Osteopontin Stimulates Preneoplastic Cellular Proliferation Through Activation of the MAPK Pathway

Xianmin Luo, Megan K. Ruhland, Ermira Pazolli, Anne C. Lind, and Sheila A. Stewart

Abstract

Alterations in the microenvironment collaborate with cell autonomous mutations during the transformation process. Indeed, cancer-associated fibroblasts and senescent fibroblasts stimulate tumorigenesis in xenograft models. Because senescent fibroblasts accumulate with age, these findings suggest that they contribute to age-related increases in tumorigenesis. Previously we showed that senescence-associated stromal-derived osteopontin contributes to preneoplastic cell growth in vitro and in xenografts, suggesting that it impacts neoplastic progression. Analysis of fibroblasts within premalignant and malignant skin lesions ranging from solar/actinic keratosis to squamous cell carcinoma revealed they express osteopontin. Given the stromal expression of osteopontin, we investigated how osteopontin impacts preneoplastic cell growth. We show that osteopontin promotes preneoplastic keratinocyte cell proliferation and cell survival through the CD44 cell receptor and activation of the MAPK pathway. These data suggest that stromal-derived osteopontin impacts tumorigenesis by stimulating preneoplastic cell proliferation thus allowing expansion of initiated cells in early lesions. Mol Cancer Res; 9(8): 1018–29. ©2011 AACR.

Introduction

Tumorigenesis results from the accumulation of cell autonomous mutations and concomitant changes in the surrounding stromal compartment (1–4). Whereas the importance of genetic mutations in would-be tumor cells is well established, the mechanisms by which the stromal compartment contributes to tumorigenesis are more poorly defined. Recent work has focused on the role stromal fibroblasts play in tumorigenesis and shown that cancer-associated fibroblasts stimulate preneoplastic and neoplastic cell growth in vitro and in xenograft models [reviewed in (3, 4)]. Microarray analysis of cancer-associated fibroblasts revealed numerous expression changes reminiscent of myofibroblasts found within wounds (5–11), raising the possibility that these factors contribute to tumorigenesis.

Senescent fibroblasts function analogously to cancer-associated fibroblasts in that they stimulate neoplastic and preneoplastic cell growth in vitro and in xenografts (12–14). Importantly, both senescent cells and genetic mutations accumulate in human tissue with age (1, 2, 15–21), raising the possibility that together they impact tumorigenic rates in aged human populations. This hypothesis led several groups to investigate how senescent fibroblasts stimulate preneoplastic cell growth. This work showed that several senescence-associated fibroblast-derived factors impact preneoplastic cell growth in different models. Indeed, amipregulin impacts preneoplastic prostate growth whereas GRO-1 enhances ovarian cell growth (22). Our group found that senescence-associated fibroblast-derived osteopontin was necessary to promote preneoplastic keratinocyte cell growth (14). Together these studies clearly show that several senescence-associated, fibroblast-derived factors impact preneoplastic cell growth.

Osteopontin is an acidic glycoprotein ascribed numerous functions in normal and pathologic physiologies. osteopontin is a noncollagenous bone matrix protein that functions in bone homeostasis, the immune system, cell trafficking, cell adhesion, cell survival, and the vascular system (23–26). Osteopontin is expressed in many cell types including macrophages, endothelial cells, smooth muscle cells, and epithelial cells (27). In addition, osteopontin functions as an inflammatory cytokine that participates in acute and chronic inflammation where it is expressed by resident epithelial, endothelial, and smooth muscle cells, infiltrating macrophages and T cells (25, 26). Finally, osteopontin is expressed in epithelial and immune cells from a wide variety of normal and tumor tissues (28–31).

Osteopontin’s ability to influence numerous biological activities is at least partially due to the diverse set of receptors it utilizes. Through its arginine-glycine-aspartic acid (RGD) motif, osteopontin engages various integrins including α5β1, αvβ3, αvβ5, αvβ6, and αvβ1 (25, 26). The α4β1 and α9β1 integrins bind osteopontin independently of its
RGD motif (32). In addition to the integrins, osteopontin interacts with CD44 independently of its RGD motif (33). Osteopontin's receptor interaction is further controlled by posttranslational modifications. For example, osteopontin can be processed by MMP3, MMP7, and thrombin, which alter its binding characteristics and biological functions (32).

Recent work has focused on osteopontin's role in skin carcinogenesis and examined osteopontin expression in lesions ranging from benign solar/actinic keratosis (AK) to squamous (SCC) and basal cell carcinomas (34). Analysis of these lesions revealed only limited osteopontin expression in the epithelial layer of early lesions but readily detectable levels within SCC (34–37). Whereas osteopontin's association with the latter stages of tumorigenesis has been studied extensively (38), its impact on the early stages of transformation is only now being appreciated. For example, osteopontin was shown to convert a preneoplastic keratinocyte to a tumorigenic cell through an unknown mechanism(s) (39). Osteopontin also promotes cell survival in a skin tumor model (40) and osteopontin activation of the PI3K and MAPK pathways is important for tumor formation in PTEN knockout mice (41). Finally, a reduction in tumor penetrance and increase in tumor latency was observed when osteopontin knockout mice were subject to a classic multistage skin carcinogenesis model. Indeed, when the carcinogenic agent, 7,12-dimethylbenz(a)anthracene (DMBA) was applied as an initiator followed by subsequent TPA treatments, osteopontin null mice displayed a significant delay in the appearance and number of skin papillomas (42). Analysis of the papillomas revealed a higher degree of apoptosis in lesions from osteopontin null animals compared with wild-type controls, arguing that osteopontin supplies an important survival signal. These data suggest that osteopontin is critical in the early stages of tumorigenesis.

Given our previous findings that senescence-associated osteopontin plays an important role in preneoplastic cell growth (14), we sought to determine how it stimulates preneoplastic keratinocyte cell growth. Here we show that osteopontin was shown to convert a preneoplastic keratinocyte to a tumorigenic cell through an unknown mechanism(s) (39). Osteopontin also promotes cell survival in a skin tumor model (40) and osteopontin activation of the PI3K and MAPK pathways is important for tumor formation in PTEN knockout mice (41). Finally, a reduction in tumor penetrance and increase in tumor latency was observed when osteopontin knockout mice were subject to a classic multistage skin carcinogenesis model. Indeed, when the carcinogenic agent, 7,12-dimethylbenz(a)anthracene (DMBA) was applied as an initiator followed by subsequent TPA treatments, osteopontin null mice displayed a significant delay in the appearance and number of skin papillomas (42). Analysis of the papillomas revealed a higher degree of apoptosis in lesions from osteopontin null animals compared with wild-type controls, arguing that osteopontin supplies an important survival signal. These data suggest that osteopontin is critical in the early stages of tumorigenesis.

Given our previous findings that senescence-associated osteopontin plays an important role in preneoplastic cell growth (14), we sought to determine how it stimulates preneoplastic keratinocyte cell growth. Here we show that recombinant human osteopontin (rhOPN) stimulates cell growth via the CD44 cell surface protein and activation of the MAPK pathway. Analysis of human skin samples ranging from premalignant actinic keratosis to malignant in situ and in vivo revealed osteopontin expression in fibroblasts present in the stromal compartment in regions coincident with the cell cycle inhibitors p21 and p16, indicating that these fibroblasts were noncycling and likely senescent. Taken together, these findings suggest that senescence-associated, stromal-derived osteopontin promotes preneoplastic and neoplastic cellular proliferation through activation of the MAPK pathway and thus contributes to tumorigenesis.

Materials and Methods

Reagents and antibodies

rhOPN was purchased from R&D system (1433-OP), FD98059 (MEK1 inhibitor), U0126 (MEK1/2 inhibitor), Ly294002 (PI3K inhibitor), and Wortmanin (PI3K inhibitor) were purchased from Sigma. The peptide integrin αβ3 inhibitor, RGD (AM-100) and negative control peptide RAD (AM-101) were from Enzo Life Science. Neutralizing antibody against CD44 (HCAM) was obtained from Santa Cruz Biotechnology Inc. (SC-7946) (43).

Fibroblasts and induction of senescence

Human dermal BJ fibroblasts were induced to undergo stress-induced premature senescence (SIPS) as previously described (14). Briefly, BJ fibroblasts were treated with 100 μg bleomycin for 18 hours and allowed to recover for 72 hours in serum-free medium. Cocultures were carried out as previously described (14).

rhOPN and cell growth measurements

HaCAT cells (preneoplastic human keratinocytes originally obtained from Norbert Fusenig at DKFZ) were transduced with a Moloney Type C virus expressing click beetle red luciferase (CBR), which led to stable transduction of CBR (14). HaCAT-CBR cells were grown as previously described (1). Briefly, cells were plated in 96-well plates (2000 cells/well) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum for 24 hours. Serum containing medium was removed, cells were washed with serum-free medium and incubated for 3 to 5 hours prior to addition of rhosteopontin (1000 ng/ml). Following addition of rhosteopontin, cells were incubated for an additional 72 hours. Cell growth was measured by luciferase activity as previously reported (14). Increases in luciferase activity were shown to be a direct measure of cell number (Supplementary Fig. S2).

BrdU incorporation and TUNEL assay

HaCAT proliferation analysis was carried out by BrdU incorporation (Roche Applied Science) and apoptosis was assessed by TUNEL assay (Roche Applied Science). Briefly, HaCAT-CBRs (2000 cells/well) were plated in a 96-well plate and starved of serum overnight before addition of rhOPN (1000 ng/ml). BrdU incorporation and apoptosis were assessed 24, 48, and 72 hours later. For the BrdU incorporation assay, 20 umol/L BrdU was added 24 hours before cell fixation. Both the BrdU and apoptosis assays were carried out per manufacturer's instructions. For coculture experiments, HaCAT-CBR cells were identified with a pan-cytokeratin antibody (Invitrogen).

CD44 blocking experiments

HaCAT-CBR cells were plated in 96-well plates (2000 cells/well) and grown in DMEM containing 10% fetal bovine serum for 24 hours. Serum containing medium was removed, cells were washed with serum-free medium and incubated in the presence of 5 μg of anti-CD44 blocking antibody or 100 μmol/L of the RAD or RGD blocking peptides for 3 to 5 hours prior to addition of rhOPN (1000 ng/ml). Following addition of rhOPN, cells were incubated for an additional 72 hours. Cell growth was measured by luciferase activity as previously reported (14).
pERK and pAKT translocation assay

 Serum starved HaCaT-CBR cells or HaCaT-CBR ectopically expressing dominant negative MEK (MEK-DN) (10,000 cells/well) were incubated with the MEK inhibitors PD98059 (40 μmol/L) or U0126 (0.3 μmol/L) or the PI3K inhibitors Ly294002 (1 μmol/L) or Wortmannin (40 nmol/L) for 1 hour, then rhOPN (1000 ng/ml) or human recombinant EGF (hEGF; 25 ng/ml) was added for 60 and 10 minutes, respectively prior to fixing cells. Cells were washed with PBS and fixed in 4% paraformaldehyde followed by immunofluorescence staining. For pERK and pAKT immunofluorescence staining, HaCaT-CBRs were permeabilized with 0.1% Triton X-100 for 20 minutes, and incubated with primary antibodies against pERK (1:50, Cell Signaling Technology), or pAKT (1:50, Cell Signaling Technology) in PBS containing 1% bovine serum albumin, 5% normal goat serum overnight at 4°C. Following a PBS wash, cells were incubated with fluorescent secondary antibodies Alexa 488 or Alexa 594 (1:500, Invitrogen Molecular Probe) for 20 minutes at room temperature and cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; 1 μg/ml, Sigma-Aldrich). Cells were imaged using the IN cell analyzer 1000 (GE Healthcare Life Science).

Immunohistochemistry

Archival human AK and SCC skin sections were deparaffinized and rehydrated after antigen retrieval in citrate buffer at pH = 6. Skin sections were quenched and incubated with 3% hydrogen peroxides for 30 minutes, followed by incubation in a protein block solution (Dako, X0909) for 30 minutes at room temperature. For single staining, human AK and normal sections were incubated with the following antibodies in antibody diluent solution (Dako, S3022): mouse anti-human osteopontin (clone 53, 1:150, Assay Design Inc), p16 (1:200, Santa Cruz Biotechnology), or p21 (1:200, Santa Cruz Biotechnology) overnight at 4°C. Primary antibodies were detected using secondary antibodies and the Dako Cytomation EnVision+ System HRP kit, visualized with DAB according to manufacturer’s instructions, and the sections were counterstained with hematoxylin.

Immunofluorescence

Archival human skin sections were deparaffinized and rehydrated after antigen retrieval in citrate buffer at pH = 6. Skin sections were quenched and incubated with protein block solution (Dako, X0909) for 30 minutes at room temperature and then incubated in prediluted vimentin antibody (ab8545, Abcam) and one of the following: p21 (1:200, Santa Cruz Biotechnology) or human osteopontin (clone 53, 1:300, Assay Design Inc.) overnight at 4°C. Tissues were washed in PBS and then incubated with the following secondary antibodies at a concentration of 1:500 for 30 minutes at room temperature (RT): goat anti-mouse Alexa-488 (A1001, Invitrogen) and goat anti-rabbit Alexa-546 (A11010, Invitrogen). Sections were evaluated under a light microscope (Nikon Eclipse 50i) or with fluorescence microscopy (Nikon).

Western blotting analysis

 Serum starved HaCAT-CBR cells or HaCAT-CBR cells ectopically expressing dominant negative MEK (MEK-DN) were pretreated with the MEK inhibitors PD98059 (50 μmol/L) for 2 hours prior to the addition of rhOPN (1000 ng/ml) for 10 minutes or 60 minutes. Cells were washed with PBS and directly lysed in dishes on ice with mammalian cell lysis buffer (MCLB; 50 mMTris-HCl pH8.0, 5mMEDTA, 0.5%NP-40, 100 mmol/L NaCl) supplemented with protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail (Calbiochem), 2 mmol/L DTT, 1mmol/L microcystin (Sigma), 1 mmol/L sodium orthovanadate, 2 mmol/L phenylmethylsulfonyl fluoride and 1μM beta-glycerophosphate. The cell lysates then were rocked at 4°C for 10 minutes, centrifuged at 14,000 rpm for 20 minutes at 4°C, and the supernatants were collected for protein quantification. The protein concentration was determined by Bradford assay. 150 μg protein lysates were diluted in MCLB and sample loading buffer and boiled for 10 minutes. Protein was separated on 12% SDS-PAGE gels followed by transfer to polyvinylidine difluoride (PVDF) membrane (Millipore). The membrane was incubated with primary antibodies phospho-p44/p42 MAPK (Erk1/2; Thr202/Tyr204; 1:1000, Cell Signaling Technology), total p44/p42 MAPK (Erk1/2) antibody (1:1000, Cell Signaling Technology), phosphor-Akt (Ser473) antibody (1:1000, Cell Signaling Technology), and total Akt antibody (1:1000, Cell Signaling Technology) overnight at 4°C. For detection, horseradish peroxidase-conjugated anti-rabbit secondary antibody and the ECL system were used with standard techniques.

Statistical analysis

Data represent the mean ± SEM; statistical significance was assessed by the Student’s t test and values were considered significant if P < 0.05.

Results

Osteopontin is expressed in the stroma of premalignant and malignant skin lesions

Osteopontin is a pleiotropic protein that impacts diverse biological processes ranging from wound healing to tumorigenesis (23–26). Previous studies have shown that in human skin osteopontin is expressed in the epithelial compartment and in infiltrating immune cells of premalignant and malignant skin lesions including AK and SCC, supporting a role for osteopontin in tumorigenesis. Whereas these studies clearly showed that osteopontin is expressed in these lesions, the analysis was focused on the epithelial compartment. To investigate whether stromal-derived osteopontin was present in early, premalignant lesions, we stained sections from human skin including AK and SCC with an antibody against osteopontin. We observed that 10 of 10 AK and 7 of 7 invasive SCC contained osteopontin-positive stromal cells whose morphology was consistent with dermal fibroblasts (Fig. 1 and data not shown). To establish that at least some of the osteopontin-positive cells were fibroblasts, serial sections
were costained with an antibody against osteopontin and one against vimentin, which is highly expressed in fibroblasts (22). As shown in Fig. 2A, vimentin-positive cells expressing osteopontin are evident in the stromal compartment of 5 of 5 AK and 5 of 5 SCC. In agreement with previous studies, we failed to detect significant levels of osteopontin in the epithelial or stromal compartment of normal human skin (Fig. 1; ref. 34).

In xenograft studies, senescent dermal fibroblasts expressing osteopontin play an important role in tumorigenesis (14). These findings raise the intriguing possibility that osteopontin-expressing senescent fibroblasts play a functional role in human AK and SCC. Expression of the cell cycle inhibitors p16 and p21 correlate with senescence in vivo (44–48). Therefore, we next analyzed serial sections from AK for coincident expression of osteopontin with p16 and p21 within the stromal compartment. We found that fibroblast-derived osteopontin expression was coincident with the cell-cycle inhibitors p16 and p21 in 12 of the 12 AK (Fig. 1A). We also noted coincident expression in SCC (Fig. 1A). Immunofluorescent staining of 5 AK and 3 SCC confirmed that human osteopontin and p21 expression was coincident (Fig. 2B). We also observed osteopontin expression in the epithelial layer of AK and SCC, which was in agreement with a previous study (34). Together, these findings show that osteopontin expression is complex and can be found in epithelial and stromal compartments.

Osteopontin stimulates preneoplastic cell growth
Osteopontin can impact cellular proliferation and survival (i.e., apoptosis) (49–51) and senescence-associated stromal-derived osteopontin stimulates cell growth in vitro and in xenografts (14). Given osteopontin’s pleiotropic functions, we next asked whether osteopontin directly impacted preneoplastic keratinocyte proliferation and/or survival. To distinguish between these two possibilities, we measured cellular proliferation and apoptosis by carrying out a BrdU incorporation assay and TUNEL assay, respectively. To determine whether fibroblasts...
impacted proliferation and/or survival we first examined the growth of HaCAT-CBR cells plated on young versus senescent fibroblasts in serum-free conditions. We restricted our analysis to HaCAT-CBR cells by first staining the cocultures with a pancytokeratin antibody and quantitating BrdU incorporation and TUNEL-positive cells only in cytokeratin-positive cells. As shown in Fig. 3A, the presence of fibroblasts, young or senescent, protected cells from apoptotic death as indicated by the drastically lower percentage of TUNEL-positive HaCAT-CBR cells compared with cells plated alone. Analysis of BrdU incorporation revealed a two-fold increase in proliferation when HaCAT-CBR cells were plated on senescent fibroblasts versus young fibroblasts (Fig. 3A). Together these data indicate that senescent fibroblasts provide a potent proliferative signal to preneoplastic epithelial cells.

Above we show that senescent fibroblasts stimulate HaCAT-CBR cell proliferation and previous work showed that fibroblast-derived osteopontin is necessary to stimulate HaCAT-CBR cell growth in vitro and in xenografts (14). We next sought to determine whether osteopontin was sufficient to stimulate HaCAT-CBR cell proliferation and/or survival. Therefore, we carried out BrdU and TUNEL analysis of HaCAT-CBR cells treated with rhOPN. Using the BrdU incorporation assay, we observed a statistically significant increase in HaCAT-CBR cell proliferation when HaCAT-CBR cells were treated with rhOPN for 72 hours compared with HaCAT-CBR cells plated alone \( (P < 0.05; \text{Fig. 3B}) \). In addition, the TUNEL assay revealed that rhOPN protected HaCAT-CBR cells from apoptotic cell death. Indeed, we observed a lower percentage of TUNEL-positive HaCAT-CBR cells in the presence of rhOPN compared with cells plated alone \( (P < 0.05; \text{Fig. 3B}) \). These data indicate that rhOPN stimulates HaCAT cell growth (Fig. 3C) by providing both a moderate proliferative and survival signal.

Osteopontin-induced HaCAT growth is mediated by the MAPK pathway

Osteopontin activates several downstream signaling cascades including the MAPK, PI3K, and NFκB pathways in a cell-specific manner [reviewed in (51)]. Given osteopontin’s ability to stimulate cellular proliferation and survival, it was possible that rhOPN stimulated multiple downstream signaling pathways. To determine which pathways were activated following treatment with rhOPN, we used several well-characterized MAPK and PI3K chemical inhibitors. To examine the importance of these signaling pathways, serum starved HaCAT-CBR cells were treated with the

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**Figure 2.** Osteopontin is expressed in stromal fibroblasts coincident with p21 in preneoplastic and neoplastic skin lesions. A, representative immunofluorescent staining of hOPN (green) and vimentin (red) from AK \( (n = 5) \) and SCC \( (n = 3) \) lesions show that fibroblasts (vimentin-positive) express hOPN. B, representative immunofluorescent staining of hOPN (green) and p21 (red) from AK \( (n = 5) \) and SCC \( (n = 3) \) lesions show co-localization within the stromal compartment.
MEK inhibitors PD98059 or U0126 or the PI3K inhibitors Ly294002 or Wortmannin were sufficient to block osteopontin-induced MAPK and PI3K activation.

We next assessed the impact of MAPK or PI3K activation on HaCAT cell growth. Serum starved HaCAT-CBR cells were treated with a MAPK or PI3K inhibitor and rhOPN and cell growth was assessed 72 hours later. As shown in Fig. 4D, both MEK inhibitors (PD98059 and U0126) significantly inhibited rhOPN-induced HaCAT-CBR cell growth. In contrast to that observed with the MAPK inhibitors, the PI3K inhibitors Ly294002 and Wortmannin had no impact on rhOPN-induced HaCAT cell growth. This finding indicates that osteopontin-mediated activation of the PI3K pathway is not responsible for the increased cell growth observed upon rhOPN treatment. Rather, it is osteopontin-mediated activation of the MAPK pathway that is important for the increased HaCAT cell growth observed upon rhOPN treatment.

Above we show that the MAPK pathway plays a critical role in osteopontin-mediated preneoplastic cell growth. To confirm the importance of the MAPK pathway in osteopontin-mediated cell growth we introduced a dominant-negatively acting allele of MEK1 (MEK-DN) into HaCAT-CBR cells. As expected, introduction of the MEK-DN allele into HaCAT-CBR cells rendered the cells insensitive to epidermal growth factor (EGF) stimulation. Indeed, we found that in contrast to control HaCAT-CBR cells, HaCAT-CBR cells expressing MEK-DN displayed a dramatic reduction in phospho-ERK (pERK) in response to EGF stimulation. These findings were confirmed by western blotting analysis of HaCAT-CBR (mock) and HaCAT-CBR-MEK-DN (MEK-DN) cells. Similar results were obtained when rhOPN was used to stimulate ERK phosphorylation. Given the impact of MEK-DN expression on ERK phosphorylation, we next examined whether it impacted HaCAT cell growth. As shown in Fig. 5C, expression of MEK-DN significantly blocked osteopontin-induced proliferation of HaCAT-CBR-MEK-DN cells. Taken together with the drug inhibitor experiments presented above, these results show that the MAPK pathway plays a critical role in osteopontin-mediated stimulation of HaCAT cell growth.

Osteopontin stimulates HaCAT growth through CD44 activation

Above we show that the MAPK pathway plays a critical role in osteopontin-mediated preneoplastic cell growth. Numerous studies have shown that osteopontin can utilize...
several cell surface receptors to mediate its effects in a target cell dependent manner (41, 51). Indeed, osteopontin binds cell surface receptors including several integrins and CD44 as well as components of the extracellular matrix. Osteopontin can bind to a variety of cell types through RGD-mediated interaction with integrins at its NH2-terminal region and non-RGD-mediated interactions with CD44 at its COOH-terminal region (53, 54). Given the complexity that surrounds osteopontin receptor utilization, we next assessed the cell receptors utilized by osteopontin to impact HaCAT cell growth.

To determine which receptor complex is activated by osteopontin and contributes to osteopontin’s growth-promoting activities, we used well-characterized blocking antibodies and peptide inhibitors. Reverse-transcription PCR (RT-PCR) analysis of HaCAT cells revealed that they do not express the α5 or β3 integrin subunits (data not shown). However, these cells do express the αv, β1, and β5 subunits as well as CD44 (data not shown). Therefore, we used RGD peptides to block interaction between rhOPN and integrins to determine its impact on HaCAT cell growth. HaCAT-CBR cells were plated and serum starved prior to the

![Figure 4. MAPK activation contributes to osteopontin-induced HaCAT cell growth. A, MAPK inhibitors effectively block phosphorylation and activation of ERK.](image-url)
addition of an RGD peptide or RAD control peptide that does not interact with integrins (25, 51, 55–60). Following serum deprivation, blocking peptides were added to the cells in the presence or absence of rhOPN and cell growth was assessed 72 hours later. As shown in Fig. 6, the presence of neither the RGD nor control RAD peptides impacted osteopontin-mediated cell growth, indicating that osteopontin’s impact on cellular growth was not mediated through activation of surface integrins.

Because RGD-containing peptides failed to block osteopontin-mediated growth, we next examined the impact of blocking CD44 activation. To determine if osteopontin impacted cell growth through the CD44 receptor, cell growth was assessed in HaCAT-CBR cells following incubation with a well-characterized CD44 blocking antibody (43) and rhOPN. As shown in Fig. 6, the presence of the neutralizing antibody against CD44 resulted in a significant inhibition of HaCAT cell growth compared with cells exposed to a control antibody or rhOPN alone ($P < 0.05$). Together these data show that osteopontin-mediated cell growth requires binding through the CD44 cell receptor.

**Discussion**

Age is the single largest risk factor for the development of neoplasia (61, 62). Investigations into the mechanisms that impact this increase have revealed that both the accumulation of cell autonomous mutations within the incipient tumor cell and changes in the stromal compartment are important. Of particular interest is accumulating evidence that senescent cells positively impact tumorigenesis (12–14). Given that senescent cells accumulate with age in both the epithelial and stromal compartments (17–19, 21, 63–66), this finding raises the possibility that accumulation of senescent cells contributes to age-related increases...
in tumorigenesis. Previously, we showed that senescence-associated, fibroblast-derived osteopontin stimulates preneoplastic cell growth in vitro and in xenografts (14). Here, we show that fibroblasts in preneoplastic AK and SCC in human skin express osteopontin, suggesting that it plays a role in both the early and later stages of tumorigenesis. Given the importance of senescence-associated osteopontin in our model and the presence of stromal-derived osteopontin in preneoplastic and neoplastic human skin samples, we investigated how osteopontin impacts preneoplastic cell growth. We show that osteopontin exerts its activity through binding the CD44 cell surface receptor. Furthermore, we show that osteopontin-mediated activation of the MAPK pathway stimulates cellular proliferation and cell survival, which contribute to increased cell growth.

Osteopontin is a pleiotropic protein that binds numerous cellular receptors and activates several downstream signaling cascades. Osteopontin expression is found in a variety of normal tissues including bone (24, 27) but osteopontin knockout mice appear normal, arguing for redundancy in the animal under normal physiologic settings (67, 68). However, osteopontin expression is increased in malignant tissues from brain, breast, mouth, prostate, liver, kidney, pancreas, ovary, and lung (28, 37, 69), arguing that it is particularly important in pathologic conditions including tumorigenesis. Indeed, recombinant osteopontin is sufficient to transform JB6 murine keratinocytes (39) and osteopontin knockout mice display reduced tumorigenesis when exposed to a classic two-step carcinogenesis protocol (67). Analysis of papillomas formed in osteopontin knockout mice revealed an increase in apoptosis compared with wild-type mice, indicating that osteopontin provides an important survival signal to initiated keratinocytes in this model. More recent work has shown that cancer-associated fibroblasts express several proteins including osteopontin that drive tumorigenesis (70). Our study adds an additional level of complexity by suggesting that osteopontin impacts cell proliferation and survival via CD44 engagement and activation of the MAPK cascade.

Osteopontin can utilize a wide variety of cell-surface receptors including several integrin pairs and CD44. Interestingly, the impact of osteopontin binding to each receptor is unique and cell type dependent and can result in activation of a unique combination of intracellular signaling pathways [for review see (60)]. Previous work has shown that CD44 downstream of osteopontin activates the PI3K pathway and this enhances cell migration (51, 43). In other systems, osteopontin’s engagement of CD44 can mediate MAPK activation leading to increased proliferation (71). CD44 can also cooperate with the HGF receptor to activate MEK in tumor cell lines as well as primary keratinocytes (72). In our work, osteopontin activates both the MAPK and PI3K pathways as evidenced by phosphorylation of ERK and AKT, respectively following osteopontin treatment. However, only inhibition of the MAPK pathway impacts cell growth, suggesting that osteopontin-mediated activation of the PI3K pathway is dispensable for increased cell growth. Because osteopontin-mediated cell growth is also inhibited in the presence of a specific CD44 blocking antibody, our data suggest that osteopontin promotes cell growth by activating the MAPK pathway via CD44.

Our analysis of how osteopontin stimulates cell growth indicated that both cellular proliferation and survival are affected. Furthermore, inhibitor analysis and use of the MEK-DN construct showed that it was activation of the MAPK pathway that was critical to increased cell growth whereas inhibition of the PI3K pathway had no impact on cell growth. Classically activation of the MAPK pathway is associated with increases in cellular proliferation whereas the PI3K pathway is more often associated with reduced apoptosis. However, more recent work has shown that the downstream target of the MAPK pathway, ERK has cytoplasmic targets including RSK that target the proapoptotic protein Bad, leading to its inactivation and reduced apoptosis (73).

The importance of the MAPK pathway to tumorigenesis, specifically in the skin, is well documented [reviewed in (74)]. Indeed, in the DMBA-TPA model the Ras oncogene is the primary target for mutation (75), which results in constitutive MAPK activation within the initiated keratinocyte. Our findings suggest osteopontin-mediated activation of the MAPK pathway is also important for sustained growth of an initiated keratinocyte, raising the possibility that stromal-derived osteopontin may activate the MAPK pathway prior to the initiating event thus providing a critical growth stimulus. This could be critical in the early stages of transformation where activation of oncogenes such as Ras are known to induce senescence within wild-type epithelial cells thus inhibiting further progression. Alternatively, it is possible that the source as well as duration of MAPK

![Figure 6. Osteopontin-induced cell growth is mediated through the CD44 surface receptor. HaCAT-CBR cells were untreated (mock) or preincubated with 5 μg/ml rIgG or anti-CD44 or 10 μmol/L RAD or RGD peptides. Following antibody or peptide treatment, serum free medium (medium) or osteopontin (1000 ng/ml) was added to cells and cell growth was assessed 72 hours later. Antibodies and peptides had no impact on cells grown in serum-free conditions (medium). HaCAT-CBR cell growth was significantly inhibited (P < 0.05) when treated with CD44 blocking antibodies whereas the integrin blocking peptide, RGD has not impact on HaCAT cell growth. The experiment presented is representative of 5 and each condition was carried out with 6 replicas.](image-url)

[^ astronomical]: Not present in the text.
activation is critical in the early stages of tumorigenesis. Indeed, accumulating evidence suggests that both the magnitude as well as duration of MAPK activation has a profound impact in the biological outcome of MAPK signaling (76–78). Future studies will need to focus on delineating the qualitative as well as quantitative impact of differential MAPK activation.

Our work and that of others clearly show that osteopontin is expressed in both the epithelial (35–37) and stromal compartments (this study) of preneoplastic and neoplastic lesions. Whether epithelial versus stromal-derived osteopontin has distinct roles during the tumorigenic process will be an important question to address. In addition to being expressed in the stromal and epithelial compartments of early lesions, osteopontin is an important metastasis marker (29, 38, 79). Whether the temporal induction of stromal-derived versus epithelial-derived osteopontin is important during the tumorigenic process is a second important question. Given that senescent cells are present in a variety of early, premalignant lesions (63–66), it is possible that senescence-derived osteopontin stimulates growth of initiated cells by increasing proliferation and/or cell survival allowing the establishment of early transforming mutations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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