Int J Clin Exp Med 2016;9(9):17203-17218
www.ijcem.com /ISSN:1940-5901/IJCEM0032712

Original Article

Osteopontin influences cell migration dynamics in human salivary gland cancer

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Received May 24, 2016; Accepted August 3, 2016; Epub September 15, 2016; Published September 30, 2016

Abstract: Salivary gland cancer although rare, is a disease with a high mortality rate, and a survival rate ranging from 20 to 40% at five or more years after diagnosis. A family of secreted glycoproteins known as SIBLING proteins has shown potential to be viable biomarkers in human salivary gland cancer. SIBLINGs are small, integrin-binding ligands with a soluble RGD motif that are expressed in mineralized tissue such as bone and teeth, and also in epithelial cells. They include DMP-1, DSPP, BSP, MEPE, and OPN. In human epithelial cancers such as breast, lung, and prostate, these proteins are known to play an important role in cell survival, proliferation, and migration. Our study of SIBLING proteins as potential biomarkers suggest that osteopontin (OPN) may be responsible for enhancing cancer progression. The current study focuses on the role of OPN in the microenvironment of human salivary gland cancer cells, especially with regard to cancer cell migration. Evaluation of cancer tissue samples, cancer cell lines, and the cancer extracellular matrix indicate that OPN is present in abundance. Our evaluation of OPN's role in influencing cell migration reveals that OPN may be responsible for cell spreading characteristics, as well the enhanced cell migration seen in cancer. Additionally, silencing the expression of OPN resulted in abrogating specific changes seen in human salivary gland cancer cell migration. The data suggest that OPN has a very critical role to play in progression and potential metastasis of human salivary gland cancers, and could serve as a valuable prognostic indicator.

Keywords: Osteopontin, migration, salivary gland cancer, SIBLINGs, immunofluorescence, extracellular matrix

Introduction

Salivary gland cancer is a rare disease that can occur in both the major and minor human salivary glands. At a statistical incidence rate of 1:100,000 in the United States per year, this pathology can in most cases have a fatal outcome. Salivary gland cancer tumors pose a considerable concern for doctors because techniques for successful diagnoses and management are limited. One of the reasons that salivary gland cancers are aggressive and potentially fatal is because of their extremely variable pathogenesis and histological presentation. The prognosis of the patient and therapy utilized for the patient is based on the stage, grade, and histology of the cancerous tumor. Treatment for this cancer is limited and despite radiotherapy and surgical procedures, the recurrence rate of these tumors is high [1, 2]. To combat this serious cancer, extended options for diagnoses and therapeutic strategies are the need of the hour, requiring continued research.

In the quest for potential diagnostic and prognostic biomarkers of salivary gland and other epithelial-based cancers, an often studied group of proteins is the family of proteins known as Small Integrin Binding N-Linked Glycoproteins (SIBLINGs). The SIBLING family consists of five proteins encoded by tandem genes, including, Dentin Matrix Protein-1 (DMP-1), Dentin Sialophosphoprotein (DSPP), Bone Sialoprotein (BSP), Matrix Extracellular Phosphoglycoprotein (MEPE), and Osteopontin (OPN). They are located on chromosome 4 within region 375,000 bp and display genetic and collective biochemical attributes that allow for easy identification [3]. The small, integrin binding ligands in SIBLINGs possess the dissolvable recognition sequence RGD (Arg-Gly-Asp) which aids in their binding process to extracellular matrix proteins. SIBLINGs affect behavior of cancer cells by reg-

ulating matrix degradation, adhesion, and migration of cancerous cells which suggests that they have important roles to play in cancer progression and metastasis [3-5]. One of the key hallmarks that cancer cells, including salivary gland cancer cells exhibit, is the potential for increased migration, tissue invasion, and metastasis. We hypothesized that osteopontin (OPN), one of the members of the SIBLING protein family, can stimulate the enhanced migration seen in human salivary gland cancer cells.

OPN is a phosphorylated sialoprotein of the SIBLING (Small Integrin-Binding Ligand N-linked Glycoprotein) protein family that is highly expressed in the mineralized extracellular matrix (ECM) of bone and teeth [6-14]. In addition to mineralized tissue, OPN is expressed in various cell types including macrophages, endothelial cells, smooth muscle cells, and epithelial cells [15-17]. OPN is characterized by the presence of a polyaspartic acid sequence and sites of Ser/Thr phosphorylation that mediate hydroxyapatite binding, and a highly conserved RGD motif [3, 8]. Through both RGD-dependent and RGD-independent interactions, cells are able to bind to the ECM via OPN-mediated integrin interactions [3], which then allows biological activity, including complement-mediated lysis, evasion, and degradation of the ECM. Since integrins are the primary receptors for cell-ECM adhesion, they act as crucial transducers of bidirectional cell signaling, regulates cell survival, differentiation, proliferation, migration, and tissue remodeling [18-25]. Thus, OPN is considered to be a multifunctional protein involved in many cellular processes including, tissue repair, developmental processes, immunity, inflammation, biomineralization, and carcinogenesis [26]. In numerous cancers, OPN is highly expressed and is believed to enrich their cellular invasion and there is growing evidence that OPN is significantly upregulated in, and plays critical roles in cancer progression. The current study, evaluates the role of OPN in enhancing cell spreading and migration of human salivary gland cancer cells.

Material and methods

Cell culture

A human salivary gland cancer cell line, HTB-41, was acquired from the American Type Culture Collection (ATCC, Manassas, VA). A normal human salivary gland cell line, HSG, was received as a generous gift from Dr. Baum B (NIH, Bethesda, MA). Routine cell culture techniques were used to maintain normal and cancerous human salivary gland cell lines. Specifically, normal HSG cells were aseptically cultured in DMEM/F-12 media (Corning Cellgro, Manassas, VA), supplemented with 10% FBS (Sigma Aldrich, St. Louis, MO) and 1% penicillin-streptomycin-amphotericin (Sigma Aldrich, St. Louis, MO). Cancer HTB-41 cells were aseptically cultured in McCoys5A media (Corning Cellgro, Manassas, VA), supplemented with 10% FBS (Sigma Aldrich, St. Louis, MO) and 1% penicillin-streptomycin-amphotericin (Sigma Aldrich, St. Louis, MO). All cells were maintained in a 5% CO_2 atmosphere at 37°C, and growth media was replaced with serum-free media prior to all experimentation.

Extracellular matrix preservation

Cancer HTB-41 cells were cultured till 90% confluence. Cells were selectively removed while preserving the ECM using a series of buffers, followed by DNA digestion to remove any remnant DNA, and a freeze-thaw cycle in liquid nitrogen to ensure cell lysis. Briefly, media was removed followed by a thorough rinse with 1X PBS. Cells were incubated in a cytoplasmic fractionation buffer for 30 minutes, followed by incubation in a nuclear fractionation buffer for 20 minutes, and thoroughly washed with Mg and Ca-free 1X PBS buffer to facilitate cell detachment and selective preservation of the extracellular matrix (ECM) [27].

Immunohistochemistry

Immunohistochemistry procedures were carried out on human salivary gland tissue as well as on extracellular matrix derived from human salivary gland cells. Tissue sections obtained through the National Disease Research Interchange (NDRI, Philadelphia, PA) were immunostained with specific antibodies against OPN. Briefly, tissue sections were rehydrated through decreasing concentrations of a graded ethanol series, endogenous peroxidase activity was quenched using 3% hydrogen peroxide (Bloxall, Vector Laboratories, Burlingame, CA), and sections were reacted against anti-OPN primary antibody (Santa Cruz Biotechnology, Inc., Dallas, TX/1:100). Sections were then conju-

gated with biotinylated secondary antibodies and reacted against an avidin-biotin complex (ABC kit, Vector Laboratories, Burlingame, CA). The ABC complex was then reacted against diaminobenzidine/DAB, a horseradish peroxidase enzyme for the specific antigen being tested. Sections were counterstained with Hematoxylin, dehydrated through a graded ethanol series, mounted on glass slides and sealed with glass cover slips.

Normal HSG and cancer HTB-41 cells were aseptically grown on tissue culture grade glass cover slips in sterile 12-well plates, till they reached 90% confluence. Cells were selectively detached while preserving the extracellular matrix (ECM) as described previously. The preserved ECM on cover slips was fixed in 3% paraformaldehyde and immunostaining with specific antibodies against OPN was carried out. Immunostained ECM on glass cover slips were sealed onto glass slides. All immunohistochemistry images were acquired using a light microscope (Leica DM2500, Leica Microsystems, Buffalo Grove, IL).

Immunofluorescence

Appropriate cell lines were aseptically cultured on uncoated sterile, culture-grade glass cover slips in appropriate growth media. On reaching about 80% confluence, cells were serum-starved for appropriate time points, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in 0.07 M PBS, and rinsed in 0.07 M PBS. After blocking with 10% blocking solution (made from donkey normal serum) for 30 min, cells were incubated overnight in appropriate primary antibodies. Antibodies used included, anti-FAK and anti-OPN (1:500; Santa Cruz Biotechnology Inc., Dallas, TX). Cells were incubated in appropriate Dylight 488 or TRITC conjugated secondary antibodies for 2 hours (1:1000; Jackson Immunoresearch Laboratories Inc., West Grove, PA), following which they were incubated with Rhodamine-Phalloidin or FITC-Phalloidin (Cytoskeleton Inc., Denver, CO) to visualize the actin cytoskeleton. Cover glasses were mounted with mounting medium containing DAPI as nuclear counter stain (Vectashield, Vector Laboratories, Burlingame, CA). Immunofluorescence images were acquired using a confocal microscope (Olympus FluoView FV300, Leeds Precision Instruments, MN). All immunofluorescence experiments were carried out in triplicate.

Western blotting

Total proteins were extracted from appropriate cells using M-PER mammalian protein extraction reagent (Thermo Scientific/Pierce, Rockford, IL) at pre-determined time points. Following protein estimation using the RC DC protein assay (Bio-Rad, Hercules, CA), equal amounts of proteins were resolved by 10% SDS-PAGE under reducing conditions. After electrophoresis, the proteins were electrotransferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA), blocked with 5% non-fat milk in 1× PBS, and probed with anti-OPN primary antibody (1:500; Santa Cruz Biotechnology, Dallas, TX). HRP-conjugated goat anti-rabbit IgG was used for detection (Cell Signaling Technology, Danvers, MA). Super Signal West Pico Chemiluminescent substrate (Thermo Scientific Pierce, Rockford, IL) was used for HRP detection. Each membrane was carefully washed, treated for 5 min with stripping buffer (Thermo Scientific/Pierce, Rockford, IL) to eliminate the previous reaction, washed with PBS, and processed as above with anti-tubulin antibody (1:10,000; Sigma Aldrich, St. Louis, MO) and HRP-conjugated goat anti-mouse IgG. Experiments were carried out in triplicate and Image Studio Lite software (LI-COR Biotechnology, Lincoln, NE) was used for semi-quantitative analyses of relative band intensities, followed by statistical analyses.

RNA interference/OPN silencing

RNA interference is a biological process in which RNA molecules inhibit gene expression, typically by causing the destruction of specific messenger RNA (mRNA) molecules. Two types of small ribonucleic acid (RNA) molecules-microRNA (miRNA) and small interfering RNA (siRNA), can be used for RNA interference. RNAs are the direct products of genes, and these small RNAs can bind to other specific messenger mRNA molecules and either increase or decrease their activity. Using manufacturer's instructions, OPN expression was silenced in normal HSG and cancer HTB-41 cells using siRNA specific for OPN (Santa Cruz Biotechnology, Inc., Dallas, TX). A scrambled/nonsense sequence of RNA was used to trans-

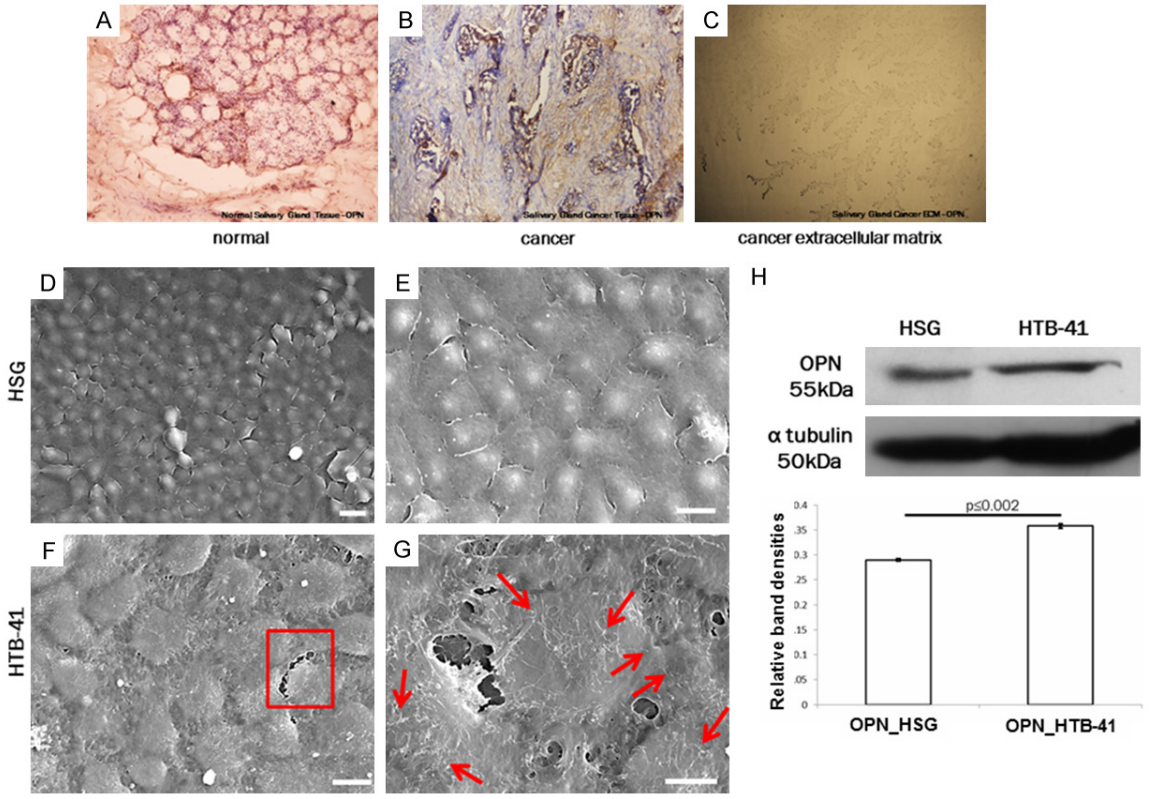

Figure 1. Human salivary gland cancer cells exhibit elevated OPN and a migratory phenotype. A: Normal salivary gland tissue section showing positive OPN expression in the cell stroma (Scale bar = 100 μm). B: Section from human salivary gland cancer shows robust, dark staining against OPN (Scale bar = 100 μm). C: Cancer HTB-41 cells were grown to confluence and the ECM was selectively preserved. The delicate, mesh-like ECM of the cancer HTB-41 cells show strong positive staining against OPN. D and E: Low and high magnifications of normal HSG cells visualized under a scanning electron microscope show uniform size, shape, and arrangement. Cells do not show significant spacing between each other. F and G: Low and high magnifications of cancer HTB-41 cells visualized under a scanning electron microcope show extensive spacing between cells with numerous multi-directional filopodia extending from all cell surfaces (red arrows). H: Western blotting against OPN and analyses of relative band intensities using Li-Cor's Image Studio Lite show significantly elevated OPN levels in cancer cells as compared to normal cells as evidenced by a student t-test (*P≤0.05; data expressed as mean ± SD).

fect both normal and cancer human SG cells and served as appropriate negative controls.

2-dimensional cell migration assay

Appropriate cell lines were seeded on sterile, culture-grade glass coverslips, and grown to confluence. Normal HSG cells were also seeded on sterile, culture-grade cover slips that are previously coated with rhOPN (1:1000 dilution in serum-free DMEM/F-12 media; R&D Systems, Minneapolis, MN), and grown to confluence. A uniform "scratch" was created in a straight line using a sterile micropipette tip on Day 0. Cells were gently washed with 1X PBS to remove any debris, respective growth media was replaced in each well, and incubated at 37°C in a 5% CO_2 environment. Starting at Day 0, images of the "scratch" were acquired from the same field every 24 hours, using an inverted microscope (Nikon Eclipse E600; Nikon, Melville, NY), until the "scratch" was completely eliminated by migrating cells. The experiment was repeated in triplicate.

3-dimensional cell migration assay

Appropriate cell lines were incubated in 1 μM of CM-Dil (Life Technologies, Grand Island, NY) tracking dye as per manufacturer's instructions. Cells were washed in 1X PBS and resuspended in respective serum-free media. Sterile, culture-grade glass cover slips (uncoated or coated with rhOPN) were placed at the bottom of each well of 6-well transwell plates. 500 μl of DMEM/F-12 serum-free media was added in

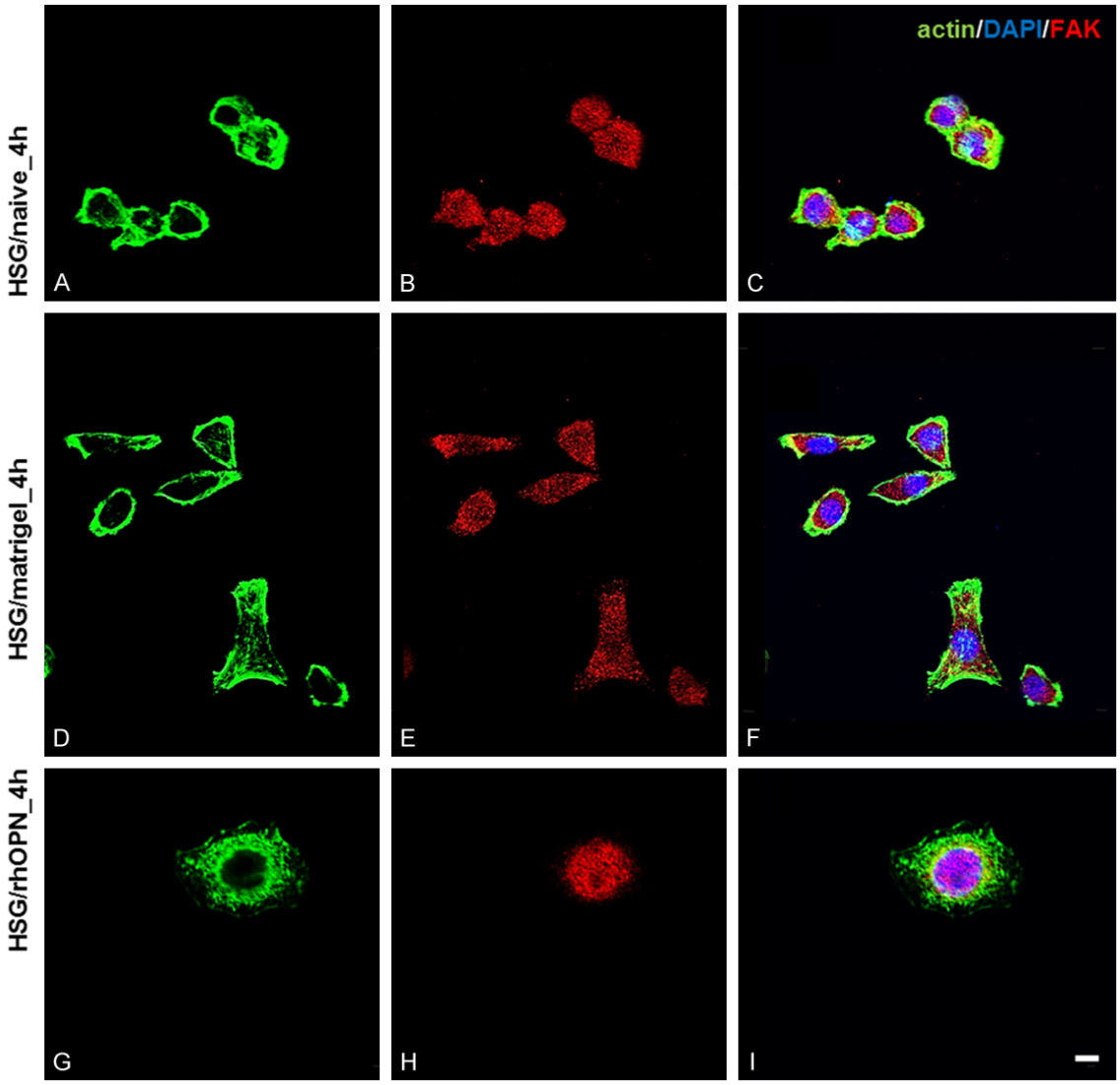

Figure 2. Confocal images showing cell morphology and cell spreading characteristics at 4 hours (Scale bar = 10 μm). A-C: Normal HSG cells show a rounded cell morphology with no evidence of cell spreading characteristics. Cells show cytoplasmic expression of FAK and cortical actin. D-F: Normal HSG cells grown on a growth-factor rich substrate predominantly show passive cell spreading characteristics. Cells show cytoplasmic expression of FAK, cortical actin, and few actin stress fibers. G-I: Normal HSG cells grown on an rhOPN substrate show passive cell spreading characteristics. Cells show cytoplasmic expression of FAK, cortical actin, and cytoplasmic fibrils.

the basolateral chamber and HSG cell suspension in 2 ml DMEM/F-12 serum-free media was added to the central well. One 6-well transwell plate received a 1:1000 dilution of rhOPN (R&D Systems, Minneapolis, MN) in the basolateral chamber, and HSG cell suspension in the central well, while one 6-well transwell plate received 500 μl of serum-free McCoys 5A media in the basolateral chamber and HTB-41, HTB-41_siOPN and HTB-41_siSS cell suspensions in 2 ml McCoys 5A serum-free media in the central well. Transwell plates were incubated at 37°C in a 5% CO_2 environment for 4 and 24 hour time points, and cover slips were imaged using confocal microscopy (Olympus FluoView FV300, Leeds Precision Instruments, MN) to quantify the adherent labeled cells. The experiment was repeated in triplicate and statistical analyses were performed.

Statistical analyses

To test for differences between groups for the Western Blotting data as well as for the

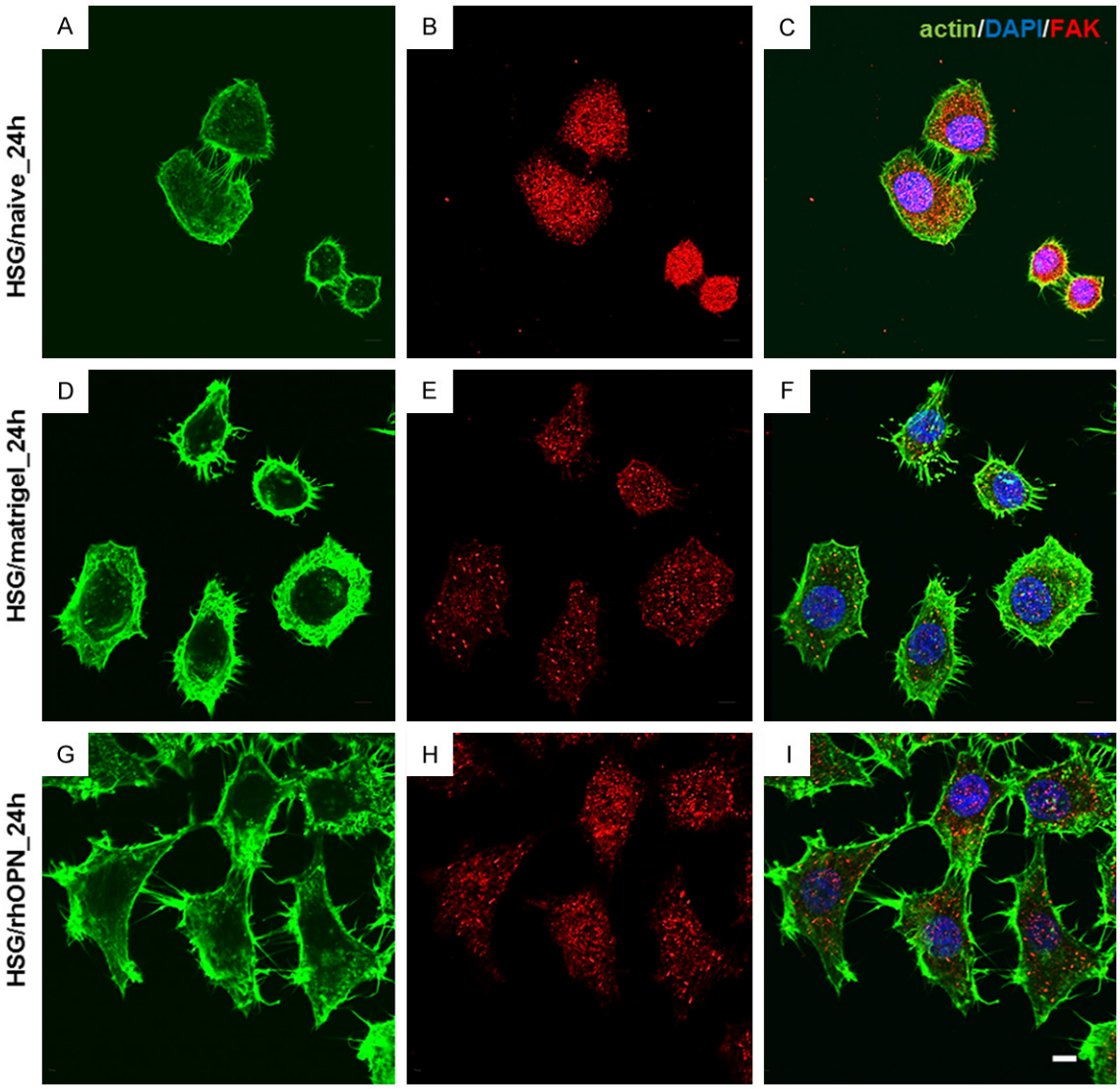

Figure 3. Confocal images showing cell morphology and cell spreading characteristics at 24 hours (Scale bar = 10 μm). A-C: Normal HSG cells continue to show a rounded cell morphology with passive cell spreading characteristics. Cells show cytoplasmic expression of FAK and cortical actin. Few filopodial extensions between neighboring cells are also visible. D-F: Normal HSG cells grown on a growth-factor rich substrate predominantly show active cell spreading characteristics. Cells show discrete cytoplasmic punctae of FAK and several filopodial extensions. G-I: Normal HSG cells grown on an rhOPN substrate show active cell spreading characteristics resulting in stellate cell morphologies. Cells show discrete cytoplasmic punctae of FAK and predominantly unidirectional filopodia.

3-dimensional migration data, repeated measures analysis of variance (ANOVA) with group as the between-group factor and one within-subject (repeated-measures) factor (time). At each time point, the mean relative density from each of 6 blots was entered into analyses. All experiments were repeated six times, so the sample size was 6. For these analyses, we used multivariate ANOVA (MANOVA) which does not require the assumptions of compound symmetry (homogeneous pooled within-group variances and across-subjects covariances) and sphericity (orthogonal components) [28].

Results

Salivary gland cancer cells exhibit elevated levels of intracellular and extracellular OPN

Evaluation of normal HSG and cancer HTB-41 human salivary gland cells revealed that cancer cells showed significantly elevated level of OPN

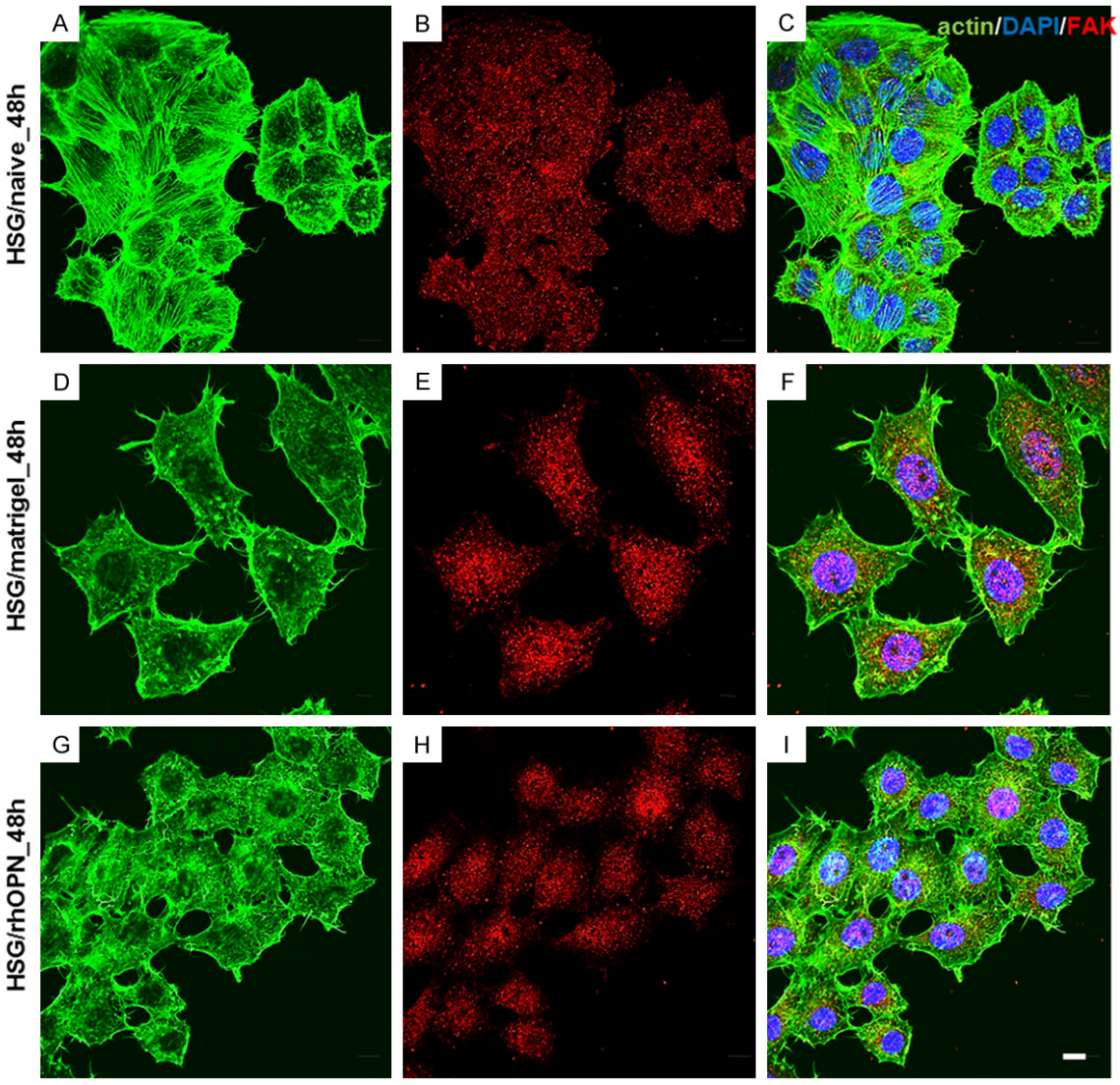

Figure 4. Confocal images showing cell morphology and cell spreading characteristics at 48 hours (Scale bar = 10 μm). A-C: Normal HSG cells continue to proliferate as a cohesive cohort with robust actin stress fiber formations. Cells show cytoplasmic expression of FAK. D-F: Normal HSG cells grown on a growth-factor rich substrate are actively spreading with numerous filopodial extensions. Cells are not maintained as a cohesive cohort, and FAK continues to be in the cytoplasm. G-I: Normal HSG cells grown on an rhOPN substrate show active cell spreading characteristics with separation between cells and filopodia. Cells show cytoplasmic expression of FAK with nuclear translocation.

within tissue sections, as well as in the extracellular matrix (ECM) of cultured cancer HTB-41 cells (**Figure 1A-C**, **1H**).

Salivary gland cancer cells exhibit morphology demonstrating a migratory phenotype

Evaluation of normal HSG and cancer HTB-41 human salivary gland cells revealed that they exhibit distinct cell morphologies and cytoarchitecture. On visualizing cells using scanning electron microscopy, normal HSG cells demonstrated a cobblestone-like appearance (**Figure 1D**, **1E**). The cells were uniform in size and shape and they exhibited cohesive connections with neighboring cells, with minimal to no intercellular spacing. In contrast, cancer HTB-41 cells showed significant separation between neighboring cells (**Figure 1F**, **1G**). In addition, cancer cells exhibited extensive and multidirectional filopodial projections. The data indicate that salivary gland cancer HTB-41 cells seem to exhibit a migratory phenotype as compared with normal HSG cells.

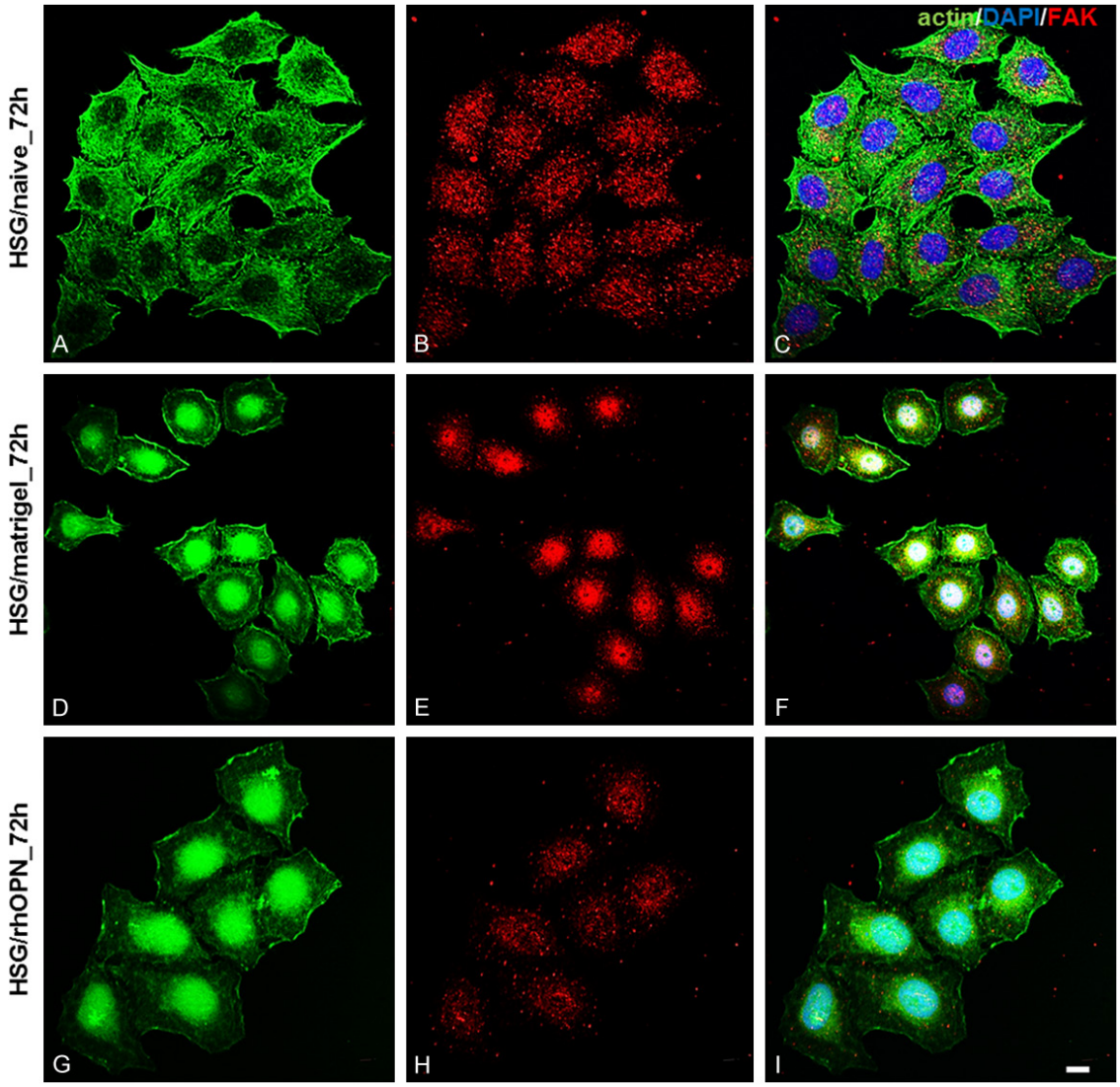

Figure 5. Confocal images showing cell morphology and cell spreading characteristics at 72 hours (Scale bar = 10 μm). A-C: Normal HSG cells show active cell spreading characteristic, but continue to remain as a cohesive cohort. Cells show cytoplasmic expression of FAK. D-F: Normal HSG cells grown on a growth-factor rich substrate have reverted back to predominantly passive cell spreading characteristics. Cells show nuclear expression of FAK and actin. G-I: Normal HSG cells grown on an rhOPN substrate have reverted to passive cell spreading characteristics. Cells show nuclear expression of FAK and actin.

Normal salivary gland cells exhibit altered morphology and migratory cytoarchitecture under the influence of OPN

Normal HSG cells were aseptically cultured on sterile, culture-grade glass cover slips that were either uncoated, coated with growth-factor rich substrate (thin coat Matrigel; Corning Inc., Tewksbury, MA), or coated with recombinant human OPN (rhOPN; 1:1000 dilution in serum-free DMEM/F-12 media; R&D Systems, Minneapolis, MN). Cells were grown to about 70% confluence, serum-starved for 24 hours, and immunostained against focal adhesion kinase (FAK) and actin to visualize the cell morphology and cell spreading characteristics using confocal microscopy at pre-determined time points of 4, 24, 48 and 72 hours (**Figures 2**-**5**). Data indicate that as early as 4 hours, the presence of a growth factor-rich substrate and an rhOPN substrate influence cells to exhibit 'spreading characteristics' (**Figure 2**). Although cell spreading may precede cell movement, mechanistically they are two very discrete phe-

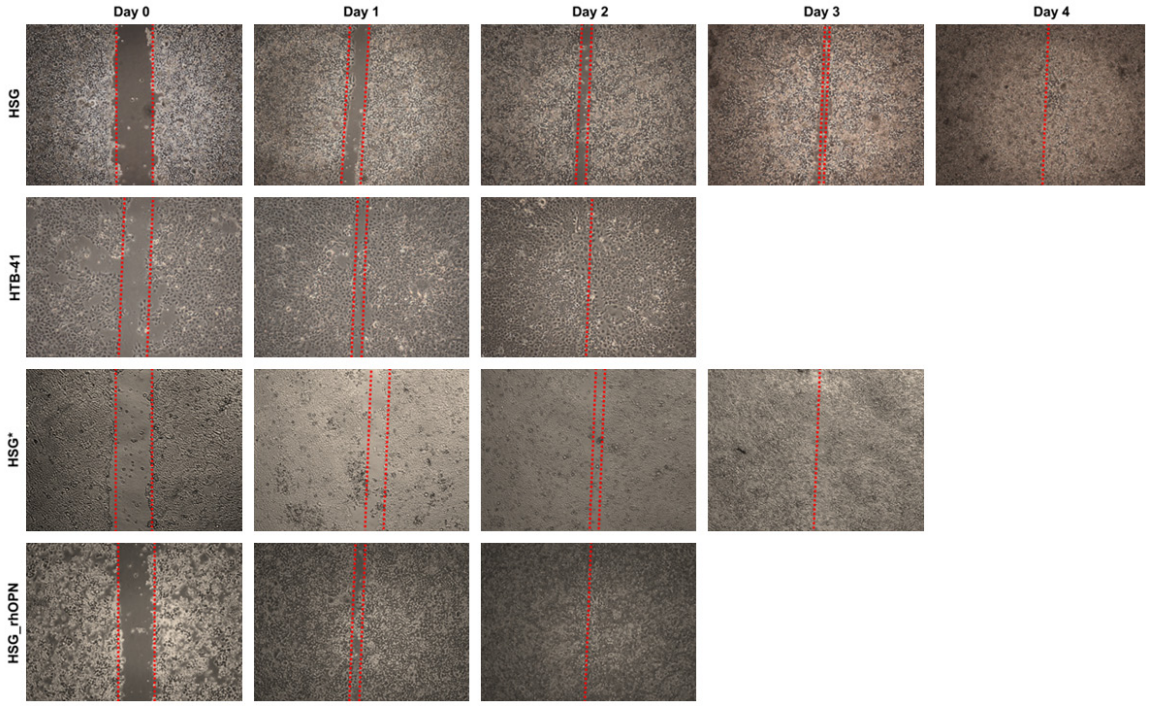

Figure 6. Human salivary gland cells exhibit increased cell migration under the influence of OPN. HSG, HTB-41 and HSG_rhOPN cells were cultured till they reached 90% confluency. A uniform "scratch" was created with a sterile micropipette tip on Day 0 (red dotted lines depict the "gap" or "cell-free" region). Cells were imaged every 24 hours post-Day 0 (4× magnification). HSG cells migrated and almost infiltrated the "scratch" by Day 4. HTB-41 cells migrated at a faster rate than HSG cells to obliterate the "scratch" in less than 48 hours, while normal HSG cells under the influence of OPN mimicked the migration rate exhibited by the cancer cells, eliminating the "scratch" by Day 3.

nomena. Both processes involve the deformation of the plasma membrane and formation of cell-substrate attachments, but cell movement is an active process which requires energy expenditure (McGrath, 2012). At 4 hours, normal HSG cells in contact with the growth factor-rich substrate and rhOPN exhibit passive adhesion and spreading (**Figure 2**), but normal HSG cells on uncoated surfaces required upto 24 hours to exhibit passive adhesion and spreading (**Figure 3**). All cells at 4 hours exhibited cytoplasmic FAK indicating passive adhesion (**Figure 2**), but the definitive punctate expression of FAK, indicating active adhesion was seen in normal HSG cells contact with the growth factor-rich substrate and rhOPN at 24 hours (**Figure 3**), but took almost 72 hours to be seen in HSG cells on uncoated surfaces (**Figure 5**). By 24 hours, cells grown on the growth factor-rich substrate and rhOPN demonstrated filopodial extensions, with the cells growing on the rhOPN substrate exhibiting longer filopodia in the same direction (**Figure 3**). Cells grown on uncoated coverslips also had a few filopodia, but all filopodial extensions were found located only between neighboring cells indicating that cells seemed to be forming a cohesive cluster or cohort. By 48 hours, in addition to filopodial extensions, there was a nuclear translocation of FAK in cells grown on the growth factor-rich substrate and rhOPN indicating that cell de-adhesion from the substrate is occurring resulting in nuclear FAK translocation (**Figure 4**). Cells grown on uncoated coverslips continued to exhibit cytoplasmic FAK and robust actin stress fibers. By 72 hours, in addition to nuclear translocation of FAK, there was also nuclear import of actin (**Figure 5**). Our data suggest that OPN in the ECM is capable of enhancing cell spreading characteristics and a migratory phenotype similar to that induced under the influence of a growth factor-rich substrate.

Normal salivary gland cells exhibit increased migration under the influence of OPN

Normal HSG cells, cancer HTB-41 cells, and normal HSG cells under the influence of recombinant human OPN were subjected to 2-D and

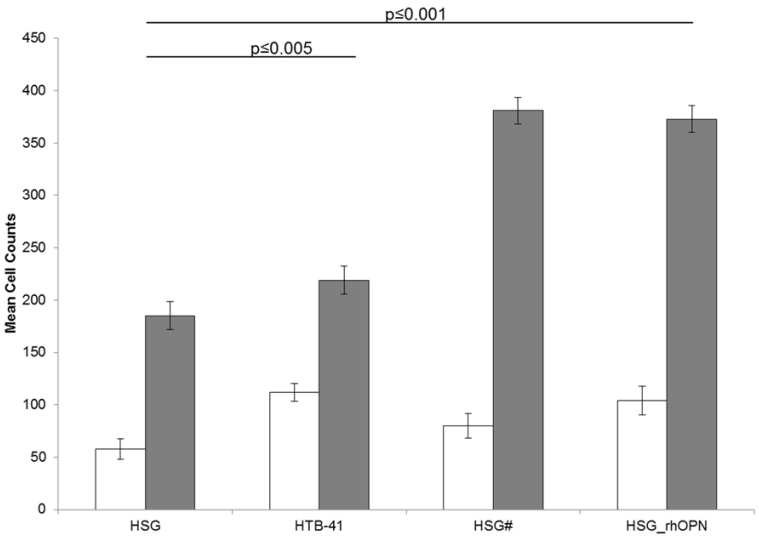

Figure 7. Data from 3-D Migration Transwell Assays at 4 and 24 hours reveals that normal HSG cells under the influence of rhOPN showed significantly higher migration rates as compared to untreated/naïve HSG cells. This was similar to the migration exhibited by normal cells under the influence of the cancer ECM (HSG#); P≤0.005 and P≤0.001.

3-D migration assays. Cancer HTB-41 cells exhibited high migration rates, and under the influence of OPN, normal HSG cells mimicked the behavior of cancer cells, demonstrating enhanced migration capabilities (**Figures 6** and **7**). The increased migration was similar to that exhibited by normal HSG cells under the influence of cancer secretome (HSG* cells).

The increased migration exhibited by cancer salivary gland cells is abrogated by silencing OPN

Following silencing OPN using RNAi techniques, 2D migration assays show that the increased migration exhibited by cancer cells is inhibited (**Figure 8**). The data indicate that silencing OPN in cancer HTB-41 cells results in decreased migration. Silencing cancer HTB-41 cells using a scrambled sequence (negative control) did not inhibit migration, rather the cells continued to migrate as untreated cancer cells and closed the created gap by Day 2. This confirms that the decreased migration following siRNA treatment of cancer HTB-41 cells is not a result of the RNA interference, but rather is a direct result of inhibiting OPN expression.

Silencing normal HSG cells with siOPN had a null effect on migration, with cells continuing to exhibit the same migration rates as seen in untreated normal HSG cells. Our data thus seem to suggest that in normal HSG cells, OPN could be involved with alternate cellular functions and not entirely with regulating cell migration.

Discussion

Osteopontin (OPN), one of the SIBLING proteins, is an integrin-binding protein that has been shown to be overexpressed in various experimental models of malignancy, and appears to be involved in tumorigenesis and metastasis. Literature has ample evidence that OPN is broadly expressed in human tumors from various organs, suggesting involvement of this protein in tumor formation [5, 25, 29-42]. While evaluating the expression of SIBLING proteins in salivary gland cancers, it is evident from our data that OPN is strongly expressed in human salivary gland cancers, supporting existing evidence that OPN is important in various aspects of carcinogenesis. The presence of OPN in salivary gland cancers thus indicates that similar to other epithelial-based cancers such as those originating in the breast, lung or prostate, OPN may be responsible for the various hallmarks of cancer, most importantly, cancer progression and metastasis in human salivary gland cancers. Most interestingly, our data show that in addition to the intracellular expression of OPN, there is a strong expression of OPN in the extracellular matrix (ECM) of cancer cells. Although the ECM is tightly controlled during normal embryonic development, the ECM is commonly dysregulated and becomes disorganized in diseases such as cancer. Abnormal ECM affects cancer progression by directly promoting cellular transformation and metastasis [18, 25, 43, 44]. Thus, our evidence of the expression of OPN in the ECM of salivary gland cancers strongly suggests that OPN in salivary gland cells, as well as within the ECM may be responsible for cancer progression, specifically cancer cell migration that promotes metastasis.

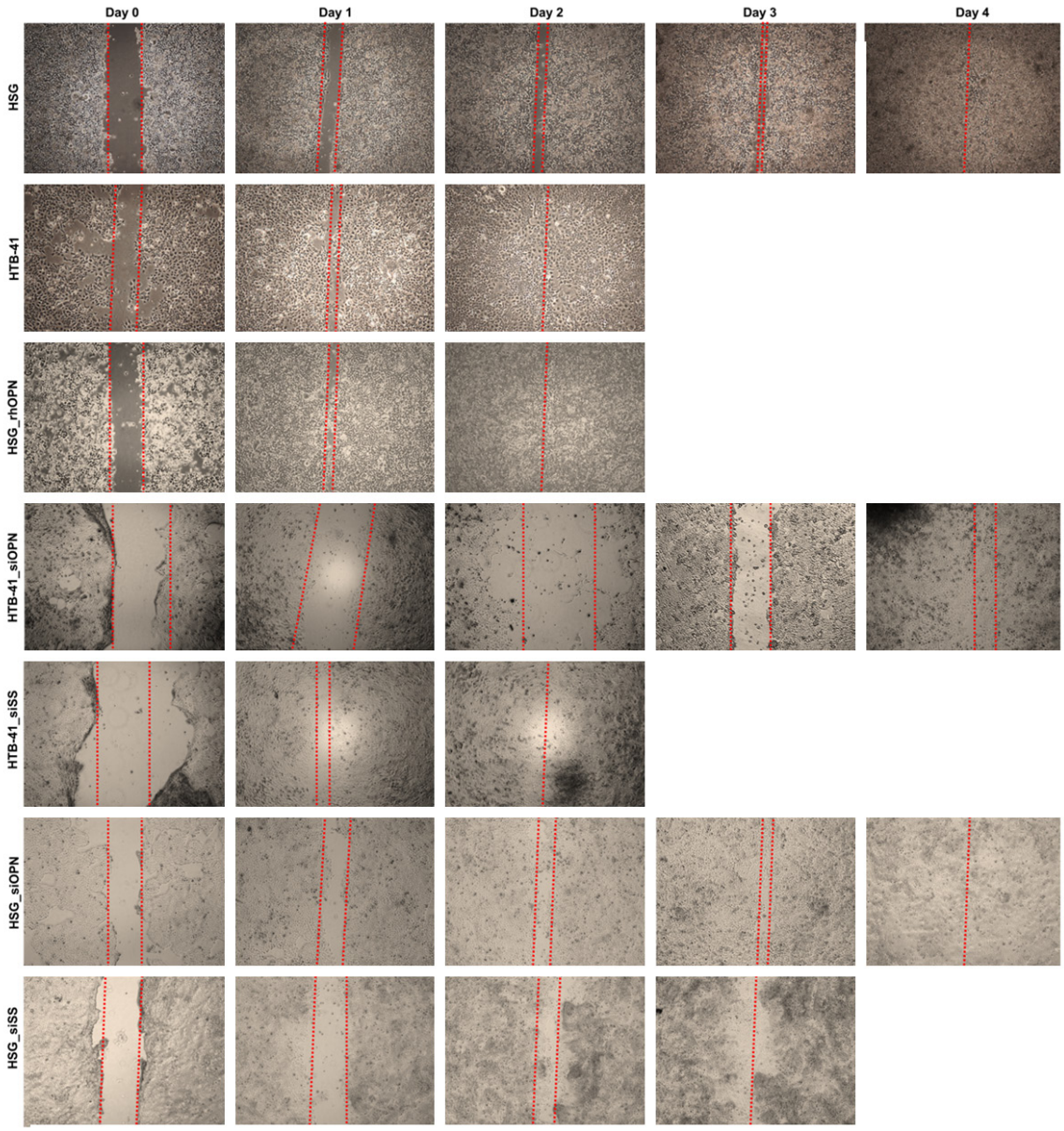

Figure 8. Human salivary gland cells exhibit decreased cell migration following silencing of OPN expression. HSG, HTB-41, HTB-41_siOPN, HTB-41_siSS, HSG_siOPN and HSG_siSS cells were cultured till they reached 90% confluency. A uniform "scratch" was created with a sterile micropipette tip on Day 0 (red dotted lines depict the "gap" or "cell-free" region). Cells were imaged every 24 hours post-Day 0 (4× magnification). HTB-41 cells that were OPN silenced showed decreased migration compared to untreated HTB-41 cells. HSG cells that were OPN silenced showed a null effect. Negative controls of both HTB-41 and HSG cells exhibited no change in their expected migration.

Cell migration, whether associated with normal migratory processes or associated with pathological processes, is the process by where cells can translocate from one location to another in the body. Cell migration is a dynamic process where several well-orchestrated molecular mechanisms are responsible for a series of coordinated steps leading to movement. The individual processes involved in cell migration include, (i) cell polarization to define leading/front and rear ends, (ii) formation of cell protrusions/membrane extensions, (iii) cell adhesion, and (iv) cell de-adhesion, cell body translocation, and retraction of the rear end. Cell migration studies carried out on fibroblasts have shown that until stimulated, a cell maintains its position by adhesive forces between its neighbors and the extracellular matrix. On receiving

chemotactic impulses, a cell undergoes polarization during which the 'front end' and 'rear end' of a cell are defined. Membrane extensions in the form of lammelipodia or filopodia are formed by cross-linking of actin filaments at the leading edge, directed by intracellular signaling pathways mediated by Rac, Rho and Cdc42 [40]. Protrusive forces for membrane extension are generated by the 'actin polymerization machinery' because of the elasticity of the actin filament. Extension is followed by adhesion of the cell to the extracellular matrix or to neighboring cells. Focal adhesions are formed by orderly organization of the actin network, which prevents the cell from retracing its steps and also offers the cell a 'foot-hold' to push itself forward again, a process termed as translocation. Translocation is accompanied by a simultaneous detachment or retraction of the trailing edge of the cell. Thus cell migration requires a coordinated sequence of events that include cell polarization, membrane extensions at the leading edge, adhesion, and translocation and retraction of the trailing edge [45-49].

Our data from scanning electron microscopy studies of cell morphology clearly show that as compared with normal salivary gland cells, cancer salivary gland cells exhibit the formation of cell protrusions or membrane extensions. These extensions are clearly visualized as filopodial extensions. In addition to the presence of filopodia, cancer cells also exhibit cell-cell separation that indicates potential cell movement away from each other. Studies on migrating fibroblasts have shown that normal cells usually migrate as a cohort or a cohesive sheet. Unlike this cohesive movement, cancer cells seem to exhibit the tendency for independent movement away from the primary cohort of cells. Our data closely parallels cancer cell behavior in patients, where individual cells from within the primary tumor exhibit the ability to migrate independently, breaking off from the primary tumor to migrate and eventually metastasize to secondary locations.

Using confocal microscopy and immunostaining techniques, our data confirm that the filopodial membrane extensions exhibited by cancer cells are rich in actin. These actin rich filaments act as environmental investigators that can probe the cell surroundings and are instructed by signaling pathways arbitrated by Rho, Rac, and Cdc42. Filopodia act as protruding sensory antennae to survey the surrounding environment. Membrane extensions aid in the translocation process allowing for the cell to progress to distant locations in the body, thus enabling cell migration. As cells engage in the migratory process, they initially exhibit passive spreading followed by active spreading [50, 51]. Our data show that in the presence of a growth factor-rich substrate, normal salivary gland cells show passive spreading as early as 4 hours, and continue on to exhibit active spreading, followed by membrane extensions and altered morphology. This is in contrast to cells that did not receive stimuli through growth factor enrichment. The cancer microenvironment is rich in various growth factors, and therefore the altered morphology and the active cell spreading characteristics seen with salivary gland cells is expected cell behavior when exposed to a growth factor enriched microenvironment, and is indicative of cell behavior *in vivo*. Interestingly, by exposing normal salivary gland cells to a microenvironment enriched with OPN also resulted in altered cell morphology, elaborate filopodial extensions, and cell-cell separations. Our data thus suggest that OPN in the microenvironment of salivary gland cells, specifically in the ECM, can influence normal and cancer cells to exhibit a cytoarchitecture conducive to cell migration. Under the influence of OPN, salivary gland cells rapidly exhibit active spreading and exhibit filopodial extensions that allow them to 'break-off' from the primary cohort and migrate independently. Thus, in a patient's primary tumor, presence of OPN in the microenvironment can be a strong stimulant to promote cancer progression through cell migration and eventual metastasis.

In addition to the altered cell morphology and migratory cytoarchitecture, our data show that under the influence of OPN, focal adhesion kinase (FAK) expression is accelerated. In a normal growth condition, FAK's localization is prominent in focal adhesions and the cytoplasm. However, cell de-adhesion from the underlying substrate or stress signals such as a chemical stress or an oxidative stress promote FAK mobilization from integrin adhesion sites to the nuclei [52]. As expected, our data show FAK expression in the cytoplasm as early as 4 hours even without exposure to growth factors or OPN. By 24 hours, although salivary gland cells show FAK expression, those cells exposed to growth factors, as well as OPN show discrete

punctae of FAK indicating formation of focal adhesions during the migratory process. By 48 hours and even at 72 hours, in addition to the cytoplasmic FAK expression there is also an evident nuclear import of FAK. Our data supports the evidence that FAK translocates into the nucleus during episodes of cell de-adhesion. Our data indicate that the nuclear translocation of FAK that is seen in normal salivary gland cells exposed to growth factors or OPN happens at 48 hours and 72 hours. This clearly coincides with the de-adhesion step of the migratory process, indicating that the cells are actively migrating under the influence of growth factors or OPN.

In order to determine if OPN can influence the migration of normal cells to mimic that seen with cancer cells, 2-dimensional and 3-dimensional migration assays were carried out. Our data from both 2-dimensional and 3-dimensional migration assays reveal that normal salivary gland cells that were cultured on or exposed to the ECM derived from cancer cells, as well as normal cells cultured on or exposed to OPN exhibited a significant increase in migration that mimics the migration exhibited by cancer salivary gland cells. Our data suggests that cells interacting with OPN at their basal surfaces are stimulated to migrate faster. This is concurrent with the role that OPN plays in cancer, where it can be responsible for cancer progression. Our data confirm that OPN expression in cancer states can promote cancer progression by allowing cells to migrate significantly faster than cells that are not stimulated by OPN. Interestingly, when normal cells were exposed to the secretome derived from cancer cells, a similar increase in migration was noted. Our data therefore indicate that the enhanced expression of OPN within the cells, as well as the presence of OPN in the cancer microenvironment, specifically the cancer ECM, can significantly promote cancer progression by driving cell migration.

In order to conclusively link OPN with increased cell migration, cancer salivary gland cells were subjected to RNA interference techniques to silence the expression of OPN. Following silencing, cancer salivary gland cells did not exhibit the enhanced migration seen in untreated salivary gland cells. On examining the leading edge of cells, active migration was confirmed by the presence of numerous filopodial extensions at the leading end of cells all across the migrating edge of the assay. Our data therefore confirm that OPN has a direct role in interacting with the ECM and promoting cell migration. Silencing OPN in normal salivary gland cells did not affect migration, and therefore suggests that in normal cells, the OPN expression is responsible for normal cellular functions, but the overexpression in cancer cells results in OPN dysregulation and a resultant function that seem to promote cell migration.

Taken together, all our data indicate that OPN plays very critical roles in cancer progression. Specifically, our data indicate that increased OPN expression in cancer cells and the cancer microenvironment is responsible for promoting cancer progression via increased cell migration. Histopathological analyses of cancer tissue samples that reveal robust OPN expression in the stroma as well as in the cells could be indicative of the potential for further progression of the primary tumor, as well as could serve as a prognostic predictor indicating the potential for metastasis. Future studies that investigate the role of OPN in cell invasion are warranted and planned. In addition, the expression of OPN in the ECM of benign, pre-malignant, and malignant cancers will be comparatively evaluated in future studies.

Acknowledgements

The authors acknowledge the technical assistance provided by Ms. Samantha Hamilton and Dr. Adrianna Marcuzzi. Human tissue samples were acquired through the National Disease Research Interchange (NDRI). The study was funded by an Advanced Investigation Award from the Southern Illinois University School of Dental Medicine (AIA16-01; PI: AJ) and through the Research Grants for Graduate Students Award from the Southern Illinois University Edwardsville to KC (PI and faculty mentor: AJ). The data presented in this manuscript was presented as part of the Master's thesis of KC.

Disclosure of conflict of interest

None.

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