ARTICLE
Journal of Cellular Biochemistry 107:1118–1128 (2009)

Journal of Cellular Biochemistry

# Bone Sialoprotein Stimulates Focal Adhesion-Related Signaling Pathways: Role in Migration and Survival of Breast and Prostate Cancer Cells

Jonathan A.R. Gordon,[1] Jaro Sodek,[2†] Graeme K. Hunter,[1] and Harvey A. Goldberg[1*]

[1] *CIHR Group in Skeletal Development and Remodeling, Department of Biochemistry and Division of Oral Biology, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario, Canada*

[2] *Faculty of Dentistry, CIHR Group in Matrix Dynamics, University of Toronto, Toronto, Ontario, Canada*

## ABSTRACT

Bone sialoprotein (BSP) is a secreted glycoprotein found in mineralized tissues however, BSP is aberrantly expressed in a variety of osteotropic tumors. Elevated BSP expression in breast and prostate primary carcinomas is directly correlated with increased bone metastases and tumor progression. In this study, the intracellular signaling pathways responsible for BSP-induced migration and tumor survival were examined in breast and prostate cancer cells (MDA-MB-231, Hs578T and PC3). Additionally, the effects of exogenous TGF-β1 and EGF, cytokines associated with tumor metastasis and present in high-levels in the bone microenvironment, were examined in BSP-expressing cancer cells. Expression of BSP but not an integrin-binding mutant (BSP-KAE) in tumor cell lines resulted in increased levels of $\alpha_v$-containing integrins and number of mature focal adhesions. Adhesion of cells to recombinant BSP or the expression of BSP stimulated focal adhesion kinase and ERK phosphorylation, as well as activated AP-1-family proteins. Activation of these pathways by BSP expression increased the expression of the matrix metalloproteinases MMP-2, MMP-9, and MMP-14. The BSP-mediated activation of the FAK-associated pathway resulted in increased cancer cell invasion in a Matrigel-coated Boyden-chamber assay and increased cell survival upon withdrawal of serum. Addition of EGF or TGF-β1 to the BSP-expressing cell lines significantly increased ERK phosphorylation, AP-1 activation, MMP-2 expression, cell migration and survival compared to untreated cells expressing BSP. This study thus defines the cooperative mechanisms by which BSP can enhance specific factors associated with a metastatic phenotype in tumor cell lines, an effect that is increased by circulating TGF-β1 and EGF. J. Cell. Biochem. 107: 1118–1128, 2009. 

**KEY WORDS:** BONE SIALOPROTEIN; MIGRATION; CANCER; MMPs

Bone sialoprotein (BSP) is an acidic glycoprotein that is generally restricted to mineralized tissue such as bone and dentin. BSP is a member of the Small Integrin-Binding LIgand N-linked Glycoprotein (SIBLING) family of proteins that also includes osteopontin (OPN), dentin matrix protein 1 (DMP1), dentin sialophosphoprotein, and matrix extracellular phosphoglycoprotein [Fisher and Fedarko, 2003]. Although these proteins are not close enough to be considered a genetic family, the chromosomal location, expression patterns and shared characteristics such as the presence of an integrin-binding motif (RGD) and post-translation modifications would suggest members of this family have related functions. Although many of the SIBLING proteins are predominately expressed in bone, several of these proteins have been shown to be aberrantly expressed in a variety of malignant tumors [Fisher

Abbreviations used: BSA, bovine serum albumin; BSP, bone sialoprotein; CMV, cytomegalovirus; αMEM, minimum essential medium α medium; ECM, extracellular matrix; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FBS, fetal bovine serum; FRNK, FAK-related non-kinase; HA, hemagglutinin tag; HBSS, Hank's buffered salt solution; HEK, human embryonic kidney; MAPK, mitogen activated protein kinase; MEK, extracellular signal-regulated kinase; MMP, matrix metalloproteinase; OPN, osteopontin; p.f.u., plaque forming unit; SIBLING, small integrin-binding ligand N-linked glycoprotein; RGD, arginine-glycine-aspartic acid; TGF-β, transforming growth factor beta; TRITC, tetramethylrhodamine isothiocyanate.

[†] Deceased.

Grant sponsor: Canadian Institutes of Health Research; Grant number: 12592.

*Correspondence to: Dr. Harvey A. Goldberg, CIHR Group in Skeletal Development and Remodeling, Schulich School of Medicine & Dentistry, Dental Sciences Building, University of Western Ontario, London, Ontario, Canada N6A 5C1. E-mail: hagoldbe@uwo.ca

Received 15 April 2009; Accepted 17 April 2009 • DOI 10.1002/jcb.22211 • © 2009 Wiley-Liss, Inc.

Published online 2 June 2009 in Wiley InterScience (www.interscience.wiley.com).

et al., 2004]. BSP is expressed by many malignant tissues, including breast, prostate [Waltregny et al., 2000], lung [Bellahcene et al., 1997], and several other cancer types [Fisher et al., 2004]. The expression of BSP has been associated with increased osteotropic metastasis, increased risk of malignancy and poor survival among patients with breast or prostate cancer [Bellahcene et al., 1996b; Waltregny et al., 1998]. BSP has been suggested to play a direct role in promoting skeletal metastasis as expression of BSP has been demonstrated to be sufficient to promote skeletal metastasis in non-osteotropic cells [Zhang et al., 2004]. A recent study has demonstrated that the transgenic overexpression of BSP resulted in increased skeletal as well as systemic metastasis of murine breast cancer cells [Tu et al., 2009]. This evidence would strongly implicate BSP in promoting skeletal metastasis in malignant cells. Although much is known about mechanisms and signaling pathways by which other SIBLING proteins (specifically OPN) regulate cancer progression, little is known about the mechanisms or signaling pathways involved in BSP-mediated metastasis and tumor survival [Bellahcene et al., 2008].

BSP normally interacts with heterodimeric cell-surface integrins, such as $\alpha_v\beta_3$. Integrins are crucial in cancer metastasis as regulators of cell invasion and migration, facilitating cell adhesion to the extracellular matrix, and for sending and receiving molecular signals that regulate these processes [Hood and Cheresh, 2002]. The level of $\alpha_v\beta_3$ expression in breast cancers is correlated with the aggressiveness of the disease in patients [Gasparini et al., 1998]. BSP has been shown to facilitate the adhesion of a variety of cell types through a common integrin ligand, an RGD sequence found near the C-terminus of the protein that is highly conserved (>80%) among species [Chenu et al., 1994]. BSP-integrin interactions (primarily through the $\alpha_v\beta_3$ integrin) have been shown to be important to stimulating the migration of tumor-derived cells [Byzova et al., 2000; Sharp et al., 2004]. Although the specific mechanisms by which BSP stimulates migration are not known, it has been reported that BSP can increase the invasiveness of cancer cells by forming a trimolecular complex with $\alpha_v\beta_3$ and the matrix metalloproteinase MMP-2 (gelatinase A), increasing localized matrix degradation [Karadag et al., 2004]. Matrix metalloproteinases such as MMP-2 and MMP-9 (gelatinase B) are initially secreted as inactive zymogens which, following cleavage by the membrane-bound MT1-MMP (MMP-14), play a critical role in the destruction of the ECM [Sato et al., 1994] facilitating invasion. BSP has also been shown to interact with complement factor H, forming a cell surface-associated complex that protects cells from complement-mediated lysis [Fedarko et al., 2000]. While BSP may play an important role as an adaptor molecule participating in the attachment of proteins to the cell surface of migrating cells, the protein may also play a more direct role, stimulating molecular signals at the focal adhesion resulting in expression of pro-metastatic factors.

One of the signaling molecules responsible for the transduction of signals from integrins is the tyrosine kinase focal adhesion kinase (FAK) [Schaller, 2001]. The phosphorylation of FAK at $Tyr_{397}$, stimulating kinase activity, is required to promote an invasive cell phenotype in malignant cells [Hauck et al., 2002; Hsia et al., 2003]. Many malignant human tumor samples exhibit increased FAK expression and tyrosine phosphorylation [Xu et al., 2000]. The interaction of FAK with downstream effectors Src and Ras can activate members of the mitogen-activated protein kinase (MAPK) pathway, such as extracellular signal-regulated kinase (ERK). ERK has been demonstrated to be important for initiating and regulating cellular events associated with cell migration and proliferation through activation of downstream targets such as members of the activator protein-1 (AP-1) along with many others [Byun et al., 2006]. It has been demonstrated that the activation of the MAPK pathways can mediate the expression of MMPs, which in turn is partially responsible for the malignant progression of several cancers [Song et al., 2006].

Members of the MAP kinase family, specifically ERK, have been shown to be important modulators involved in transmitting signals from growth factors such as epidermal growth factor (EGF) and transforming growth factor (TGF)-β [reviewed in Navolanic et al., 2003]. These growth factors have long been established to be important regulators of tumor metastasis and growth. EGF has been shown to act as an autocrine factor in many tumors [Normanno et al., 2001]. EGF can act as a stimulus facilitating the migration of tumor cells and aid in their survival. Similarly, TGF-β signaling has been demonstrated to influence tumor progression and metastasis [Bierie and Moses, 2006].

The purpose of this study was to examine the mechanism by which BSP expression directly promotes migration and survival of breast cancer and prostate carcinoma cells in established cell models. As integrins are crucial not only to BSP–cell interactions but also to metastasis and activation of intracellular signaling pathways in response to growth factors, BSP-expressing cancer cells were examined for integrin expression, focal adhesion formation and activation of focal adhesion-associated kinases in the presence of growth factors normally associated with metastasis and tumor survival.

## MATERIALS AND METHODS

### REAGENTS

Human embryonic kidney cells (HEK-293), MDA-MB-231, Hs578T and PC3 cells were obtained from ATCC (Manassas, VA). All cell culture reagents including culture media, trypsin, hygromycin, fetal bovine serum (FBS), L-glutamine (Glutamax II), penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). All tissue culture dishes were from Becton-Dickinson (Franklin Lakes, NJ) unless otherwise specified. Insulin was obtained from Sigma (St. Louis, MO). Fugene 6 transfection reagent was acquired from Roche Applied Science (Basel, Switzerland). Adenoviral vectors, pShuttle and pAdeno-X were from BD Biosciences (San Jose, CA). The vectors pCDNA3.1-Hygro and pCDNA3.1-FRNK were from Invitrogen and Dr. D. Schlaepfer (Scripps Research Institute, La Jolla, CA), respectively. Pharmacological inhibitors PP2 and PD98059 were acquired from Calbiochem (La Jolla, CA), as were EGF and TGF-β1. BCA protein assay reagent kit was obtained from Pierce Biotechnology (Rockford, IL). Western immunoblotting reagents: Hybond-P PVDF membranes, enhanced chemiluminescence (ECL) reagents, Hyperfilm-ECL film were acquired from GE Healthcare (Buckinghamshire, United Kingdom). Antibodies were obtained as follows: anti-FAK and anti-phosphoFAK$^{Y397}$ from Chemicon (Temecula, CA), anti-ERK2, anti-phospho-ERK1/2 from

Cell Signaling (Beverly, MA), anti-c-Fos and anti-phospho-c-Fos antibodies from Santa Cruz Biotechnology (Santa Cruz, CA), anti-rabbit-HRP from GE Healthcare, anti-β-actin antibody from Sigma and anti-mouse IgG-Alexa 488 from Invitrogen (Molecular Probes). Dual Luciferase assay kit was obtained from Promega. Reagents for real-time PCR, including Taqman polymerase, MMP-2, MMP-9, and MMP-14 primers and probes (Assays-on-Demand) were obtained from Applied Biosystems (Foster City, CA). All other chemicals and reagents were obtained from Sigma unless otherwise noted.

### CELL CULTURE

HEK-293 cells used for generating recombinant adenovirus were maintained in modified alpha medium (αMEM) supplemented with 10% FBS, 2 mM glutamine, 100 U/ml of penicillin and streptomycin. MDA-MB-231 and PC3 cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS, 2 mM glutamine and 100 U/ml of penicillin and streptomycin. Hs578T cells were maintained in the same medium with the additional supplementation of 10 μg/ml insulin. For routine subculturing or experiments, cells were detached using 0.25% trypsin and 0.53 mM EDTA in Hank's buffered salt solution (HBSS) and then plated at the specified cell densities in 6- or 24-well plates. For some experiments, cells were plated on glass coverslips. All cells were maintained at 37°C in a humidified 5% $CO_2$ environment. To generate stable cell lines expressing the dominant-negative kinase-inactive C-terminal fragment of FAK (FRNK) [Sieg et al., 1999], MDA-MB-231 cells were transfected with pCDNA3.1-FRNK or pCDNA3.1-hygro plasmids using Fugene 6 reagent. Cells were then selected using 600 μg/ml hygromycin. After approximately 14 days of selection, 24 clones expressing target and control plasmids were screened by PCR for plasmid-based DNA and then analyzed for FRNK expression by Western blotting using an anti-HA and anti-FAK antibodies. Pooled clonal cell lines expressing FRNK were subsequently used for experimentation.

### GENERATION OF BSP AND BSP MUTANT-ENCODING ADENOVIRUS AND RECOMBINANT PROTEINS

For expression of BSP in carcinoma cell models, BSP adenoviral constructs containing full-length rat BSP or an Arg-Gly-Asp (RGD) to Lys-Ala-Glu (KAE)-mutated BSP (BSP-KAE) cDNA was cloned into pShuttle vector downstream of the cytomegalovirus (CMV) promoter and then sub-cloned into the linearized pAdeno-X vector. Linearized adenoviral vectors were then transfected into sub-confluent HEK-293 cells using Fugene 6 transfection reagent. The generated BSP, BSP-KAE, or empty vector (EV) virus was generated in HEK-293 cells and titered to determine infection efficiency. Sub-confluent cell cultures were incubated with viral stock diluted to $3.0 \times 10^7$ p.f.u./ml in serum-free DMEM for 2 h at 37°C and 5% $CO_2$. At 24 h post-infection cells were assayed for BSP or mutant expression by Western blotting using anti-BSP antibodies and/or by qPCR using BSP-specific primers. The expression level of the BSP and BSP-KAE mRNA/protein was determined to be approximately equal at this timepoint. These cells were subsequently used for the experiments described below. The prokaryotic recombinant rat BSP proteins (rBSP and rBSP-KAE) were expressed and purified as previously described [Tye et al., 2003].

### IMMUNOPRECIPITATION AND WESTERN BLOTTING

For specific kinase activation analysis, 6-well plates of sub-confluent cells were infected with EV, BSP or BSP-KAE viral constructs as described above. At 24 h post-infection, where specified, cells were treated with vehicle or 50 ng/ml EGF or 10 ng/ml TGF-β1 for 1 h and then washed twice with cold PBS and dislodged by scraping and/or vigorously resuspended in RIPA lysis buffer (150 mM NaCl, 10 mM Tris, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mM EDTA, pH 7.4) containing proteinase inhibitors. Lysates were cleared by centrifugation and stored at −80°C until use. Protein concentrations were determined by the BCA protein assay. For immunoprecipitation, cleared lysates were incubated with the indicated primary antibody for 2 h at 4°C under constant agitation. Protein A-Agarose (Sigma) was added to lysates and samples incubated with agitation for 1 h at 4°C. Samples were then collected by centrifugation and washed with excess cold IP buffer several times.

For immunoblotting, protein samples were separated under reducing conditions using 7.5 or 10% SDS–PAGE gel. After electrophoresis, proteins were immobilized on polyvinylidene fluoride membranes (Hybond-P PVDF), blocked in either 5% non-fat milk/0.1% Tween-20/PBS solution or 2–5% BSA/0.1% Tween-20/PBS solution and incubated overnight at 4°C or at room temperature for 1 h. Proteins were then detected by incubating the membrane for 1 h with specified primary antibody as indicated, followed by several washes with 0.1% Tween-20/PBS and incubation with anti-rabbit-HRP (1:15,000) dissolved in 0.5% BSA/0.1% Tween-20/TBS. Antibody binding to membranes was detected by incubation with ECL reagents and then visualized on Hyperfilm-ECL film. As a control for protein loading, blots were stripped (2% SDS, 100 mM β-mercaptoethanol, 50 mM Tris) and reprobed with anti-β-actin antibody. Resulting films were scanned on a flatbed scanner and densitometry preformed using the ImageJ program.

### IMMUNOCYTOCHEMISTRY AND CONFOCAL MICROSCOPY

Cells that had been previously infected as described above were fixed with 3.7% paraformaldehyde in PBS and permeabilized with 0.5% Triton X-100 in PBS at 24 h post-infection. Coverslips or chamber slides were then blocked with 2% BSA/PBS and incubated with anti-vinculin antibody (1:200 dilution) in 0.5% BSA/PBS for 1 h. Subsequently, cells were washed and incubated with anti-mouse IgG-Alexa 488 (1:1,000) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (1:500) in 0.2% BSA/PBS. Slides were imaged using a Zeiss Axiovert LSM510Meta laser scanning confocal microscope using a 40× objective.

### LUCIFERASE REPORTER ASSAY

Cells were transfected with 0.25 μg pGL3-AP-1-Luc reporter plasmid plus 0.01 μg pRL-SV40 containing a cDNA for *Renilla reniformis* luciferase to control for transfection efficiency. Cells were then infected with EV, BSP-KAE or BSP viral constructs as described above. At 24 h post-infection, cell media was exchanged for αMEM supplemented with 10% FBS, 2 mM glutamine. Cells were then treated with 50 μM PD98059 or 0.5 μM PP2 and then treated with vehicle (0.05% DMSO), 50 ng/ml EGF or 10 ng/ml TGF-β for

1 h. Cells were harvested by passive lysis and assayed using the Dual Luciferase assay kit on a LMaxII luminometer (Molecular Devices).

### QUANTITATIVE REAL-TIME PCR

Total RNA was extracted from 24 h-post infection cell lysates using RNAeasy kit with DNase I treatment as directed by the manufacturer. The total RNA in each sample was quantified by absorbance readings at 260 nm using a Beckman-Coulter DU 530 spectrophotometer. Following RNA purification, qPCR analysis was performed using 50 ng total RNA in 15 μl reactions containing Taqman® one-step RT-PCR master mix kit and gene-specific primers and probes for MMP-2, MMP-9, and MMP-14. As an internal control, 18S RNA of each sample was simultaneously quantified using eukaryotic 18S primers and VIC probes. Amplification was performed in an ABI Prism 7900 HT Sequence Detector using standard settings of 40 cycles with an annealing temperature of 60°C. All samples were amplified in five parallel reactions and experiments were performed at least twice with similar results.

### INVASION AND MIGRATION ASSAYS

For invasion assays, modified Boyden transwell chambers (Becton-Dickinson, 3 μm pore size) were coated with a thin layer of ECM-Matrigel (Sigma). Matrigel was diluted in water and 25 μg in 100 μl of gel suspension was added to the upper chamber. After polymerization for 1 h at 37°C, and drying, the Matrigel was reconstituted with 100 μl serum-free DMEM. The transwell chambers were placed into 24-well dishes containing 0.4 ml of serum-free DMEM with or without 2% FBS, 50 ng/ml EGF or 10 ng/ml TGF-β1. Cells were added to the upper compartment at a concentration of $1 \times 10^5$ cells in 0.3 ml of serum-free media. After 24 h at 37°C cells on the upper membrane surface were removed by swabbing and transwell chambers were washed with PBS. Cells on the lower membrane surface were fixed by treatment with 3.7% formaldehyde. Cells were stained (0.1% crystal violet, 2% ethanol) and migration values were determined either by dye elution and absorbance at 600 nm or by counting nine random high-power (40×) fields per chamber. For pathway-inhibition studies, suspended cells were treated with 200 μM RGD peptides (GRGDSP), 0.5 μM PP2, 50 μM PD98059 or respective controls [GRADSP peptides, 0.05% DMSO (vehicle)] for 30 min and used for migration assays as described. Migration was quantified as number of cells migrating/control cells migrating × 100% ± SD. Experiments were performed using three replicates and independently repeated at least three times. The scratch assay is a common method to determine cell migration [Coomber and Gotlieb, 1990]. Cells were plated in 24-well plates or glass coverslips and grown to confluence. A sterile pipette tip was used to create an approximate 1 mm gap in the cell layer cell monolayer and an image was taken using an inverted microscope (0 h). Cells were then treated with 20 μg/ml BSA, rBSP or rBSP-KAE and incubated for 8 h at 37°C (5% $CO_2$) with images captured on an inverted microscope.

### CELL SURVIVAL ASSAY

Cells that had been previously infected with EV, BSP or BSP-KAE were plated at a density of $1 \times 10^5$ cells/cm$^2$ in 12-well plates. Cells were then grown to 80% confluence and media replaced by serum-free DMEM supplemented with antibiotics (as described) with or without 50 ng/ml EGF or 10 ng/ml TGF-β1. At specified time-points, cells were repeatedly washed with PBS and gently trypsinized from the culture surface, neutralized with serum, and a 1% solution of trypan blue was added to the dish. Viable cells were then counted on a hemocytometer, an average of three counts being performed on each well. The mean number of viable cells from three replicate wells ± SD and experiments were independently determined at least three times. For pathway-inhibition studies, culture media was supplemented with 200 μM synthetic RGD peptides (GRGDSP), 0.5 μM PP2, 25 μM PD98059 or respective controls (GRADSP peptides, DMSO (vehicle)) and exchanged every 24 h.

### STATISTICAL ANALYSIS

All data were analyzed using Prism 4.0 (GraphPad). Statistical significance was determined by one- or two-way analysis of variance (ANOVA) and Bonferonni post-test where stated. Data are presented as means ± SD.

## RESULTS

### BSP EXPRESSION IN BREAST AND PROSTATE CANCER CELL MODELS INCREASES EXPRESSION OF $\alpha_V$-INTEGRINS AND PROMOTES MATURE FOCAL CONTACT FORMATION

It has been suggested that changes in integrin expression and function may be an important contributing factor to metastasis, tumor growth and development [Knudsen and Miranti, 2006]. Furthermore, the interaction of BSP with cell-surface integrins has been demonstrated to facilitate cancer cell migration and invasion presumably through formation of a trimolecular complex with $\alpha_v\beta_3$ and MMP-2 [Karadag et al., 2004]. To determine if expression of BSP had a direct effect on integrin expression and function in carcinoma cell lines, the total cellular $\alpha_V$, $\beta_3$, and $\beta_5$ contents of BSP-infected or EV-infected MDA-MB-231, Hs578T, and PC3 cells were determined by Western-blot analysis (Fig. 1A). Other than $\beta_5$ levels in the Hs578T cell line, significant ($P < 0.01$) increases in $\alpha_V$, $\beta_3$, and $\beta_5$ integrin subunits were shown in all cells infected with BSP compared to EV-infected cells (Fig. 1B). Cells infected with virus encoding an RGD to KAE mutant of BSP (BSP-KAE), did not demonstrate any increase in integrin subunit protein levels (data not shown).

To assess if the observed increase in integrin expression resulted in increased focal adhesion formation, immunofluorescence microscopy was used to visualize the association of vinculin with phalloidin-stained stress fibers in MDA-MB-231 cells upon adhesion. Mature focal adhesions were characterized as vinculin-positive adhesive structures in contact with stress fibers (co-localization), as opposed to focal complexes that were assembled at the cell periphery and were not in contact with stress fibers. Cells infected with BSP virus displayed a higher number of mature focal adhesions compared to cells infected with EV or BSP-KAE (Fig. 1C,D).

It has been demonstrated that TGF-β1 can regulate the number of mature focal adhesions in fibroblastic cell types [Dabiri et al., 2006]. To determine the effect of BSP on focal adhesion formation in cancer cells, EV, BSP-, or BSP-KAE-infected MDA-MB-231 cells were

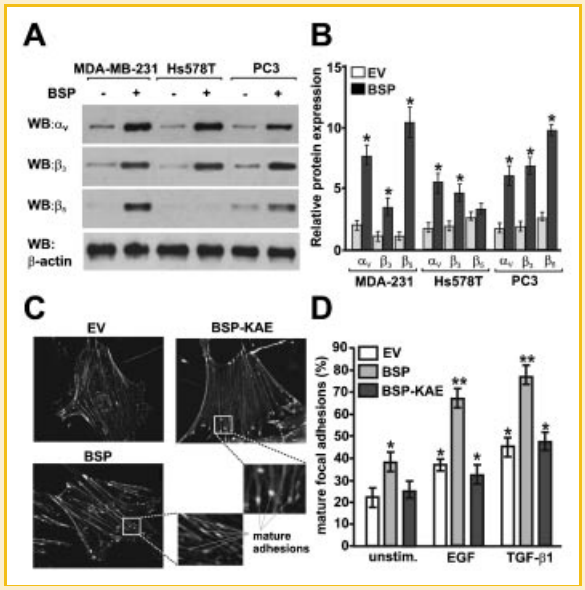


Fig. 1. Integrin expression and focal adhesion formation in carcinoma cell models. Adenovirus-infected (BSP) or control-infected (EV) MDA-MB-231, Hs578T and PC3 cells were analyzed for expression of specific integrin subunits. A: Western blot analysis using anti-$\alpha_v$, anti-$\beta_3$, or anti-$\beta_5$ antibodies demonstrated that cells overexpressing BSP (lanes 2, 4, and 6) displayed increases in specific integrin protein levels compared to EV controls (lanes 1, 3, and 5). As a protein loading control, blots were stripped and re-probed with anti-β-actin antibodies. B: Three replicate blots were subjected to densitometric analysis to determine relative levels of integrin subunits. Data are the average of triplicate determination ± SD ($^{*}P < 0.01$ vs. control). C: MDA-MB-231 cells were infected with EV, BSP, or BSP-KAE, fixed and stained with anti-vinculin followed by anti-rabbit Alexa 488 and TRITC-phalloidin. Mature focal adhesions are represented as co-localization of vinculin with actin stress fibers (bright region: Inset). D: The number of mature focal adhesions (vinculin-positive adhesion in contact with actin stress fiber) in infected MDA-MB-231 cells with or without EGF and TGF-β1 were counted and expressed as a percentage of all focal contacts in each cell. Data shown are averages of 20 representative cells from three replicate experiments ± SD ($^{*}P < 0.01$ vs. EV, $^{**}P < 0.01$ vs. unstimulated BSP-infected).

cultured with or without EGF (50 ng/ml) or TGF-β1 (10 ng/ml) treatment. EGF or TGF-β1 treatment significantly increased the ratio of mature focal adhesions in all transfected MDA-MB-231 cells (treated vs. unstimulated; Fig. 1D).

## INTERACTION OF BSP WITH CANCER CELL LINES INVOLVES ACTIVATION OF FAK AND MAPK PATHWAYS

To investigate the downstream signaling pathways involved in the integrin-mediated interaction of breast and prostate carcinoma cells with BSP, the phosphorylation and activation of focal adhesion-associated intracellular kinases were determined using phospho-specific antibodies. Following adherence to BSP for 1 h, MDA-MB-231, Hs578T or PC3 cells demonstrated an increase phosphorylation of FAK as assayed by immunoprecipitation/Western blot analysis using anti-FAK and anti-phosphotyrosine (PY20) antibodies, respectively (Fig. 2A). Similarly, an increase of FAK phosphorylation was detected by Western blotting with anti-phosphoFAK$^{Y397}$ antibody in BSP-infected MDA-MB-231 cells (Fig. 2B,C). This FAK activation was not observed in EV or BSP-KAE-infected cells. The effects of EGF and TGF-β1 in stimulating FAK phosphorylation in BSP or BSP-KAE-expressing MDA-MB-231 cells were also investigated. Addition of exogenous EGF and TGF-β1 had no effect on FAK$^{Y397}$ phosphorylation in EV or BSP-KAE-infected cells whereas both cytokines significantly increased ($P < 0.05$) FAK phosphorylation in BSP-infected cells (Fig. 2B,C).

One of the major downstream effectors of FAK phosphorylation is the MAP kinase ERK. MDA-MB-231, Hs578T, or PC3 adhering to rBSP for 1 h exhibited increased ERK phosphorylation compared to cells left in suspension or to cells that adhered to poly-L-lysine (Fig. 2D). MDA-MB-231 cells infected with EV, BSP, or BSP-KAE were also assessed for ERK phosphorylation. The basal level of ERK phosphorylation was significantly ($P < 0.05$) higher in BSP-infected cells compared to EV and BSP-KAE-infected cells (Fig. 2E,F). Addition of exogenous EGF and TGF-β1 increased ERK phosphorylation in all cells, and significantly increased ($P < 0.05$) ERK phosphorylation in BSP-infected MDA-MB-231 cells compared to untreated BSP-infected cells (Fig. 2E,F).

## BSP EXPRESSION INCREASES AP-1 ACTIVITY IN CANCER CELL LINES

One of the major downstream targets for ERK is components of the AP-1 family. Members of the AP-1 transcriptional-activator family such as c-Fos have been demonstrated to regulate migration as well as the expression of integrin sub-units [Eriksson et al., 2005; Liu et al., 2006]. Infection of MDA-MB-231, Hs578T, and PC3 cells with BSP virus increased ($P < 0.01$) AP-1-mediated luciferase activity (Fig. 3A–C). This increased activity was dependent on the Src-activated MAP kinase pathway as addition of PP2 (inhibitor of Src-kinase) or PD98059 (inhibitor of MEK/ERK) decreased luciferase activity to control levels (adCMV-infected). The addition of EGF or TGF-β1 to BSP-infected cells resulted in a significant increase in AP-1-mediated activity compared to cells expressing BSP alone. Similarly, in MDA-MB-231 cells expressing BSP there was an increase in phosphorylation of the AP-1 component c-Fos as detected by immunoprecipitation followed by Western blotting; this was not observed in EV-infected cells or in cells expressing BSP-KAE (Fig. 3D).

## BSP-MEDIATED ACTIVATION OF FAK-ERK STIMULATES EXPRESSION OF MMP-2, MMP-9, AND MMP-14

A distinct hallmark of highly metastatic cells is the increase expression of MMPs. As well, AP-1 activity has been shown to regulate the expression of specific MMPs in certain cancers [Han et al., 2006]. Cells expressing BSP demonstrated an increase in levels of MMP-2, MMP-9, and MMP-14 mRNA (Fig. 4A–C). Furthermore, TGF-β1 and EGF treatment of BSP-infected MDA-MB-231 cells significantly increased levels of all MMP's studied compared to untreated cells expressing BSP (Fig. 4D). The relative increase in MMP transcriptional activity in BSP infected cells was abolished by the addition of PP2 or PD98059, suggesting that this expression was MAPK-pathway dependent (Fig. 4D).

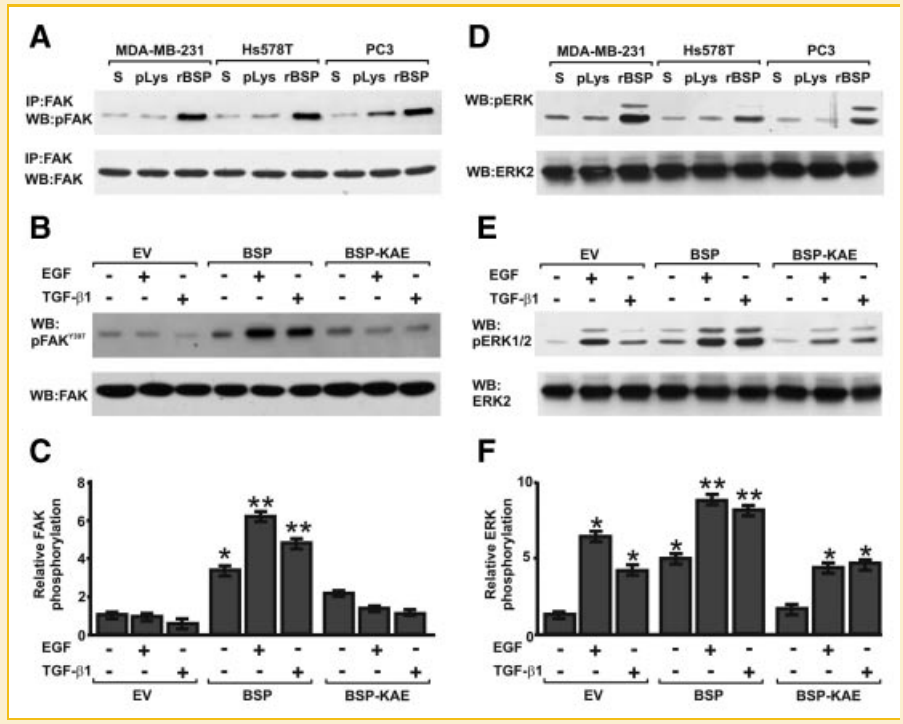


Fig. 2. BSP-mediated activation of FAK and ERK in cancer cells. A: MDA-MB-231, Hs578T, and PC3 cells were suspended in serum-free media and then left in suspension (S), plated on poly-L-lysine (pLys) or plated on rBSP. FAK protein was immunoprecipitated with anti-FAK antibody and phosphorylated FAK was detected by blotting with anti-phosphotyrosine antibody (PY20). B: Adenovirus-infected MDA-MB-231 cells were treated with 50 ng/ml EGF or 10 ng/ml TGF-β1 and 1 h post-treatment total cell lysates were analyzed for FAK phosphorylation using anti-phosphoFAK$^{Y397}$ antibody. C: Three replicate gels were analyzed by densitometry and presented as phosphorylated/total FAK ± SD ($^{*}P < 0.05$ vs. EV (adCMV alone); $^{**}P < 0.05$ vs. unstimulated adCMV-BSP). D: Cell lysates were assayed for ERK phosphorylation using a phospho-specific p42/44 (ERK) antibody. E: Adenovirus-infected MDA-MB-231 cells were treated with EGF or TGF-β1, lysed and total cell lysates analyzed for ERK phosphorylation using anti-phospho-ERK antibody at 1 h post-treatment. F: Three replicate gels were analyzed by densitometry and data presented as phosphorylated/total ERK ± SD ($^{*}P < 0.05$ vs. EV; $^{**}P < 0.05$ vs. unstimulated BSP-infected).

### TGF-β1- AND EGF-MEDIATED MIGRATION OF BREAST AND PROSTATE CELL LINES IS INCREASED BY BSP EXPRESSION

To confirm the effect of BSP expression on invasion of cancer cell lines, a Matrigel-coated Boyden chamber assay was used to determine cell migration. Cells were suspended in serum-free medium and allowed to migrate towards 2% serum-containing media. Cells infected with BSP virus demonstrated significant ($P < 0.01$) increase in migration compared to cells infected with EV or BSP-KAE (Fig. 5A,B). To determine if the effects of BSP in stimulating migration could also be facilitated by exogenous BSP, MDA-MB-231 cells were treated with recombinant BSP and cell migration activity was assessed using a scratch assay. Cells treated with 20 μg/ml rBSP demonstrated increased cell migration resulting in almost complete coverage of the approximately 1 mm gap in the cell monolayer after 8 h (Fig. 5C). In comparison, cells treated with rBSP-KAE or BSA (control) displayed less motility than BSP-treated cells and did not fill the monolayer gap in the 8 h time-period. This same trend was observed in cells that had been infected with BSP virus, with cells filling the 1 mm gap in 8 h whereas EV or BSP-KAE infected MDA-MB-231 cells demonstrated a slower rate of migration (data not shown).

To determine the effect of BSP expression on the EGF- and TGF-β1-stimulated invasion by cancer cell lines, a Matrigel-coated Boyden chamber assay was used to determine cell migration. Cells were suspended in serum-free medium and allowed to migrate towards 2% serum-containing medium containing 50 ng/ml EGF, 10 ng/ml TGF-β1 or no growth factor (unstimulated). Cells expressing BSP demonstrated significant ($P < 0.01$) increase in migration upon stimulation with EGF and TGF-β1 compared to cells infected with EV or BSP-KAE virus (Fig. 5D–F). In order to determine the relationship of integrin-related signaling pathways to the stimulation of migration in BSP-expressing MDA-MB-231 cells, several pathway-specific inhibitors were used. Inhibitors of integrin binding (GRGDSP peptides), FAK kinase activity (FRNK), Src activity (PP2), or ERK activity (PD98059) significantly ($P < 0.01$) reduced EGF- and TGF-β1-stimulated migration (Fig. 5G,H).

### BSP EXPRESSION IN CANCER CELL LINES INCREASES CELL SURVIVAL AFTER SERUM WITHDRAWAL

One of the hallmarks of tumor progression to distal sites is the survival of cells in avascular conditions. The three cell lines used in this study all demonstrated serum dependence for their continued growth, such that within 3 days of serum withdrawal, only 38–45% of MDA-MB-231 cells were viable (Fig. 6A). However, BSP-infected cells showed a survival rate of 55–65% at a period of 3 days. Similar results were obtained for Hs578t and PC3 cells (Fig. 7A). Over the period of 7 days, the BSP expressing cells did display a decrease in cell viability; however, this decrease was much

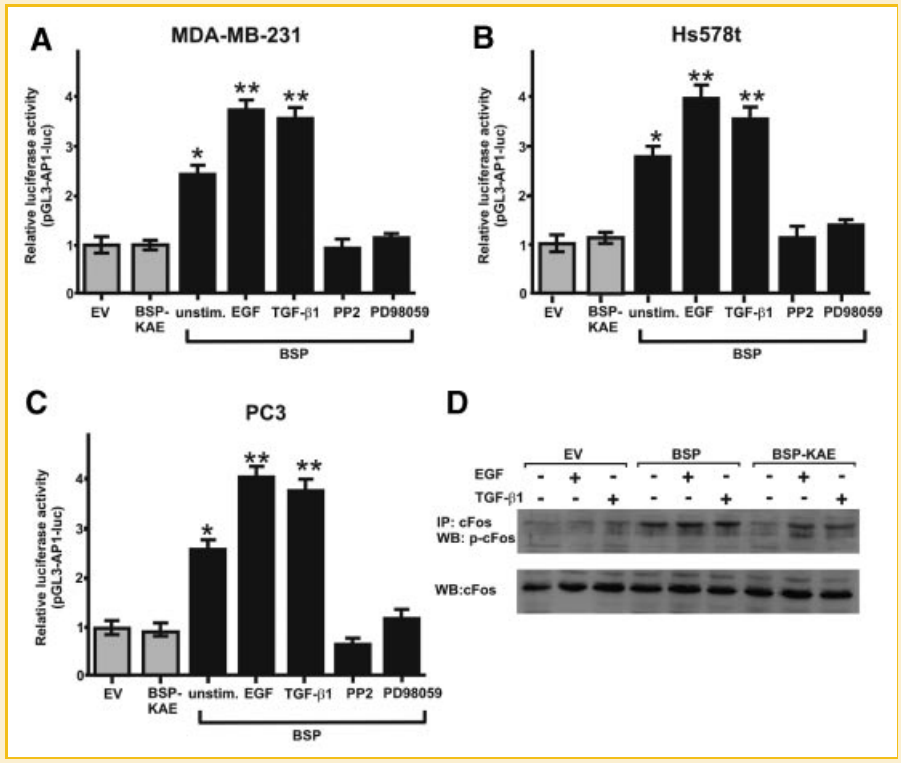


Fig. 3. AP-1-mediated luciferase reporter activity in BSP expressing cells. A: MDA-MB-231, (B) Hs578T, or (C) PC3 cells were transfected with pGL3-AP-1-Luc reporter plasmid to determine AP-1-mediated activity in cells. Cells were then infected with EV, BSP-KAE or BSP viral constructs. BSP expressing cells were then treated with TGF-β1 or EGF for 3 h or inhibitors of Src kinases (PP2) or MEK (PD98059) for 30 min and luciferase activity in each cell line quantified. Statistical significance was determined by one-way ANOVA followed by a Bonferroni post-test ($^{*}P < 0.01$ vs. EV; $^{**}P < 0.05$ vs. BSP-infected). Data presented as representative values ± SD from at least three independent experiments. D: Adenovirus-infected MDA-MB-231 cells were treated with 50 ng/ml EGF or 10 ng/ml TGF-β1 for 3 h and cell lysates were immunoprecipitated with anti-c-Fos antibody and assayed for phosphorylation using an anti-phospho-c-Fos antibody. The blots were stripped and reprobed with anti c-Fos.

less than those observed with either EV or BSP-KAE-infected cells. At all points after 3 days, there was a significantly ($P < 0.05$) extended survival of BSP-infected cells. Addition of 50 ng/ml EGF (Fig. 6B) or 10 ng/ml TGF-β1 (Fig. 6C) to serum-free media of BSP-infected MDA-MB-231 cells significantly increased cell survival, compared to untreated BSP-infected cells (unstimulated), after 3 days of culture. Similar effects were seen in Hs578T and PC3 cells upon addition of EGF (Fig. 7B) or TGF-β1 (Fig. 7C). The effects of integrin signaling-related inhibitors were also tested on BSP-infected MDA-MB-231 cells. Addition of GRGDSP, PP2, or PD98059 as well as the stably expressed FRNK construct all reduced cell survival in EGF and TGF-β1 stimulated BSP-expressing MDA-MB-231 cells (Fig. 6D,E).

## DISCUSSION

In this study we have demonstrated that increased BSP expression in three different cancer cell lines, including the breast cancer cell lines MDA-MB-231 and Hs578T, and the prostate cancer cell line PC3, results in increased metastatic markers and cell survival. Expression of BSP resulted in (i) an increased number of integrin subunits associated with metastasis as well as an elevated number of focal adhesions responsible for cell motility; (ii) activation of metastasis-associated signaling pathways which includes increased phosphorylation/activation of FAK, ERK, and AP-1-associated proteins; (iii) heightened expression of prometastatic markers which include several MMPs; (iv) enhanced cellular sensitivity and responsiveness to cytokines (TGF-β and EGF); (v) and elevated cell migration and cell survival after serum withdrawal. These findings demonstrate a novel mechanism by which BSP expression can promote metastasis and tumor cell survival (as summarized in Fig. 7D).

We have also shown that BSP promotes cell migration in these three cancer cell lines. These cell lines have been previously demonstrated to express varying amounts of BSP and several studies, using these cell lines and in primary tumors derived from these cells, have shown that relative BSP expression levels correlates with metastatic potential [Bellahcene et al., 1996a; Bellahcene and Castronovo, 1997]. BSP has been demonstrated to stimulate the in vitro migration of breast cancer cells via a mechanism involving $\alpha_v$-containing integrins [Sung et al., 1998]. In our study, the expression of BSP in these three distinct cancer cell lines resulted in elevated expression of integrin subunits specifically involved in BSP-mediated interaction. This finding is particularly important given the wide-ranging roles integrins play in tumor progression as the severity and metastatic potential of numerous cancers are associated with the expression and activation of integrins, including $\alpha_v\beta_3$

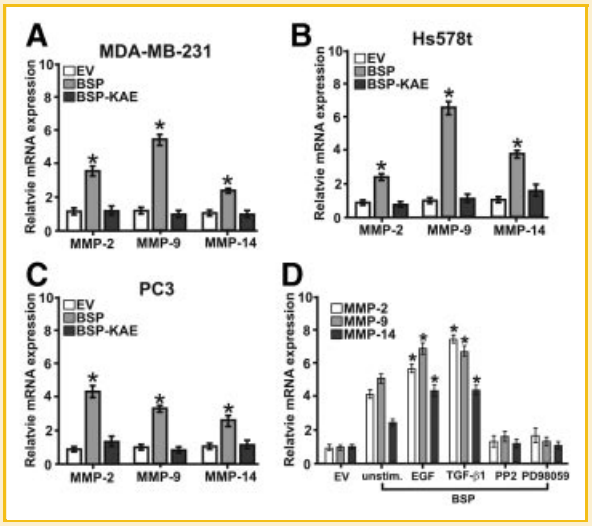


Fig. 4. BSP expression increases MMP-2, MMP-9, and MMP-14 expression in breast and prostate cell lines. A: MDA-MB-231, (B) Hs578T, or (C) PC3 cells were infected with EV, BSP-KAE, or BSP viral construct. Total mRNA was subjected to qPCR analysis using specific primer and probe sets for MMP-2, MMP-9, and MMP-14. Results were normalized and expressed as fold increase over adCMV infected cells. D: MDA-MB-231 cells were infected with adCMV-BSP and treated with 50 ng/ml EGF, 10 ng/ml TGF-β1, PP2 (0.5 μM), or PD98059 (50 μM). Statistical significance was determined by one-way ANOVA followed by a Bonferroni post-test ($^{*}P < 0.05$ vs. untreated, BSP-infected). Data represents three independent experiments ± SEM.

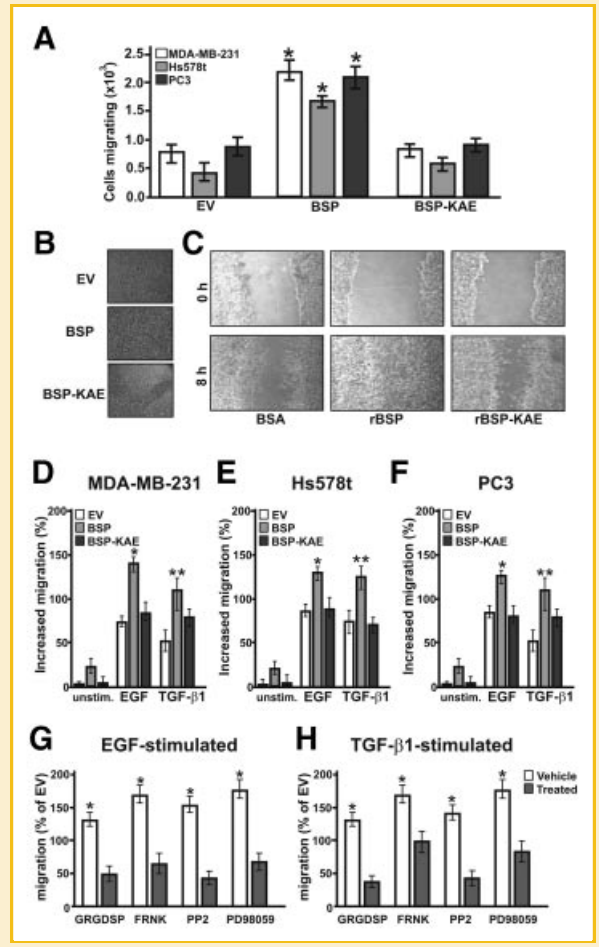


Fig. 5. BSP expression increases growth factor-mediated migration of breast and prostate cell lines. A: Migration of MDA-MB-231, Hs578T, and PC3 cells treated with EV, BSP, or BSP-KAE was assayed using Matrigel-coated 3 μm filter Boyden chambers. Cells were allowed to migrate for 8 h to 2% serum-containing media. Cells migrating to lower chamber were counted ±SD for three replicate experiments ($^{*}P < 0.01$ vs. adCMV). B: Representative crystal violet stained fields of view of migrating cells. C: A 1 mm gap was generated in a confluent monolayer of MDA-MB-231 cells and cells were treated with 20 μg/ml rBSP, rBSP-KAE or BSA. Phase contrast images were taken at time of scratching (0 h) and after 8 h incubation. Migration of (D) MDA-MB-231, (E) Hs578T, and (F) PC3 cells was assayed using Matrigel-coated 3 μm filter Boyden chambers. Cells were allowed to migrate for 24 h to 2% serum-containing media (unstimulated), serum-free media supplemented with 50 ng/ml EGF or 10 ng/ml TGF-β1. Cells migrating to lower chamber were counted and expressed as the mean percent increase over control (EV unstimulated) ± SD for three replicate experiments ($^{*}P < 0.01$ vs. EGF-stimulated EV; $^{***}P < 0.05$ vs. TGF-β1-stimulated EV, respectively). BSP-infected MDA-MB-231 cells were treated with pathway-specific inhibitors GRGDSP (100 mM), PP2 (0.5 μM), or PD98059 (50 μM), or were stably transfected with the FRNK construct. Cells migrating to lower chamber in response to (G) EGF or (H) TGF-β1 were counted and expressed as the average percentage of control (EV unstimulated) ± SD for three replicate experiments. Statistical significance was determined by *t*-test ($^{*}P < 0.01$ vs. uninhibited).

[Pecheur et al., 2002]. Integrins have been demonstrated to regulate metastasis by several wide-ranging mechanisms, including the activation and distribution of MMPs involved in the degradation of the extracellular matrix during invasion [Morini et al., 2000; Karadag et al., 2004] and transmitting signals responsible for focal adhesion formation and turnover [Basson, 2008]. We have demonstrated that the increase in integrin subunits in response to BSP expression results in the formation of a greater number of actin stress fiber-associated focal adhesions. As focal adhesions are characterized not only by an attachment of cells with the extracellular matrix but also by sites of assembly for many intracellular kinases, the increase in focal adhesions represents an initial step in activation of specific kinases involved in regulating migration [McLean et al., 2005].

One of the key mediators of integrin signaling, FAK, is a critical regulator of cell migration [Hsia et al., 2003]. In this study we have demonstrated that BSP can stimulate the activation of FAK upon cell adhesion in the three cancer cell lines. Furthermore, BSP expression in MDA-MB-231 cells resulted in an increased basal level of FAK $Tyr_{397}$ phosphorylation. These findings are particularly relevant given the role of phosphorylation of FAK at $Tyr_{397}$ in the regulation of the disassembly of focal adhesions thereby increasing cell motility [Hamadi et al., 2005]. As well, the phosphorylation of FAK at $Tyr_{397}$ has been shown to activate the Src-mediated Ras-Raf pathway, which in turn is a key regulator of the important MAP kinase ERK [Giehl, 2005]. While the mechanism by which ERK activity regulates migration is unclear, the activation of ERK from a wide-range of stimuli results in elevated migration activity [Tremblay et al., 2006]. The increased ERK activity demonstrated in malignant cells expressing BSP may contribute to a progressively motile cell phenotype, increasing both random migration and invasive properties. The activation of AP-1-mediated luciferase

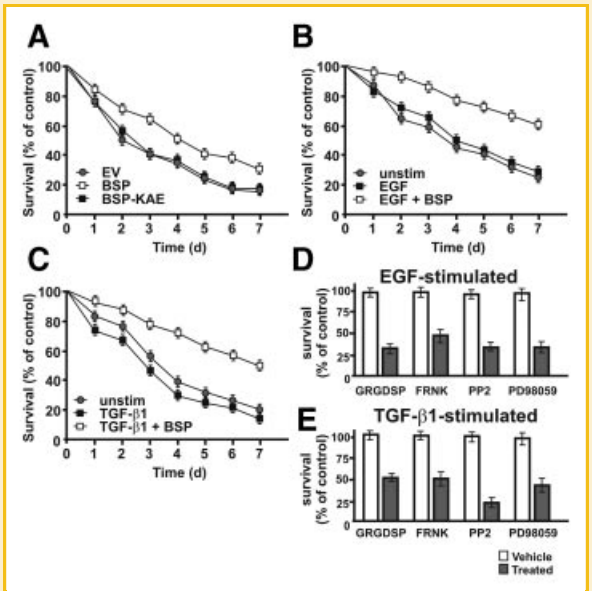


Fig. 6. Quantitation of MDA-MB-231 cell survival after serum withdrawal. A: MDA-MB-231 cells were infected with EV, BSP, or BSP-KAE adenovirus. After serum withdrawal cell survival was determined by counting viable cells. The values are expressed as the percentage of cell survival compared to day 0 ± SD from three independent experiments. Survival of adCMV-BSP-infected cell lines was determined upon addition of (B) EGF or (C) TGF-β1. The values are expressed as the percentage of cell survival compared with day 0 ± SD derived from three independent experiments. Addition of GRGDSP peptide (100 nM) or PP2 (0.5 μM), or use of stably-expressed FRNK and PD98059 (50 μM), reduced cell survival after 3 days in serum-free media containing (D) EGF or (E) TGF-β1.

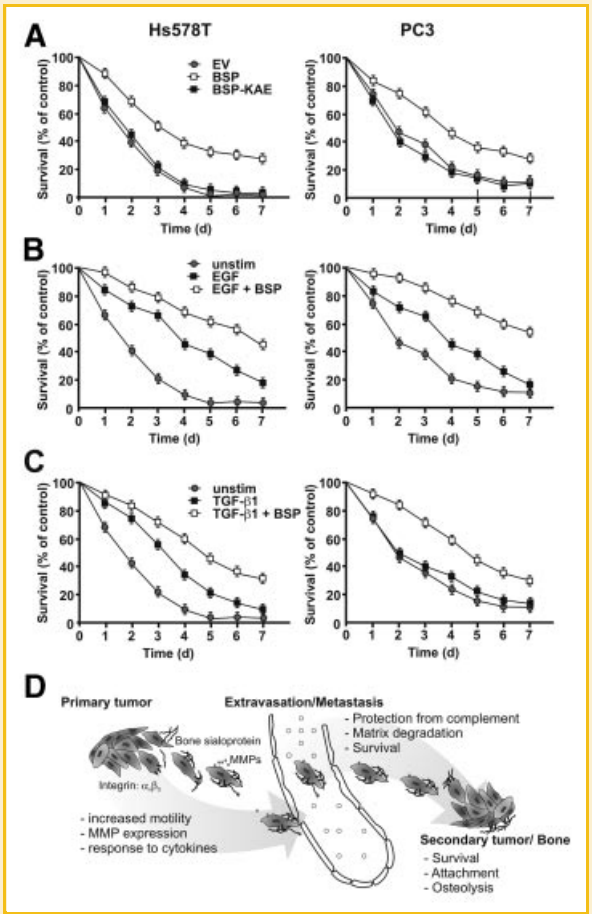


Fig. 7. Quantitation of Hs578T and PC3 cell survival after serum withdrawal. A: Hs578T or PC3 cells were infected with EV, BSP, or BSP-KAE adenovirus. After serum withdrawal cell survival was determined by counting viable cells. The values are expressed as the percentage of cell survival compared to day 0 ± SD from three independent experiments. Survival of adCMV-BSP-infected cell lines was determined upon addition of (B) EGF or (C) TGF-β1. The values are expressed as the percentage of cell survival compared with day 0 ± SD derived from three independent experiments. D: Summary of BSP involvement in promoting distinct stages of metastasis leading to secondary tumor formation in bone.

reporter activity and specifically the activation of c-Fos in cells expressing BSP would suggest that the nuclear targets of ERK activity in malignant cells include members of the AP-1 family. The diverse functions ascribed to the activation of members of the AP-1 pathway suggests that AP-1 in part controls the invasive phenotype, as it has been shown to regulate functions such as cell migration, integrin expression and cell survival [Libermann and Zerbini, 2006; Ozanne et al., 2007].

One of the most intriguing findings of this study is the synergistic effects on cancer cells of BSP and the growth factors EGF and TGF-β1. Several studies have demonstrated that integrin signaling co-ordinates with signaling originating from growth factor receptors in the co-operative control of cell migration [Kawahara et al., 2002; Cabodi et al., 2004]. Exposure of cells to EGF and TGF-β1 has been demonstrated to phosphorylate ERK [Ellenrieder et al., 2001; Hong et al., 2005]. In our study, FAK and ERK phosphorylation levels were enhanced in BSP-expressing cells treated with EGF or TGF-β1. The increase in ERK phosphorylation was cumulative to the observed increases in cells infected with BSP adenovirus. The expression of BSP in the breast and prostate cancer cells also increased invasion of cells in response to EGF and TGF-β1 stimulation. Based on findings from other labs and the data presented in this study, invasion activity in cells expressing BSP may be the product of two separate effects, the first an increase in MMP levels, presumably resulting in degradation of the surrounding ECM, and the second, a stimulation of migration due to ERK activation. The effect of BSP expression in stimulating migrational activity was demonstrated to be dependent on the RGD integrin-binding motif, as infection of cells with a BSP-KAE adenovirus did not stimulate invasion or random migration as observed in the wild-type BSP-infected cells. The addition of integrin-blocking peptides, as well as specific inhibitors of Src and MEK/ERK activity (PP2, PD98059) or a dominate-negative kinase-inactive FAK (FRNK), all reduced invasion of cells expressing BSP, demonstrating the dependence on signals elicited from focal adhesions to regulate migrational response to growth factor stimulation.

One of the major hallmarks of tumor progression is cell survival. Other studies have demonstrated that integrin-associated signaling activity through FAK is important in regulating breast cancer cell survival [Park et al., 2004]. While expression of BSP was sufficient to

increase cell survival over controls, the addition of EGF or TGF-β1 further increased survival of cells following serum withdrawal. Similar to the effects observed in promoting cell migration, cell survival was dependent on integrin-mediated signaling, as the BSP-KAE-expressing cells demonstrated no alteration in cell survival. Addition of integrin-blocking peptides, as well as PP2 or PD98059 or expressing FRNK all reduced cell survival in BSP-expressing cells, suggesting that these observed effects are dependent on activation of integrin-mediated events. BSP has been previously shown to form a trimolecular complex with $\alpha_v\beta_3$ and complement Factor H that protects cells from complement-mediated lysis [Fedarko et al., 2000]. The increased cell survival of BSP-expressing cells would suggest that the protein may also aid in cell survival by directly initiating integrin-mediated cell signaling and increasing cell responsiveness to circulating growth factors.

Although there is significant amount of evidence demonstrating that BSP plays a multifaceted role in cancer metastasis it is still unclear why this protein is specifically upregulated in osteotropic cancers. Several reports have demonstrated that Runx2, the master regulator of bone formation and a strong transcriptional regulator of BSP expression, has a primary role in promoting tumor osteotropism and osteolytic disease [Young et al., 2005]. In another study, BSP and MMP expression was increased upon TGF-β1 stimulation in metastatic 4T1 murine breast cancer cells [Nam et al., 2006]. This would suggest that there are multiple events that can upregulate BSP expression in primary tumors. In this study we have presented data demonstrating that increased BSP expression directly stimulates the activation of integrin-related intracellular signaling pathways regulating cell migration and BSP expression was sufficient to induce expression of MMP-2, MMP-9, and MMP-14. Our studies thus provide a mechanism to explain the relationship of BSP expression and aggressiveness or metastatic potential of tumor cells and could be a suitable target to prevent osteotropic tumor metastasis.

## ACKNOWLEDGMENTS

This work was supported by a Canadian Institutes of Health Research grant to H.A.G. and G.K.H. The authors would like to thank Honghong Chen (University of Western Ontario), Dr. T.M. Underhill (University of British Columbia, Vancouver, BC) and Dr. C.E. Tye (Forsyth Institute, Boston, MA) for technical support and helpful input.

## REFERENCES

Basson MD. 2008. An intracellular signal pathway that regulates cancer cell adhesion in response to extracellular forces. Cancer Res 68:2–4.

Bellahcene A, Castronovo V. 1997. Expression of bone matrix proteins in human breast cancer: Potential roles in microcalcification formation and in the genesis of bone metastases. Bull Cancer 84:17–24.

Bellahcene A, Antoine N, Clausse N, Tagliabue E, Fisher LW, Kerr JM, Jares P, Castronovo V. 1996a. Detection of bone sialoprotein in human breast cancer tissue and cell lines at both protein and messenger ribonucleic acid levels. Lab Invest 75:203–210.

Bellahcene A, Kroll M, Liebens F, Castronovo V. 1996b. Bone sialoprotein expression in primary human breast cancer is associated with bone metastases development. J Bone Miner Res 11:665–670.

Bellahcene A, Maloujahmoum N, Fisher LW, Pastorino H, Tagliabue E, Menard S, Castronovo V. 1997. Expression of bone sialoprotein in human lung cancer. Calcif Tissue Int 61:183–188.

Bellahcene A, Castronovo V, Ogbureke KU, Fisher LW, Fedarko NS. 2008. Small integrin-binding ligand N-linked glycoproteins (SIBLINGs): Multifunctional proteins in cancer. Nat Rev Cancer 8:212–226.

Bierie B, Moses HL. 2006. TGF-beta and cancer. Cytokine Growth Factor Rev 17:29–40.

Byun HJ, Hong IK, Kim E, Jin YJ, Jeoung DI, Hahn JH, Kim YM, Park SH, Lee H. 2006. A splice variant of CD99 increases motility and MMP-9 expression of human breast cancer cells through the AKT-, ERK-, and JNK-dependent AP-1 activation signaling pathways. J Biol Chem 281:34833–34847.

Byzova TV, Kim W, Midura RJ, Plow EF. 2000. Activation of integrin alpha(V)beta(3) regulates cell adhesion and migration to bone sialoprotein. Exp Cell Res 254:299–308.

Cabodi S, Moro L, Bergatto E, Boeri Erba E, Di Stefano P, Turco E, Tarone G, Defilippi P. 2004. Integrin regulation of epidermal growth factor (EGF) receptor and of EGF-dependent responses. Biochem Soc Trans 32:438–442.

Chenu C, Ibaraki K, Gehron Robey P, Delmas PD, Young MF. 1994. Cloning and sequence analysis of bovine bone sialoprotein cDNA: Conservation of acidic domains, tyrosine sulfation consensus repeats, and RGD cell attachment domain. J Bone Miner Res 9:417–421.

Coomber BL, Gotlieb AI. 1990. In vitro endothelial wound repair. Interaction of cell migration and proliferation. Arteriosclerosis 10:215–222.

Dabiri G, Campaner A, Morgan JR, Van De Water L. 2006. A TGF-beta1-dependent autocrine loop regulates the structure of focal adhesions in hypertrophic scar fibroblasts. J Invest Dermatol 126:963–970.

Ellenrieder V, Hendler SF, Boeck W, Seufferlein T, Menke A, Ruhland C, Adler G, Gress TM. 2001. Transforming growth factor beta1 treatment leads to an epithelial-mesenchymal transdifferentiation of pancreatic cancer cells requiring extracellular signal-regulated kinase 2 activation. Cancer Res 61:4222–4228.

Eriksson M, Arminen L, Karjalainen-Lindsberg ML, Leppa S. 2005. AP-1 regulates alpha2beta1 integrin expression by ERK-dependent signals during megakaryocytic differentiation of K562 cells. Exp Cell Res 304:175–186.

Fedarko NS, Fohr B, Robey PG, Young MF, Fisher LW. 2000. Factor H binding to bone sialoprotein and osteopontin enables tumor cell evasion of complement-mediated attack. J Biol Chem 275:16666–16672.

Fisher LW, Fedarko NS. 2003. Six genes expressed in bones and teeth encode the current members of the SIBLING family of proteins. Connect Tissue Res 1:33–40.

Fisher LW, Jain A, Tayback M, Fedarko NS. 2004. Small integrin binding ligand N-linked glycoprotein gene family expression in different cancers. Clin Cancer Res 10:8501–8511.

Gasparini G, Brooks PC, Biganzoli E, Vermeulen PB, Bonoldi E, Dirix LY, Ranieri G, Miceli R, Cheresh DA. 1998. Vascular integrin alpha(v)beta3: A new prognostic indicator in breast cancer. Clin Cancer Res 4:2625–2634.

Giehl K. 2005. Oncogenic Ras in tumour progression and metastasis. Biol Chem 386:193–205.

Hamadi A, Bouali M, Dontenwill M, Stoeckel H, Takeda K, Ronde P. 2005. Regulation of focal adhesion dynamics and disassembly by phosphorylation of FAK at tyrosine 397. J Cell Sci 118:4415–4425.

Han S, Ritzenthaler JD, Sitaraman SV, Roman J. 2006. Fibronectin increases matrix metalloproteinase 9 expression through activation of c-Fos via extracellular-regulated kinase and phosphatidylinositol 3-kinase pathways in human lung carcinoma cells. J Biol Chem 281:29614–29624.

Hauck CR, Hsia DA, Puente XS, Cheresh DA, Schlaepfer DD. 2002. FRNK blocks v-Src-stimulated invasion and experimental metastases without effects on cell motility or growth. EMBO J 21:6289–6302.

Hong IK, Kim YM, Jeoung DI, Kim KC, Lee H. 2005. Tetraspanin CD9 induces MMP-2 expression by activating p38 MAPK, JNK and c-Jun pathways in human melanoma cells. Exp Mol Med 37:230–239.

Hood JD, Cheresh DA. 2002. Role of integrins in cell invasion and migration. Nat Rev Cancer 2:91–100.

Hsia DA, Mitra SK, Hauck CR, Streblow DN, Nelson JA, Ilic D, Huang S, Li E, Nemerow GR, Leng J, Spencer KS, Cheresh DA, Schlaepfer DD. 2003. Differential regulation of cell motility and invasion by FAK. J Cell Biol 160:753–767.

Karadag A, Ogbureke KU, Fedarko NS, Fisher LW. 2004. Bone sialoprotein, matrix metalloproteinase 2, and alpha(v)beta3 integrin in osteotropic cancer cell invasion. J Natl Cancer Inst 96:956–965.

Kawahara E, Nakada N, Hikichi T, Kobayashi J, Nakanishi I. 2002. EGF and beta1 integrin convergently regulate migration of A431 carcinoma cell through MAP kinase activation. Exp Cell Res 272:84–91.

Knudsen BS, Miranti CK. 2006. The impact of cell adhesion changes on proliferation and survival during prostate cancer development and progression. J Cell Biochem 99:345–361.

Libermann TA, Zerbini LF. 2006. Targeting transcription factors for cancer gene therapy. Curr Gene Ther 6:17–33.

Liu G, Ding W, Liu X, Mulder KM. 2006. c-Fos is required for TGFbeta1 production and the associated paracrine migratory effects of human colon carcinoma cells. Mol Carcinog 45:582–593.

McLean GW, Carragher NO, Avizienyte E, Evans J, Brunton VG, Frame MC. 2005. The role of focal-adhesion kinase in cancer - a new therapeutic opportunity. Nat Rev Cancer 5:505–515.

Morini M, Mottolese M, Ferrari N, Ghiorzo F, Buglioni S, Mortarini R, Noonan DM, Natali PG, Albini A. 2000. The alpha 3 beta 1 integrin is associated with mammary carcinoma cell metastasis, invasion, and gelatinase B (MMP-9) activity. Int J Cancer 87:336–342.

Nam JS, Suchar AM, Kang MJ, Stuelten CH, Tang B, Michalowska AM, Fisher LW, Fedarko NS, Jain A, Pinkas J, Lonning S, Wakefield LM. 2006. Bone sialoprotein mediates the tumor cell-targeted prometastatic activity of transforming growth factor beta in a mouse model of breast cancer. Cancer Res 66:6327–6335.

Navolanic PM, Steelman LS, McCubrey JA. 2003. EGFR family signaling and its association with breast cancer development and resistance to chemotherapy (Review). Int J Oncol 22:237–252.

Normanno N, Bianco C, De Luca A, Salomon DS. 2001. The role of EGF-related peptides in tumor growth. Front Biosci 6:D685–D707.

Ozanne BW, Spence HJ, McGarry LC, Hennigan RF. 2007. Transcription factors control invasion: AP-1 the first among equals. Oncogene 26:1–10.

Park HB, Golubovskaya V, Xu L, Yang X, Lee JW, Scully S II, Craven RJ, Cance WG. 2004. Activated Src increases adhesion, survival and alpha2-integrin expression in human breast cancer cells. Biochem J 378:559–567.

Pecheur I, Peyruchaud O, Serre CM, Guglielmi J, Voland C, Bourre F, Margue C, Cohen-Solal M, Buffet A, Kieffer N, Clezardin P. 2002. Integrin alpha(v) beta3 expression confers on tumor cells a greater propensity to metastasize to bone. FASEB J 16:1266–1268.

Sato H, Takino T, Okada Y, Cao J, Shinagawa A, Yamamoto E, Seiki M. 1994. A matrix metalloproteinase expressed on the surface of invasive tumour cells. Nature 370:61–65.

Schaller MD. 2001. Biochemical signals and biological responses elicited by the focal adhesion kinase. Biochim Biophys Acta 1540:1–21.

Sharp JA, Waltham M, Williams ED, Henderson MA, Thompson EW. 2004. Transfection of MDA-MB-231 human breast carcinoma cells with bone sialoprotein (BSP) stimulates migration and invasion in vitro and growth of primary and secondary tumors in nude mice. Clin Exp Metastasis 21:19–29.

Sieg DJ, Hauck CR, Schlaepfer DD. 1999. Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. J Cell Sci 112:2677–2691.

Song H, Ki SH, Kim SG, Moon A. 2006. Activating transcription factor 2 mediates matrix metalloproteinase-2 transcriptional activation induced by p38 in breast epithelial cells. Cancer Res 66:10487–10496.

Sung V, Stubbs JT III, Fisher L, Aaron AD, Thompson EW. 1998. Bone sialoprotein supports breast cancer cell adhesion proliferation and migration through differential usage of the alpha(v)beta3 and alpha(v)beta5 integrins. J Cell Physiol 176:482–494.

Tremblay PL, Auger FA, Huot J. 2006. Regulation of transendothelial migration of colon cancer cells by E-selectin-mediated activation of p38 and ERK MAP kinases. Oncogene 25:6563–6573.

Tu Q, Zhang J, Fix A, Brewer E, Li YP, Zhang ZY, Chen J. 2009. Targeted overexpression of BSP in osteoclasts promotes bone metastasis of breast cancer cells. J Cell Physiol 218:135–145.

Tye CE, Rattray KR, Warner KJ, Gordon JA, Sodek J, Hunter GK, Goldberg HA. 2003. Delineation of the hydroxyapatite-nucleating domains of bone sialoprotein. J Biol Chem 278:7949–7955.

Waltregny D, Bellahcene A, Van Riet I, Fisher LW, Young M, Fernandez P, Dewe W, de Leval J, Castronovo V. 1998. Prognostic value of bone sialoprotein expression in clinically localized human prostate cancer. J Natl Cancer Inst 90:1000–1008.

Waltregny D, Bellahcene A, de Leval X, Florkin B, Weidle U, Castronovo V. 2000. Increased expression of bone sialoprotein in bone metastases compared with visceral metastases in human breast and prostate cancers. J Bone Miner Res 15:834–843.

Xu LH, Yang X, Bradham CA, Brenner DA, Baldwin AS, Jr., Craven RJ, Cance WG. 2000. The focal adhesion kinase suppresses transformation-associated, anchorage-independent apoptosis in human breast cancer cells. Involvement of death receptor-related signaling pathways. J Biol Chem 275:30597–33604.

Young DW, Pratap J, Javed A, Weiner B, Ohkawa Y, van Wijnen A, Montecino M, Stein GS, Stein JL, Imbalzano AN, Lian JB. 2005. SWI/SNF chromatin remodeling complex is obligatory for BMP2-induced, Runx2-dependent skeletal gene expression that controls osteoblast differentiation. J Cell Biochem 94:720–730.

Zhang JH, Wang J, Tang J, Barnett B, Dickson J, Hahsimoto N, Williams P, Ma W, Zheng W, Yoneda T, Pageau S, Chen J. 2004. Bone sialoprotein promotes bone metastasis of a non-bone-seeking clone of human breast cancer cells. Anticancer Res 24:1361–1368.