9. Assessment of epidermal hyperplasia and apoptotic cells by TUNEL analyses

To assess UVB-induced epidermal hyperplasia, dorsal skin of mice was exposed to UVB two times (every other day). After 48 h, 2-3 pieces of skin/mouse (n=3 mice/treatment) were harvested and embedded in paraffin. Epidermal thickness was assessed from H&E stained sections by counting the layers of cells in the epidermis. Dorsal skin and skin surrounding the tumors were also harvested from WT and OPN-null mice exposed to UVB for 43 weeks.

To determine the number UVB-induced apoptotic basal keratinocytes, skin of mice was exposed to UVB at 200 mJ/cm² and harvested at 11 and 24 h after exposure. Tissue sections were used to detect apoptotic signals by TUNEL analyses as previously described [9].

3. Results

3. 1. Ablation of OPN suppresses UVB-induced tumor incidence and multiplicity

Female WT and OPN-null mice were subjected to UVB radiation for 43 weeks. Compared to WT mice, OPN-null mice showed a lower tumor incidence and multiplicity ($p = 0.026$) (Figure 1A and B, respectively). For the two groups, there were no significant differences in body weights throughout the course of the experiment (Figure 1C). Eight of ten WT mice developed tumors. Of these seven were *in situ* or early invasive cSCCs and one was a sarcoma. cSCC *in situ* is defined as changes in the skin containing sufficient cell proliferation and atypia, but the growth is contained within the epidermis. Invasive cSCC consists of irregular nests and cords of highly atypical (pleomorphic, disorganized and more of them contained mitotic figures) epidermal cells infiltrating the dermis to various depths. In contrast to WT mice, no cSCCs developed in OPN-null mice. Of the eight OPN-null mice one developed a sarcoma and one a benign melanoma (Figure 2A). These data indicate that OPN facilitates UVB-induced cSCC development.

3. 2. OPN expression in tumors of WT mice

All cSCCs showed OPN expression in the cytoplasm of stratum granulosum and spinous layer rather than in the basal cell layer of the tumors (Figure 2B). The surrounding skin chronically exposed to UVB also expressed OPN (data not shown). In sarcoma, minimal to no OPN expression was detected (Figure 2C). To confirm antibody specificity to OPN, kidneys from WT and OPN-null mice served as positive and negative controls, respectively (Figure 2D). To further verify OPN expression semi-quantitative RT-PCR assays of OPN mRNA were performed using total RNA extracted from regions containing cSCCs and sarcomas. The sarcoma from a WT mouse showed no detectable OPN mRNA (Figure 2E). Since the cSCCs were small (0.5-5 mm³ compared to 550 mm³ of sarcoma), insufficient samples were available for determination of OPN mRNA.

3. 3. UVB stimulates OPN expression

To determine if UVB-induces OPN expression in the epidermis, the dorsal skin of 10-week-old WT mice were exposed to 200 mJ/cm² of UVB and OPN protein and mRNA expression were determined after 11, 12, 24 or 25 h of single exposure. Western blot analyses showed that after 11 or 12 h of UVB exposure low expression of OPN protein was observed and its expression increased by 24 and 25 h (Figure 3A, left and right panels). The specificity of the OPN band was confirmed using OPN-null mice, as negative control, treated with and without UVB for 25 h, Figure 3A, right panel. Immunoprecipitation analyses of epidermal lysates from WT and OPN-null mice exposed to UVB showed a significant induction of OPN expression in WT mice after 24 h UVB exposure compared to those not treated, Figure 3B, further confirming UVB-induced OPN expression. Additionally, ELISA analyses, which has greater sensitivity, were also performed. We observed basal level of OPN expression in the epidermis of non-exposed mice (256 ± 84 pg/mg, $n = 4$). After a single UVB exposure,

the expression of OPN protein increased at 11, 24, and 48 h post-exposure (944 ± 299 pg/mg, $p = 0.006$; $1,006 \pm 102$ pg/mg, $p = 0.057$; $1,824 \pm 197$ pg/mg, respectively, $p < 0.001$, $n = 3$). Likewise, by RT-PCR analyses, no OPN mRNA was detected in OPN-null or non-exposed WT mice or in WT mice examined at 4 or 8 h after exposure to UVB, but it was detected at 24 h post-exposure (Figure 3C). As oppose to the presence of basal OPN protein, the lack of basal mRNA OPN detection is partly due to the greater instability of total RNA during its retrieval from the tissue.

3. 4. Ablation of OPN does not affect UVB-induced epidermal hyperplasia

Because tumor development is dependent on the survival and proliferation of initiated cells, we first determined if UVB-induced secretion of OPN into the microenvironment of epidermal cells stimulated epidermal hyperplasia and/or enhanced cell survival upon UVB exposure during the early stages of photocarcinogenesis. The dorsal skin of WT and OPN-null mice was exposed to UVB two times (once every other day) and harvested 48 h after the last exposure. Although UVB stimulated epidermal hyperplasia in WT and OPN-null mice relative to control mice, there were no significant differences in the number of epidermal cell layers between these two groups (WT, 5.75 ± 0.3 and OPN-null, 5.43 ± 0.32 , $n = 3$). Further, exposure to UVB for 43 weeks did not show a significant difference in epidermal thickness between the two groups (Figure 4A, B). These data indicate that increase of OPN expression does not mediate UVB-induced epidermal hyperplasia.

3. 5. OPN enhances the survival of basal keratinocytes exposed to UVB

In contrast to its lack of effect on cell proliferation, OPN suppressed UVB-induced apoptosis [33] of basal keratinocytes in the skin of WT mice. TUNEL analyses of dorsal skin of WT and OPN-null mice harvested at 11 and 24 h after a single UVB exposure showed increases, relative to their respective controls, in the numbers of apoptotic basal keratinocytes in the interfollicular regions (Figure 4C and D). By 24 h there is an overall increase in apoptotic basal keratinocytes in WT and OPN-null mice compared to that at 11h after exposure (WT, $p < 0.001$; OPN-null, $p < 0.001$). This is likely attributed to increases in acute UVB-induced inflammation with cytokine secretion produced by keratinocytes and/or infiltrating neutrophils and macrophages [33-35]. Furthermore, there were significantly higher numbers of apoptotic basal keratinocytes of OPN-null mice compared with WT mice. The findings suggests that UVB-induced OPN expression observed by 11h and further increased at 24 to 48 h enhances the survival of normal and, likely, UVB-induced initiated cells.

Acute UVB irradiation triggers infiltration of polymorphonuclear cells (PMNs) into the dermis [36]. Since OPN is reported to act as a chemoattractant for neutrophils [37, 38], a major subpopulation of PMNs that could also directly or indirectly contribute to an increase in apoptosis of basal keratinocytes, we assessed the levels of PMNs in the dermis of OPN-null and WT mice. The number of dermal PMNs is increased by 11 h and persists at 24 h after UVB exposure in both WT and OPN-null mice compared to their respective controls (Figure 4E). At 11 h the number of infiltrating PMNs did not differ between WT and OPN mice, but by 24 h, there were significantly more PMNs in the OPN-null mice. These findings suggests that the increase in OPN expression during these time points do not play a

significant role in the recruitment of PMNs, but likely in enhancing cell survival. Also the higher levels of dermal PMNs at 24 h after UVB exposure in OPN-null may, in addition to the lack of OPN, contribute to the increase in apoptotic basal keratinocytes. Interestingly though, preliminary data showed that the level of myeloperoxidase, IP-10 and MIP-1 α indicative of dermal neutrophils [39] in OPN-null mice at 24 h after UVB exposure were lower than that of WT mice (data not shown), suggesting that further validation studies are necessary on the number of dermal neutrophils within PMNs.

3. 6. OPN suppresses UVB-induced ONSC cell apoptosis and caspase-9 activity

To more directly determine whether OPN enhances cell survival UVB-induced apoptosis assays were performed using the osteopontin-null squamous cell carcinoma cell line (ONSC) established in our laboratory [25]. The main reason for using these cells, which lack OPN expression, is to prevent the possibility of UVB inducing OPN synthesis. ONSC cells were exposed to UVB at 0, 25 and 50 mJ/cm² followed by the addition of OPN or 1% BSA as negative control for 24 h. In BSA-treated cells, 50 mJ/cm² of UVB irradiation significantly induced cell apoptosis compared to those exposed to 25 mJ/cm² of UVB and non-irradiated cells ($p < 0.01$), Figure 5A. The addition of 3 μ g/ml of OPN to cells irradiated with 50 mJ/cm² of UVB significantly, increased the survival of ONSC cells, while there was no effect at 25 mJ/cm². Furthermore, OPN at high concentration (5 μ g/ml) also enhanced ONSC survival at 50 mJ/m² of UVB compared to BSA-treated cells, but there was no additional increase in cell survival when compared to cell treated with 3 μ g/ml of OPN (Figure 5B), suggesting a saturation effect by OPN.

To determine the potential mechanisms of OPN in preventing UVB-induced cell apoptosis, we assessed whether OPN can suppress UVB-induced activation of caspase-9, an initiator caspase leading to cell death, in ONSC cells. Cells with single exposure of 50 mJ/cm² UVB followed by the addition of 1, 3 or 5 μ g/ml of OPN or BSA for 6 h indicated that OPN dose-dependently suppressed the activation of caspase-9 when compared to controls (Figure 5C). Likewise, as above, cells treated with 5 μ g/ml of OPN showed no additional suppression of caspase-9 activity when compared with that of 3 μ g/ml of OPN.

3.7. OPN enhances the activation or phosphorylation of FAK in UVB-induced ONSC cell apoptosis

We have previously shown that OPN suppresses anoikis of murine pre-neoplastic epidermal-like cells (JB6 Cl41.5a), by attenuating caspase-8 activation and that this is mediated in part through integrin-binding and activation of FAK phosphorylation at Y861 [40]. FAK, a nonreceptor protein-tyrosine kinase, is one of the main signaling molecules that mediate cell survival following engagement of matrix proteins to integrins and hyaluronic acid to CD44 [41, 42]. Here, we determined whether extracellular OPN regulates FAK expression and/or its activation in UVB-induced apoptosis of ONSC cells and thereby, supports the possibility of secreted OPN engaging with integrin(s) to enhance cell survival.

ONSC cells were treated with 50 mJ/cm² of UVB followed by the addition of 5 μ g/ml of OPN or 1% BSA and harvested at 3 and 5 h after exposure. Control ONSC cells showed activated FAK with phosphorylation at Y397, while UVB exposure decreased its

phosphorylation status (Figure 5D). The addition of OPN appears to increase FAK activation as shown by an increase in phosphorylated FAK compared to BSA control at 5 h after single UVB exposure. However, OPN did not increase total FAK expression.

3. 8. Epidermal expression of FAK in OPN-null mice of controls and after acute UVB treatment was less than those of WT mice

To extend our *in vitro* finding of OPN stimulating FAK phosphorylation/activation to *in vivo model*, we determined whether total FAK and activated phospho[Y397]-FAK in the epidermis correlated with UVB-induced OPN expression during acute exposure of skin to UVB. In non-irradiated skin, the expression of total FAK in the epidermis was much more prominent in WT compared to OPN-null mice (Figure 6A). Western blot analysis of total FAK (Figure 6B) verified the expression of FAK in the epidermis shown by immunohistochemical analyses. Furthermore, FAK expression in the primary fetal keratinocytes was greater in WT mice compared to that of OPN-null mice. In the epidermis of control mice, FAK expression is strongly localized in the nuclei and less in the cytoplasm of the keratinocytes. At 11 h after single UVB exposure, total FAK expression has mainly moved away from the nucleus to the cytoplasm. Similarly to unexposed skin, epidermal total FAK expression was higher in WT than that of OPN-null mice (Figure 6C).

In contrast to total FAK, the expression of phospho[Y397]-FAK or activated FAK was not detected in control and UVB-exposed skin of WT or OPN-null mice using three different anti-phospho[Y397]-FAK antibodies. We reasoned that activated FAK is present, but that these antibodies were not suitable to detect its expression in paraffin-embedded tissues.

Although we were unable to determine the expression of activated FAK *in vivo*, the findings that total FAK expression was higher in the epidermis of WT mice compared to OPN-null mice at no treatment and even at 11 h after UVB exposure, suggest the possibility of OPN in either regulating or enhancing the expression of FAK. Additionally, the higher expression of FAK in WT mice compared to OPN-null mice implies that UVB-induced OPN can more rapidly through its interaction with integrins activate FAK and elicit biological responses that includes suppression of UVB-induced apoptosis of keratinocytes in WT mice.

We were unsuccessful in determining the expression of various integrins previously shown to engage with OPN *in vitro* in the epidermis of WT and OPN-null mice by immunohistochemical analyses. This is likely due to 1) inappropriate antibodies for paraffin-embedded skin specimens, or 2) the need for additional optimization to detection various integrin subunits.

3. 9. Epidermal expression of CD44 in OPN-null mice of controls and after acute UVB treatment was significantly less than those of WT mice

The mechanism for OPN-enhanced survival of cells *in vitro* involves its interaction with not only surface receptors, such as integrins, but also CD44 [40, 43-47]. CD44 is a family of transmembrane receptors consisting of CD44 standard (CD44s) and its variant isoforms, CD44v1-v10 [48]. CD44s has been shown to bind to OPN [49]. Among the various isoforms of CD44, extensive studies using expression vector of different isoforms of CD44

also support the interaction of OPN with CD44v6 and CD44v7 [50]. Here, we determined if the presence of CD44 correlated with UVB-induced OPN expression during acute exposure of skin to UVB using antibody that recognizes all isoforms of CD44 [51] and a specific antibody to CD44v6, which is expressed in mouse epidermis [52].

In skin of non-irradiated mice, minimal numbers of keratinocytes expressing CD44 were observed, consistent with previous study [53]. Relative to OPN-null mice, however, there were significantly greater numbers of such keratinocytes in the interfollicular regions of WT mice (Figure 6D). CD44 was mainly localized in the plasma membranes, but some were in the cytoplasm of cells in the basal and spinous layer of the epidermis. At 11 h after single UVB exposure, the expression of CD44 in keratinocytes of WT mice was similar to that of controls, but, relative to OPN-null mice at 11 h, the number of keratinocytes expressing CD44 remained greater in WT mice (Figure 6D, E). At 24 h after single UVB exposure, the expression of CD44 in keratinocytes of WT mice was significantly decreased relative to controls, and relative to OPN-null mice, no differences were observed.

The higher expression of CD44 in the epidermis of WT compared to OPN-null mice at no treatment and 11 h after UVB irradiation, suggests the possibility of OPN in stimulating expression and/or maintaining the stability of CD44 (see Discussion). Additionally, the higher levels of CD44 also facilitated a greater possibility of induced-OPN in engaging with CD44 to enhance the survival of stressed cells. It is not clear, why a decrease in CD44 expression in the epidermis of WT at 24 h is observed compared to those of control and 11 h post UVB irradiation. We speculate that it is partially attributed by the dramatic increase in the number of apoptotic cells at 24 h (11-21 cells/field/section) as compared to that of earlier time points of no treatment (0 cells) and 11 h (2-7 cells) after UVB treatment (Figure 4D).

In contrast to CD44 expression, the antibody to CD44v6 showed no expression in the keratinocytes of WT or OPN-null mice (data not shown). We believe that mouse skin do express CD44v6 as previously reported, since antibody to CD44 showed positive signals, but that the specific antibody to CD44v6 is not suitable for detection in paraffin-embedded tissues.

4. Discussion

In our previous study, the two-stage model for skin chemical carcinogenesis was used to address the function of induced OPN expression in tumor promotion. That study showed that OPN-null mice resulted in marked decrease in papilloma incidence and multiplicity compared to WT mice [9]. In the present study, the photocarcinogenesis model addresses the function of OPN in tumor progression or the development of cSCC. Here, we showed that the lack of UVB-induced OPN expression in OPN-null mice resulted in suppressed tumor formation relative to WT mice. In contrast to WT mice, OPN-null mice developed no cSCCs after 43 wks of UVB exposure. Because in this study only female mice were used, which normally develop fewer tumors than males [9], it is likely that UVB exposed OPN-null male mice will develop cSCC, but significantly less than that of WT mice. Additionally, in this photocarcinogenesis model, cSCC in WT mice expressed OPN consistent with that of

human cSCC [20]. Thus, the data from these two carcinogenesis models support the concept that induced-OPN facilitates development of epithelial/keratinocyte-derived tumors.

One may argue that the decreased development of cSCC in OPN-null mice compared to WT mice is due to expression of compensatory protein(s) that prevents tumor development. Bone sialoprotein (BSP) is a secreted protein with several features common to OPN (binds to hydroxyapatite and interacts with integrin through its RGD motif) and like OPN [9, 54-56], is minimally expressed in normal skin [57]; therefore, it is a likely candidate to compensate for OPN in OPN-null mice [58, 59]. However, with respect to its regulation by calcitriol, BSP is suppressed [59] while OPN is stimulated by calcitriol, thus upon UVB-induced production of calcitriol in the skin, BSP expression in OPN-null and WT mice is possibly downregulated. We conclude that in photocarcinogenesis suppressed tumor development in OPN-null mice is not likely due to compensatory effect of BSP. Instead, we believe that elevated- or UVB induced-OPN is critical in driving cSCC development.

We postulate that induced-OPN expression and secretion into the microenvironment of the epidermis is involved in the early progression stage by maintaining survival of UVB-induced initiated cells. The present findings support the role of UVB-induced de novo synthesis of OPN in preventing UVB-induced apoptosis of basal keratinocytes. A single exposure of UVB increases the expression of OPN in the epidermis by 11 h, with higher expression at 24 h that is sustained until at least 48 h. This correlates with the greater survival rate of basal keratinocytes in WT mice at 11 and 24 h when compared with that of OPN-null mice.

The possibility of greater basal keratinocyte survival in WT mice is further supported by our *in vitro* studies, indicating that the addition of OPN suppresses UVB-induced cell apoptosis in cells lacking OPN expression. Additionally, the increased cell survival of UVB-induced apoptosis of ONSC cells is likely mediated upstream by OPN activation of FAK through phosphorylation at Y397 and attenuation of caspase-9 activation. Thus, the persistent exposure of mouse skin to UVB, which generates initiated basal keratinocytes, is likely protected by continuous UVB-induced OPN secreted into the microenvironment. Since cSCCs are postulated to be derived from basal keratinocytes [60], the lack of cSCC development in OPN-null mice underscores the importance of OPN in facilitating basal cell survival.

The mechanisms by which induced OPN prevents apoptosis of UVB-irradiated keratinocytes *in vivo* remains to be addressed. Nevertheless, *in vitro* studies suggest that the survival of various cell types involves the interaction of OPN with integrins and/or CD44, CD44v6 and other variants [43-47, 61]. Specifically, OPN ligation with variants of CD44 (CD44v) has been shown to suppress UVB-induced apoptosis that is mediated through integrin activation in gastrointestinal cancer cells [61].

CD44v6 and CD44v10 are expressed in keratinocytes of pre-natal and adult mice [52, 62]. Although we did not detect the expression of CD44v6 using this specific antibody, we were able to show CD44 expression, using antibody that recognizes all isoforms of CD44, suggesting the possible presence of CD44v6 and/or CD44v10. We found that, at 11 h after

single UVB exposure, the greater expression of CD44 in WT mice relative to OPN-null mice correlates with induced OPN suppression of UVB-induced apoptosis of basal keratinocytes. This observation suggests, but does not establish, that OPN suppression of UVB-induced apoptosis of keratinocytes is mediated through CD44 *in vivo*.

The decreased level of CD44 expression mainly localized in the plasma membrane of keratinocytes of OPN-null compared to WT mice has been previously observed in OPN-null osteoclasts [63]. It is possible that basal level of OPN in the epidermis of WT mice is either stimulating CD44 synthesis, maintaining its stability, as previously shown in human liver cancer cell line, HepG2 [64], and/or stimulating CD44 to migrate to the surface of the plasma membrane [63-65] and consequently, enhancing the interaction of OPN with CD44 receptor. Further studies are necessary to establish whether OPN stimulates the expression and/or stability of CD44 protein in keratinocytes.

In addition to the possibility of CD44 mediating OPN enhanced cell survival, its engagement with integrins on keratinocytes to prevent apoptosis of keratinocytes is likely, but remains to be determined *in vivo*. The mouse epidermis, consistent with mouse epidermal-like JB6 cell line, does not express $\beta 3$, an integrin subunit shown to bind OPN through $\alpha \nu \beta 3$ in various cell lines. Extensive integrin characterization and investigation on the engagement of OPN with various integrin subunits in TPA-activated JB6 cells suggest that it likely interacts with $\alpha \nu \beta 5$ integrin [66], which is also expressed in the epidermis [67]. Therefore, in addition to interacting with CD44, it may also engage with $\alpha \nu \beta 5$ *in vivo* and thereby, stimulate FAK activation.

In mouse skin, the greater expression of total FAK in the epidermis of non-exposed and 11 h after acute UVB-treated WT mice compared to that of OPN-null mice, allows the induced-OPN to rapidly interact with integrin and activate FAK to enhance cell survival of WT keratinocytes as oppose those of OPN-null mice. The possibility of FAK mediating OPN induced survival of initiated cells is strongly supported by previous studies where suppression or deletion of FAK expression in the basal keratinocytes also resulted in decreasing their survival and subsequently, attenuating papilloma formation and malignant transformation in two-stage skin carcinogenesis model [68, 69]. Thus, we postulate that induced-OPN through engagement with integrin/CD44 stimulates FAK activation *in vivo*, and thereby, facilitates the development of cSCC in photocarcinogenesis.

Besides enhancing cell survival, OPN is reported to promote cell proliferation *in vitro* [47, 70-72]. Although, the use of *in vitro* UVB-induced apoptosis model supports OPN's role in enhancing cell survival, this model does not rule out its potential role in driving cell proliferation in other cell types *in vitro*. Nevertheless, we found that OPN does not stimulate cell proliferation *in vivo*. UVB-induced epidermal hyperplasia indicate that the lack of OPN expression does not decrease the number of epidermal cell layers. Furthermore, chronic UVB exposure for 43 weeks also shows no difference in epidermal cell layers between the two groups. These data corroborate with our previous studies indicating no differences in tumor promoter-induced epidermal hyperplasia between WT and OPN-null mice [9]. Therefore, in the context of two skin carcinogenesis systems, induced OPN does not drive proliferation of keratinocytes *in vivo*.

In summary, the present and previous findings [9] underscore the importance of changes in the microenvironment, either by chemical- or UVB-induced expression of the matricellular protein, OPN, in facilitating tumor promotion and progression. This is the first study using the photocarcinogenesis model to demonstrate that induced expression of OPN is necessary for UVB-induced development of cSCCs. We hypothesize that, prior to clonal expansion of initiated cells, the capacity of these cells to survive or to die in a hostile environment is a major limiting factor in tumor development, and that UVB-induced OPN, secreted into the microenvironment of basal keratinocytes, is required to prolong the survival of normal and initiated basal keratinocytes, which eventually develop into cSCCs.

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Highlights

- We investigate the role of osteopontin (OPN) in photocarcinogenesis.
- OPN-null female mice did not develop cutaneous SCC compared to wild-type mice.
- Secreted OPN enhances the survival of initiated keratinocytes.
- Favorable microenvironment of initiated cells is necessary for SCC development.

Fig. 1. Tumor incidence and multiplicity and body weights of UVB-treated OPN-null and WT female mice

Dorsal skin of WT (n = 10) and OPN-null (n = 8) mice was treated with UVB for 43 weeks as described in Materials and Methods. (A) Percent of mice with tumors, (B) Number of tumors per mouse, significantly different between groups, $p = 0.026$. Comparisons between groups were analyzed by Mann-Whitney Rank Sum Test. (C) Body weight.

Fig. 2. Histology of tumors from UVB-treated OPN-null and WT mice and OPN expression in tumors of WT mice

(A) Histology of an early invasive cSCC, a cSCC *in situ*, and a sarcoma from WT mice, and a benign melanoma from OPN-null mouse. *, region of invasive cSCC.

Immunohistochemical analyses of OPN expression in a cSCC of WT mouse (B), and sarcomas (C) and kidneys (D) of WT and OPN-null mice. Tissue sections were incubated with anti-OPN or its isotype control, IgG1, κ . In cSCC (B), top right panel, OPN staining without H&E stain; lower right panel, OPN staining with H&E stain. In sarcoma (C), lower right panel, OPN-null sarcoma serves as negative control for OPN expression. In kidney (D), WT (left panels) and OPN-null kidneys (right panels) served as positive and negative

controls for OPN expression, respectively. **(E)** OPN mRNA expression by semi-quantitative RT-PCR. OPN mRNA expression was not observed in WT sarcoma. TPA-induced OPN mRNA expression of JB6 Cl41.5a cells served as a positive control. RT-PCR for GAPDH served as control for RNA integrity. M, molecular weights of DNA ladder.

Fig. 3. UVB-induced OPN expression in the epidermis

(A) Dorsal skin of WT and OPN-null mice was exposed to a single dose of 200 mJ/cm² of UVB. The epidermises were harvested after 11 and 24 h (left panel) or 12 and 25 h (right panel) of exposure for Western blot analyses (60 and 50 µg/sample, respectively). Left panel, rat recombinant OPN (rOPN), approximate relative MW 75 kDa, from the same blot with X-ray film from decreased exposure time. Right panel, rOPN was overloaded and degraded. Top panels, probed with mouse OPN antibody (Akm2A1); lower panel, probed with antibody to GAPDH. kDa, kilodalton. (B) Dorsal skin was exposed to a single exposure of UVB as above. After 24 h epidermal lysates were immunoprecipitated with antibody to OPN. Dorsal skin of WT mice treated with TPA for 24 h served as positive control for OPN expression. (C) Similar experiments were performed as (A), and total RNA isolated from WT and OPN-null (OPN) mouse epidermis at 0 (C-1 and C-2), 4, 8, and 24 h after UVB exposure were analyzed for OPN and GAPDH by semi-quantitative RT-PCR. N = 2 mice/ time point. M, molecular weights of DNA ladder.

Fig. 4. UVB-induced OPN expression does not mediate UVB-induced epidermal hyperplasia, but suppresses UVB-induced apoptosis of basal keratinocytes. UVB increased dermal infiltration of PMNs

(A) Top panels, dorsal skin of WT and OPN-null mice was exposed to UVB twice (2x, every other day) and harvested at 48 h post exposure. Skin sections were stained with H&E. Lower panels, skin adjacent to the tumor after 43 wks of UVB exposure. Original magnification, 40x. (B) Histogram of the average number of epidermal cell layers per field per section (± SD) from WT and OPN-null mice not exposed (CT) or exposed to UVB, as indicated in (A). From each mouse, 2-3 skin sections were analyzed; 6-7 fields at a magnification of 40x were assessed per piece of skin, and the average number of layers/field/mouse was recorded. CT, n = 3; 2x, n = 3; 43 wk, WT, n = 4, OPN-null, n = 5. **, p < 0.001, significantly different from their corresponding CT and 43 wk groups. OO, p < 0.001, significantly different from their corresponding CT groups. Comparisons between

groups were analyzed by the paired t-test, since these samples were shown to be normally distributed (Shapiro-Wilk method) and with equal variance. Software for the statistical analyses was from Sigma Plot 11. (C) TUNEL analyses of UVB-induced basal cell apoptosis. Dorsal skin of mice was treated with or without UVB and harvested after 11 h. TUNEL-positive cells are indicated by arrows. (D) Dot plot of the average number of apoptotic signals per skin section per mouse. To score the number of TUNEL-positive cells, only basal cells at the interfollicular region in the epidermis were analyzed. The numbers of apoptotic cells/skin were scored from ten fields at 40x magnification. 2-3 skin samples/mouse were assessed and averaged. Comparisons between groups at 11 h and between no exposure and WT or OPN-null of 11 h were analyzed by Mann-Whitney Rank Sum Test. Comparison between groups at 24 h was analyzed by the paired t-test. (E) Dot plot of the number of PMNs in 1 cm of dermis per mouse. The number of dermal PMNs were scored from H&E stained skin sections. Comparison between groups was analyzed by the paired t-test.

Fig. 5. OPN suppresses UVB-induced ONSC cell apoptosis and caspase-9 activity, and enhances the activation of FAK

(A) Histogram of the average number of live ONSC cells after single UVB exposure in the presence or absence of OPN (average of four independent experiments). Cells were exposed to UVB at 0, 25 and 50 mJ/cm², and then treated with 1% BSA or 3 μg/ml of OPN. After 24 h, live cells were counted by hemocytometer (quadruplicated dishes/treatment/experiment). (B) Experiments were performed as above, except cells were exposed to a signal dose of 50 mJ/cm² of UVB followed by the addition of 0.1, 1, 3 or 5 μg/ml of OPN or 1% of BSA for 24 h. (C) Experiments were performed as that of (B) and harvested after 6 h and analyzed for caspase-9 activity. Comparison between groups was analyzed by the paired t-test in (A-C). (D) ONSC cells were exposed to 50 mJ/cm² of UVB as above and treated with 5 μg/ml of OPN or BSA for 3 and 5 h. HaCaT cells not exposed to UVB served as positive control for FAK expression [73]. Cell lysates were immunoprecipitated with anti-FAK antibody and resolved in 7.5% gel for detection of phosphorylated FAK (pY³⁹⁷-FAK) and total FAK. Top

panel, Western blot analyses, lower panel, histogram of relative amount of pY³⁹⁷-FAK normalized to total FAK.

Fig. 6. FAK and CD44 expression in the epidermis of UVB-treated WT and OPN-null mice (A) Photos are representative of immunohistochemical analyses of FAK expression in WT and OPN null mice of control (n = 4/group). Nonspecific binding by IgG is observed in the sebaceous glands. (B) Western blot analyses of total FAK from primary keratinocytes of WT and OPN-null mice. (C) Immunohistochemical analyses of epidermal expression of FAK of WT (n = 5) and OPN null (n = 3) mice after 11 h of a single UVB exposure. IgG staining are not shown. (D) Dot plot of the average number of CD44-expressing keratinocytes per mouse in the interfollicular epidermis of WT and OPN-null mice of control and UVB-treated skin at 11 and 24 h after exposure. The average numbers of CD44 expressing keratinocytes per mouse were derived from 2-3 skin sections/mouse. In each skin section, the total number of CD44 expression keratinocytes in the interfollicular epidermis of 8-mm skin sections was assessed. Comparison between groups was analyzed by the paired t-test. (E) Photos are representative of CD44 expression in WT and OPN-null

epidermis after 11 h of a single UVB exposure. Parallel skin sections were incubated with antibody to CD44 and isotype control, IgG_{2b,κ}.