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Bone sialoprotein in cancer metastasis and bone remodelling: Effects on cancer cell motility, osteoclast differentiation, and activity

Bone Sialoprotein in der Krebsmetastasierung und Knochenremodellierung: Einfluss auf die Motilität von Krebszellen sowie die Differenzierung und Aktivität von Osteoklasten

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List of abbreviations

Abbreviation	Definition
ALP	Alkaline phosphatase
BMD	Bone mineral density
BMP	Bone morphogenetic protein
BMM	Bone marrow-derived macrophage
BMSC	Bone marrow stromal cell
BSA	Bovine serum albumin
BSP	Bone sialoprotein
BSP2	Bone sialoprotein-2
Ca ²⁺	Calcium
CaP	Calcium phosphate
CATSK	Cathepsin K
COL	Collagen
CV	Crystal violet
dNTP	Deoxynucleoside triphosphate
DMSO	Dimethyl sulfoxide
DTC	Disseminated tumour cell
EC ₈₀	80% maximal effective concentration
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ETDA	Ethylenediaminetetraacetic acid
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FN	Fibronectin
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GOI	Gene of interest
GRGDSP	Glycine-Arginine-Glycine-Aspartic acid-Serine-Proline
HA	Hydroxyapatite
HSC	Haematopoietic stem cell
IBSP	Integrin Binding Sialoprotein
IC ₅₀	Half-maximal inhibitory concentration
LHRRVKI	Leucine-Histidine-Arginine-Arginine-Valine-Lysine-Isoleucine
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MET	Metastasis
MM	Multiple myeloma
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
NaOH	Sodium hydroxide
NaH ₂ PO ₄	Monosodium phosphate
Na ₂ HPO ₄	Disodium phosphate
NSCLC	Non-small cell lung cancer
OC	Osteoclast
OCN	Osteocalcin

OPG	Osteoprotegerin
OPN	Osteopontin
OS	Overall survival
pAdC	Prostatic adenocarcinoma
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PDAC	Pancreatic ductal adenocarcinoma
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
PTM	Post-translational modification
P/S	Penicillin/Streptomycin
QOL	Quality of life
RANK	Receptor activator of nuclear factor kB
RANKL	Receptor activator of nuclear factor kB ligand
RFS	Recurrence-free survival
RGD	Arginine-glycine-aspartic acid
Runx2/Cbfa1	Runt-related transcription factor 2
SIBLING	Small integrin binding ligand N-linked glycoprotein
TGF- β	Transforming growth factor beta
TNF- α	Tumour necrosis factor alpha
TRAF6	Tumour necrosis factor receptor-associated factor 6
TRAcP/TRAP	Tartrate-resistant acid phosphatase
VN	Vitronectin
VNR	Vitronectin receptor

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1. Introduction

Bone remodelling is a cyclical process involving the resorption of old or damaged bone and the subsequent formation of new bone. This process, which is tightly regulated by local and systemic signals as well as by three principal bone cell types—osteocytes (which sense mechanical and hormonal cues), osteoclasts (responsible for bone degradation), and osteoblasts [which synthesise and mineralise the bone extracellular matrix (ECM)]—is essential for maintaining skeletal integrity. Bone remodelling supports, for instance, fracture healing and mineral homeostasis; however, imbalances in bone remodelling facilitate not only osteolytic or osteopetrotic disorders, but also the growth of secondary bone tumours.

Metastases account for approximately 90% of cancer-related deaths, with bone being one of the most frequent sites for secondary tumour formation. Advanced breast and prostate cancers, and to a lesser extent lung cancers, frequently metastasise to bone, where they disrupt the balance of bone remodelling, leading to osteolytic, osteoblastic, or mixed lesions. The impact of secondary bone tumours on bone remodelling and the patient itself is vast, leading to skeletal related events (SREs) such as fractures, hypercalcaemia, and severe pain, significantly impairing a patient's quality of life (QOL) and overall survival (OS).

Originally recognised as a regulator of bone formation and matrix mineralisation, bone sialoprotein (BSP) is now emerging as a multifaceted protein involved in tumour progression and skeletal metastasis. Elevated BSP levels have been observed in the sera of cancer patients, and its expression in primary tumour tissues—both at the mRNA and protein levels—corroborates its potential role in mediating angiogenesis, proliferation, survival, adhesion, migration invasion, and immune evasion of cancer cells. Notably, the presence of an RGD sequence in BSP suggests that integrin-mediated interactions may underpin several of these activities. Furthermore, *in vitro* and *in vivo* animal studies demonstrate that BSP stimulates the differentiation and activity of osteoclasts, frequently in synergy with RANKL.

Despite these insights, it remains unclear whether metastatic sites, particularly secondary bone tumours, express BSP at the gene level. Moreover, the effects of human BSP on the adhesion of lung cancer and osteosarcoma cells, as well as the specific contribution of integrin $\alpha\beta 5$ to BSP-induced cell attachment, are inadequately characterised. Most existing studies have focused on BSP-induced invasion rather than migration, and evidence regarding BSP-mediated osteoclastogenesis has solely stemmed from non-human models. Elucidating BSP's expression and its function in tumour cells and within the bone microenvironment will refine our understanding of the interplay between metastasis development and bone resorption, thereby advancing our grasp of the complex processes that drive skeletal metastatic progression.

Accordingly, this project aims to elucidate the role of recombinant human BSP in modulating cancer cell adhesion, migration, and osteoclast differentiation and activity. A series of *in vitro* experiments will be employed to achieve these objectives. Specifically, we will assess the adhesive properties of BSP in breast adenocarcinoma, prostate

adenocarcinoma, large-cell lung cancer, and osteosarcoma cell lines. We will also evaluate the functional significance of human BSP's heparin-binding motif LHRRVKI in these interactions, as the corresponding sequence in rat BSP (FHRRKA) has been shown to promote the adhesion of rat osteoblastic cells. Cell adhesion assays incorporating blocking studies with RGD-containing peptides, anti-integrin antibodies, and LHRRVKI-containing peptides will provide insights into BSP-mediated cell-extracellular matrix (ECM) interactions. Furthermore, multiple anti-BSP antibodies will be evaluated for their ability to inhibit the adhesion of breast cancer cells to BSP, aiding in the characterisation of potential therapeutic candidates. The effects of exogenous BSP on breast and prostate cancer cell migration will be assessed using transwell (Boyden chamber) assays. In addition, to investigate BSP's influence on human osteoclast differentiation and activity *in vitro*, human primary macrophages derived from peripheral blood monocytes will be cultured for 10 days with macrophage colony-stimulating factor (M-CSF) before BSP treatment. Synthetic calcium-phosphate discs will serve as a model of the mineralised bone ECM. The effects of different culture conditions, including varying concentrations of BSP, RANKL, and the anti-human BSP antibody FP-21, on osteoclast differentiation and/or activity will be examined. To further explore BSP's significance in tumour progression and bone remodelling, gene expression analysis will be performed on bone metastases and healthy bone tissue using RT-PCR.

Overall, these findings will enhance our understanding of BSP's role in cancer cell motility and bone resorption, potentially supporting the development of novel therapeutic strategies, such as anti-BSP antibodies, to improve patient outcomes.

1.1 Bone

1.1.1 Bone structure

The human body has approximately 270 bones at birth; however, as bones fuse during growth, the adult human skeleton typically comprises 206 bones [1]. Bone tissue consists of specialised cells, blood vessels, nerves, an organic matrix, and inorganic minerals [2].

Chondrocytes, found in cartilage and the epiphyseal growth plates of developing bone, are essential for cartilage formation and endochondral ossification, the process by which cartilage is replaced by bone during skeletal development [3]. Their lifespan can vary significantly, ranging from a few weeks to several years [4, 5]. Osteoblasts, constituting approximately 4% to 6% of bone cells, are cuboidal cells located on bone surfaces [6]. These short-lived cells (days to months) coordinate the synthesis of the bone matrix and its subsequent mineralisation during both embryonic development and adulthood [7-10]. Osteocytes, comprising over 90% of the cellular composition in adult bone, are terminally differentiated osteoblasts embedded within the mineralised bone matrix [7]. These long-lived cells (10-20 years) function as mechanosensors and play critical roles in orchestrating bone remodelling and homeostasis [11, 12]. Osteoclasts (OCs), which account for about 1% to 2% of bone cells, are large, multinucleated cells responsible for resorbing the organic and

inorganic bone matrix through acidification and proteolysis at resorption sites (Howship's lacunae) [13, 14]. These cells are short-lived, with a lifespan of only a few days to weeks [7, 15, 16].

The organic extracellular matrix (ECM), or osteoid, primarily consists of type I collagen (COL1) which accounts for approximately 90% of its composition and provides tensile strength and flexibility [17, 18]. The remainder includes non-collagenous proteins such as osteocalcin (OCN), osteonectin (ON/SPARC), and SIBLING family proteins, including bone sialoprotein (BSP), which regulate matrix mineralisation [19-23] and facilitate cell signalling [21, 23]. The inorganic ECM is predominantly (ca. 95%) hydroxyapatite (HA) crystals $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$, formed by nucleated calcium and phosphate ions [22, 24-26]. These crystals contribute to the rigidity and compressive strength of bone [17]. Trace elements such as magnesium and sodium enhance structural stability and support metabolic processes involved in bone health [27, 28].

Cortical (compact) bone constitutes approximately 80% of the mature skeleton and is found in the outer layers of bones [29, 30]. Its dense structure provides strength and support [29, 31, 32], although it has a lower metabolic rate and slower remodelling activity compared to trabecular (spongy) bone [33, 34]. Trabecular bone, found in the interior of bones, has a porous, lattice-like architecture that enhances resistance to compressive forces and supports a higher rate of metabolic turnover due to its increased surface area for mineral exchange [31, 35].

1.1.2 Bone development

Bone development occurs through two distinct processes: Intramembranous (direct bone formation) and endochondral ossification (bone formation from cartilage) [36]. In intramembranous ossification, mesenchymal stem cells (MSCs) differentiate directly into bone-forming osteoblasts [37], which secrete both the organic and inorganic matrix [38]. This process forms flat bones, such as those in the skull and clavicle. Endochondral ossification, responsible for the formation of long bones, such as the femur and humerus, involves a sequence of chondrocyte proliferation, matrix mineralisation, and replacement of cartilage with bone tissue [3, 39]. This growth occurs at the epiphyseal plates (growth plates) at the proximal and distal ends of long bones, which remain active during childhood and adolescence to facilitate bone elongation [36].

1.1.3 Bone remodelling

Bone remodelling in the adult skeleton is a continuous process involving the removal of old or damaged bone and the formation of new bone. This process is regulated by three cell types: Osteocytes (mechanosensory cells), osteoclasts (bone resorbing cells), and osteoblasts (bone forming cells), whose proliferation, differentiation, and activity are regulated by local and systemic factors. Physiological bone remodelling supports fracture healing [40], maintains bone strength [41, 42], adapts to mechanical stimuli [43], and ensures

mineral homeostasis [44]. However, imbalances in this process can lead to diseases, such as Paget's disease [45] and rheumatoid arthritis [46] from excessive bone resorption [47], or osteopetrosis from irregular bone formation [48]. Insights into bone homeostasis and remodelling mechanisms are essential for preventing and treating these disorders.

Bone remodelling consists of four key phases: Activation, resorption, reversal (transition) and formation. The resorption of old bone and the formation of new bone occurs over the course of approximately 3-6 months, whereby cells directly and indirectly communicate with each other to regulate a cycle.

Activation

Osteocytes play a central role in bone remodelling [49], reacting to biochemical signals and sensing mechanical stress [50-52]. Fatigue or unloading of osteocytes leads to osteocyte apoptosis, attracting osteoclast precursors and osteoclasts to sites of bone damage [53-55]. Osteoclast precursors, derived from the haematopoietic stem cell niche in the bone marrow, must migrate through the bloodstream and across the endothelial cell layer via either paracellular or transcellular routes. Stromal cell-derived factor-1 (SDF-1, also known as C-X-C ligand 12 or CXCL12), secreted by endothelial cells and bone marrow stromal cells (BMSCs), binds to C-X-C chemokine receptor type 4 (CXCR4) on osteoclast precursors, facilitating their migration from the bloodstream to the bone [56].

Once osteoclast precursors reach the bone surface, they undergo differentiation into mature osteoclasts, driven by the signalling molecules receptor activator of nuclear factor κ B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). M-CSF, secreted by osteoblasts and BMSCs, binds to its receptor c-FMS on osteoclast precursors, promoting their proliferation and survival by activating the PI3K/Akt and extracellular signal-regulated kinase (ERK) pathways [57-61]. RANKL, expressed by osteoblasts and BMSCs, binds to RANK on precursor cells, activating several signalling pathways essential for osteoclast differentiation and activation, including proto-oncogene tyrosine-protein kinase (Src) pathway, and inhibitor of NF- κ B kinase (IKK) pathways, as well as the three mitogen-activated protein kinase (MAPK) pathways: c-Jun N-terminal kinase (JNK) [62-64], p38 and ERK [57, 58, 65, 66], and calcium signalling [67-69]. TRAF6-dependent RANK signalling upregulates the master transcription factor NFATc1, essential for osteoclastogenesis [66, 67, 70, 71]. NFATc1 drives the expression of genes involved in osteoclast differentiation and function [60], including dendritic cell-specific transmembrane protein (DC-STAMP) and c-Fos, which regulate tartrate-resistant acid phosphatase positive (TRAP⁺/TRAcP⁺) mononuclear cell (TRAP⁺ pre-OCs) fusion and multinucleation into large, multinucleated osteoclasts [72-74], as well as the bone resorption process [75, 76]. Key markers of osteoclast development are vitronectin (VN) receptor (α v β 3), TRAP, and matrix metalloproteinase 9 (MMP-9) [77, 78]. TRAP is expressed in both precursors and mature osteoclasts [79, 80], integrin α v β 3 is abundant in mature, resorbing osteoclasts [81, 82], and MMP-9 is present throughout osteoclast development [83], and aids ECM

degradation by osteoclasts [84]. The activation phase of bone remodelling typically lasts from several hours to a few days [85].

Resorption

Mature osteoclasts adhere to the bone surface via integrin receptors, primarily $\alpha v \beta 3$ integrin, binding to the arginine–glycine–aspartic acid (RGD)-sequence of ECM proteins and forming sealing zones that isolate the resorption area [86, 87]. Upon attachment, mature osteoclasts develop a ruffled border facing the bone matrix, which increases surface area to facilitate enzyme secretion [88, 89]. Through this ruffled border, osteoclasts release hydrogen (H^+) ions via vacuolar-type ATPase (V-ATPase) proton pumps [90-93], acidifying the resorption area, and secrete enzymes such as TRAP, cathepsin K (CATSK) and MMPs into the resorption pits (Howship's lacunae) to degrade both the mineralised and organic components of bone [13, 89, 94-96]. Chloride channels, such as CLC-7, maintain electrochemical balance [97, 98]. Bone degradation products are then endocytosed at the ruffled border and exocytosed at the basolateral membrane for removal [89, 92, 93, 99, 100].

Osteoclast attachment to and detachment from the bone surface is a dynamic process mediated by integrin engagement and Pyk2/Src/Cbl complex formation, which enables osteoclasts to migrate during bone resorption [87, 101]. Additionally, RANK/RANKL signalling, mediated by TRAF6, supports osteoclast activation and resorptive function [70], while DC-STAMP [76, 102] and osteoclast stimulatory transmembrane protein (OC-STAMP) [102] regulate resorptive activity, particularly in physiological and pathological contexts. Bone resorption can occur in both cyclic and continuous modes [103]. In cyclic bone resorption, osteoclasts follow discrete phases of adhesion, resorption, detachment, migration to a new site, and resorption at the new location [99]. In continuous resorption, osteoclasts maintain constant contact with the bone surface, gradually progressing along the bone and creating continuous resorption tracks known as trenches [99, 104]. The RANKL to OPG ratio critically regulates the formation of mature, multinucleated osteoclasts and subsequent bone resorption [105, 106]. OPG, a decoy receptor secreted by osteoblasts and osteogenic stem cells [107-110], binds to RANKL, preventing its interaction with RANK and thereby inhibiting RANK/RANKL signalling, which suppresses osteoclastogenesis and bone resorption. RANKL expression is stimulated by factors like interleukin (IL)-1 β , IL-6, IL-11, IL-17, tumour necrosis factor (TNF)- α , parathyroid hormone (PTH), 1,25-Dihydroxy-Vitamin D3 [$1\alpha,25(OH)_2D_3$] and Wnt family member 5A (Wnt5a) [111-116]. OPG expression is induced by bone morphogenic protein (BMP)-2, IL-1 β , IL-4, IL-13, 1,25-Dihydroxy-Vitamin D3 [$1\alpha,25(OH)_2D_3$] and TNF- α [117-120].

During resorption, osteoblast differentiation and activity are actively suppressed to allow complete removal of old bone. Osteoclasts and osteoclast precursors release semaphorin-4D (Sema4D) to inhibit osteoblastogenesis [121, 122], ensuring effective bone resorption. The bone resorption phase typically spans approximately 22 to 30 days [123, 124].

Reversal (transition)

Once bone resorption is complete, osteoclasts typically undergo apoptosis, a process regulated by signals from osteoblasts and the bone environment. Osteoblasts release factors like Fas ligand (FasL) [125, 126] and Semaphorin-3A (Sema3A) [127] to inhibit bone resorption and promote osteoclast apoptosis. The Ephrin-B2 (EFNB2)/Ephrin type-B receptor 4 (EPHB4) pathway also aids the reversal phase by inhibiting osteoclast differentiation and stimulating osteoblast differentiation and survival [128]. The transition from resorption to formation typically spans approximately 5-8 days (short reversal phase) [123, 129] but can extend to 2-5 weeks in trabecular bone or during pathological bone remodelling (long reversal phase) [129, 130].

Formation

During bone formation, mesenchymal progenitor cells differentiate into pre-osteoblasts and then mature osteoblasts, that proliferate, synthesise the organic matrix, and mineralise the bone matrix [8-10]. This process is regulated by signalling pathways like Wnt/ β -catenin [131, 132], BMP [133], Hedgehog (Hh) [134, 135] and Notch [136], alongside transcription factors runt-related transcription factor 2 (Runx2/Cbfa1) [137, 138], osterix (Osx/SP7) [139], and activating transcription factor 4 (ATF4) [140]. Osteoblasts secrete osteoid (unmineralised bone matrix) and ECM proteins such as alkaline phosphatase (ALP) [141, 142], COL [143-146], OCN [147] and BSP [148, 149]. Mineralisation involves calcium and phosphate ion nucleation and HA crystal formation $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ [19, 22, 24], facilitated by ALP [150-152] and regulated by OCN, which can bind to HA [23, 25].

Activated osteoblasts have four cell fates and can: 1) terminally differentiate into osteocytes by becoming embedded in the mineralised bone matrix [153]; 2) remain as quiescent bone-lining cells with potential for reactivation [154, 155]; 3) undergo apoptosis [126]; or 4) de-differentiate into osteoprogenitor-like cells [156]. Bone formation typically lasts 89 days [123], but can range from 28 days [157] to four months [123].

1.2 Cancer

Cancer is a leading cause of death worldwide, responsible for approximately 10 million cancer deaths (9.74 million deaths) in 2022 [158], an increase from 7.6 million in 2008 [159]. Globally, nearly 20 million new cancer cases (19.96 million) were recorded in 2022 (including keratinocyte carcinomas), with lung cancer being the most commonly diagnosed (12.4% of all sites), followed by female breast (11.6%), colorectal (9.6%), prostate (7.3%), and stomach (4.9%) cancers [158]. Lung cancer also caused the most cancer deaths, with approximately 1.8 million fatalities (18.7% of all sites) in 2022 [158]. This was followed by 904 000 deaths from colorectal (9.3%), 757 000 deaths from liver (7.8% of all sites), 666 000 deaths from female breast (6.9% of all sites), 660 000 deaths from stomach (6.8% of all sites), and 397 000 deaths from prostate cancers [158]. By 2050, new cancer cases are expected to surpass 35 million

(35.3 million) [158], emphasising the critical need for innovative approaches in cancer screening, diagnosis, treatment, and enhanced healthcare infrastructure.

Approximately 90% of cancer deaths are caused by metastasis rather than the primary tumour [160]. Tumour metastasis involves multiple steps including cancer cell dissemination from the primary tumour site, entry into the blood stream (intravasation), survival and arrest in the circulation, exit from the blood stream (extravasation), infiltration, and colonisation of distant organs, thereby allowing cancer to spread from its primary site to another organ of the body (metastasis). This metastatic cascade is quite complex and highly inefficient, with not even 0.1% of circulating cancer cells successfully forming metastases [161].

1.2.1 Osteosarcoma

Osteosarcoma is the most common primary malignant bone tumour [162], although it is considered a rare form of cancer, with an annual incidence of 3.4 cases per million people [162]. It mainly affects young individuals aged 5–25 years, commonly targeting the metaphyseal plates of the distal femur or proximal tibia (74.5%), with less frequent involvement of the proximal humerus (11%) [162]. A smaller incidence peak occurs in adults aged 70–84 years, with common sites including the femur, tibia, fibula (27%), and pelvis (19%) [162]. Osteosarcoma can be categorised into central, intramedullary, and surface subtypes, with conventional osteosarcoma, a central subtype, accounting for 80% of all diagnoses [163].

The disease is characterised by abnormal osteoid (unmineralised matrix) production by transformed osteoblasts (bone-forming cells), disrupting ECM remodelling via MMPs and heparinases, reducing bone mineral density (BMD), and increasing fracture risk [162, 164]. Genetic and epigenetic alterations affecting osteoblast differentiation from MSCs contribute to its pathogenesis, and some cancer-initiating cells may resemble osteoprogenitors [165]. Once diagnosed, the mortality rate is 41.2% [162]. The average 5-year survival rate is 57.7%, with better outcomes for localised cases (61.6%) compared to metastatic cases, especially with lung involvement (20–30%) [162]. Late-onset osteosarcoma has a lower survival rate (27%) than young-onset disease (61.6%) [162].

1.2.2 Cancer metastasis

Extracellular matrix composition in healthy lung, breast, and prostate tissues

The connective tissue matrix and basement membrane, collectively the ECM, supports organ function in lung, breast, and prostate tissues, enabling mammary gland development and lactation cycles [166], alveolar gas exchange [167], and glandular function [168]. Composed primarily of collagens [COL type 1 and VI (COL1 and COLVI)], elastin, proteoglycans, and glycoproteins [166–168], it provides strength, elasticity, adhesion, and growth factor regulation [169]. Notably, McCabe *et al* [170] showed in mice that ECM protein composition can vary nearly 4000-fold between organs, highlighting its tissue-specificity.

Cancer cell dissemination and intravasation

Cancer cells escape primary tumours by exhibiting motility [171], degrading the ECM [172, 173], and resisting anoikis – a form of programmed cell death triggered when anchorage-dependent cells detach from the ECM [174]. This process often involves full or partial epithelial-to-mesenchymal transition (EMT) [171], enabling both collective and individual (single cell) migration with significant plasticity [175-178]. Collective migration, reliant on cell-cell junctions, occurs during embryogenesis [179], wound healing [180], and cancer metastasis [176]. While it resembles single cell migration [181], it can proceed independently of focal adhesions [182]. Conversely, single-cell migration involves integrin-mediated mesenchymal and amoeboid modes. Mesenchymal single-cell migration, better termed invasion, relies on active ECM degradation via MMPs [173] and urokinase-type plasminogen activator (PLAU) [172], alongside cycles of protrusion, integrin engagement, focal adhesion formation, actomyosin contraction, and retraction [183-185]. In contrast, amoeboid migration involves squeezing through the narrow gaps of the ECM without degradation [175, 177]. Integrins, which consist of an α and a β subunit, form 24 distinct heterodimer combinations in humans, with 18 different α and eight β subunits [186]. They mediate adhesion and migration by binding ECM components, stabilising the cell membrane, and connecting to the actin cytoskeleton via focal adhesion proteins [187, 188]. Integrin bidirectional signalling regulates adhesion, motility, and survival, with temporal FAK/Rho GTPase signalling balancing attachment and detachment during migration [189, 190]. Integrin activity and affinity can be suppressed by Ras/Raf/MAPK signalling [191, 192]. Additionally, tumour cells recruit endothelial cells [193], leukocytes, and tumour-associated macrophages (TAMs) [194, 195] to remodel the microenvironment and enhance dissemination.

Survival in transit

In the circulation, tumour cells face shear forces, immune attacks, and substrate absence [196, 197]. They employ immune escape strategies like platelet or macrophage interactions, and produce cytokines to evade natural killer (NK) cell attacks [195, 198-200], and activate survival pathways, such as transforming growth factor beta (TGF)- β signalling [201, 202]. Dormancy [203, 204], and altered gene expression enable cells to resist apoptosis [205, 206], increasing survival chances in transit.

Arrest and extravasation at the secondary site

Tumour cells arrest in the bloodstream, adhere to endothelial cells, and invade the basement membrane (trans-endothelial migration) using adhesion molecules like integrins and selectins [207]. This process depends on capillary bed size, local blood flow, and coagulation factors such as thrombin and von Willebrand factor (vWF), which stimulate blood clot formation and tumour cell adhesion to the vessel bed [208]. Signalling pathways like PI3K/Akt [209] and host cell interactions (e.g., with platelets or astrocytes) [210, 211] further facilitate extravasation.

Tumour colonisation and dormancy

Successful metastasis requires disseminated tumour cells (DTCs) to grow at secondary sites [212]. Many DTCs enter dormancy (G0-G1 cell cycle arrest) [213], evading treatment [203], and enabling recurrence. ECM remodelling and its degradation products can reactivate dormant cells [214-217]. In bone, osteoblasts (bone-forming cells) maintain dormancy, while osteoclasts (bone-resorbing cells) can reactivate dormant tumour cells [218], potentially leading to disease progression.

Seed and soil hypothesis

Paget's seed and soil hypothesis proposed in 1889 suggests that metastasis is non-random, with tumour cells (the 'seeds') preferentially metastasising to specific organs (the 'soil') that provide a favourable microenvironment for their growth [219]. Primary tumours can initiate pre-metastatic niche formation in specific organs by releasing exosomes and extracellular vesicles (EVs) into the blood stream [220, 221]. Cancer-associated fibroblasts (CAFs), key components of the tumour stroma, further prime the seed and soil by secreting ECM components [222, 223], MMPs [222, 224], cytokines [225-227], chemokines [228], growth factors [229-231], exosomes [232], and immunosuppressive factors [233, 234]. Together, these molecules drive immune evasion, angiogenesis, and ECM remodelling, creating a supportive environment for tumour growth and colonisation. Mechanical and anatomical factors like vascular and lymphatic systems also influence metastasis patterns.

1.2.3 Skeletal metastases

Cancer commonly disseminates to the lymph nodes, lungs, liver and bones [235-237]. Skeletal metastases are frequently detected in advanced stages of breast (70-100%) and prostate cancer (68-100%), with lower incidences reported in lung (26-50%), kidney (26-50%), and thyroid cancer (42%) [238, 239]. Metastatic bone disease also occurs in nearly every patient with multiple myeloma ($\geq 95\%$) [240]. Bone is among the most frequent sites for cancer metastasis for several reasons. The fenestrated capillaries in the bone marrow regions provide extensive blood flow and exchange of molecules, which support the formation and growth of bone metastases [241, 242]. Adhesive molecules synthesised by tumour cells can bind to the bone matrix and to cells in the bone marrow stroma [243-245]. Bone stores a lot of growth factors [e.g., BMP, fibroblast growth factor (FGF), TGF- β] and calcium [246]. When the inorganic and organic ECM are degraded during bone resorption, these factors are released and stimulated [242, 246]. For instance, TGF- β not only couples bone resorption and bone formation [133], but also enhances tumour cell proliferation [247] and invasion [248], thereby promoting tumour progression. In breast cancer patients, metastatic bone lesions are commonly found in the skull, followed by the proximal femur and humerus [219]. Prostate cancer tends to metastasise to the spine and pelvis, but lesions can also appear in the ribs, humerus, sternum, and femur [249]. In lung cancer, skeletal metastases are predominantly located in the spine, ribs, and pelvis [250-253]. Secondary bone cancers can cause severe

skeletal complications, substantially impairing the patient's quality of life (QOL). The median overall survival (OS) following a diagnosis of bone metastases is approximately 6 months [254]. Though prognosis varies widely depending on the primary malignancy, with life expectancy ranging from 2.8 months to nearly 8 years (57 months) [254, 255]. While curative treatments for bone metastases remain elusive, disease progression and symptoms can often be controlled. Thus, a more comprehensive analysis of metastatic progression could contribute to the development of more effective treatment approaches, ultimately benefiting patient QOL.

Classification of bone metastases

Secondary bone cancer exerts distinct effects on bone remodelling—osteolytic, osteosclerotic, or mixed—which vary not only between tumour types but also among patients. Osteolytic (bone-resorbing) skeletal metastases are characterised by excessive bone resorption, while osteoblastic or osteosclerotic (bone-forming) skeletal metastases exhibit enhanced bone formation. Mixed skeletal metastases display both osteolytic and osteoblastic features.

Approximately 10-20% of bone metastases from breast cancer are osteoblastic [256, 257], 48% are osteolytic, and 38% are mixed [257, 258]. However, an earlier study by Walther *et al* [259] reported a considerably higher proportion of osteolytic metastases (80-90%). The majority of skeletal metastases in prostate cancer patients are considered osteosclerotic [249, 260, 261]. Yet, osteolysis may actually precede the dominant osteoblastic activity [262, 263], suggesting the presence of a mixed phenotype in patients with prostate cancer bone metastases. In lung cancer patients, skeletal metastases are predominantly osteolytic (ca. 65%), followed by mixed bone metastases (around 24%). Osteoblastic metastases are less frequently detected in lung cancer cases (around 11%) [264, 265]. Notably, cancer metastases to bone can also be heterogenous within an individual patient, with some skeletal metastatic sites displaying an osteolytic phenotype and others showing osteoblastic or mixed lesions [266].

Skeletal related events

Skeletal metastases disrupt the bone remodelling process and can have a significant impact on the skeleton, ultimately affecting a patient's QOL. Bone degradation, mixed or sclerotic lesions, cytokine release, and tumour invasion into surrounding nervous tissue can lead to various skeletal complications, known as skeletal related events (SREs). SREs include, among others, microfractures, pathological fractures [267], hypercalcaemia [268], spinal instability [269] and spinal cord compression [267]. In addition, patients experience metastatic cancer-induced bone pain [270-272] and the pain may be so intense that the patient becomes unable to sit, stand, or walk [271].

Therefore, enhancing our understanding of the complex mechanisms underlying cancer metastasis to bone is essential for translating these insights into clinical practice. Such

advancements could improve not only the care and QOL of patients, but also early detection, diagnosis, and overall disease outcomes. Among the molecular factors implicated in tumour progression and skeletal metastases, BSP has emerged as an interesting candidate. Initially characterised as a mediator of bone formation and matrix mineralisation, BSP has also been increasingly implicated in processes such as bone resorption and cancer biology. BSP may influence tumour cells by affecting their growth and spread, potentially contributing to the development of skeletal metastases and impacting bone remodelling dynamics. These dual roles underscore the importance of BSP as an important focus for advancing our understanding of skeletal metastasis.

1.3 Bone sialoprotein

Bone sialoprotein, also known as integrin-binding sialoprotein (IBSP) or bone sialoprotein-2 (BSP2), is part of the small integrin-binding ligand, N-linked glycoprotein (SIBLING) family, which includes OPN, dentin matrix protein 1 (DMP-1), dentin sialophosphoprotein (DSSP) and matrix extracellular phosphoglycoprotein (MEPE) [273]. The human SIBLING genes are located within a 375 kb region on chromosome 4q21 [274]. Despite low sequence homology, SIBLING proteins share similar exon structures and a conserved arginine–glycine–aspartic acid (RGD) cell-attachment sequence, which facilitates cell attachment and signalling [274]. These proteins are mainly expressed in bone and dentin, and secreted into the osteoid [38, 148]. Their activity and function are influenced by post-translational modifications (PTMs) like glycosylation, phosphorylation and sulphation [275].

1.3.1 Gene structure of bone sialoprotein

Bone sialoprotein is encoded by the 15-kb IBSP gene on chromosome 4q21.1 [276], consisting of seven exons and six introns. Exon 1 is non-coding, while exons 2-7 encode functional elements: the signal sequence (exon 2), phosphorylation sites by casein kinase II (CK2) (exons 3 and 5), a proline-rich region (exon 4) [277], a glycosaminoglycan (GAG)-binding domain (exon 6) [278], and over 50% of the protein, including the RGD sequence, in exon 7. Polyglutamic acid residues are encoded in exons 5 and 7 [279]. BSP transcription is regulated by transcription factors like CRE binding protein 1 (CREB1), phosphorylated CREB1, Runx2, Msh homeobox 2 (Msx2), distal-less homeobox 5 (Dlx5), SMAD family member 1 (SMAD1), activator protein 1 (AP-1) family members (c-Fos, c-Jun, JunD, Fra2) [280-283], and co-activator p300 [281]. The gene structure of BSP is shown in figure 1.

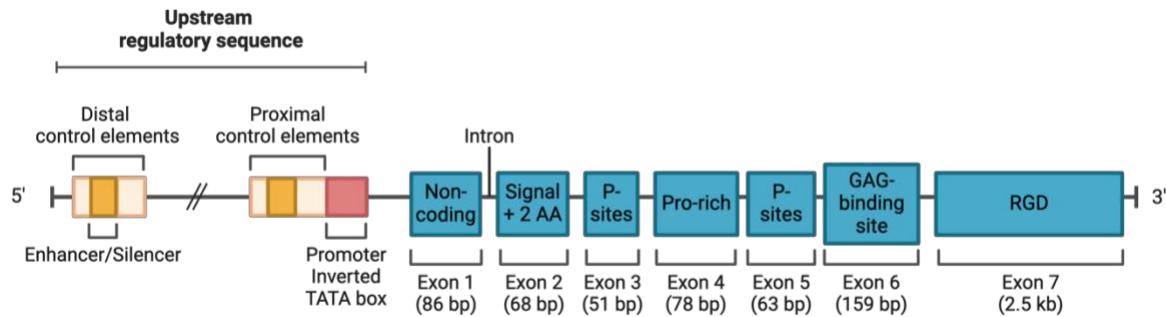


Figure 1. Human BSP gene structure with the proximal and distal promoter.

AA, amino acids; bp, base pairs; BSP, bone sialoprotein; GAG, glycosaminoglycan; P, phosphorylation; Pro, proline; RGD, arginine–glycine–aspartic acid. Modified from Bouleftour *et al* [278] and Ogata *et al* [277]. Created with BioRender.com.

1.3.2 Protein structure of bone sialoprotein

Native human BSP is a 317-amino acid protein, including a 16-amino-acid signal peptide [284], with a core molecular weight of 33.6 kDa [285]. PTMs like N- and O-linked glycosylation, serine (Ser) and/or threonine (Thr) phosphorylation, and tyrosine (Tyr) residue sulphation, increase its weight to 70-80 kDa [286, 287]. Three polyglutamic acid-rich regions [288] — comprise ~22% of the protein [289], contribute to its acidity and roles in osteoblast differentiation [149], mineralisation [149, 290], and osteoclast regulation [291].

The N-terminal region has an L-H-R-R-V-K-I (Lys-His-Arg-Arg-Lys-Val-Ile) sequence that binds to heparin and heparan sulphate proteoglycans [284], potentially enhancing RGD-integrin interactions [292]. Additionally, a collagen-binding site [293], and tyrosine-rich areas [290], which may be sulphated, are located near the N-terminus. The central and N-terminal domains host polyglutamic acid-rich sequences [288] that regulate HA nucleation and binding [294-298]. The C-terminal domain contains the RGD motif critical for cell adhesion and integrin-mediated signalling [278, 299, 300], supported by Tyr-rich regions [285, 301] and dependent on native protein structure [287]. Structurally, BSP is highly flexible, with low α -helix (5%), high β -sheet (32%), and substantial random coil (46%) [287], enabling interactions with binding partners [302, 303] and supporting roles in matrix mineralisation, cell-matrix interactions, and adhesion. This open structure underpins BSP's versatility in biological functions. A simple model of the unfolded human BSP protein structure is shown in figure 2.

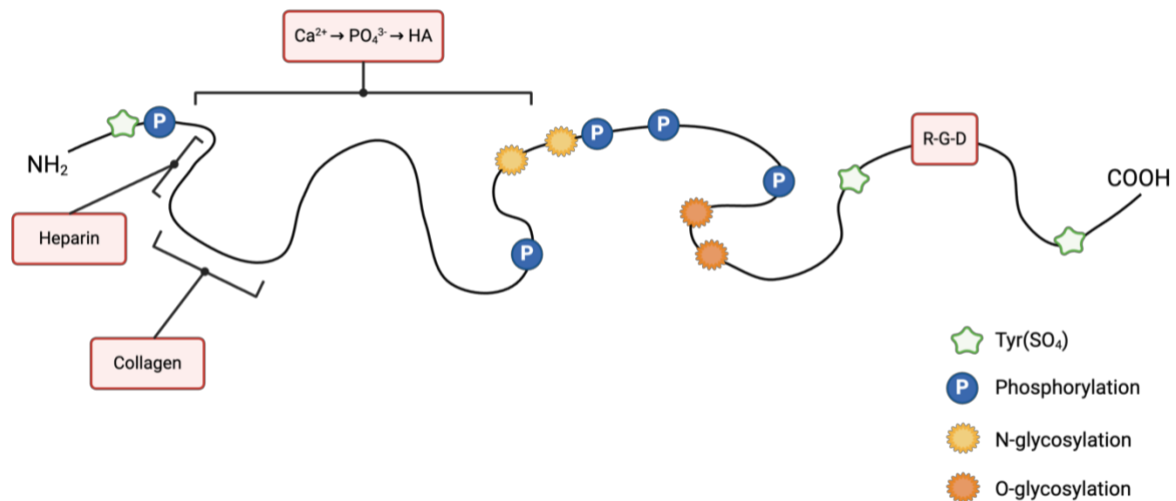


Figure 2. Unfolded protein structure of human BSP.

BSP, bone sialoprotein; HA, hydroxyapatite; RGD, arginine–glycine–aspartic acid; Tyr(SO₄), tyrosine sulfation. Modified from Bouleltour *et al* [278] and Ganss *et al* [304]. Created with BioRender.com.

1.3.3 Synthesis and expression of bone sialoprotein

BSP is produced by various cell types including osteoblasts [148, 305], osteoclasts [148], osteocytes [148, 305], BMSCs [306], chondrocytes [148], platelets [307], and placental trophoblast cells [148]. Its mRNA and protein expression are regulated by hormones, growth factors, cytokines and other factors, such as PTH [308, 309], PTH-related peptide (PTHrP) [310], oestrogen receptor alpha (ERα) [311], IL-8 [312], 1,25-dihydroxyvitamin D3 (calcitriol) [313], IL-11 [314, 315], FGF-2 [281, 283, 316], platelet-derived growth factor BB (PDGF-BB) [282], insulin-like growth factor 2 (IGF-2) [317], TNF-α/TNF [318], calcium hydroxide [Ca(OH)₂] [319, 320], MMP-2 [321], and MMP-9 [322].

BSP expression is predominantly found in mineralised connective tissues. It is highly abundant in the bone ECM [148], comprising approximately 11-15% of the non-collagenous proteins [323, 324], compared to less than 1% in dentin [324]. BSP is expressed by osteoblasts [148, 325, 326], osteoclasts [148], and osteocytes [148, 325]. BSP is also detected in dentin [274], chondrocytes [148], as well as in placental trophoblast cells [148, 325], kidney macrophages and tubular cells [327], and pancreatic islet, ductal, and smooth muscle cells [328]. Notably, BSP is also synthesised and expressed in (osteotropic) tumours.

1.3.4 Expression of bone sialoprotein in cancer

Expression of bone sialoprotein in primary and secondary cancer tissue and tumour cells

BSP is expressed in a wide range of cancer cell lines and tumour cells associated with primary and secondary malignancies. Positive BSP protein expression is detected in glioma, breast, oral squamous cell, kidney, multiple myeloma, lung, osteosarcoma, ovarian, pancreatic, and prostate cancers, with predominantly cytoplasmic, heterogenous staining of tumour cells (table 1). Notably, primary breast tumours from patients who develop bone

metastases show significantly higher BSP expression compared to those without metastases [329]. Healthy or benign tissues generally exhibit minimal or no BSP protein expression.

Cancer (Subtype)	Protein expression of bone sialoprotein		Quantification	Reference
	Normal (n)	Tumour (n)		
Bone (OS)	–/o ^{Matched tissue}	+++* (32)	NA	[330]
Brain (HGG, LGG)	(16)	100% positive* (270) 73% + 27% +++	IHC	[331]
Breast (IDC, ILC)		87.7% positive Heterogenous staining intensity (+/+/+/+++)	IHC	[329]
Breast	–/o	89% positive (454)	IHC	[332]
Breast (in situ carcinoma, invasive carcinoma)	–/o ^{Matched tissue}	87% positive* (51) Cytoplasmic	IHC	[333]
Oral mucosa (OSCC)	–	90% positive (87) Cytoplasmic and perinuclear	IHC	[334]
Kidney with bone MET, non-bone MET or no MET	Macrophages and tubular cells ^{Matched tissue}	85% positive (75) Heterogenous staining intensity (+/+/+/+++)	IHC	[327]
Bone marrow (MM)		100% positive (5) Heterogenous staining intensity (+/+/+/+++)	Double IF FACS	[335]
Lung (LUSC, LUAD, BAC)	–/o Cartilage	100% positive* (48) Heterogenous staining intensity Cytoplasmic and membrane- bound	IHC	[336]
Ovary		21% positive (38) (+/+/+++) 79% –/o	IHC	[336]
Pancreas (PDAC)	80% – (10) 20% +/++ Cytoplasmic in islet, ductal, and smooth muscle cells	30% positive (20) Diffuse staining of nearby non- target cells	IHC	[328]
Prostate (negative BPH, pAdC)	16% (64) Cytoplasmic and perinuclear	80% positive Cytoplasmic and perinuclear	IHC	[337]
Prostate		100% positive (5) Cytoplasmic with diffuse staining of nearby non-target cells and ECM	IHC	[338]
Prostate	–/o	78.9% positive (180) Heterogenous staining intensity (+/+/+/+++) Mainly cytoplasmic	IHC	[339]

Table 1. Protein expression of BSP in primary tumours and healthy tissue.

–, undetectable/no expression; o, very low expression; +, low expression; ++, moderate expression; +++, high expression. *P<0.05 compared to control. BAC, bronchioalveolar; BPH, benign prostate hyperplasia; BSP, bone sialoprotein; ECM, extracellular matrix; FACS, fluorescence-activated cell sorting; HGG, high-grade glioma; IF, immunofluorescence; IDC, invasive ductal carcinoma; IHC, immunohistochemistry; ILC, invasive lobular carcinoma; LGG, low-grade glioma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell; MM, multiple myeloma; OS, osteosarcoma; OSCC, oral squamous cell carcinoma; pAdC, prostatic adenocarcinoma; PDAC, pancreatic ductal adenocarcinoma.

Tumours actively synthesise BSP, with elevated mRNA levels observed in glioma, breast, colon, gastric, kidney, lung, osteosarcoma, pancreatic, rectal, and thyroid tumour

tissue compared to normal tissue (table 2). Moderate to strong BSP mRNA is particularly evident in prostate cancer [339] and multiple myeloma cells [335]. Table 3 summarises BSP protein and mRNA expression in human tumorigenic and non-tumorigenic cell lines. Limited data exists on BSP protein expression in pancreatic cancer and mRNA expression in lung cancer cell lines.

Cancer (Subtype)	mRNA expression of bone sialoprotein		Source (n)	Quantification	Reference
	Normal (n)	Tumour (n)			
Bone (OS)	+	+++* (85)	TARGET database		[330]
Bone (OS)	–/o	+++*	Matched tissue (15)		[330]
Brain	++				
(HGG)		+++*† (30)	Patients	RT-PCR	[331]
(LGG)		+++* (25)			
Brain (GBM)	++	+++ ^{NA}	TCGA		[340]
Breast	o				
(Ductal)		+++* (31)	Matched tissue (53)	Cancer profiling array	[341]
(Lobular)		+ (31)			
Breast with bone MET and non-bone MET		+++ (6)	Patients	RNA sequencing	[342]
Breast		+++	GEPIA2 database		[342]
Breast		+ / +++ / +++ (18)	Patients	RT-PCR	[343]
Colon	o	+++*	Matched tissue (39)	Cancer profiling array	[341]
Gastric (stomach)	o	+++*	Matched tissue (27)	Cancer profiling array	[341]
Kidney	o	+++*	Matched tissue (21)	Cancer profiling array	[341]
Bone marrow (MM)		++ / +++ (5)		<i>In situ</i> hybridisation	[335]
Lung	o	+ ^{NS}	Matched tissue (21)	Cancer profiling array	[341]
Lung (LUAD)	o	+++*	TCGA		[344]
Lung (LUSC)	o	+++*			
Lung (LUAD)		80% + / +++ / +++ (10)	Patients	RT-PCR	[336]
Ovary	o	++ ^{NS}	Matched tissue (17)	Cancer profiling array	[341]
Pancreas (PDAC)	41% o 59% – (27)	86% +* (29)	Patients Organ donors	RT-PCR	[328]
Prostate	o	++	Matched tissue (4)	Cancer profiling array	[338]
Prostate	–/o	+++ (8)	Patients	<i>In situ</i> hybridisation	[339]
Rectum	o	+++*		Cancer profiling array	[341]
Thyroid	o	+++*		Cancer profiling array	[341]
Uterine (Endometrium)	o	+ ^{NS}		Cancer profiling array	[341]

Table 2. mRNA expression of BSP in primary tumours and healthy tissue.

–, undetectable/no expression; o, very low expression; +, low expression; ++, moderate expression; +++, high expression. *P<0.05 compared to control. †compared to LGG. GBM, glioblastoma; GEPIA2, gene expression profiling interactive analysis 2; HGG, high-grade glioma; LGG, low-grade glioma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell; MM, multiple myeloma; NA, not available; NS, non-significant; OS, osteosarcoma;

PDAC, pancreatic ductal adenocarcinoma; TARGET, therapeutically applicable research to generate effective treatments; TCGA, the cancer genome atlas.

Cell/Tumour Type	Cell (line)	BSP expression		Quantification	Reference
		mRNA	Protein		
Bone					
Primary osteoblast	hOB	+++		RT-PCR	[330]
Bone cancer					
OS	MG-63	+++		RT-PCR	[330, 345]
OS	SaOs-2	+++			
Bone marrow					
MM	L363	+ / ++	+++	In situ hybridisation FACS	[335]
MM	LP-1	+ / ++	o		
MM	MMS-1	+ / ++	+		
MM	OPM-1	+ / ++	++		
MM	U266	+ / ++	+++		
Breast					
Immortalised breast epithelial	MCF-10A		o	Western blot	[346]
Breast cancer					
BAC	MCF-7	++		RT-PCR	[343, 346, 347]
BAC	MDA-MB-231	o	+++		
BDC	T-47D	+			
Lung cancer					
NSCLC	A-549		+++	Western blot	[344]
NSCLC	CL1-5		++		
Pancreas	Activated PSCs	o		RT-PCR	[328]
Pancreatic cancer					
PDAC	ASPC-1	+		RT-PCR	[328]
PDAC	BxPc-3	+			
PDAC	Capan-1	–			
PDAC	Colo-357	+			
PDAC	MIA PaCa-2	–			
PDAC	Panc-1	–			
PDAC	SU86.86	+			
PDAC	T3M4	+			
Prostate					
Primary epithelial	HuPepiC	+	–	RT-PCR	[312, 337]
Immortalised epithelial	RWPE1	– / +	o / –	Western blot	
Immortalised BPH	WPE1-NB26	+	+		
Prostate cancer					
Derived from LNCaP	C4-2B	–		RT-PCR Western blot	[312, 337, 345, 348]
pAdC	DU145	o	+++		
pAdC	LNCaP	– / o / +++	+		
pAdC	PC-3	+++	+		

Table 3. Protein and mRNA expression of BSP in primary cells and cell lines of healthy and tumorigenic origin. –, undetectable/no expression; o, very low expression; +, low expression; ++, moderate expression; +++, high expression. BAC, breast adenocarcinoma; BDC, breast ductal carcinoma; BPH, benign prostatic hyperplasia; BSP, bone sialoprotein; hOB, human osteoblast; HuPepiC, human prostate epithelial cell; MM, multiple myeloma; NSCLC, non-small cell lung cancer; OS, osteosarcoma; pAdC, prostatic adenocarcinoma; PDAC, pancreatic ductal adenocarcinoma; PSC, pancreatic stellate cell.

BSP expression is also present in metastatic tumour cells. Moderate to strong BSP protein expression is observed in 100% ($n=42$) of tumour cells of bone metastases from breast cancer including invasive ductal carcinoma [347, 349]. 20% (i.e., 6 out of total 30) of primary non-small cell lung cancer (NSCLC) tumours stain positive for BSP protein compared to 80% ($n=24$) of NSCLC bone metastases [350]. 31% (i.e., 8 out of total 28) of NSCLC non-bone metastases [e.g., liver, central nervous system (CNS), adrenal glands] stain positive for BSP

protein [350]. Furthermore, BSP protein is expressed in 100% ($n=9$) of pancreatic ductal adenocarcinoma (PDAC) lymph node and liver metastases [328]. In breast cancer, BSP mRNA levels in bone metastases are significantly higher than in non-bone metastases. This was demonstrated by *in situ* hybridisation, which showed positive, heterogeneous BSP staining in 100% ($n=10$) of tumour cells from intraductal breast cancer bone metastases [347]. RNA sequencing data also support elevated BSP mRNA expression in breast cancer bone metastases [342], though the small sample size ($n=6$) and unreported statistical significance limit interpretation. In contrast, weak BSP mRNA expression is observed in pancreatic cancer lymph node metastases, comparable to primary pancreatic tumours [328]. Overall, research on BSP mRNA synthesis in metastases, particularly bone metastases, remains limited, and variability in quantification methods complicates characterisation.

Interestingly, BSP protein expression is significantly elevated in breast and lung tumours associated with microcalcifications [333, 336], potentially facilitating HA nucleation [295]. This process may alter the tumour microenvironment and prime tumour cells for the bone microenvironment, contributing to the development of bone metastases [333].

Expression of bone sialoprotein in the serum

BSP expression has been quantified in the sera of cancer patients, both with and without metastases, as well as in healthy subjects. Cancer patients generally show higher serum BSP levels than healthy individuals, with further increases in those with bone or non-bone metastases (table 4). However, one study found a trend toward lower serum BSP in PDAC patients compared to controls [328].

Serum BSP levels vary widely between experimental groups and studies, likely due to differences in detection methods. Concentrations above 100 ng/ml were typically reported using competitive enzyme-linked immunosorbent assay (ELISA), which offers high sensitivity but risks cross-reactivity [351]. In contrast, concentrations below 100 ng/ml were generally measured with standard ELISA or radioimmunoassay (RIA). Standard ELISA, employing two antibodies, is highly specific but may underestimate levels if epitopes are inaccessible [352]. RIA, similar to competitive ELISA, uses radiolabelled antigens for detection [353]. The choice of detection method may have significantly affected the sensitivity, specificity, and reliability of BSP quantification.

Cancer (Subtype)	Mean $\pm$ SD (ng/ml)		Quantification	Reference
	Normal (n)	Tumour (n)		
Bone marrow (MM)	$\approx 10.5 \pm 12.97$ (133)	$\approx 39.6 \pm 26.98^*$ (32)	RIA	[354]
Bone marrow (MM)	$\approx 10.1 \pm 6.0$ (139)	$\approx 27.6 \pm 28.8^*$ (62)	RIA	[355]
Bone marrow (MM)	$\approx 12 \pm 2.5$ (68)	$\approx 13.4 \pm 7.5^{NA}$ (16)	RIA	[356]
Breast	154 ± 13 (77)	318 ± 18^{NA} (20)	Competitive ELISA	[357]
Breast	$\approx 10.5 \pm 12.97$ (133)		RIA	[354]
with bone MET		$\approx 17.8 \pm 21.85^*$ (19)		
Colon	154 ± 13 (77)	373 ± 19^{NA} (20)	Competitive ELISA	[357]
Lung	154 ± 13 (77)	155 ± 11^{NA} (20)	Competitive ELISA	[357]
Lung (NSCLC)	12.33 ± 5.29 (110)		ELISA	[358]
with bone MET		$44.61 \pm 15.38^{*†}$ (72)		
with non-bone MET		$22.22 \pm 13.88^*$ (74)		
Pancreas (PDAC)	$\approx 8.0 \pm 1.63$ (8)	$\approx 6.5 \pm 2.58^{NA}$ (8)	RIA	[328]
Pancreas (PDAC)	$\approx 0.79 \pm 1.48$ (39)	$\approx 1.72 \pm 2.07^*$ (132)	ELISA	[359]
Prostate	114 ± 63 (110)	222 ± 98^{NA} (102)	Competitive ELISA	[338]
Prostate	154 ± 13 (77)	285 ± 19^{NA} (20)	Competitive ELISA	[357]
Prostate	7.51 ± 1.31 (42)		ELISA	[360]
with bone MET		$38.93 \pm 9.12^{*†}$ (42)		
with non-bone MET		$9.23 \pm 3.12^*$ (41)		

Table 4. BSP serum levels collected from cancer patients and healthy controls.

* $P < 0.05$ compared to control; † compared to non-bone MET. BSP, bone sialoprotein; ELISA, enzyme-linked immunosorbent assay; PDAC, pancreatic ductal adenocarcinoma; MET, metastasis; MM, multiple myeloma; NA, not available; NSCLC, non-small cell lung cancer; RIA, radioimmunoassay.

1.3.5 Functions of bone sialoprotein

Bone sialoprotein in angiogenesis

BSP promotes angiogenesis *in vitro* (2D and 3D), and *in ovo* through RGD-integrin $\alpha v \beta 3$ interactions [361, 362], MMP-2 activation [362], and increased expression of pro-angiogenic genes [363]. Immobilised BSP in COL1 enhances the proliferation, adhesion and migration of human umbilical vein endothelial cells (HUVECs) [361, 363]. BSP potentially couples angiogenesis with osteogenesis, as it is involved in bone formation, and COL is a key component of the bone ECM [17, 18].

Bone sialoprotein in bone remodelling

Cytomegalovirus (CMV) BSP-overexpressing mice (8-weeks-old) show osteopenia, reduced trabecular bone volume and thickness, greater osteoclast numbers, decreased osteoblast markers (ALP, OCN), and elevated osteoclast markers (RANKL, TRAP) in serum, with increased serum calcium and unchanged phosphorus and magnesium levels compared to wild-type mice [364]. BSP overexpression does not affect osteoblast surface or number, likely due to reduced trabecular bone volume [364]. BSP-deficient mice (4-month-old) show reduced cortical thickness, increased trabecular bone density, delayed mineralisation, and low bone turnover [365]. These results indicate that BSP expression regulates bone remodelling. Further *ex vivo* and *in vivo* studies have investigated its effects on bone formation, osteoclastogenesis, and osteoclast activity.

Osteoblast formation and bone matrix mineralisation

BSP expression is absent in early osteoblast precursors [148, 326], but expressed in mature osteoblasts [148, 326], suggesting a role in late-stage differentiation and mineralisation. *Ex vivo* and *in vitro* studies show that BSP facilitates osteoblast adhesion [326], late-stage osteoblast differentiation [365] and bone matrix synthesis via its RGD [149] and COL-binding regions [293], activating ERK1/2 and Akt signalling [293]. BSP also nucleates HA [366], supporting inorganic bone formation [149, 364, 365].

Osteoclast differentiation and activity

Current evidence indicates that BSP significantly influences osteoclast differentiation and activity, often synergising with RANKL, but sometimes acting independently. At low concentrations (≤ 50 ng/ml), BSP alone has minimal effects on osteoclastogenesis or resorption, but higher concentrations or overexpression can enhance these processes even in the absence of RANKL [291, 344, 367]. For example, high BSP levels or transfection-induced overexpression promote osteoclastogenesis and bone resorption in murine monocyte/macrophage-like RAW264.7 cells without requiring RANKL [367]. Conversely, BSP knockdown or deficiency impairs differentiation and motility, but not resorption activity [365, 368], suggesting BSP is more critical for osteoclast differentiation than activity.

BSP's effects depend on the experimental context. Co-treatment with RANKL enhances osteoclastogenesis and resorption in murine bone marrow derived macrophages (BMMs) and RAW264.7 cells [364], showing synergistic effects compared to BSP or RANKL alone [344, 367]. However, bovine BSP inhibits differentiation in murine BMMs and osteoblast-like cell co-cultures [291], suggesting its impact is modulated by cell-cell interactions and signalling pathways. Mechanistically, BSP enhances RANKL-induced osteoclast differentiation and activity via integrin-dependent and integrin-independent mechanisms, potentially activating calcium channels and triggering intracellular calcium signalling, calcineurin activation, and nuclear translocation of NFATc1 in osteoclast precursors [367]. Activation of NFATc1, a key osteoclastogenic transcription factor, upregulates osteoclast-related genes such as RANK, TRAP, and CTSK [367, 368]. BSP may also engage RANK-associated PI3K/Akt and JNK pathways [367], which regulate differentiation [369, 370] and confer anti-apoptotic effects in mature osteoclasts [367]. Interestingly, BSP-deficiency in murine osteoclasts or pre-osteoclasts does not affect NFATc1 transcription, warranting further investigation into gene regulation [368].

Regarding activity, BSP binds integrin $\alpha v \beta 3$ via its RGD motif at the C-terminal, activating Src kinase and intracellular calcium signalling pathways to support cytoskeletal reorganisation, podosome stability, and bone resorption [291, 367, 368, 371]. Interestingly, the RGD motif appears less important in certain contexts, such as PTH-stimulated bone resorption in mouse BMM-derived osteoclasts [372], where other BSP regions like polyglutamic acid-rich domains near the N-terminus of BSP or PTMs such as phosphorylation may enhance osteoclast activity [291, 372]. Dephosphorylated BSP, for instance, reduces osteoclast resorptive capacity, highlighting the importance of PTMs [372].

Experimental variability complicates conclusions, with studies differing in BSP sources (e.g., human recombinant vs. bovine), BSP concentrations (e.g., 0.0001 ng/ml to 90 µg/ml), cell models, and substrates (natural vs. synthetic). RAW264.7 cells, often used due to their convenience, do not reflect primary human osteoclast biology, as their osteoclastogenesis is rapid and does not require M-CSF [373, 374]. The lack of studies using human cell models like THP-1 (human leukaemia monocytic cell line) [375] or primary human osteoclasts [376] further limits translational relevance. Substrate choice also affects results. While natural mineralised substrates like dentin mimic bone environments, they can influence osteoclast activity differently than synthetic alternatives. For instance, enzyme levels of TRAP and CTSK are higher on natural substrates compared to synthetic alternatives [377], but resorption is often consistent across mineralised substrates (osteologic vs. dentin) [367].

In summary, BSP plays a multifaceted role in osteoclast biology across animal models, promoting differentiation and activity through multiple pathways and interactions with RANKL. Its effects are influenced by concentration, experimental context, and regulatory mechanisms. Standardised methodologies and human-centric models are essential to fully elucidate BSP's mechanisms and clinical relevance.

Bone sialoprotein in cancer progression and immune evasion

Cancer cell proliferation

Cancer cell proliferation drives tumour growth, metastasis, and aggressiveness, influencing disease progression and treatment response [378]. *In vitro* and *in vivo* evidence indicate that exogenous BSP or its overexpression promotes cancer cell proliferation [330, 342, 379-381], while BSP knockdown reduces it [342, 382, 383]. BSP may achieve this by interacting with integrins αv and $\alpha v\beta 5$ [381] and upregulating SMAD family member 4 (SMAD4) [342] and Poly(ADP-Ribose) Polymerase 1 (PARP-1) activity [379]. Though SMAD4 often acts as a tumour suppressor [384], it also supports cancer cell proliferation, migration, and invasion [342]. PARP-1, critical for DNA repair and cell cycle regulation, further facilitates cancer growth [385]. Most findings are based on MDA-MB-231 breast cancer cells; further research is needed to determine BSP's effects on prostate and lung cancer proliferation.

Cancer cell survival

Cancer cell survival enables tumour progression and metastasis by resisting apoptosis, tolerating ECM detachment, evading immunity, enduring hypoxia, and adapting to new microenvironments [174, 386, 387]. BSP enhances breast and prostate cancer cell survival by activating RGD-integrin MEK/ERK (MAPK) signalling [299] and regulating PARP-1 activity to control DNA repair and survival [379, 388]. It may also enable anoikis resistance in lung cancer via the microRNA (miR)-150-5p/MMP-14 axis [389], facilitating metastasis. However, the long-term effects of BSP on cancer cell survival and apoptosis remain underexplored [389].

Complement evasion by binding to factor H

The complement cascade mediates cell lysis to defend against infections and maintain tissue homeostasis [390, 391]. Only one study has explored BSP's role in cancer immune evasion. Fedarko *et al* [392] showed that BSP binds integrin $\alpha\beta 3$ via its RGD motif and recruits factor H, a complement regulatory protein, to inhibit complement-mediated cell lysis. This mechanism, observed in multiple myeloma and breast cancer cells [392], may contribute to immune evasion and tumour metastasis. Further research is needed to confirm BSP's role in cancer cell immune evasion.

Cancer cell adhesion

Cell adhesion refers to the adhesion of cells to each other (cell-cell adhesion) or the ECM (cell-matrix adhesion) and is critical for tissue integrity, repair and function [393, 394], as well as for cell motility [171], including in cancer.

Rat BSP promotes the adhesion of various cancer cell types, including triple-negative breast cancer (TNBC) (MDA-MB-231 and Hs578t), HER2-positive breast cancer (SK-BR-3), melanoma (M21, MDA-MB-435, LCC15-MB), and rat osteosarcoma (UMR-106) cells, across a wide concentration range (0.3–43 $\mu\text{g/ml}$) and short incubation periods (≤ 1 h) [381, 395]. Recombinant rat BSP enhances attachment of MDA-MB-231, Hs578t, and prostate adenocarcinoma (PC-3) cells [299], while human BSP (recombinant or fragments), though less studied, promotes adhesion of MDA-MB-231 and melanoma (MDA-MB-435, LCC15-MB) cells [381], with cyclic peptides showing stronger adhesive properties than linear peptides [300]. Overexpression of BSP in MDA-MB-231 cells enhances adhesion compared to controls [299]. However, recombinant human BSP does not affect the adhesion of PDAC (Capan-1 and SU88686) cells to solubilised basement membrane matrix [328].

BSP-mediated adhesion is primarily driven by its RGD sequence, which binds integrin receptors ($\alpha\beta 3$, $\alpha\beta 5$, and $\beta 3$) on various cancer cell lines (M21, LCC15-MB, MDA-MB-231, MDA-MB-435, SK-BR-3) [381, 395]. This RGD-specific interaction seems to be unique to BSP, as FN-derived GRGDSP peptide does not affect MDA-MB-231 cell attachment [300, 381]. BSP binding activates integrin-dependent signalling pathways, inducing (Src)-MEK/ERK, PI3K/Akt and calpain (calcium-dependent protease family), which promote focal adhesion complex formation that includes vinculin and actin and enhanced attachment [299, 395, 396]. The heparin-binding site LHRRVKI near BSP's N-terminus may synergise with the RGD motif to facilitate adhesion [292], although its function in humans remains unclear, while the collagen-binding site in the N-terminal domain seems to play a minimal role [397]. Notably, the mechanisms of integrin engagement and downstream signalling, such as MEK/ERK (MAPK), may depend on the cancer cell type [299, 395], and the specific integrin receptors involved in osteosarcoma and prostate cancer cell adhesion are still unidentified. It is currently unknown whether BSP stimulates the adhesion of lung cancer cells, emphasising the need for further investigation.

Cancer cell migration and invasion

Cell motility, essential for embryogenesis [179] and tissue regeneration [180], facilitates tumour growth, invasion and metastasis [171, 176]. Migration and invasion are two distinct but related processes: Migration refers to movement of cells through tissue or spaces, which may occur without active matrix degradation (e.g., amoeboid-like movement). In contrast, invasion involves the active transversal of cells through physical barriers, such as collagen IV or Matrigel, through proteolytic degradation of the ECM.

Studies show that endogenous and exogenous BSP enhances motility *in vitro* (scratch, transwell, and matrigel invasion assays) and metastasis *in vivo*. BSP silencing inhibits migration [342, 344, 379, 383] and invasion [342, 344] *in vitro*, and reduces skeletal metastases *in vivo* [344, 383]. BSP stimulates migration and/or invasion in various cancer cell types, including glioblastoma [340], melanoma (M21, MDA-MB-435S) [395, 398], breast (MDA-MB-231, MDA-MB-231-BAG, Hs578t, MCF-7) [299, 342, 380, 398], NSCLC (A-549, CL1-5, NCI-H520) [344] [398], prostate (PC-3, DU-145) [398], thyroid (SW-579) [398] and rat osteosarcoma (UMR-106) cells [395]. However, exogenous BSP does not affect prostate cancer (LNCaP) [398] or osteosarcoma (MG-63, SaOs-2, SK-ES-1) [398] cell invasion and has been shown to suppress invasion in PDAC (Capan-1, SU.86.86) cells [328]. Notably, most studies have focused on exogenous BSP's effects on invasion rather than migration. Additionally, while BSP's role in MDA-MB-231 cell motility is well-documented, research on the impact of endogenous BSP expression on cell motility in other cancer cell lines remains limited.

BSP regulates cancer cell migration and invasion mainly via RGD-integrin interactions. It binds integrins like integrin $\alpha\beta 3$ (M21, SW-579) [395, 398], α_v (glioblastoma) [340], and $\alpha_v/\alpha\beta 3$, but not $\alpha\beta 5$ (MDA-MB-231) [381], activating pathways such as FAK [299], PI3K/Akt [344, 395], (Src)-MEK/ERK (MAPK) [299, 395], and calpain [395, 399]. These pathways promote cytoskeletal reorganisation and motility. Additionally, BSP facilitates cell motility and metastasis formation through the miR-150-5p/MMP-14 axis [344, 389]. It may also enhance MMP expression (e.g., MMP-2, MMP-9, and MMP-14) and translocation [299, 344], forming integrin-MMP complexes to degrade the ECM and enable invasion [173, 398]. BSP and MMP-2 are co-expressed in prostate neoplasm [337] and several cancers [398, 400]. Moreover, BSP promotes invasion through RGD-independent mechanisms, as a recombinant human BSP fragment with a KAE substitution for RGD similarly promotes breast cancer cell invasion as one with the RGD sequence [381]. These mechanisms highlight BSP's multifaceted role in cancer cell motility and tumour progression, with signalling pathways and MMP activity depending on the cell type [299, 344, 380].

Further research is needed to uncover how BSP mediates cancer cell motility, particularly in prostate cancer, where its specific contributions and underlying mechanisms remain poorly understood. An overview of the proposed role of BSP in tumour progression and metastasis is shown in figure 3.

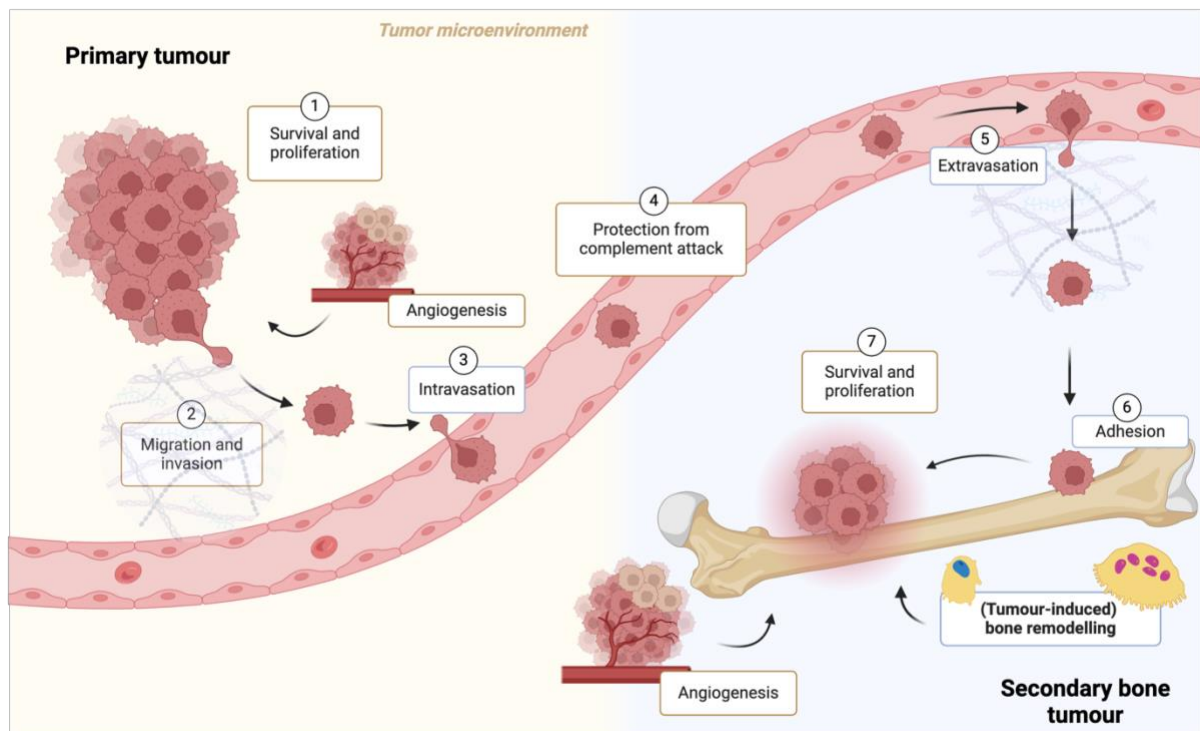


Figure 3. The proposed roles of BSP in tumour progression and skeletal metastasis development.

In the tumour microenvironment, BSP promotes cancer cell proliferation, potentially through RGD-integrin interactions and by stimulating the expression of SMAD4 and PARP-1. BSP enhances cancer cell survival via RGD-integrin-mediated MEK/ERK signalling and supports anoikis resistance through the miR-150-5p/MMP-14 axis. These effects on survival and EMT enable cancer cell dissemination, migration, and invasion, facilitating the establishment of metastases at secondary sites, particularly in bone. Upon entry into the bloodstream, BSP protects DTCs from complement-mediated destruction. This occurs through its initial binding to integrin receptors on DTCs, followed by interaction with factor H to block complement activation and suppress cell lysis. This immune escape mechanism further enhances cancer cell survival and dissemination. At secondary sites such as bone, BSP supports extravasation and tumour colonisation by promoting adhesion, proliferation, survival, migration, and invasion of cancer cells. Both endogenous and exogenous BSP expression contribute to cancer cell adhesion and motility via RGD-integrin binding, which activates signalling pathways including FAK, Src-MEK/ERK (MAPK), PI3K/Akt, and calpain-mediated cascades. Once tumour cells colonise the bone, they drive bone remodelling. BSP expression by both tumour and bone cells enhances osteoblast formation, matrix mineralisation, osteoclast differentiation, and activity. The extent of bone formation and resorption varies depending on the primary tumour type and individual patient characteristics. In summary, BSP orchestrates multiple processes that collectively promote tumour progression, skeletal metastasis, and tumour-induced bone remodelling. BSP, bone sialoprotein; DTCs, disseminating tumour cells; EMT, epithelial-mesenchymal transition; FAK, focal adhesion kinase; miR-150-5p, microRNA-150-5p; MMP, matrix metalloproteinase; PARP-1, Poly(ADP-Ribose) Polymerase 1; RGD, arginine-glycine-aspartic acid; SMAD4, SMAD family member 4. Created with BioRender.com.

Bone sialoprotein as a potential cancer biomarker

Quantifying BSP in serum holds potential as a cancer biomarker. Elevated serum BSP levels correlate with tumour grade, cancer stage, and secondary developments like skeletal metastases in patients with multiple myeloma [355], lung [358], and prostate cancers [338, 360]. However, one study found no significant correlation between serum BSP levels and TNM staging, including tumour size, lymph node status, and metastasis, in pancreatic adenocarcinoma patients [359], suggesting that BSP may not correlate with all clinicopathological features across cancers. Despite this, elevated serum BSP is linked to

poorer survival in multiple myeloma [355], lung [358], and pancreatic [359] cancers. BSP protein expression in primary tumours of breast [329, 332, 349] and lung [350] cancers is associated with bone and non-bone metastasis development, including to the lungs and potentially lymph nodes. BSP synthesis in primary tumours of osteosarcoma [330], glioma [331], breast [341, 342], colon [341], lung [341, 344], pancreatic [359], and rectum [341] cancers correlates with TNM staging, recurrence free survival (RFS), and OS, highlighting its prognostic value. Thus, BSP, especially when assessed with other biomarkers, may aid early cancer detection, guide treatment, and provide insights into treatment response and disease progression.

1.4 Rationale

Collectively, the current literature highlights BSP as a central regulator of tumour progression, influencing angiogenesis, cancer cell proliferation, survival, adhesion, migration, and invasion. BSP is frequently detected at the gene and protein levels in primary tumour tissues across various cancer types. However, studies examining BSP expression in bone metastases remain limited, particularly regarding its mRNA levels in secondary tumours.

Interactions between BSP and $\alpha\beta3$ integrins are critical mediators of cancer cell adhesion and motility, potentially facilitating bone metastasis. While BSP-integrin-driven cancer cell adhesion and motility appear to be predominantly RGD-dependent, the specific contributions of BSP binding to $\alpha\beta5$ integrins and other motifs, such as the heparin-binding sequence, remain poorly understood. Additionally, despite extensive research into exogenous BSP's role in cancer cell invasion, its effects on cancer cell migration are insufficiently explored.

Beyond tumour biology, BSP plays a dual role in bone remodelling, promoting both bone formation (osteoblast differentiation and matrix mineralisation) and resorption (osteoclast differentiation and activity). Evidence suggests BSP synergises with RANKL to facilitate the activation and resorption phases of bone remodelling. However, existing studies rely on animal models, leaving the role of BSP in human osteoclastogenesis and resorption activity unclear.

Thus, BSP may influence both tumour cell behaviour and bone-resorbing cell function, contributing to secondary bone tumour development and associated remodelling processes. Uncovering the mechanisms underlying BSP's roles in tumour progression and bone degradation—whether physiological or pathological—could pave the way for improved strategies in detecting, preventing, and treating cancer and bone diseases, such as osteoporosis or Paget's disease.

Specifically, the aims of this project are:

1. To investigate whether human BSP enhances the adhesion of human breast adenocarcinoma, prostate adenocarcinoma, large-cell lung cancer, and osteosarcoma cells through interactions of the R-G-D sequence with the integrin receptors $\alpha\beta3$ and

- $\alpha v \beta 5$. This will be assessed using *in vitro* cell adhesion assays and blocking studies using RGD-containing peptides and anti-integrin antibodies.
2. To assess the role of the heparin-binding sequence (L-H-R-R-V-K-I) of human BSP in the adhesion of breast and prostate cancer cells. *In vitro* cell adhesion assays and blocking studies with LHRRVKI-containing peptides will be carried out.
 3. To investigate the effectiveness of anti-BSP antibodies in blocking the adhesion of breast adenocarcinoma cells to human BSP. *In vitro* cell adhesion assays and blocking studies using anti-BSP antibodies will be carried out.
 4. To evaluate whether human BSP functions as a chemoattractant, stimulating the migration of breast and prostate cancer cells. *In vitro* cell migration assays (Boyden chamber) will be conducted for 24 h to quantify cell movement.
 5. To determine if human BSP alone promotes the differentiation of human primary macrophages into multinucleated osteoclasts and stimulates osteoclast activity. Peripheral blood mononuclear cells will be differentiated into macrophages and used in *in vitro* osteoclast differentiation and bone resorption assays, with synthetic calcium-phosphate discs serving as a substrate for resorption over 24 days. The effectiveness of an anti-BSP antibody in suppressing osteoclast differentiation will also be evaluated.
 6. To evaluate the synergistic effects of human BSP and RANKL in inducing osteoclast differentiation and activity. Macrophages, differentiated from peripheral blood mononuclear cells, will be used in assays for osteoclast differentiation and resorption activity (24 days). Synthetic calcium-phosphate discs will be used to mimic the bone ECM for bone resorption.
 7. To quantify the gene expression levels of BSP and other proteins or enzymes associated with tumour progression and bone remodelling in bone metastases and healthy bone tissue. RT-PCR will be performed to measure gene expression in breast and lung cancer bone metastases, as well as in normal bone tissue.

The hypotheses of this project are:

1. Human BSP functions as an anchor-protein that induces the adhesion of breast adenocarcinoma, prostate adenocarcinoma, large-cell lung cancer, and osteosarcoma cells. The adhesion of these cancer cells to BSP is mediated through RGD-integrin $\alpha v \beta 3$ and/or $\alpha v \beta 5$ binding interactions.
2. The heparin-binding domain of human BSP enhances cancer cell adhesion.
3. The anti-BSP antibodies effectively inhibit breast cancer cell adhesion to human BSP, with varying potency among the different antibodies.
4. Human BSP acts as a chemoattractant and promotes the migration of breast and prostate cancer cells.

5. Human BSP alone stimulates the differentiation and activity of human primary osteoclasts, while an anti-BSP antibody suppresses BSP-induced osteoclastogenesis.
6. Human BSP and RANKL synergistically enhance osteoclast differentiation and activity compared to BSP or RANKL alone.
7. BSP mRNA is highly expressed in bone metastases from breast and lung cancer patients. BSP expression is expected to be elevated in malignant tissue compared to healthy bone tissue.

Collectively, we hypothesise that BSP is a key regulator of human cancer cell adhesion and migration, as well as human osteoclastogenesis and bone resorption.

2. Materials and methods

An overview of the experimental design of the study is shown in figure 4.

2.1 Materials

A detailed description of all the materials used in this study can be found in the appendix.

2.2 Cell culture

MG-63. The human osteosarcoma cell line MG-63 (ATCC CRL-1427) was cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) supplemented with 10% heat-inactivated foetal calf serum (FCS) and 1% penicillin/streptomycin (P/S) solution.

MDA-MB-231 and MCF-10A. The human breast adenocarcinoma cell line MDA-MB-231 [DSMZ ACC 732 (RRID:CVCL 0062)] was originally established from the pleural effusion of a patient with TNBC. Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% heat-inactivated FCS, 1% P/S, 2mM L-Alanyl-L-Glutamine, 5 ml Minimum Essential Medium non-essential amino acids (MEM NEAA) 100X and 1 mM sodium pyruvate. The human non-tumorigenic mammary gland epithelial cell line MCF-10A (ATCC CRL-10317) was cultured in Mammary Epithelial Cell Basal Medium (MEBM). The medium was supplemented with 10% heat-inactivated FCS, 1% P/S and the Mammary Epithelial Cell Growth Medium (MEGM) SingleQuots Kit containing 2 ml bovine pituitary extract (BPE), 0.5 ml human epidermal growth factor (hEGF), 0.5 ml insulin, 0.5 ml hydrocortisone and 0.5 ml gentamicin sulphate-amphotericin (GA-1000).

PC-3. The human prostate adenocarcinoma cell line PC-3, originally established from a bone metastasis of a grade IV prostate cancer patient, was kindly provided by [REDACTED]. Cells were cultured in Iscove's Basal Medium, supplemented with 10% heat-inactivated FCS and 1% P/S.

A-549 and NCI-H460. The human non-small cell lung adenocarcinoma cell line A-549 was kindly provided by [REDACTED] and was cultured in DMEM, supplemented with 5% FCS and 1% P/S. The human large cell lung carcinoma cell line NCI-H460 [H460] (ATCC HTB-177) was grown in RPMI 1640 medium, supplemented with 10% heat-inactivated FCS and 1% P/S.

All cell lines were cultured in a humidified environment at 37°C and 5% CO₂. Medium was renewed twice a week.

2.3 Cell handling

All procedures were carried out in a sterile cell culture environment under a laminar flow hood, using autoclaved and disinfected materials. All methods incorporated aseptic techniques, sterile media, reagents, consumables, and sterile handling while wearing appropriate personal protective equipment (PPE). The work area was routinely disinfected with 70% ethanol to maintain a clean and contamination-free environment.

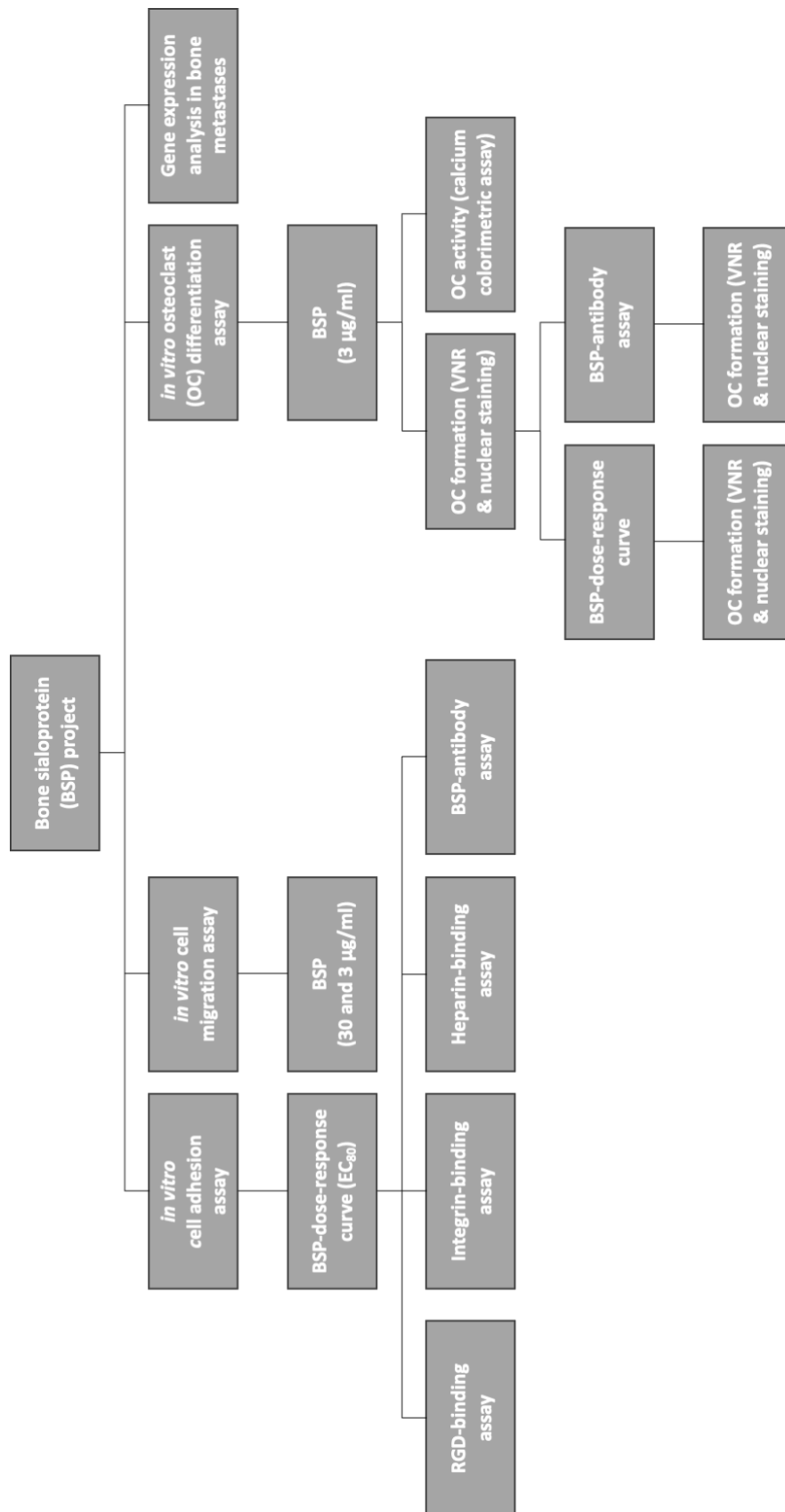


Figure 4. Overview of the experiments and methods used in the study. BSP, bone sialoprotein; EC₈₀, 80% maximal effective concentration; RGD, arginine-glycine-aspartic acid; OC, osteoclast; VNR, vitronectin receptor.

2.3.1 Cell counting

Living cells were counted with an automated cell counter or manually with a Neubauer haemocytometer. Trypan blue or Erythrosin B was used stain cells. Trypan blue staining is one of the most common methods to determine cell viability. Trypan blue is a colorimetric dye that distinguishes between viable and nonviable cells by staining only the cytoplasm of dead cells [401]. The selective dye incorporation occurs due to the loss of plasma membrane integrity of non-viable cells. The cell membrane of living cells remains intact [401]. Non-viable cells will appear blue whilst viable cells will appear clear (i.e. unstained) when viewed under a microscope. However, Trypan blue is carcinogenic, hazardous and cytotoxic. A safer, but less popular alternative to count viable cells is Erythrosin B. Erythrosin B stain is a safe and non-toxic dye [402]. Erythrosin B assesses cell viability based on a similar principle as Trypan blue [402].

Cell viability of peripheral blood mononuclear cells (PBMCs) and macrophages was assessed using Acridine Orange/Propidium Iodide (AO/PI). AO/PI is an orange-red dye that differentiates viable from non-viable cells by assessing their membrane integrity and nucleic acid content. AO can permeate the cell membrane and bind to nucleic acids [403]. PI only binds to non-viable nucleic acids, as it is membrane impermeable [403]. Co-staining of cells with AO and PI causes viable nucleated cells to fluoresce green, while non-viable nucleated cells will fluoresce orange to red [403]. PI was handled with care as it is carcinogenic [403].

Cultured cells were washed with Dulbecco's phosphate-buffered saline (PBS), treated with Accutase® solution for 5-10 min and resuspended in medium. Cells were centrifuged at room temperature (RT) at 353 x g for 5 min. The supernatant was discarded and cells were resuspended in specified medium, buffer or solution.

For automatic cell counting, cell suspensions (10 µl) were diluted in Trypan blue solution (0.4%) or Erythrosin B (dilution factor 1:2). 18 µl PBMC suspensions were diluted in 2 µl AO/PI. 10 µl of the mixture was added into each of the two sample loading chambers of a LUNA™ cell counting slide. The cell counting slide was then stuck into the LUNA™ for brightfield cell counting. From the two automated cell counting readouts, the average cell concentration (living cells per ml cell suspension) was calculated and used in experiments.

For manual cell counting, a haemocytometer and a haemocytometer glass cover slip were cleaned with 70% ethanol prior to use. The coverslip was slightly moistened and placed on top of the centre of the haemocytometer. Cell suspensions (10 µl) were diluted in Trypan blue solution (0.4%) or Erythrosin B (dilution factor 1:2) and 10 µl of the mixture was slowly pipetted into the chamber of the haemocytometer. The Neubauer cell chamber was then viewed under a microscope. Viable cells were manually counted in the 16 quadrants of each corner square. The total concentration of living cells per ml cell suspension was calculated using the following equation:

$$\text{Living cells per ml} = \left(\frac{\text{Sum of counted viable cells in the four corner squares}}{4} \right) \times 2 \times 10^4$$

2.3.2 Freezing of cells

Cells were washed with PBS, detached using Accutase® for 5-10 min and resuspended in medium. Following centrifugation at RT (353 x g, 5 min), the supernatant was carefully removed by vacuum suction. In general, cells were resuspended in 95% medium and 5% dimethyl sulfoxide (DMSO). The vials were placed into a freezing container and stored at -80°C for at least 4 h to freeze cells in a slow and gentle process (-1°C/min). For long-term storage, the cryogenic vials were transferred to a cryogenic storage tank.

2.3.3 Thawing of cells

Frozen cells were rapidly defrosted by immersing the cryogenic vial in a water bath at 37°C. When almost completely defrosted, cells were added to a 50 ml falcon with 9 ml complete medium and centrifuged (353 x g, 5 min, RT). Following centrifugation, the supernatant was discarded and the cell pellet was resuspended in 10-20 ml complete medium. Cells were then seeded on tissue culture flasks.

2.4 Cell adhesion assays

2.4.1 Standard curve

A standard curve was generated for each cell line. Cells were washed with PBS, detached using Accutase® and centrifuged at RT (353 x g, 5 min). The supernatant was discarded and cells were resuspended in reduced growth medium containing 2.5% FCS. Living cells were counted. Cells were diluted to 100 000, 50 000, 25 000, 12 500, 6 250, 3 125, 1 562.5, and 0 cells per 250 µl and then seeded onto a 24-well culture plates in quintuple (250 µl/well). Following a 2-hour-incubation period (37°C, 5% CO₂), the supernatant was carefully removed by vacuum suction. AlamarBlue™ cell viability reagent was diluted 1:10 in reduced (2.5% FCS) growth medium according to the manufacturer's instructions and added to the wells (500 µl/well). Cell viability (metabolic activity) was measured in triplicate (100 µl/well) on a 96-well microplate after 4 h (37°C, 5% CO₂) using the alamarBlue® assay. Cell viability was used to quantify cell adherence. Fluorescence was read at 540 nm excitation wavelength and 590 nm emission wavelength with a fluorescence multi-well reader. The average fluorescence of the blank (0 cells/250 µl) was subtracted from all individual readings. The mean fluorescence value was plotted against the seeded cell concentration to create a standard curve.

2.4.1.1 alamarBlue® assay

The alamarBlue® assay is a widely used method for assessing cell viability and cytotoxicity [404]. The assay measures the metabolic activity of cells by the reduction of resazurin [404]. The alamarBlue™ cell viability reagent contains resazurin. Resazurin is non-toxic, cell-permeable, redox-sensitive and stable in cell culture media [404]. Resazurin solution acts as an intermediate electron acceptor in the electron transport chain, without interrupting the electron transfer of active cells and affecting aerobic cellular respiration

[405]. When electrons are accepted, the dye changes from its initial blue, non-fluorescent state to the pink-coloured and fluorescent resorufin solution (figure 5) [406]. The fluorescence intensity is directly proportional to the quantity of living cells.

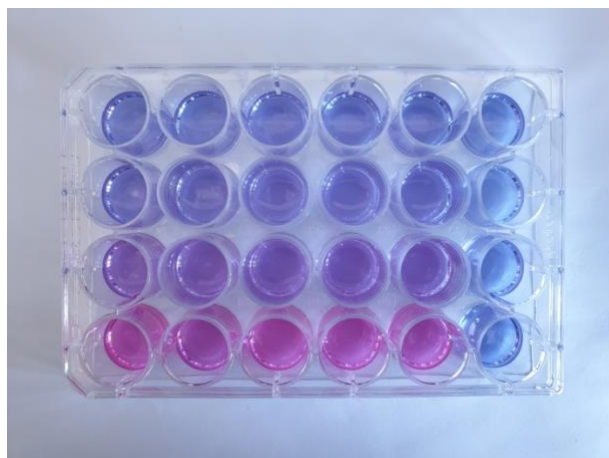


Figure 5. Example of colour changes of the alamarBlue™ cell viability reagent upon reduction.

2.4.2 Bone sialoprotein-binding assays

To explore the role of BSP in cancer cell adhesion, a BSP-binding assay was carried out once in quintuple. Here, we describe a modified protocol by Oliveira-Ferrer *et al* [407]. A 24-well suspension culture plate was pre-coated (250 µl/well) with PBS, human plasma fibronectin (FN) (10 µg/ml), human recombinant BSP CR-1 (3 µg/ml), BSP CR-3 (3 µg/ml) and human (hu) huBSP2 (3 µg/ml), diluted in PBS. The Chinese hamster ovary (CHO) cell line was used for the production of human recombinant BSP proteins. FN was used as a positive control and PBS was used as a negative control. The concentration of BSP was chosen based on initial, unpublished data from the laboratory. The 24-well suspension culture plate was incubated overnight at RT. On the subsequent day, cultured cells were washed with PBS, detached using Accutase®, centrifuged at RT (353 x g, 5 min) and diluted to 2.4×10^5 cells/ml in reduced growth medium (2.5% FCS). The supernatant of the coated plate was removed by vacuum suction. Cells were seeded onto the plate at a density of 6×10^4 cells/250 µl per well. Following a 2-hour incubation period (37°C and 5% CO₂), the supernatant was discarded. Then, alamarBlue™ cell viability reagent (1:10 in reduced growth medium) was added to the 24-well suspension culture plate (500 µl/well). A blank was also included. The alamarBlue® assay was performed after 4 h (37°C, 5% CO₂) using a 96-well microplate to measure cell viability.

2.4.3 Concentration-dependent bone sialoprotein-binding assays

Based on the preliminary results, the BSP-binding assay was discontinued or a concentration dependence and response to BSP of human cancer cell lines was investigated (dose-response curve). The following cell lines were included: MDA-MB-231, PC-3, NCI-H460, and MG-63. Briefly, huBSP2 was serially diluted in PBS to various concentrations, ranging from

10 µg/ml down to 0 µg/ml. 24-well suspension culture plates were coated with huBSP2 at 250 µl per well and incubated overnight at RT. The subsequent protocol steps were the same as those used in the previous experiment.

The BSP concentration-dependent adhesion assay was carried out in triplicate and repeated three independent times. Data from all experiments were pooled for analysis. The 80% maximal effective concentration (EC₈₀) of huBSP2 was calculated for each cell line. Briefly, BSP concentrations (X) were log-transformed using the following equation:

$$X = \text{Log}_{10}(X) = \text{Log}_{10}(\text{Concentration})$$

A sigmoidal dose-response curve with a variable slope was generated by plotting the average fluorescence readings against the log-transformed BSP concentrations. Non-linear regression (curve fitting) was used to determine EC₈₀ values. These EC₈₀ values of huBSP2 were used in the following RGD-binding, heparin-binding, integrin-binding and anti-BSP antibody assays.

2.4.3 RGD-binding assays

An RGD-binding assay was conducted to explore the mechanism behind the adhesion of human cancer cell lines to BSP and, consequently, to the ECM.

The protocol by Stachurska *et al* [245] was modified and applied in the present study. 10 µg/ml human VN (250 µl/well) and the EC₈₀ of huBSP2 (250 µl/well) were coated onto 24-well suspension culture plates. We chose VN over FN because research indicates that FN does not bind to integrin αvβ5, whereas VN can bind to both integrin αvβ3 and αvβ5 [408]. The EC₈₀ of huBSP2 was determined from the concentration-dependent BSP-binding assay. After incubation of the plate overnight at RT, the supernatant of the coating solution was removed. 3% bovine serum albumin (BSA) in PBS was added to each well (250 µl/well) to block non-specific binding sites for 30 min at RT. In the meantime, cell suspensions of cancer cells at 2.4x10⁵ cells/ml in reduced growth medium were prepared. Cell suspensions were incubated with FN-derived RGD peptide Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) for 30 min at the following initial concentrations: 200, 100, 25, and 0 µM. Once 3% BSA was discarded, the plate was rinsed two times with PBS (500 µl/well) and the incubated cell suspensions were seeded at 6x10⁴ cells per well. Cells were incubated for 2 h at 37°C and 5% CO₂. Then, the supernatant was removed and the plate was rinsed with 500 µl PBS per well. AlamarBlue™ cell viability reagent was diluted 1:10 in reduced growth medium and added to the wells (500 µl/well), including a blank. The alamarBlue® assay was performed after 4 h (37°C, 5% CO₂) to assess cell viability. The extent of cell spreading was assessed by imaging adherent cells with phase-contrast microscopy.

Based on the preliminary results, the concentration of the GRGDSP peptide was adjusted to elicit a dose-response curve of the RGD-motif containing peptide, ranging from

300 to 0 μ M. Three replicates were used per group and the experiment was repeated three times. The following cell lines were included: MDA-MB-231, PC-3, NCI-H460, and MG-63.

2.4.4 Heparin-binding assays

A heparin-binding assay was developed to define the regulatory function of the heparin sequence of BSP in mediating the adhesion of cancer cells to BSP and hence the ECM. The experiment was performed as described in section 2.4.3, with some modifications.

The LHRRVKI sequence (heparin-binding domain)-containing peptide was custom synthesised from Biomatik. GRGDSP peptide was not used in the experiment. Cell suspensions were incubated with the LHRRVKI peptide at concentrations of 100, 50, 20, and 0 μ g/ml or PBS for 30 min at 37°C and 5% CO₂. Adherent cells were assessed using the alamarBlue® Assay. Experiments were performed in triplicate and repeated three times. The breast MDA-MB-231 and prostate PC-3 adenocarcinoma cell lines were used in the experiments.

2.4.5 Integrin-binding assays

To assess the role of the cell surface integrin receptors α v β 3 and α v β 5 in BSP-induced cancer cell adhesion, a receptor inhibition assay was performed using the following cell lines: MDA-MB-231, PC-3, NCI-H460, and MG-63. The experiment followed the protocol outlined in section 2.4.3, with several modifications.

A 24-well suspension culture plate was pre-coated with 10 μ g/ml human VN and the EC₈₀ of huBSP2 at 250 μ l per well. Plates were incubated overnight at RT. On the subsequent day, the supernatant of the coating solution was discarded. The plate was incubated with 3% BSA at RT for 30 min (250 μ l/well) to block non-specific binding sites. Cell suspensions were prepared in reduced growth medium and 2.4x10⁵ cells/ml were incubated for 60 min at 37°C and 5% CO₂ with anti-integrin α v β 3 antibody (10 μ g/ml), anti-integrin α v β 5 antibody (10 μ g/ml) or isotype control (10 μ g/ml). Wells were washed twice with PBS (500 μ l/well) after incubation with 3% BSA. Cell suspensions were seeded to the pre-coated wells at 6x10⁴ cells per well. Following a 2-hour incubation period at 37°C and 5% CO₂, the supernatant was removed and wells were rinsed with 500 μ l PBS. Cell viability was measured after 4 h (37°C, 5% CO₂) using the alamarBlue® assay. Blank wells containing only the alamarBlue™ cell viability reagent, without any cells, were included as well. Experiments were performed in duplicate and repeated two times.

2.4.6 Bone sialoprotein-antibody assays

To investigate the functionality of BSP antibodies in inhibiting cancer cell adhesion to BSP, BSP-antibody assays were carried out *in vitro*.

Based on preliminary data, the EC₈₀ of huBSP2 was incubated with various concentrations of BSP-antibody mouse anti-human FP-21 (12, 9, 6, 3, and 0 μ g/ml) or rat anti-human IDK-1 (240, 120, 60, 30, and 0 μ g/ml) for 30 min at RT. Then, 24-well suspension-culture plates were pre-coated in triplicate (250 μ l/well) with FN (10 μ g/ml), PBS, BSP (EC₈₀)

and BSP-antibody combinations. Plates were incubated overnight at RT. After overnight incubation, the supernatant was removed. 60 000 cells were seeded in 250 µl reduced growth medium per well. Cells were allowed to adhere to the pre-coated surface for 2 h at 37°C, 5% CO₂. Following the incubation period, the supernatant was removed and plates were washed with PBS (500 µl/well). Diluted alamarBlue™ cell viability reagent (1:10 in reduced growth medium) was added to each well (500 µl/well). The metabolic activity of the adherent cells was measured using the alamarBlue® assay after 4 h at 37°C, 5% CO₂. Fluorescence was read in triplicate (100 µl/well) with a fluorescence multi-well reader.

The experiment was performed three times in triplicate. BSP-antibody assay with FP-21 was also carried out in triplicate once on a 96-well suspension-culture plate. The breast adenocarcinoma cell line MDA-MB-231 was used in the experiments.

Seven newer anti-BSP antibodies were also tested: YU828-A01, YU828-A06, YU828-D07, YU828-D09, YU828-H06, YU829-H06, and YU831-A05. Based on preliminary data, the EC₈₀ of huBSP2 was incubated with YU831-A05 (50, 25, 5, 2.5, 1, 0.5, and 0 µg/ml) or YU828-D09 (100, 50, 25, 5, 2.5, 0.5, and 0 µg/ml) for 30 min at RT for blocking experiments. huBSP2 (EC₈₀) was incubated with YU828-A01, YU828-A06, YU828-D07, YU828-H06, or YU829-H06 at antibody concentrations of 100, 33.3, 11.1, 3.7, 1.2, 0.4, and 0 µg/ml for 30 min (RT). A 96-well suspension-culture plate was pre-coated in triplicate (40 µl/well) with FN (10 µg/ml), PBS, BSP (EC₈₀) and BSP-antibody combinations. Plates were additionally coated with a uniform solution of 100 µg/ml anti-BSP antibody (AK 100 µg/ml). The plate was incubated overnight at RT. The supernatant of the plate was removed and 2.5x10⁴ cells in reduced growth medium were seeded at 100 µl per pre-coated well. After a 2-hour incubation period at 37°C, 5% CO₂, the supernatant was removed and the plate was washed with PBS (200 µl/well). Diluted alamarBlue™ cell viability reagent (1:10 in reduced growth medium) was added at 315 µl per well and incubated for 4 h (37°C, 5% CO₂). The alamarBlue® assay was performed in triplicate (100 µl/well). Prior to the blocking experiments, a standard curve relating fluorescent intensity to the number of seeded cells was generated.

BSP-antibody assays with YU831-A05 and YU828-D09 were performed in duplicate twice. Other experiments were carried out in triplicate and repeated three times. The human breast adenocarcinoma cell line MDA-MB-231 was used in the experiments.

The half-maximal inhibitory concentration or 50% inhibitory concentration (IC₅₀) of the anti-BSP antibodies FP-21, IDK-1, YU828-A01, YU828-A06, YU828-H06, YU829-D07, and YU829-H06 was calculated. Briefly, anti-BSP antibody concentrations (X) were log-transformed using the following equation:

$$X = \text{Log}_{10}(X) = \text{Log}_{10}(\text{Concentration})$$

A sigmoidal inhibitor-response curve with a variable slope was generated by plotting the average fluorescence readings (expressed as % control) against the log-transformed antibody concentrations. The top plateau of the curve was constrained to 100% and the

bottom plateau to 0%. Non-linear regression analysis was then performed to calculate IC₅₀ values.

2.4.7 Quantification of cancer cell adhesion

The alamarBlue® cell viability assay was used to measure cell adherence. The fluorescence readings were adjusted by subtracting the mean fluorescence value of the blank (i.e. auto-fluorescence of alamarBlue™ cell viability reagent) from each individual sample reading. To quantify cell adherence, the individual sample readings for each group were averaged. Percentages were determined in relation to the negative control using the following equation:

$$\% \text{ control} = \frac{(\text{control} - \text{treated})}{\text{control}} \times 100$$

2.5 Cell migration (Boyden chamber) assays

2.5.1 Standard curve

A standard curve was generated for following two cell lines: MDA-MB-231 and PC-3. Cells were serum-starved overnight in medium containing 0% FCS. The next day, cells were washed with PBS and detached from the tissue culture plate using Accutase®. Cells were pelleted by centrifugation (5 min, 353 x g, RT) and resuspended in medium with 1% FCS and no P/S. The viable cell count was determined using trypan blue staining. 100 000, 50 000, 25 000, 12 500, 6 250, 3 125, 1 562.5, and 0 cells in 250 µl were added to a 48-well cell culture plate in triplicate. The plate was incubated overnight at 37°C and 5% CO₂. The medium was discarded and the wells were washed twice with PBS (700 µl/well). Cells were fixed in 4.5 % formaldehyde (600 µl/well) for 10 min (RT) using a shaker. The fixative was removed and the wells were washed twice with PBS (700 µl/well). The subsequent steps were performed in the dark.

Cells were stained with 0.5% crystal violet (CV) solution for 10 min at RT using a shaker (600 µl/well). CV binds to the DNA and protein of cells [409]. Cells undergoing cell death detach. Migrated cells are quantified by extracting the dye and measuring the absorbance using a spectrophotometer [410].

The excess dye was discarded and the wells were washed three times with PBS (700 µl/well). The dye was eluted in the well with 400 µl of 33% acetic acid for 10 min (RT) using a shaker. 100 µl of the eluent was transferred to a 96-well microplate and the absorbance was measured at 600 nm using a microplate reader (triplicate readings per standard). To generate a standard curve, the mean absorbance value of the blank (0 cells/250 µl) was subtracted from all individual readings. The average of each cell concentration was calculated and plotted versus mean absorbance (600 nm). The equation of the line of best fit was used for subsequent data analysis.

2.5.2 Migration assays

To examine if BSP stimulates cancer cell migration, the migration assay protocol outlined by Feoktistova *et al* [409] was adapted and used in the present study. Prior to performing the assay, breast MDA-MB-231 and prostate PC-3 adenocarcinoma cells were starved in serum-free medium (0% FCS) overnight. Following starvation, cells were harvested and centrifuged to generate a cell pellet. The pellet was resuspended in medium with 1% FCS and no P/S and living cells were quantified. The cells were diluted to 3×10^5 cells/ml. Meanwhile, a 24-well cell culture insert companion plate was prepared with cell culture inserts (8 μ m pore size). 250 μ l of the cell suspension was seeded to the insert and incubated for 10 min at 37°C and 5% CO₂. 700 μ l of the chemoattractant was pipetted into the lower chamber of the 24-well plate. The migration assay was performed in triplicate for each experimental condition. The chemoattractant of interest was huBSP2 at 30 μ g/ml and 3 μ g/ml in 1% FCS. The concentration of 3 μ g/ml BSP was chosen because it had effectively promoted cancer cell adhesion in our experiments. A concentration of 30 μ g/ml BSP was selected as a starting point to assess a potential concentration-dependent effect of BSP on cancer cell migration. The positive controls were 10% FCS and/or 50 ng/ml epidermal growth factor (EGF) in 1% FCS, while the negative control contained only 1% FCS, without any chemoattractant or inhibitor. The use of a single purified growth factor like EGF enhances reproducibility and reduces variability compared to FCS, which is a complex mixture of growth factors, cytokines, and other molecules [411]. A blank condition (no chemoattractant and no cells) in 1% FCS was also included. The plate was incubated for 24 h (37°C and 5% CO₂) to allow cells to migrate (figure 6). After 24 h, the cell culture inserts were removed from the plate and the inserts were washed by carefully dipping them into PBS twice. The non-migrated cells inside the culture inserts were removed by using moistened cotton swabs. Cells on the lower site of the insert filters were fixed in 4.5 % formaldehyde (600 μ l/well) for 10 min at RT using a shaker. Afterwards, the inserts were washed by carefully dipping them into PBS twice. In the meantime, a new 24-well cell culture plate was prepared with 600 μ l CV dye (0.5%). The washed inserts were placed into the wells and cells were stained for 10 min at RT using a shaker. Afterwards, the inserts were quickly dipped into PBS a couple of times to remove excess (i.e. unbound) CV and to remove dead cells. The cell culture inserts were air-dried for 15 min. Images of migrated cells were taken using light microscopy (figure 7). For the quantification of cell migration, the dye was eluted in 400 μ l of 33% acetic acid for 10 min at RT using a shaker. The eluent was transferred to a 96-well microplate (100 μ l/well) and the absorbance was measured at 600 nm using a microplate reader (triplicate absorbance readings for each control and sample).

The absorbance was corrected by subtracting the mean absorbance value of the blank (no chemoattractant and no cells) from all individual sample readings. The individual sample readings were averaged and the total number of migrated cells was calculated using the

equation from the standard curve ($y = mx + b$) and taking the dilution factor into account (where applicable). Number of migrated cells:

$$y = mx + b \times \text{dilution factor}$$

Percentage of migrated cells (% migration) were determined in relation to the number of seeded cells using the following equation:

$$\% \text{ migration} = \frac{\text{number of migrated cells}}{\text{number of seeded cells}} \times 100$$

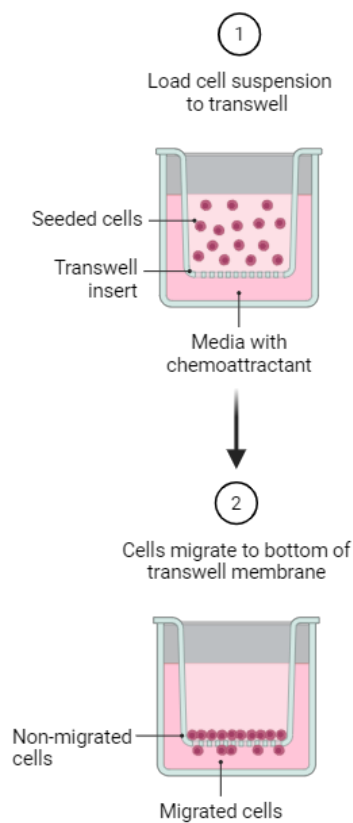


Figure 6. Overview of the cell migration (Boyden chamber) assay.

Cells are seeded on top of 8 μm cell culture inserts ①. Media with chemoattractant is in the bottom chamber ①. Cells migrate through the pores of the cell culture insert and attach at the other side of the insert ②. Created with BioRender.com.

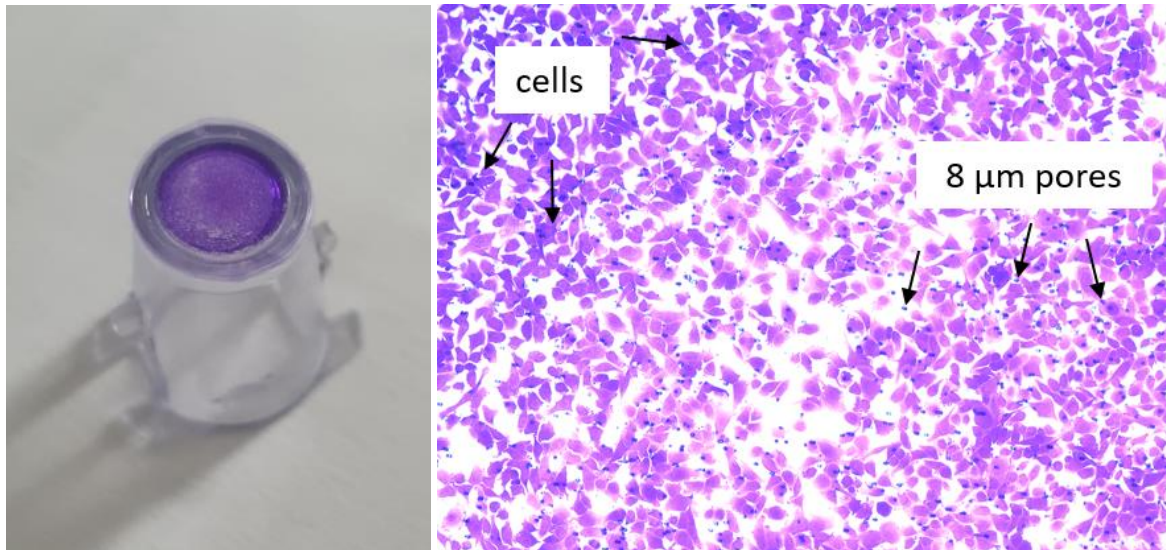


Figure 7. Example of cell staining with 0.5% CV.

Representative pictures of PC-3 cell transwell migration toward 50 ng/ml EGF (x10 magnification). CV, crystal violet; EGF, epidermal growth factor.

2.6 *In vitro* assays for osteoclast differentiation and functional activation

2.6.1 Harvest of human peripheral blood mononuclear cells from fresh whole blood

PBMCs were isolated from healthy human whole blood as described in Henriksen *et al* [376] and shown in figure 8. In short, the blood was diluted 1:2 in PBS. 30 ml of the diluted blood was carefully layered on top of 15 ml Histopaque®-1077 and PBMCs were generated using density gradient centrifugation (30 min, 400 x g, RT, no brake) (figure 9). Following centrifugation, the mononuclear cell layer was collected and washed 3-4 times with PBS (2% FCS, 2 mM EDTA) until the supernatant was clear (centrifugation: 10 min, 300 g, RT, break 9).

2.6.2 Isolation of CD14-positive monocytes using CD14 MicroBeads

After washing the mononuclear cell layer, the cell pellet was resuspended in cold buffer (0.5% BSA, 2 mM EDTA in PBS, pH 7.2) and CD14-positive (CD14⁺) monocytes were isolated using CD14 MicroBeads from Miltenyi Biotec according to the manufacturer's instructions. In brief, FcR blocking reagent was added to the cell suspension and incubated for 10 min at 2-8°C, followed by a 15-min incubation with CD14 MicroBeads at the same temperature. Cells were washed, centrifuged (10 min, 300 g, 4°C) and resuspended in cold buffer. Following preparation of the MACS® LS column, the cell suspension was applied onto the MACS® LS column and unlabelled cells were collected by a collection tube (figure 10). The MACS® LS column was then washed three times, removed from the magnet and the magnetically labelled CD14⁺ cells in the MACS® LS column were flushed out into a new collection tube.

CD14⁺ cells were centrifuged (10 min, 300 x g, RT) and resuspended in culture medium (MEM Alpha Medium + GlutaMAX), supplemented with 10% heat-inactivated FCS, 1% P/S and 20 ng/ml M-CSF. Cells were seeded at 1.5–3x10⁶ cells per 75 cm² flask. M-CSF was used to promote the differentiation of monocytes, to stimulate macrophage proliferation and to

inhibit their apoptosis [412, 413]. CD14⁺ cells were grown for 10 days at 37°C, 5% CO₂. The complete growth medium (incl. M-CSF) was changed every second or third day.

Generate Human Macrophages From Peripheral Blood

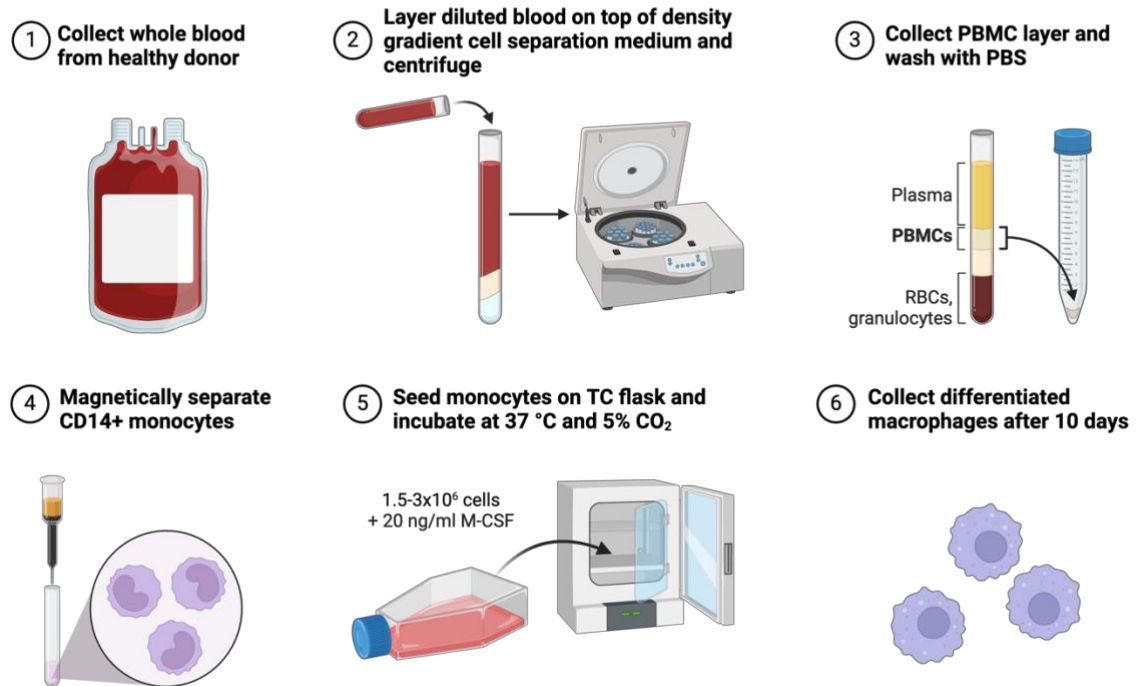


Figure 8. Schematic of the generation of human macrophages from peripheral blood. M-CSF, macrophage-colony stimulating factor; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; RBCs, red blood cells; TC, tissue culture. Created with BioRender.com.

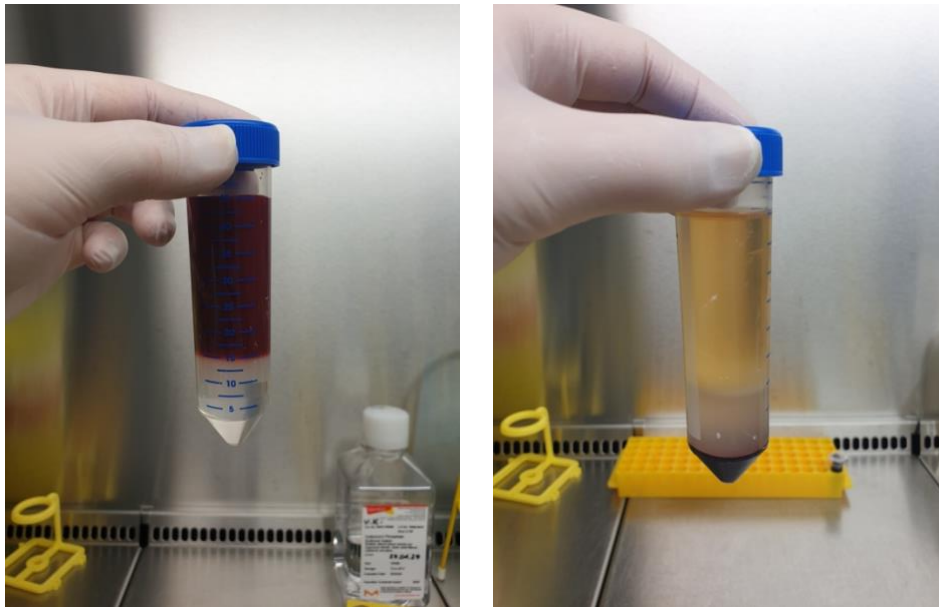


Figure 9. Example of blood separation with Histopaque®-1077.

A) Diluted blood is layered onto the density gradient cell separation medium. B) Separation of blood after Histopaque®-1077 density gradient centrifugation.

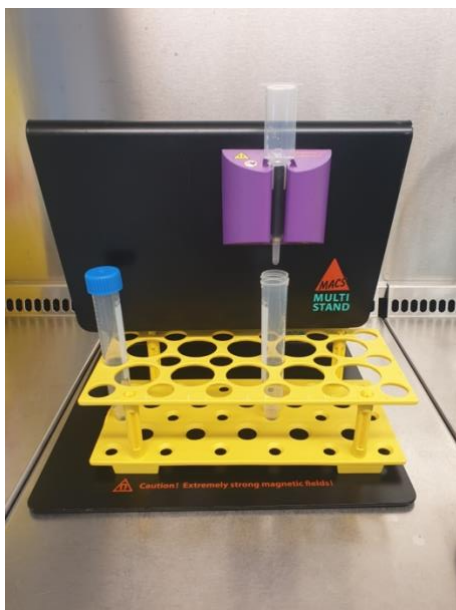


Figure 10. Set up of the magnetic separation of CD14⁺ monocytes from PBMCs using CD14 MicroBeads. PBMCs, peripheral blood mononuclear cells.

2.6.3 Osteoclast differentiation assays using bone sialoprotein

To determine whether BSP promotes osteoclast differentiation, we followed an osteoclastogenesis protocol by Henriksen *et al* [376]. In short, after 10 days in culture with M-CSF, macrophages – serving as a model for osteoclast precursors – were seeded on a 96-well cell culture plate in complete culture medium, supplemented with different growth factors and concentrations, depending on the experiment.

To harvest the macrophages, cells were washed twice with PBS, digested with Trypsin/EDTA for 5 min and detached from the flask using a cell scraper. Cells were

centrifuged (8 min, 200 x g, RT). The supernatant was discarded and cells were resuspended in growth medium (MEM Alpha Medium + GlutaMAX), supplemented with 10% FCS and 1% P/S. Macrophages were counted and the cell suspension was diluted to 2×10^5 cells/ml. Cells were seeded at 2×10^4 cells/well in a 96-well cell culture plate. Additional growth factors or BSP-antibody were added depending on the experimental condition (table 5). In previous experiments in our laboratory, a concentration of 5 $\mu\text{g/ml}$ BSP was used to enhance osteoclastogenesis (data not shown). However, in this study, 3 $\mu\text{g/ml}$ BSP was selected to promote osteoclast differentiation, as this concentration had been effective in stimulating cancer cell adhesion. Cells were cultured for 7 days and for 14 days. The complete culture medium (incl. growth factors and/or inhibitors) was changed every second or third day.

Experiment	Medium with growth factor or inhibitor (concentration)
BSP vs. RANKL treatment (<i>n</i> =3)	a. M-CSF (20 ng/ml) + RANKL (50 ng/ml) b. M-CSF (20 ng/ml) + BSP (3 $\mu\text{g/ml}$) c. M-CSF (20 ng/ml) + RANKL (50 ng/ml) + BSP (3 $\mu\text{g/ml}$)
Dose-response-curve of BSP (<i>n</i> =2)	a. M-CSF (20 ng/ml) b. M-CSF (20 ng/ml) + BSP (0.123 $\mu\text{g/ml}$) c. M-CSF (20 ng/ml) + BSP (0.37 $\mu\text{g/ml}$) d. M-CSF (20 ng/ml) + BSP (1.11 $\mu\text{g/ml}$) e. M-CSF (20 ng/ml) + BSP (3.33 $\mu\text{g/ml}$) f. M-CSF (20 ng/ml) + BSP (10 $\mu\text{g/ml}$)
Functionality of BSP-antibody (<i>n</i> =1)	a. M-CSF (20 ng/ml) + BSP (3 $\mu\text{g/ml}$) b. M-CSF (20 ng/ml) + BSP (3 $\mu\text{g/ml}$) + FP-21 (100 $\mu\text{g/ml}$) c. M-CSF (20 ng/ml) + RANKL (50 ng/ml) + BSP (3 $\mu\text{g/ml}$) d. M-CSF (20 ng/ml) + RANKL (50 ng/ml) + BSP (3 $\mu\text{g/ml}$) + FP-21 (100 $\mu\text{g/ml}$)

Table 5. Growth factors and inhibitors used in the osteoclast differentiation assays.

BSP, bone sialoprotein; M-CSF, macrophage colony stimulating factor; *n*, number of donors; RANKL, receptor activator of nuclear factor kappa-B ligand.

2.6.4 Assessment of osteoclast formation by $\alpha\text{v}\beta 3$ receptor and nuclear staining

Differentiated, mature osteoclasts are multinucleated cells and highly express the vitronectin receptor (VNR) $\alpha\text{v}\beta 3$ [414]. To assess osteoclast formation, the nuclei and $\alpha\text{v}\beta 3$ -integrin were co-stained using Hoechst dye and anti-VNR antibody. Hoechst dye (33342) is a bisbenzimidazole dye that is commonly used to stain the DNA of living cells by preferentially binding to adenine-thymine sequences in the minor groove of DNA [415]. Osteoclast development was viewed under an inverted fluorescence microscope after 7 and 14 days.

The medium was removed from the 96-well cell culture plate using vacuum suction and wells were washed with PBS (300 $\mu\text{l/well}$). Cells were fixed in 4.5 % formaldehyde at RT for 10-15 min. Following the incubation time, cells were washed three to five times (5 min/wash) in PBS (300 $\mu\text{l/well}$) and the plate was gently swirled using a laboratory shaker. Cells were blocked for 30 min at RT using 0.5% BSA in PBS (100 $\mu\text{l/well}$) before being incubated with the unconjugated primary antibody. Anti-integrin alpha V beta 3 antibody (LM609) was

diluted 1:100 in blocking agent and added at 50 µl per well. Following one hour at RT on a laboratory shaker, cells were washed three to five times in PBS (300 µl/well). The subsequent steps were performed in the dark. Cells were incubated with the fluorophore-conjugated secondary antibody [Goat anti-Mouse IgG (H+L), Alexa Fluor™ 488] at 1:1000 dilution in blocking reagent (50 µl/well) for 1 hour at RT. The secondary antibody was removed and cells were washed 3-5 times in PBS (300 µl/well). Hoechst dye (Hoechst 33342) was diluted 1:250 in PBS and added to the cells (50 µl/well). Following 10 min at RT, cells were washed once with PBS (300 µl/well). Then, new PBS was added to the wells (100 µl/well) and samples were imaged under a fluorescence microscope. Five images were taken per sample and green fluorescent protein (GFP)- and DAPI-fluorescence were overlaid to distinguish mature osteoclasts from osteoclast precursors-like cells. Osteoclasts were defined as VNR-positive and having ≥2 nuclei. Osteoclasts were manually counted and percent osteoclasts were determined using the following equation:

$$\% \text{ osteoclasts} = \frac{\text{number of osteoclasts (OC.n)}}{\text{number of cells (Cell.n)}} \times 100$$

For the BSP vs. RANKL treatment group, three independent experiments were conducted. The dose-response-curve of BSP was investigated using two independent donors. The functionality of the FP-21 BSP-antibody was evaluated in a case study. Automated image segmentation was also developed to quantify human osteoclasts.

2.6.5 *In vitro* resorption assays using calcium phosphate discs and bone sialoprotein

To examine whether BSP promotes the bone resorption activity of osteoclasts, we used synthetic calcium phosphate (CaP) discs and modified the protocol described by Bernhardt *et al* [377]. The disks (1.2 mm thick with a 4.8 mm diameter) were primarily made of alpha-tricalcium phosphate (α-TCP) and nanocrystalline calcium-deficient hydroxyapatite (CDHA). After 10 days in culture with 20 ng/ml M-CSF, macrophages – serving as a model for osteoclast precursors – were seeded on 96-well suspension culture plates with 2D synthetic CaP scaffolds. Synthetic CaP scaffolds were prepared by incubating the discs in 70% isopropanol for 15 min, followed by three wash steps with PBS. After overnight incubation in growth medium (MEM Alpha Medium + GlutaMAX, supplemented with 10% FCS and 1% P/S) at 37°C and 5% CO₂, discs were ready for use. The bone resorption assay was carried out in duplicate using the following treatments:

- a. Medium (MEM Alpha Medium + GlutaMAX, 10% FCS, 1% P/S)
- b. M-CSF (20 ng/ml)
- c. M-CSF (20 ng/ml) + RANKL (50 ng/ml)
- d. M-CSF (20 ng/ml) + BSP (3 µg/ml)
- e. M-CSF (20 ng/ml) + RANKL (50 ng/ml) + BSP (3 µg/ml)

4x10⁴ cells were seeded onto the CaP scaffolds on a 96-well suspension culture plate at 250 µl/well. After overnight incubation in growth medium with M-CSF (37°C, 5% CO₂), the discs (incl. cells) were transferred to a new 48-well suspension culture plate. Cells were cultured for 14 days (37°C, 5% CO₂). Half of the culture medium (incl. growth factors) was changed every third day. The supernatant was collected during every medium change and supernatant samples were frozen at -20°C until assayed. After 14 days, bone resorbing activity of generated osteoclasts was assessed using the calcium colorimetric assay kit (MAK022, Sigma-Aldrich) according to the manufacturer's instructions. The kit measures free calcium in culture supernatants. A standard curve was generated each time the assay was run and the concentration of calcium in the sample (nmole/µl) was calculated from the standard curve.

Calcium concentration:

$$\frac{Sa (\mu g)}{Sv (\mu l)} = C (\mu g / \mu l)$$

Sa = Calcium quantity in sample (µg), calculated from the standard curve

Sv = Sample volume in the well (µl)

C = Calcium concentration in the sample (µg/µl)

Molecular weight of calcium = 40 µg/µmole

Final calcium concentration (Cf) in the sample:

$$\frac{C (\mu g / \mu l)}{40 \mu g / \mu mole} = Cf (\mu mole / \mu l) = Cf (nmole / \mu l)$$

2.7 Gene expression of bone sialoprotein in bone metastases of cancer patients

2.7.1 Tissue sample collection

To investigate the gene expression of BSP in bone metastases of osteotropic cancer, tumour samples were collected from cancer patients with bone metastases who underwent surgical treatment at the University Medical Center Mainz. A bone sample was collected from a patient without (metastatic) bone disease at the University Medical Center Mainz. Written informed consent was obtained from all patients. The ethics committee of the Landesärztekammer Rheinland-Pfalz approved the use of residual materials, in alignment with the university medical center's regulations and the ethical standards set forth in the Declaration of Helsinki and the ICH Guidelines for Good Clinical Practice.

2.7.2 RNA extraction and quantitative real-time PCR

RNA was isolated from samples using TRIzol RNA isolation [416]. In short, the samples were snap-frozen and grinded with liquid nitrogen using a pre-cooled ceramic mortar and pestle. The tissue powder was lysed in TRIzol using a homogeniser. Following centrifugation, chloroform was added to promote phase separation. A centrifugation step separated the sample into three phases and the upper aqueous phase containing RNA was collected. 100% isopropanol was used to precipitate RNA. A centrifuge was used to pellet the RNA. Several

washes with 75% ethanol were performed to dissolve the salts, but not the RNA. The clean RNA pellet was air dried and dissolved in nuclease free water.

RNA quantity and quality (A260/280 nm and A260/230 nm) was measured by NanoDrop One ThermoFisher Scientific spectrophotometer. 1 µl nuclease free water was used as a blank. The volume of the sample solution was 1 µl. RNA was stored at -20°C for short-term storage or at -80°C for long-term storage.

RNA was diluted to 0.2 µg/µl in nuclease free water. RNA (1 µg) was reverse transcribed by random priming using deoxynucleoside triphosphates (dNTPs) and M-MuLV Reverse Transcriptase supplied with 10x M-MuLV Reverse Transcriptase Reaction Buffer. Samples were reverse transcribed into cDNA for one hour at 42°C followed by 20 min at 65°C using a thermocycler. cDNA was immediately used for real-time PCR or stored at -20°C for short-term storage.

cDNA was diluted 1:10 in nuclease free water. Quantitative real-time PCR was performed with 5 µl cDNA in a 15 µl volume with 0.1 µl forward primer, 0.1 µl reverse primer, 10 µl Blue S'Green qPCR 2x Mix and 4.8 µl RNase-free water. Sequence specific primers are shown in table 6. Samples were pre-incubated for 2 min at 95°C and then underwent 40 amplification cycles (denaturation at 95°C for 5 sec, primer-annealing and –elongation at 60°C for 25 sec) using a real-time PCR thermal cycler. Samples and controls were run in triplicate. Data quality was assessed by evaluating the individual PCR amplification curves. Replicates did not vary by more than 0.5 cycles. The Ct values of the housekeeping gene GAPDH and the gene of interests (GOIs) were averaged. Results were normalised to GAPDH:

$$\Delta Ct \text{ value} = GOI - \text{housekeeping gene}.$$

Gene name	Primer sequence (5' -> 3')
<i>BSP</i>	Forward: TGG AAA AGG TGA AAC AAA TGG AAG A Reverse: TCC CCT GAC GTG TAT GGC TA
<i>GAPDH</i>	Forward: CGA CCA CTT TGT CAA GCT CA Reverse: AGG GGA GAT TCA TGT TGG TG
<i>OPG</i>	Forward: TAA CGT GAT GAG CGT ACG GG Reverse: GAT GTC CAG AAA CAC GAG CG
<i>OPN</i>	Forward: GGT CAC TGA TTT TCC CAC GG Reverse: CTC CTC GCT TTC CAT GTG TG
<i>PTHrP</i>	Forward: TCT TGA GGG GCT GCT CTT TG Reverse: CGG AAA TGG GAG TCT CTC CG
<i>RANKL</i>	Forward: GGC ACT GCG TGG AGT AAA ATG Reverse: TGA ACG TGG GTG TCC CTT TC
<i>Runx2</i>	Forward: CTG TGG TTA CTG TCA TGG CG Reverse: AGG TAG CTA CTT GGG GAG GA
<i>TRAP</i>	Forward: AAT GGC CAA TGC CAA GGA GA Reverse: GGG AGC GGT CAG AGA ATG CG

Table 6. Sequence specific primers.

BSP, bone sialoprotein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; OPG, osteoprotegerin; OPN, osteopontin; PTHrP, parathyroid hormone-related protein; RANKL, receptor activator of nuclear factor kappa-B ligand; Runx2, runt-related transcription factor 2; TRAP, tartrate-resistant acid phosphatase.

2.8 Statistics

Values are presented as mean $\pm$ standard deviation. Statistical analyses, including One-way ANOVA and Two-way ANOVA, followed by Dunnett's multiple comparison test, were conducted using GraphPad Prism 9 software for Windows. Statistical significance was set at $P < 0.05$.

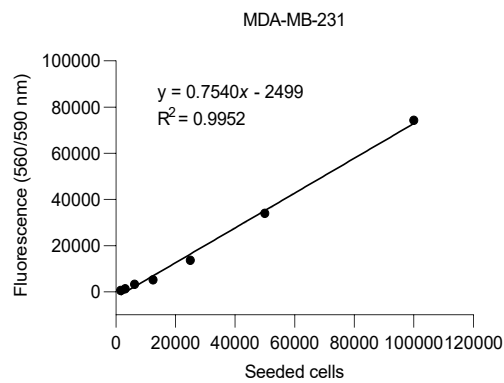
3. Results

3.1 Cell adhesion assays

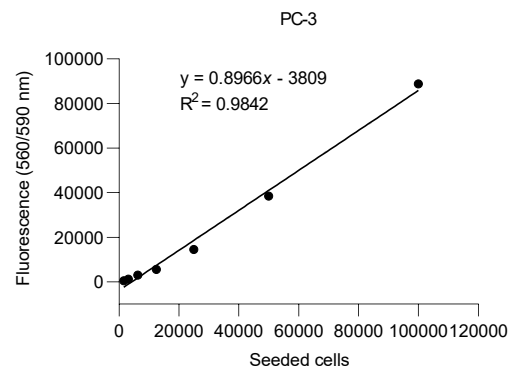
3.1.1 Standard curves generated from human cell lines

The standard curves of the human cell lines breast adenocarcinoma (MDA-MB-231), prostate adenocarcinoma (PC-3), large-cell lung cancer (NCI-H460), NSCLC (A-549), osteosarcoma (MG-63), and the non-tumorigenic, immortalised human breast epithelial cell line (MCF-10A) are depicted in figure 11. Serial dilutions of cells were seeded onto culture plates and incubated for 2 h to allow adhesion. Fluorescence intensity increased proportionally with the number of seeded cells after 2 h of adhesion for all cell lines. The coefficient of determination (R^2) was greater than 0.96 in every cell line.

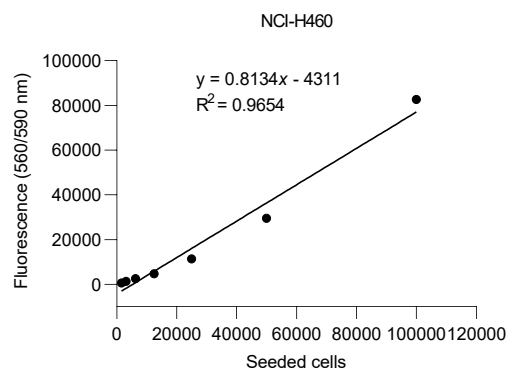
A



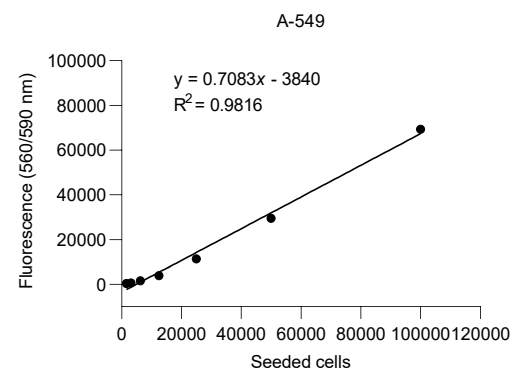
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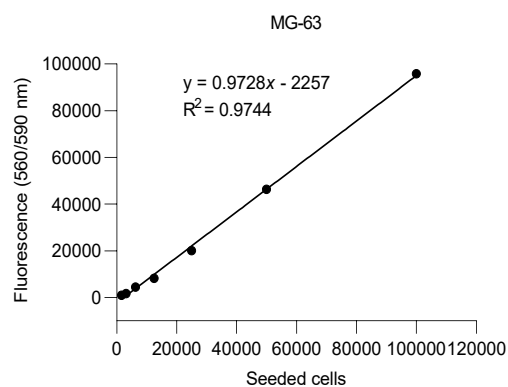
C



D



E



F

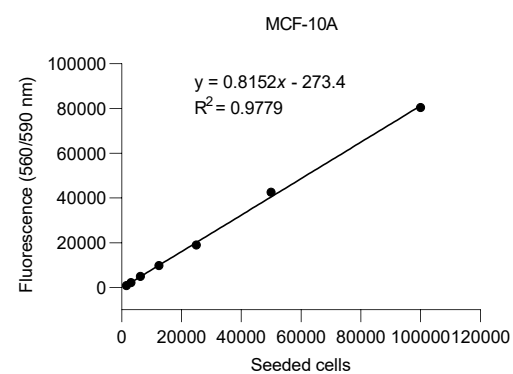


Figure 11. Fluorescence-based standard curves for cell adhesion in cancer and non-tumorigenic cell lines. Standard curves of breast (MDA-MB-231) (A), prostate (PC-3) (B), large-cell lung (NCI-H460) (C), NSCLC (A-549) (D), and osteosarcoma (MG-63) (E) cancer cell lines and non-tumorigenic mammary epithelial cell line (MCF-10A) (F) for the adhesion assay. Number of seeded cells were plotted against fluorescence intensity at 560/590 nm, measured using a fluorescence multi-well reader after a 2-h cell adhesion period followed by a 4-h incubation with alamarBlue®. The best-fit line and coefficient of determination (R^2) are presented in the graphs. One experiment was carried out. Results are expressed as mean ($n=5$). NSCLC, non-small cell lung cancer.

3.1.2 Bone sialoprotein induces human cancer and mammary epithelial cell adhesion *in vitro*

Rat or bovine BSP stimulates the adhesion of various cell types, including rabbit osteoclasts [291], rat osteosarcoma [395], human melanoma (MDA-MB-435) [395] and breast cancer (SKBR3) cells [395]. However, the role of human BSP in anchorage-dependent cell adhesion remains underexplored. The effects of human BSP on cancer cell adhesion may be cell-line specific, with some studies reporting stimulation [381] and others showing no significant impact [328]. To further investigate the role of human BSP in (cancer) cell adhesion, we carried out an adhesion assay to assess the ability of different human BSP proteins to promote the attachment of MDA-MB-231, PC-3, NCI-H460, A-549, MG-63, and MCF-10A cells. Suspension culture plates coated with 3 µg/ml BSP CR-3 and huBSP2 significantly enhanced the adhesion of cancer cell lines compared to the negative control (PBS) after 2 h (figure 12A-E). Although 3 µg/ml BSP CR-1 also promoted adhesion, it did not enhance attachment in A-549 cells (figure 12D). BSP proteins strongly induced the attachment of the human mammary epithelial cell line MCF-10A (figure 12F). The inherent adhesion capacity of the human cell lines was as follows: MG-63 > NCI-H460 > A-549 > MCF-10A > MDA-MB-231 > PC-3.

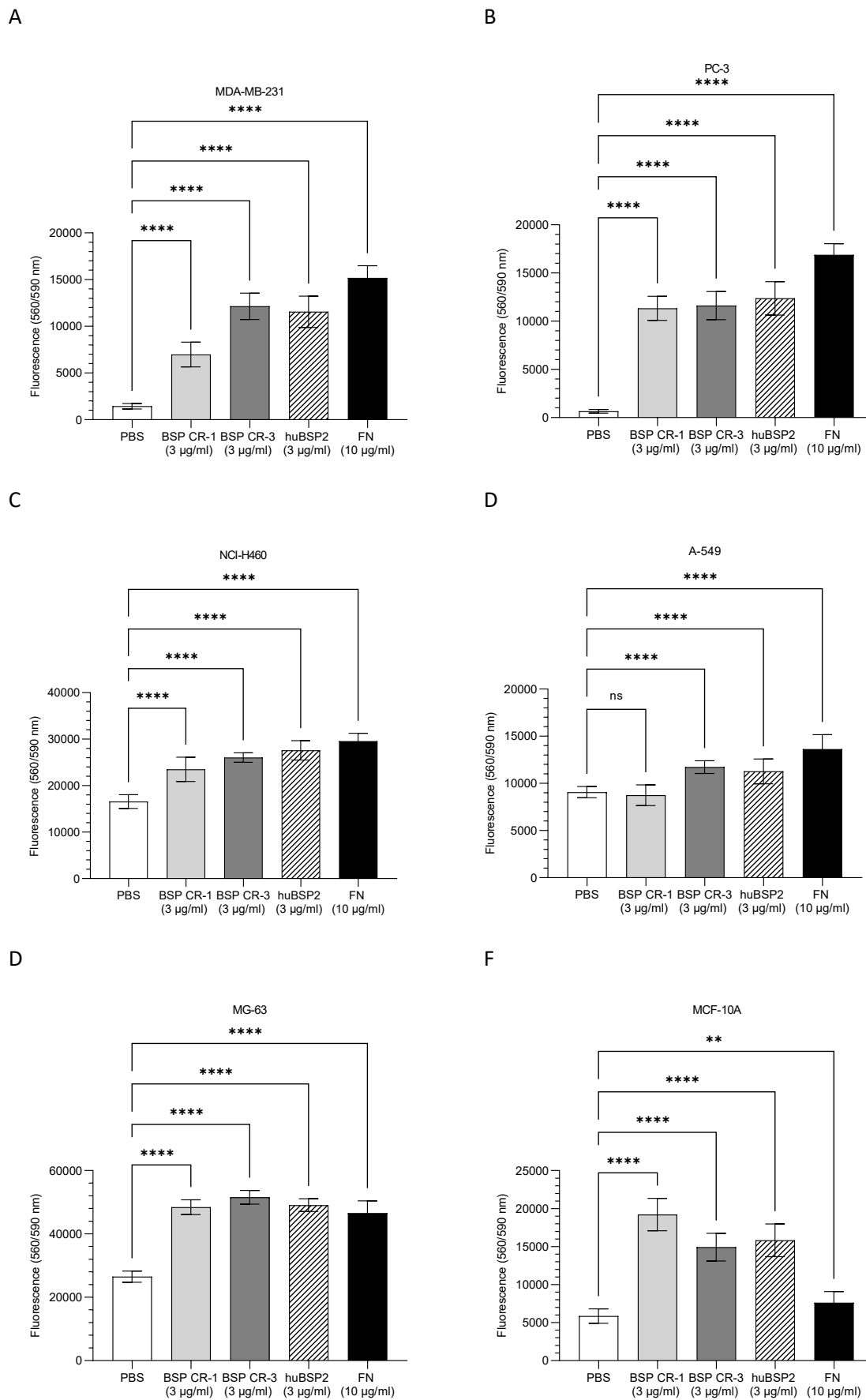


Figure 12. Adhesion of human cell lines to various surface coatings.

Attachment levels of MDA-MB-231 (A), PC-3 (B), NCI-H460 (C), A-549 (D), and MG-63 (E) cancer cells, and MCF-10A epithelial cells (F) to surfaces coated with PBS, BSP and FN after 2 h. Adherent cells were quantified using the alamarBlue® assay (4 h) and measuring fluorescence at 560/590 nm. Data are shown as mean values with standard deviation bars ($n=5$). ** $P<0.01$; **** $P<0.0001$ vs. negative control (PBS), one-way ANOVA, Dunnett's multiple comparison test. BSP, bone sialoprotein; FN, fibronectin; ns, non-significant; PBS, phosphate-buffered saline.

3.1.3 Bone sialoprotein dose-dependently increases the attachment of human cancer cells

A previous study demonstrated that MDA-MB-231 cells adhere to plates coated with linear or cyclic human BSP peptides in a dose-dependent manner [0.01-10 μM (≈ 0.6 -676 $\mu\text{g/ml}$) within 3 h [300]. Our current investigation revealed that 3 $\mu\text{g/ml}$ of human BSP protein significantly enhanced cancer cell adhesion. To expand upon these findings, we conducted a dose-response adhesion assay to determine whether human cancer cell lines also exhibit concentration-dependent adhesion to human BSP protein, specifically to huBSP2, rather than to human BSP peptides. A dose-response curve for BSP (huBSP2) and MDA-MB-231 cells was established using huBSP2 concentrations of 4, 2, 1, 0.5, 0.25, and 0 $\mu\text{g/ml}$. For PC-3 cells, huBSP2 was serially diluted to 10, 3.33, 1.11, 0.37, 0.123, and 0 $\mu\text{g/ml}$, while for NCI-H460 and MG-63 cells, the dilutions were 6, 3, 1.5, 0.75, 0.375, 0 $\mu\text{g/ml}$ and 12, 6, 3, 1.5, 0.75, and 0 $\mu\text{g/ml}$, respectively. Adhesion of MDA-MB-231, PC-3, NCI-H460 and MG-63 cancer cells to huBSP2 increased with increasing huBSP2 concentrations within 2 h (figure 13). The BSP concentrations required to achieve 80% of maximal cell adhesion (EC_{80}) were calculated from a sigmoidal curve based on fluorescence data and BSP concentrations. The EC_{80} values were 1.209 $\mu\text{g/ml}$ for MDA-MB-231, 3.5 $\mu\text{g/ml}$ for PC-3, and 1.403 $\mu\text{g/ml}$ for NCI-H460 cells. 1.1 $\mu\text{g/ml}$ huBSP2 was selected for subsequent adhesion experiments with MG-63 cells.

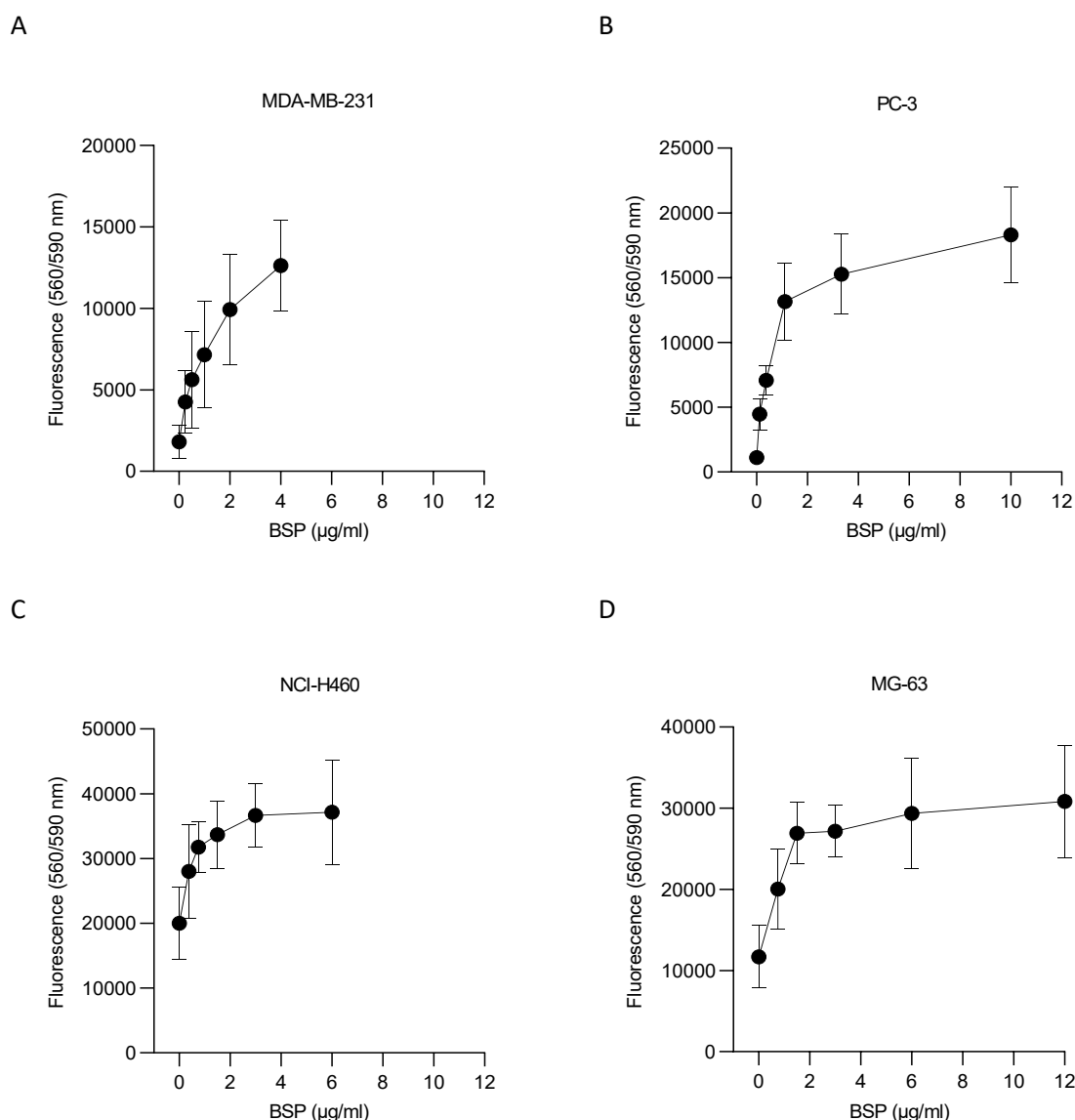


Figure 13. Enhanced attachment of human cancer cell lines to BSP at higher protein concentrations. Adhesion of MDA-MB-231 (A), PC-3 (B), NCI-H460 (C), and MG-63 (D) cancer cells to BSP at various protein concentrations after 2 h. Adherent cells were quantified using the alamarBlue® assay (4 h) and measuring fluorescence by a fluorescence multi-well reader at 560/590 nm. Values are mean $\pm$ standard deviation ($n=9$). BSP, bone sialoprotein.

3.1.4 Cancer cell adhesion to bone sialoprotein is strongly mediated by its RGD sequence

Our previous findings showed that human BSP protein dose-dependently stimulated the adhesion of human cancer cells. To further explore the binding mechanism underlying this, we focused on the RGD (Arg-Gly-Asp) sequence located in the C-terminal region of BSP. This motif is known to regulate BSP-enhanced angiogenesis [361, 362], cancer cell survival [299], and immune evasion by tumour cells [392]. Additionally, it has been shown to mediate the adhesion of MDA-MB-231 cells [381]. The literature suggests that the RGD sequence may be essential for mediating cancer cell adhesion to BSP, potentially contributing to the metastatic progression. Our current investigation into the RGD sequence's involvement revealed that pre-incubation of cells with increased concentrations of exogenous FN-derived

GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro) for 30 min strongly reduced the attachment of MDA-MB-231, PC-3, NCI-H460 and MG-63 cancer cell lines to huBSP2-coated surfaces after 2 h (figure 14). At absent or low concentrations of the RGD-containing peptide, cancer cells displayed an elongated shape (figure 15A-C). At higher concentrations of GRGDSP, the cells adopted a more rounded shape and began to detach from BSP-coated surfaces (figure 15D-E).

Prior incubation of cancer cells with exogenous GRGDSP peptide had minimal impact on the adhesion of MDA-MB-231 and NCI-H460 cells to VN (data not shown). For MDA-MB-231, attachment of cells to VN reduced by 7.6% at 12 μ M RGD peptide with respect to the control with 0 μ M GRGDSP [55723 ± 5560 (fluorescence 560/590 nm) vs. 60311 ± 8150]. Similarly, for NCI-H460, adhesion to VN was reduced by 6.3% at 120 μ M of the RGD-containing peptide (39549 ± 5697 vs. 42217 ± 6853). The exogenous RGD peptide was able to partially inhibit PC-3 and MG-63 cancer cell adhesion to VN, although complete inhibition was not achieved (data not shown). At 120 μ M GRGDSP, PC-3 cell adhesion to VN was greatly reduced by 62.8% compared to 0 μ M GRGDSP (13226 ± 7038 vs. 35575 ± 14194). For MG-63, cell attachment to VN was inhibited by 33% at 300 μ M GRGDSP (35599 ± 5856 vs. 53137 ± 9004).

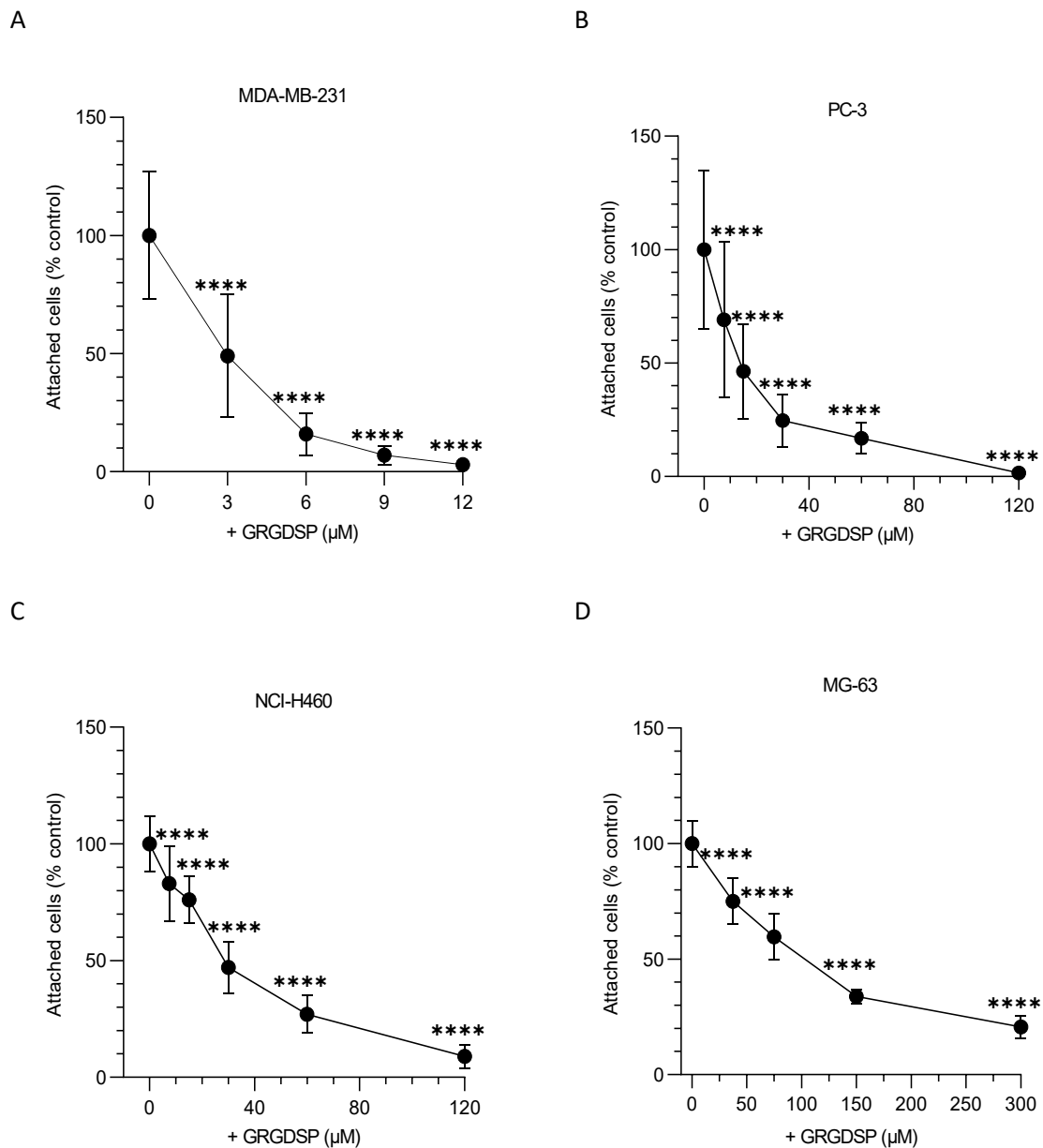


Figure 14. Impact of exogenous GRGDSP peptide on the attachment of human cancer cell lines to BSP. Adhesion of MDA-MB-231 (A), PC-3 (B), NCI-H460 (C), and MG-63 (D) cancer cells to BSP following a 30-min incubation at 37°C with increasing concentrations of GRGDSP peptide. Cells were cultured on BSP-coated plates for 2 h. Adherent cells were quantified using the alamarBlue® assay (4 h) and measuring fluorescence at 560/590 nm. Results are presented as percentages of negative control (0 μM GRGDSP) (100%) ± standard deviation ($n=9$). **** $P<0.0001$ compared to 0 μM GRGDSP, one-way ANOVA, Dunnett's multiple comparison test. BSP, bone sialoprotein; GRGDSP, Gly-Arg-Gly-Asp-Ser-Pro.

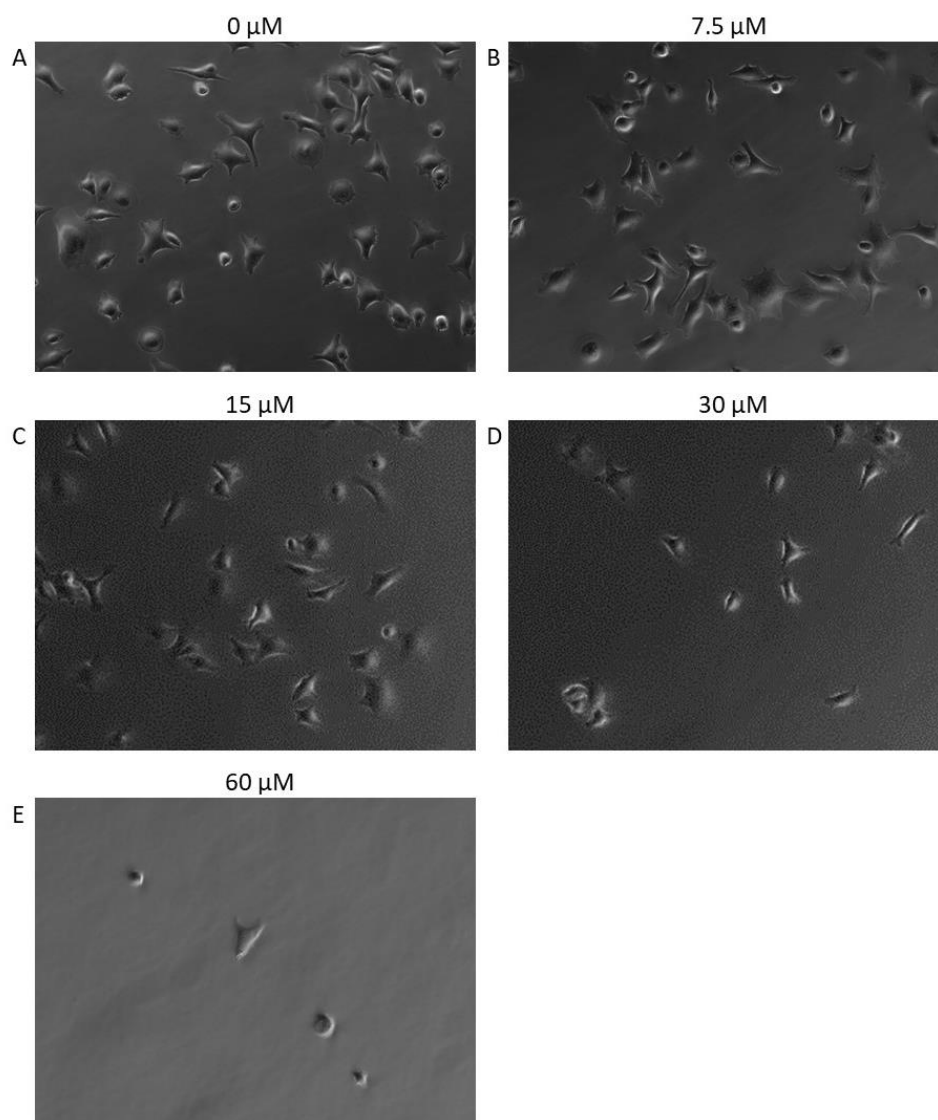


Figure 15. Dose-dependent response of PC-3 cells to GRGDSP peptide on BSP-coated surfaces. The impact of incubating PC-3 cells with varying concentrations of GRGDSP peptide—0 μM (A), 7.5 μM (B), 15 μM (C), 30 μM (D), and 60 μM (E)—for 30 min, followed by 2 h of culture on BSP-coated plates, on cell adhesion and morphology (magnification x20). BSP, bone sialoprotein; GRGDSP, Gly-Arg-Gly-Asp-Ser-Pro.

3.1.5 Differential effects of the heparin-binding sequence of bone sialoprotein on cancer cell adhesion

BSP contains a heparin-binding site that may regulate cell adhesion. In rat BSP, the heparin-binding sequence FHRRKA promotes the adhesion and spreading of rat osteoblast-like cells, but does not induce vinculin contact formation or actin cytoskeletal reorganisation [292]. In human BSP, the N-terminal heparin-binding domain LHRRVKI (Lys-His-Arg-Arg-Lys-Val-Ile) has been identified, but its role in mediating adhesion – whether of osteoblast-like cells or cancer cells – remains unclear.

We investigated the role of LHRRVKI in BSP-mediated cancer cell adhesion. Adhesion of MDA-MB-231 cells to BSP-coated surfaces increased in a dose-dependent manner with

preincubation of cells with rising exogenous LHRRVKI concentrations (figure 16A). In contrast, adhesion of PC-3 to BSP decreased at higher exogenous LHRRVKI concentrations (figure 16B).

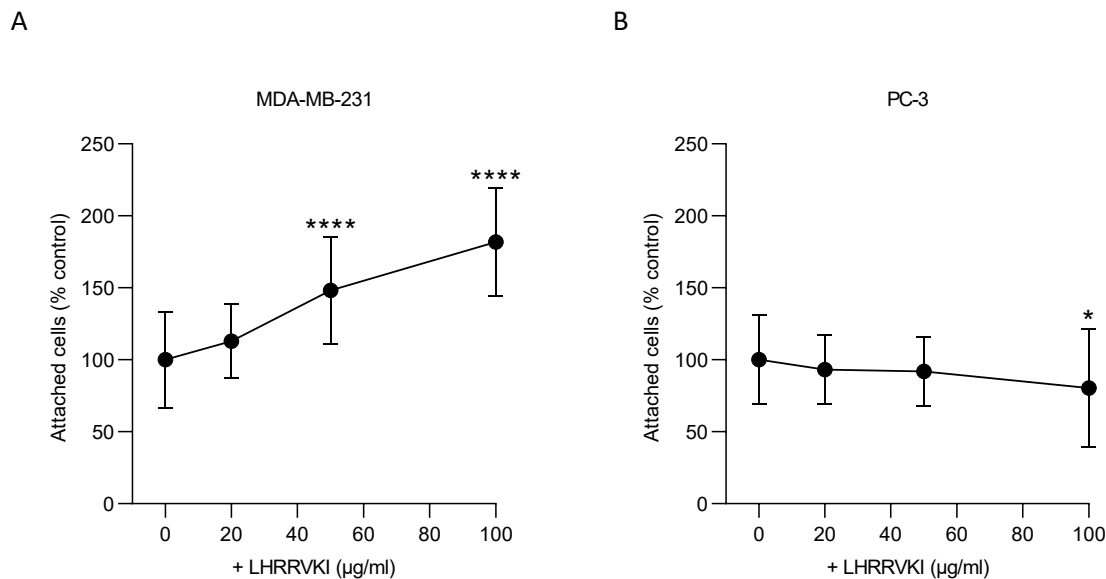


Figure 16. Effects of exogenous LHRRVKI peptide on cancer cell adhesion to BSP.

MDA-MB-231 (A), and PC-3 (B) cells were incubated for 30 min at 37°C in the presence of increasing concentrations of LHRRVKI peptide. Cells were cultured for 2 h on BSP-coated plates and attached cells were examined by the alamarBlue® assay (4 h). Data are reported as percentages compared with negative control (0 µg/ml LHRRVKI) (100%) ± standard deviation ($n=9$). * $P<0.05$; **** $P<0.0001$ compared to 0 µg/ml LHRRVKI, one-way ANOVA, Dunnett's multiple comparison test. BSP, bone sialoprotein; LHRRVKI, Lys-His-Arg-Arg-Lys-Val-Ile.

3.1.6 Integrin receptors $\alpha\beta 3$ and $\alpha\beta 5$ regulate cancer cell adhesion to bone sialoprotein

Binding of ECM ligands to RGD-binding integrins on the cell surface triggers focal adhesion formation, cytoskeletal reorganisation, and cell adhesion [190, 417]. The RGD domain of BSP may bind to integrin $\alpha\beta 3$ on osteoclasts to mediate adhesion [291, 367], while the adhesion of breast carcinoma SKBR-3 cells to BSP occurs through integrin $\alpha\beta 5$ [395].

We demonstrated that the RGD domain strongly regulates human BSP-induced cancer cell adhesion. This effect may be mediated through the interaction of integrins $\alpha\beta 3$ and $\alpha\beta 5$, which are expressed by both primary tumour cells and cancer cell lines. These integrins bind the RGD sequence of BSP, facilitating attachment [418, 419]. Preincubation of human cancer cell lines with integrin antibodies anti- $\alpha\beta 3$ or anti- $\alpha\beta 5$ prior to seeding significantly inhibited the attachment of MDA-MB-231, PC-3, and NCI-H460 to huBSP2-coated plates after 2 h, with varying degrees of inhibition observed (figure 17A-C). The integrin antibodies did not affect the adhesion of MG-63 cells to huBSP2 (figure 17D).

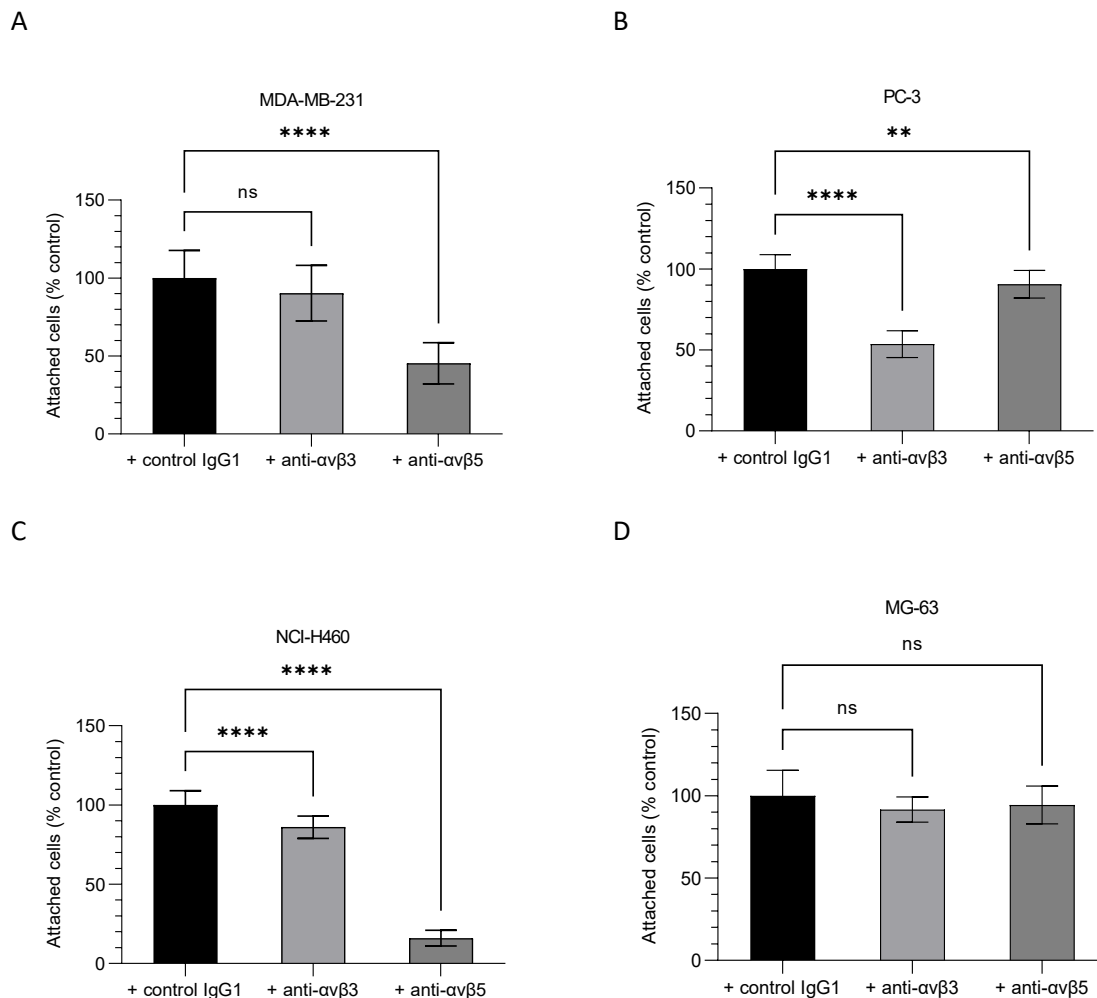


Figure 17. Effects of integrin antibodies anti- α v β 3 and anti- α v β 5 on human cancer cell adhesion to BSP. MDA-MB-231 (A), PC-3 (B), NCI-H460 (C), and MG-63 (D) cells were incubated with 10 μ g/ml of either isotype control IgG1, anti- α v β 3 antibody, or anti- α v β 5 antibody for 60 min at 37°C before being seeded onto BSP-coated plates for 2 h. Adherent cells were quantified using the alamarBlue® assay (4 h) and measuring fluorescence at 560/590 nm. Results are presented as percentages of isotype control IgG1 (100%) $\pm$ standard deviation ($n=4$). ** $P<0.01$; **** $P<0.0001$ compared to the negative control IgG1, one-way ANOVA, Dunnett's multiple comparison test. BSP, bone sialoprotein; ns, non-significant.

3.1.7 Antibodies targeting human bone sialoprotein inhibit breast cancer cell adhesion to bone sialoprotein

BSP antibodies IDK-1 and FP-21

We have inhibited BSP-mediated attachment of human cancer cells *in vitro* using exogenous peptides (GRGDSP) and antibodies targeting integrins α v β 3 and α v β 5. Building on this, we investigated whether antibodies directed against BSP could suppress its adhesive activity. Identifying effective anti-BSP antibodies through *in vitro* assays is a critical first step toward developing new cancer therapies or enhancing existing drugs targeting integrin-ECM interactions. Since no currently approved cancer drugs specifically target integrins α v β 3 or α v β 5 [420], there is a pressing need for alternative inhibitors or antibodies to disrupt this binding mechanism and potentially inhibit tumour progression and metastasis.

Based on prior experiments with a range of mouse anti-human FP-21 and rat anti-human IDK-1 BSP-antibody concentrations (300, 240, 180, 120, 60, 30, and 0 $\mu\text{g/ml}$, data not shown), subsequent blocking experiments were performed with mouse anti-human FP-21 (12 to 0 $\mu\text{g/ml}$) and rat anti-human IDK-1 (240 to 0 $\mu\text{g/ml}$) BSP-antibody concentrations. Adhesion of MDA-MB-231 cells to BSP-coated plates strongly decreased when huBSP2 (EC_{80}) was incubated with increasing concentrations of IDK-1 or FP-21 (figure 18). FP-21 effectively inhibited MDA-MB-231 cell adhesion to BSP at lower antibody concentrations.

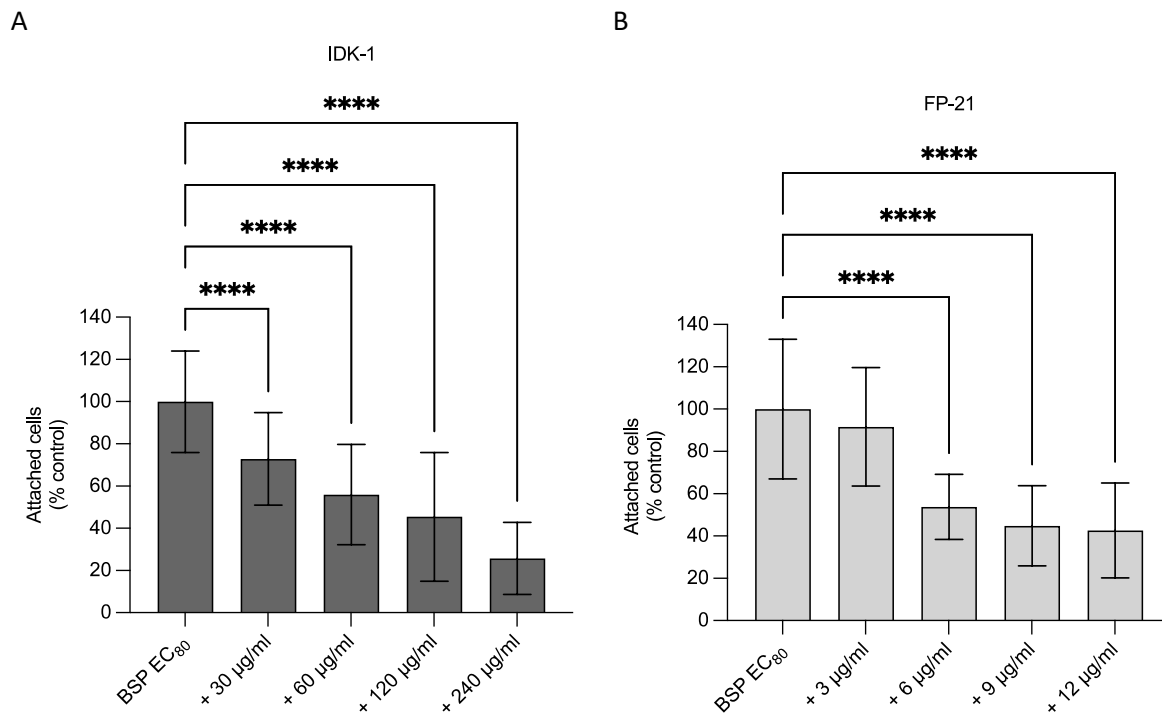


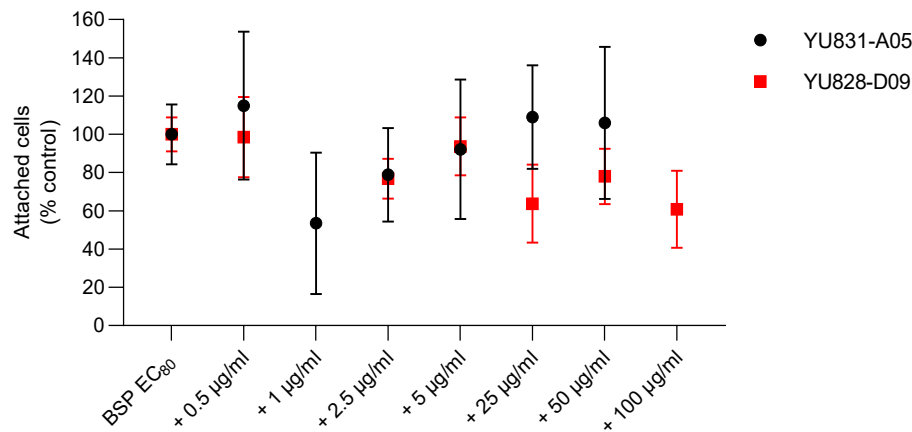
Figure 18. Anti-BSP antibodies rat anti-human IDK-1 (A) and mouse anti-human FP-21 (B) inhibit the adhesion of MDA-MB-231 cells to BSP with increasing antibody concentrations. Following a 30-min incubation with IDK-1 or FP-21 antibody concentrations, BSP-antibody mixtures were coated on suspension culture plates overnight. Cells were cultured on the BSP-coated plates for 2 h. Adherent cells were quantified using the alamarBlue[®] assay (4 h) and measuring fluorescence at 560/590 nm. Percentages were calculated relative to BSP EC_{80} (0 $\mu\text{g/ml}$ antibody), considered 100%. Results are expressed as mean $\pm$ standard deviation ($n=9$). **** $P<0.0001$ compared to the negative control (BSP EC_{80}), one-way ANOVA, Dunnett's multiple comparison test. BSP, bone sialoprotein.

BSP antibodies YU831-A05 and YU828-D09

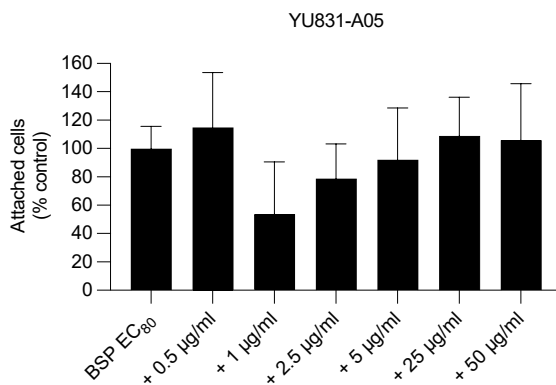
Following the successful testing of the anti-BSP antibodies FP-21 and IDK-1, we next tested whether the anti-BSP antibodies YU831-A05 and YU828-D09 could suppress BSP-induced adhesion of MDA-MB-231 breast cancer cells. Initial experiments using YU831-A05 and YU828-D09 at concentrations of 50, 5, 1, and 0 $\mu\text{g/ml}$ demonstrated the greatest inhibition of MDA-MB-231 cell adhesion to BSP at 1 $\mu\text{g/ml}$ YU831-A05 and 50 $\mu\text{g/ml}$ YU828-D09 (data not shown). Based on the results, subsequent blocking experiments used a broader range of concentrations: YU831-A05 (50, 25, 5, 2.5, 1, 0.5, and 0 $\mu\text{g/ml}$) and YU828-D09 (100, 50, 25, 5, 2.5, 0.5, and 0 $\mu\text{g/ml}$).

There was a trend for YU831-A05 to decrease MDA-MB-231 attachment to BSP at 1 $\mu\text{g/ml}$ ($p=0.0781$). YU828-D09 reduced MDA-MB-231 cell adhesion to BSP at concentrations of 100 $\mu\text{g/ml}$ and 25 $\mu\text{g/ml}$. Figure 19A-C illustrates the effects of anti-BSP antibodies YU831-A05 and YU828-D09 on MDA-MB-231 cell adhesion to BSP.

A



B



C

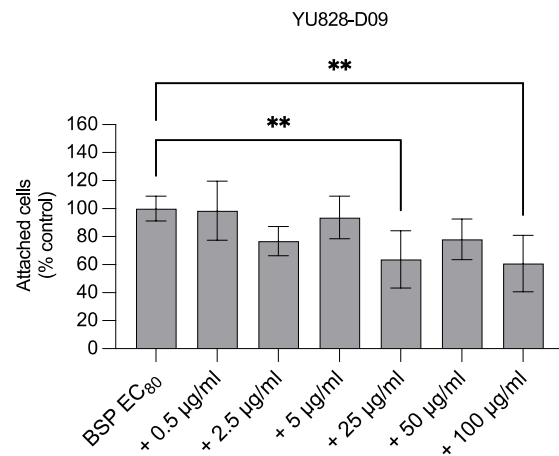


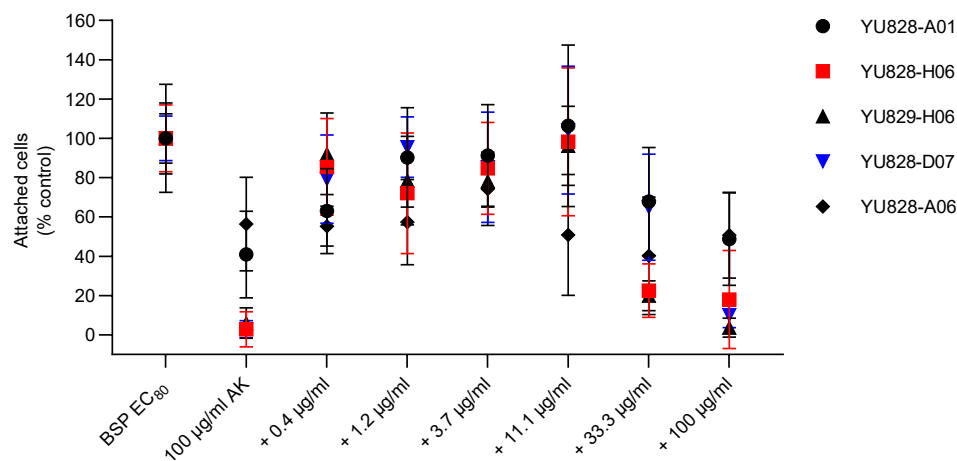
Figure 19. Effect of anti-BSP antibodies YU831-A05 and YU828-D09 on MDA-MB-231 cell adhesion to BSP. Combined results of anti-BSP antibodies YU831-A05 and YU828-D09 on breast carcinoma (MDA-MB-231) cell adhesion to BSP (A). Individual effects of various concentrations of anti-BSP antibodies YU831-A05 (B), and YU828-D09 (C) on the attachment of MDA-MB-231 cells to BSP. Following a 30-min incubation with BSP antibody concentrations, BSP-antibody mixtures were coated on 96-well suspension culture plates overnight. Cells were cultured on the BSP-coated plates for 2 h. Adherent cells were quantified using the alamarBlue® assay (4 h) and measuring fluorescence at 560/590 nm. Data are reported as percentages compared with BSP EC₈₀ (0 $\mu\text{g/ml}$ antibody) (100%). Values are mean $\pm$ standard deviation ($n=4$). ** $P<0.01$ compared to negative control (BSP EC₈₀), one-way ANOVA, Dunnett's multiple comparison test. BSP, bone sialoprotein.

BSP antibodies YU828-A01, YU828-H06, YU829-H06, YU828-D07, and YU828-A06

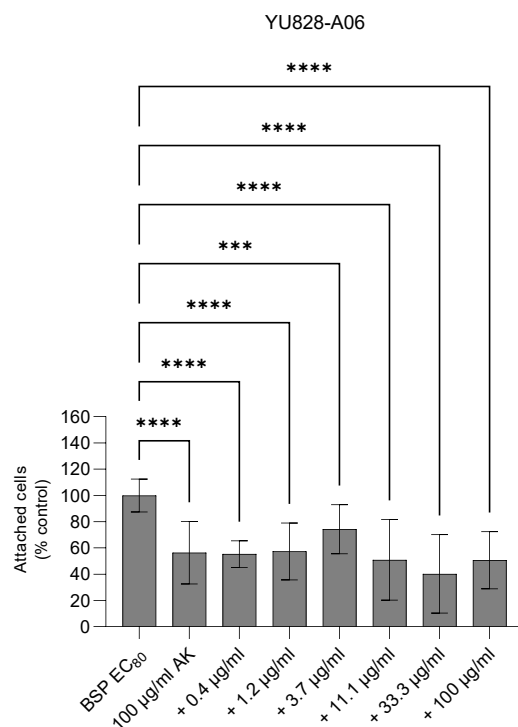
After testing the anti-BSP antibodies FP-21, IDK-1, YU831-A05 and YU828-D09, we proceeded to evaluate the inhibitory potential of additional anti-BSP antibodies YU828-A01,

YU828-H06, YU829-H06, YU828-D07, and YU828-A06 on BSP-enhanced attachment of MDA-MB-231 breast cancer cells. Blocking assays were performed using a range of antibody concentrations: 100, 33.3, 11.1, 3.7, 1.2, 0.4, and 0 $\mu\text{g/ml}$. Strong inhibition of MDA-MB-231 cell adhesion to BSP was observed at 100 $\mu\text{g/ml}$ and 33.3 $\mu\text{g/ml}$, with the highest inhibition achieved using 100 $\mu\text{g/ml}$ of YU829-H06 (-96% compared to control). Other anti-BSP antibodies were less potent at 100 $\mu\text{g/ml}$: YU829-H06 > YU828-D07 > YU828-H06 > YU828-A01 > YU828-A06. MDA-MB-231 cells did not adhere to coatings of 100 $\mu\text{g/ml}$ of anti-BSP antibodies YU828-H06, YU829-H06, YU828-D07, and YU828-A06 in the absence of BSP. Adhesion was observed with a homogenous coating of 100 $\mu\text{g/ml}$ YU828-A01. Figure 20A-F illustrates the effects of anti-BSP antibodies on MDA-MB-231 cell adhesion to BSP.

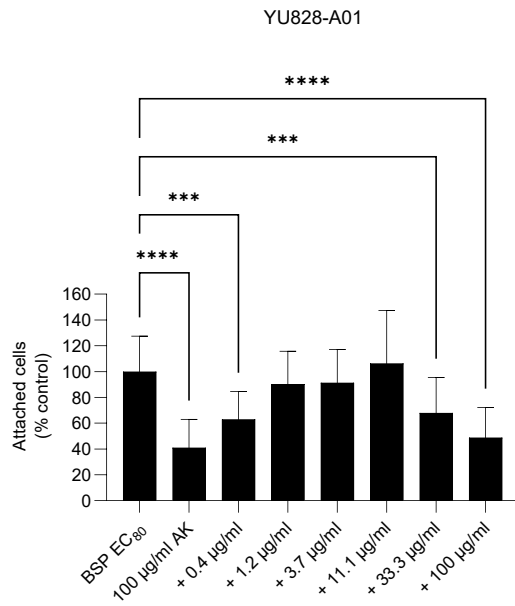
A



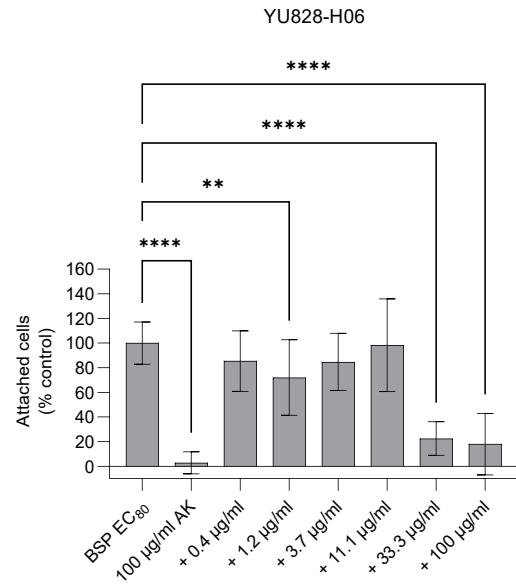
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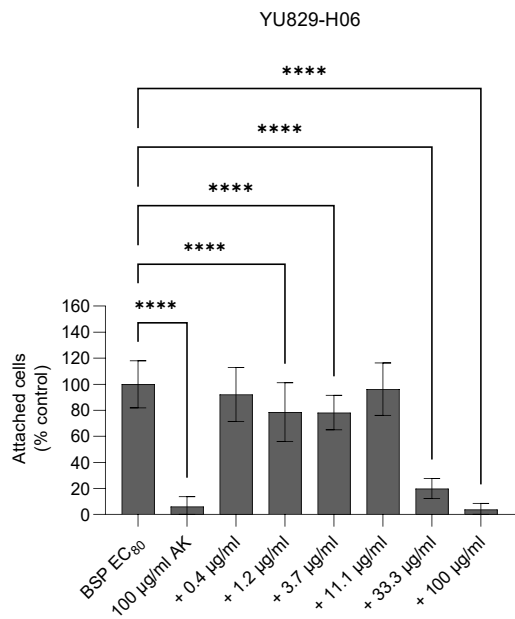
C



D



E



F

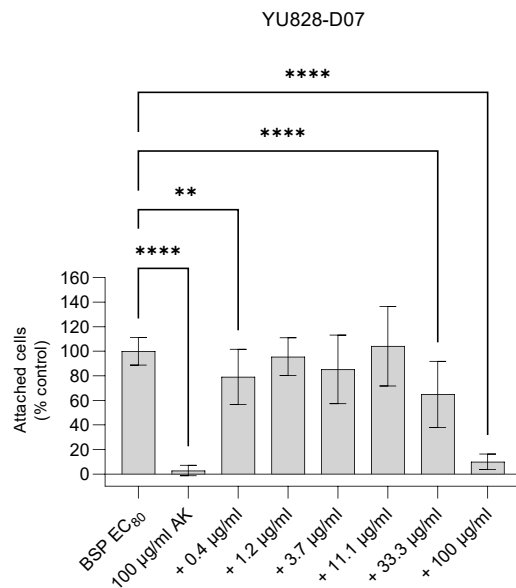


Figure 20. Anti-BSP Antibodies (YU828-A01, YU828-H06, YU829-H06, YU828-D07, YU828-A06) decrease MDA-MB-231 adhesion to BSP.

Summary of the effects of various anti-BSP antibodies on breast carcinoma MDA-MB-231 cell adhesion to BSP (A). Anti-BSP antibodies YU828-A06 (B), YU828-A01 (C), YU828-H06 (D), YU829-H06 (E), and YU828-D07 (F) decreased the adhesion of MDA-MB-231 cells to BSP at higher antibody concentrations. After a 30-min incubation with BSP antibody concentrations, BSP-antibody mixtures were coated on 96-well suspension culture plates overnight. Cells were cultured on the BSP-coated plates for 2 h. Adherent cells were quantified using the alamarBlue® assay (4 h) and measuring fluorescence at 560/590 nm. The graphs also illustrate the effect of a homogenous coating of 100 µg/ml anti-BSP antibody (100 µg/ml AK) on MDA-MB-231 cell adhesion. Percentages were calculated relative to BSP EC₈₀ (0 µg/ml antibody), considered 100%. Results are presented as mean $\pm$ standard deviation ($n=9$). ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$ compared to the negative control BSP EC₈₀ (0 µg/ml antibody), one-way ANOVA, Dunnett's multiple comparison test. AK, antibody; BSP, bone sialoprotein.

Half-maximal inhibitory concentrations

Table 7 outlines the half-maximal inhibitory concentration (IC_{50}) values of the anti-BSP antibodies FP-21, IDK-1, YU828-A01, YU828-A06, YU828-H06, YU829-D07, and YU829-H06, reflecting their effectiveness in inhibiting MDA-MB-231 cell adhesion to BSP. Based on these values, the potency of the antibodies followed the order: FP-21 > YU828-A06 > YU829-H06 > YU828-H06 > YU829-D07 > IDK-1 > YU828-A01.

Anti-BSP antibody	IC_{50} ($\mu\text{g/ml}$)
FP-21	8.064
IDK-1	84.41
YU828-A01	94.83
YU828-A06	21.28
YU828-H06	24.69
YU829-D07	42.35
YU829-H06	23.59

Table 7. Half-maximal inhibitory concentration values of the anti-BSP antibodies in MDA-MB-231 cells as determined by blocking experiments and the alamarBlue[®] assay. BSP, bone sialoprotein; IC_{50} , half-maximal inhibitory concentration.

In summary, human BSP-coated surfaces promoted the adhesion of the human mammary epithelial cell line MCF-10A and the human cancer cell lines MDA-MB-231, PC-3, NCI-H460, A-549, and MG-63 cells, with MDA-MB-231, PC-3, NCI-H460, and MG-63 cells showing increased adhesion at higher BSP concentrations. Pre-incubation with FN-derived GRGDSP peptide significantly inhibited cancer cell adhesion to human BSP in a dose-dependent manner. The heparin-binding site peptide (LHRRVKI) reduced PC-3 adhesion, while enhancing MDA-MB-231 adhesion. Anti-integrin antibodies selectively inhibited BSP-induced adhesion, with $\alpha v \beta 5$ blocking MDA-MB-231 adhesion and $\alpha v \beta 3$ and $\alpha v \beta 5$ blocking PC-3 adhesion, while MG-63 adhesion remained unaffected. BSP antibodies effectively reduced MDA-MB-231 adhesion to human BSP, with mouse anti-human FP-21 and rat anti-human IDK-1 showing dose-dependent inhibition. Low concentrations of FP-21 and high concentrations of IDK-1 were particularly effective. YU831-A05 had no significant impact, while YU828-D09 significantly inhibited MDA-MB-231 adhesion at 100 and 25 $\mu\text{g/ml}$. YU828-A01, YU828-H06, YU829-H06, YU828-D07, and YU828-A06 also strongly inhibited MDA-MB-231 adhesion at 100 and 33.3 $\mu\text{g/ml}$. FP-21 demonstrated significant inhibitory potency (IC_{50}).

3.2 Cell migration (Boyden chamber) assays

3.2.1 Standard curves generated from human cancer cell lines

Standard curves of the human cancer cell lines MDA-MB-231 and PC-3 were generated (figure 21). The increase in absorbance intensity was proportional to the increase in the number of seeded cells. The coefficient of determination (R^2) was greater than 0.94 in the two cell lines.

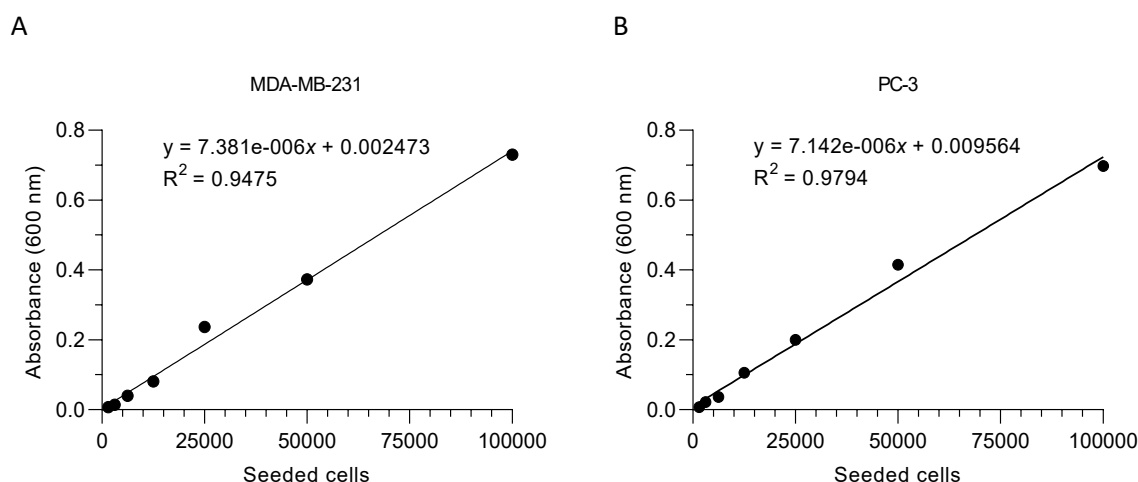


Figure 21. Standard curves of MDA-MB-231 (A), and PC-3 (B) human cancer cells for the migration assay. The graphs show the number of seeded cells against the absorbance measured at 600 nm by a spectrophotometer. Cells were stained with 0.5% CV and the dye was eluted in 33% acetic acid. The equation of the best-fit line and coefficient of determination (R^2) are presented in the graphs. One experiment was carried out. Results are mean ($n=3$). CV, crystal violet.

3.2.2 Bone sialoprotein regulates the migration of human cancer cell lines

We demonstrated that human BSP promotes the adhesion of human cancer cells, including breast, prostate, lung, and osteosarcoma, as well as non-tumorigenic mammary epithelial cells *in vitro*. While adhesion is a key step in metastasis, BSP may also regulate migration, influencing chemotaxis and tumour spread [172, 173]. Although BSP has been shown to enhance the invasion of melanoma, breast adenocarcinoma, and thyroid cancer cells [398], its role in cancer cell migration, particularly in prostate cancer, remains underexplored. To investigate its potential role in cancer cell migration, we conducted Boyden chamber assays using human cancer cell lines. Cells that migrated through the inserts were stained with CV for visualisation and quantification (figure 22). Results were inconclusive. 30 $\mu\text{g/ml}$ huBSP2 in the bottom chamber increased MDA-MB-231 cell migration (figure 23A) and inhibited PC-3 cell migration (figure 23C). 3 $\mu\text{g/ml}$ huBSP2 in the bottom chamber had no effect on MDA-MB-231 cell migration (figure 23B) and stimulated PC-3 cell migration (figure 23D).

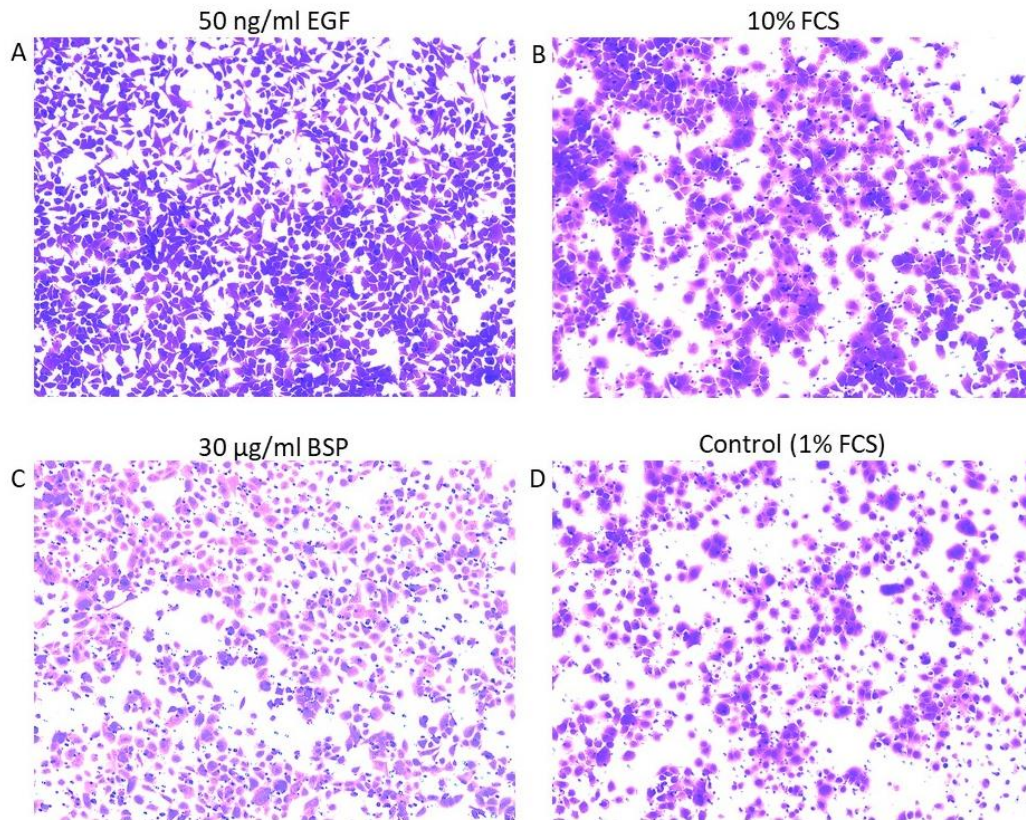


Figure 22. 30 µg/ml BSP reduces the migration of PC-3 cells.

Representative microscopic images of migrated PC-3 cells in response to 50 ng/ml EGF (A), 10% FCS (B), 30 µg/ml BSP (C), and control (1% FCS) (D) in the bottom chamber after 24 h of incubation (x10 magnification). Cancer cells migrated through the pores of the cell culture insert and migrated cells were stained with 0.5% CV. BSP, bone sialoprotein; CV, crystal violet; EGF, epidermal growth factor; FCS, foetal calf serum.

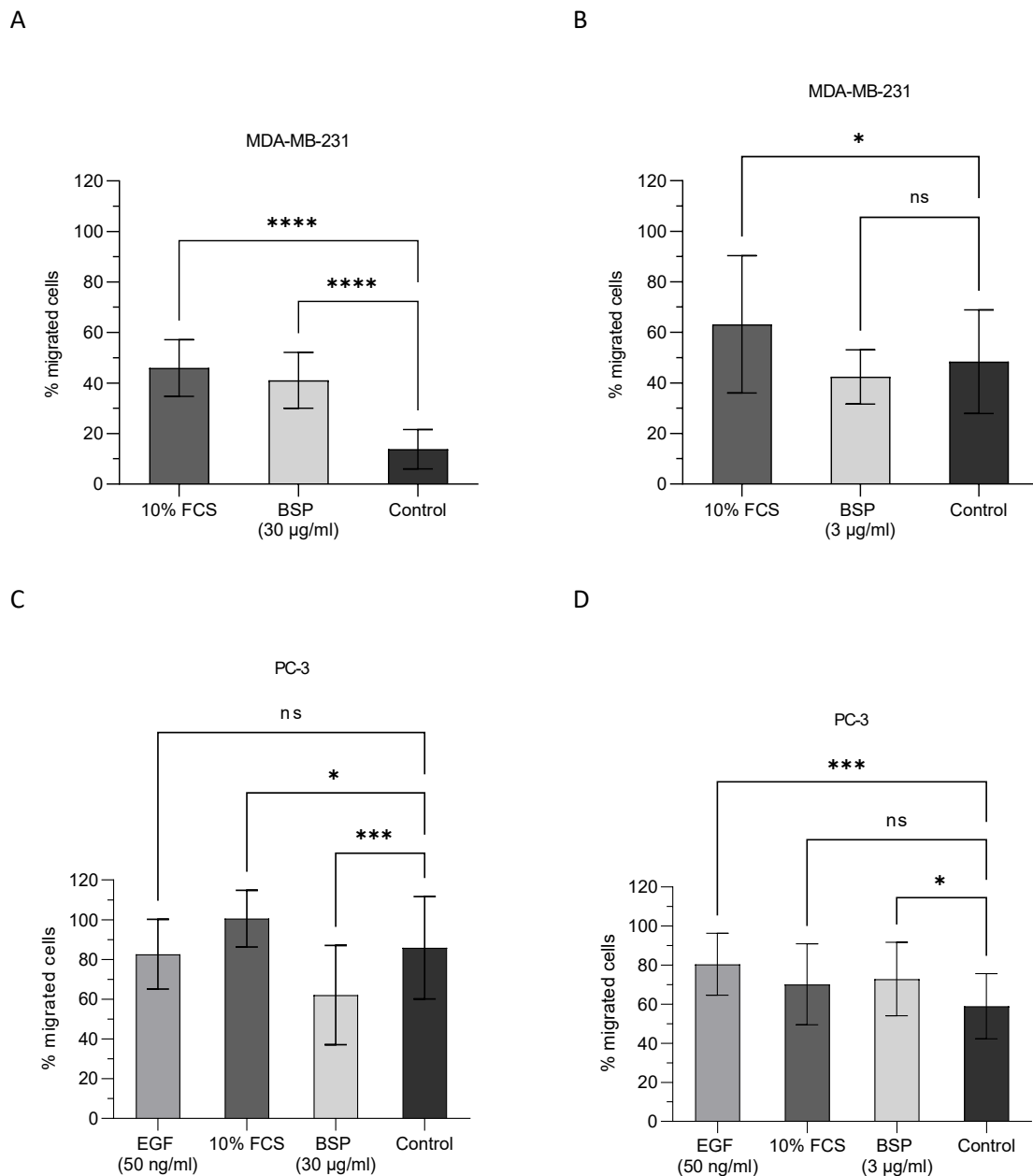


Figure 23. Percent migrated human cancer cells after 24 h. Migration of MDA-MB-231 cells to 30 µg/ml BSP (A) and 3 µg/ml BSP (B), and migration of PC-3 cells to 30 µg/ml BSP (C) and 3 µg/ml BSP (D) in the bottom chamber. Migrated cells were stained with 0.5% CV and quantified by elution in 33% acetic acid (absorbance 600 nm). Data are reported as percentages compared with seeded cells (100%). Values are mean $\pm$ standard deviation ($n=9$). * $P<0.05$; *** $P<0.001$; **** $P<0.0001$ vs. negative control (1% FCS), one-way ANOVA, Dunnett's multiple comparison test. BSP, bone sialoprotein; EGF, epidermal growth factor; FCS, foetal-calf serum; ns, non-significant.

3.3 *In vitro* evaluation of osteoclast differentiation and activation

3.3.1 Bone sialoprotein stimulates the differentiation of primary human macrophage-derived osteoclast precursor-like cells into mature human osteoclasts *in vitro*

In vivo animal studies have shown that BSP overexpression or knockdown influences bone remodelling, leading to changes in trabecular bone volume, thickness, BMD, and parameters such as osteoclast number and surface area [364, 365]. *In vitro* results suggest

that BSP's role in osteoclast differentiation depends on various factors, such as cell source, reagent concentration, and mono- or co-culture conditions. Depending on these variables, BSP has been reported to stimulate [367], inhibit [291], or have no effect [344, 367] on osteoclast differentiation. Notably, the co-treatment of BSP with RANKL often exhibits a synergistic effect on osteoclast formation [344, 367].

While these findings are insightful, they remain limited to animal-derived models. The effect of exogenous human BSP, either alone or in combination with RANKL, on osteoclastogenesis in humans is unknown. To investigate this, we carried out an osteoclast differentiation assay to evaluate the ability of BSP to promote the differentiation of primary human macrophages – serving as a model for osteoclast precursors – into mature osteoclasts *in vitro*. A higher number of osteoclasts, defined as VNR-positive cells with ≥ 2 nuclei, were detected after 24 days compared to 17 days across all experimental groups (table 8). 3 $\mu\text{g/ml}$ BSP showed a trend toward inducing osteoclast formation after both time points. Co-treatment with 50 ng/ml RANKL potentially upregulated osteoclastogenesis after 24 days compared to BSP alone treated osteoclast precursor-like cultures. The data suggests that treatment with RANKL and BSP did not further enhance osteoclast formation compared to RANKL alone at either time point.

	Day 17	Day 24
	% OC.n/Cell.n	% OC.n/Cell.n
M-CSF + RANKL	5.04 $\pm$ 1.96	7.35 $\pm$ 3.37
M-CSF + BSP	5.67 $\pm$ 1.87	6.09 $\pm$ 1.83
M-CSF + RANKL + BSP	4.09 $\pm$ 1.89	6.52 $\pm$ 2.49

Table 8. BSP stimulates the differentiation of primary human osteoclast progenitor cells into mature human osteoclasts in the presence or absence of RANKL.

10-day old osteoclast progenitors generated from PBMCs were stimulated with 20 ng/ml M-CSF and 50 ng/ml RANKL, 3 $\mu\text{g/ml}$ BSP, or 50 ng/ml RANKL and 3 $\mu\text{g/ml}$ BSP for 7 and 14 days. Cells were stained with Hoechst dye and anti- $\alpha\text{v}\beta 3$ -integrin antibody to examine osteoclast formation. VNR-positive cells with ≥ 2 nuclei were considered as osteoclasts. Five images were taken and the number and percentage of osteoclasts were determined in relation to the total cell number (% OC.n/Cell.n) by one investigator. Results are the mean from three independent donors $\pm$ SD ($n=3$). BSP, bone sialoprotein; M-CSF, macrophage colony-stimulating factor; OC, osteoclast; PBMC, peripheral blood mononuclear cell; RANKL, receptor activator of nuclear factor kappa-B ligand; VNR, vitronectin receptor.

In murine RAW264.7 cells, treatment with increasing concentrations of human recombinant BSP has been shown to progressively enhances osteoclast formation after 3 days [367]. Inspired by this dose-dependency in murine cells, we sought to determine whether a similar concentration-dependent effect of BSP occurs in primary human osteoclast progenitors. While our initial experiments indicated a trend for human BSP to stimulate osteoclast formation at a single concentration after 17 and 24 days, we next performed two independent concentration-dependent BSP-osteoclast differentiation assays to evaluate this effect in human cells.

BSP exhibited a dose-dependent trend in promoting osteoclastogenesis after 17 and 24 days. A potential plateau in osteoclastogenesis was observed at 3.33 $\mu\text{g/ml}$ BSP after 17

days (Figure 24A), whereas after 24 days, osteoclast formation appeared to continue increasing (Figure 24B). Fluorescence images depicting BSP-induced osteoclast differentiation in a concentration-dependent manner after 17 days are shown in Figure 25.

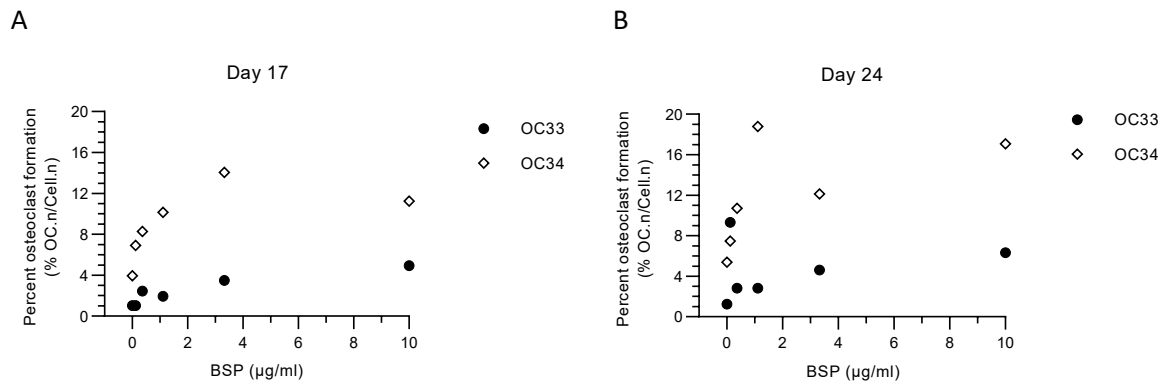


Figure 24. Percent osteoclast formation with increasing BSP concentrations after 17 days and 24 days in culture. Osteoclast precursor-like cells, derived from 10-day-old PBMC cultures, were treated with 20 ng/ml M-CSF and varying concentrations of BSP (0, 0.123, 0.37, 1.11, 3.33, and 10 μg/ml) for 7 or 14 days. Osteoclastogenesis was evaluated using Hoechst dye for nuclear staining and an anti-VNR antibody to identify VNR-positive cells. Cells with two or more nuclei and VNR positivity were classified as osteoclasts. Quantification was based on five images, calculating the total number and percentage of osteoclasts relative to the total cell count (% OC.n/Cell.n). Data points correspond to individual values from two independent donors ($n=2$). % OC.n/Cell.n, percent osteoclast formation; BSP, bone sialoprotein; M-CSF, macrophage colony-stimulating factor; OC, osteoclast; PBMC, peripheral blood mononuclear cell; VNR, vitronectin receptor.

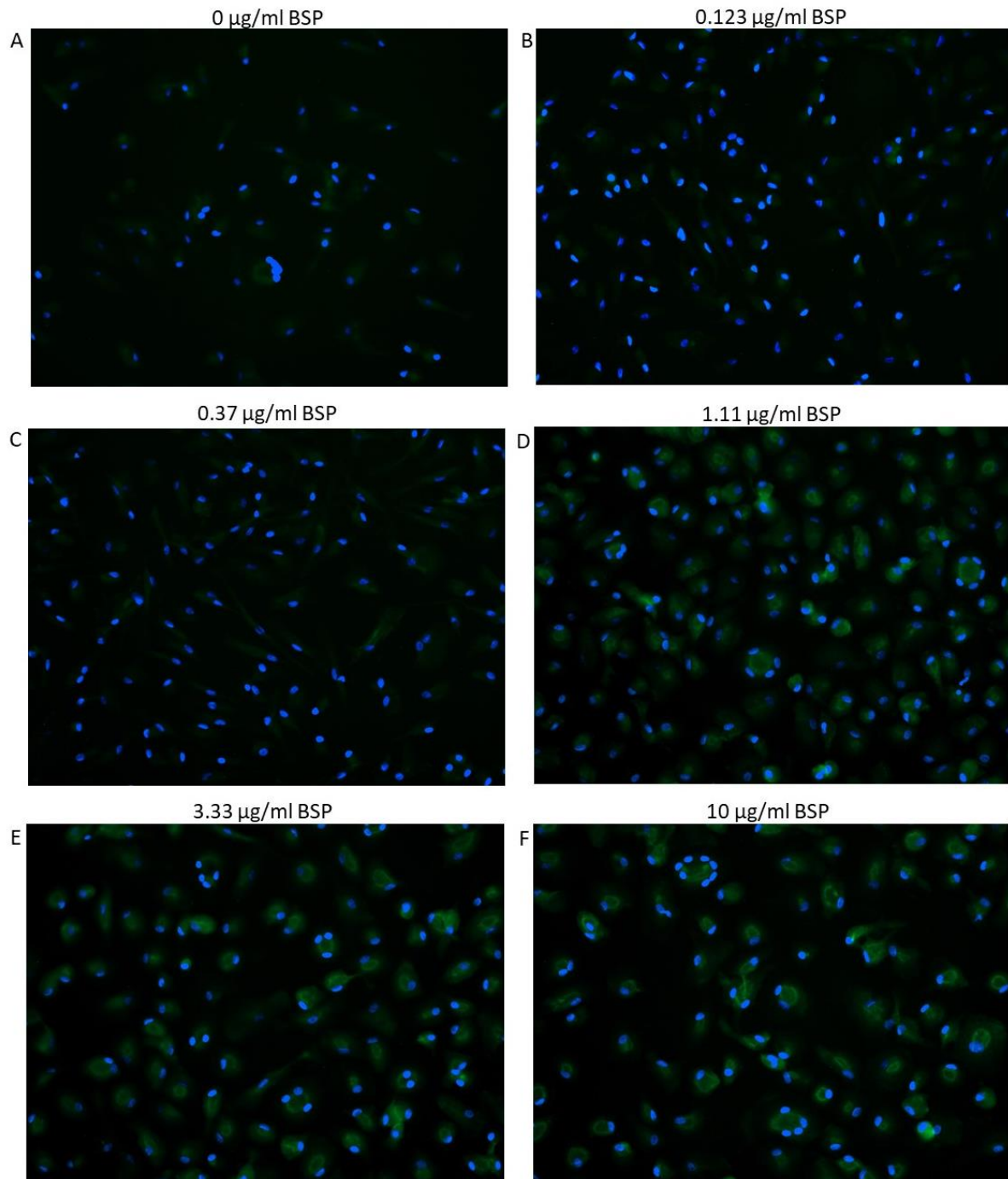


Figure 25. Representative images of BSP-induced osteoclast formation after a total of 17 days. 10-day old osteoclast precursor-like cells generated from PBMCs were cultured with 20 ng/ml M-CSF and increasing concentrations of BSP – 0 $\mu\text{g/ml}$ (A), 0.123 $\mu\text{g/ml}$ (B), 0.37 $\mu\text{g/ml}$ (C), 1.11 $\mu\text{g/ml}$ (D), 3.33 $\mu\text{g/ml}$ (E), and 10 $\mu\text{g/ml}$ (F) – for 7 days. Cells were stained with Hoechst dye and anti- $\alpha v \beta 3$ -integrin antibody to determine osteoclast differentiation. Osteoclasts were VNR-positive cells (green) and had ≥ 2 nuclei (blue). BSP, bone sialoprotein; M-CSF, macrophage colony-stimulating factor; PBMC, peripheral blood mononuclear cell; VNR, vitronectin receptor.

Our prior blocking experiments demonstrated that the mouse anti-human FP-21 BSP-antibody reduced the adhesion of breast cancer cells to BSP. The impact of this antibody on BSP's osteoclastogenic activity remains less clear. An antibody, capable of inhibiting multiple

BSP functions *in vitro*, holds potential as a therapeutic agent to mitigate tumour progression and osteolysis—whether tumour-related or not—*in vivo*.

In a case report, 100 µg/ml BSP-antibody FP-21 showed a trend toward inhibiting BSP-induced osteoclastogenesis after 24 days, but not after 17 days (table 9). There was a trend suggesting that the BSP-antibody FP-21 promotes osteoclastogenesis in osteoclast precursor-like cultures co-treated with RANKL and BSP after 17 and 24 days.

	Day 17				Day 24			
	>2 nuclei	2 nuclei	Cells OC.n/Cell.n	%	>2 nuclei	2 nuclei	Cells OC.n/Cell.n	%
M-CSF		3	289	1.04		4	320	1.25
M-CSF + RANKL		14	588	2.38	1	10	399	2.76
M-CSF+ BSP		5	300	1.67	2	17	224	8.48
M-CSF + BSP + FP-21		8	158	5.06		10	207	4.83
M-CSF + RANKL + BSP		9	500	1.80	1	6	173	4.05
M-CSF + RANKL + BSP + FP-21		17	231	7.79	6	26	400	8.00

Table 9. Case study ($n=1$) of the effect of mouse anti-human FP-21 BSP-antibody on osteoclast differentiation *in vitro*.

10-day old primary human osteoclast precursor-like cells generated from PBMCs were cultured in the presence or absence of 20 ng/ml M-CSF, 50 ng/ml RANKL, 3 µg/ml BSP, and 100 µg/ml mouse anti-human FP-21 BSP-antibody for 7 and 14 days. Osteoclastogenesis was assessed by co-staining cells with Hoechst dye and anti-VNR antibody. VNR-positive cells with ≥ 2 nuclei were considered as osteoclasts. Five images were taken and the number and percentage of osteoclasts were calculated (% OC.n/Cell.n). BSP, bone sialoprotein; M-CSF, macrophage colony-stimulating factor; OC, osteoclast; PBMC, peripheral blood mononuclear cell; RANKL, receptor activator of nuclear factor kappa-B ligand; VNR, vitronectin receptor.

3.3.2 Bone sialoprotein and RANKL induce resorption of calcium phosphate discs by primary human osteoclasts *in vitro*

Our initial experiments indicated that human BSP stimulates the differentiation of primary human osteoclast precursor-like cells into mature osteoclasts *in vitro*. To further explore its role, we examined whether BSP also influences osteoclast activity. Previous research demonstrates that BSP alone stimulates bone resorption in mature murine and rabbit osteoclasts cultured on osteologic discs or ivory slices [291, 367]. Additionally, BSP in combination with RANKL has been shown to enhance the resorptive activity in murine osteoclast precursors more effectively than BSP or RANKL alone [364, 367].

However, the current knowledge on BSP's role in osteoclast-mediated resorption is based on animal studies, leaving its effects in humans unclear. Specifically, whether BSP can induce human bone resorption *in vitro* independently or in synergy with RANKL remains to be determined. To address this gap, we treated primary human osteoclast precursor-like cells with BSP for 14 days on 2D synthetic CaP discs to assess their resorptive activity. Treatment with 3 µg/ml BSP alone had no effect on the amount of calcium in culture supernatants, an indicator of osteoclast activity, compared to cultures treated with medium (figure 26). 50 ng/ml RANKL and 3 µg/ml BSP significantly stimulated osteoclast-mediated bone resorption at 14, 17 and 23 days. There was a non-significant trend suggesting that BSP and RANKL

synergistically promoted osteoclast activity at 14 and 23 days compared to cultures treated with RANKL alone.

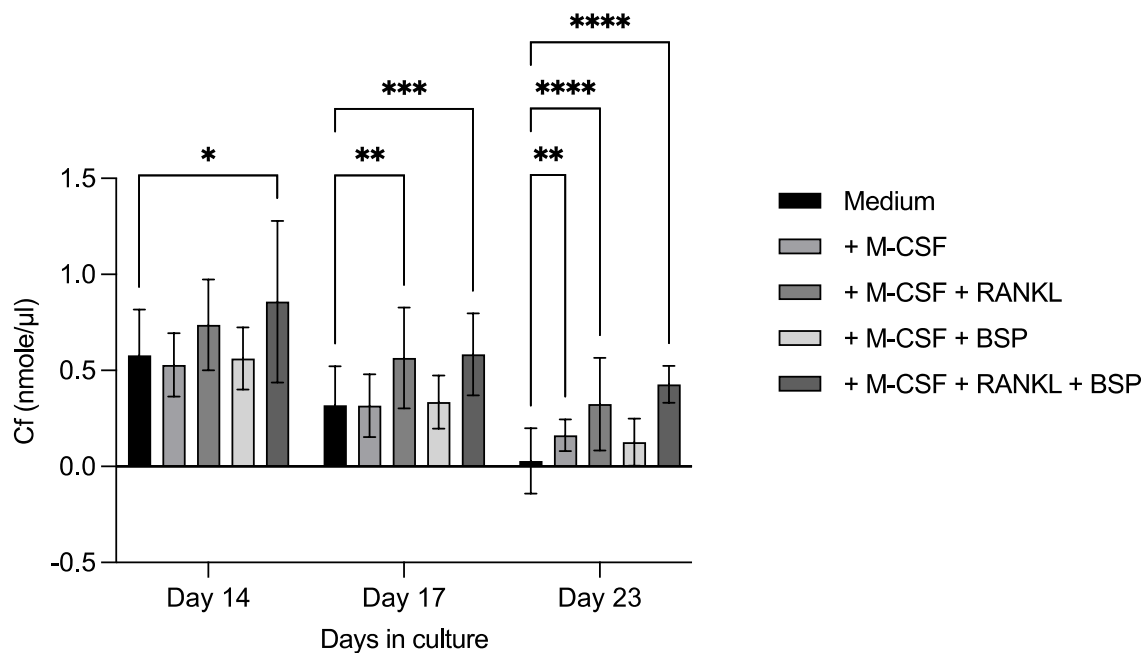


Figure 26. Effect of BSP on calcium-phosphate resorption by human osteoclasts *in vitro*.

10-day old primary human osteoclast precursor-like cells generated from PBMCs were cultured with a) medium, b) 20 ng/ml M-CSF, c) 20 ng/ml M-CSF and 50 ng/ml RANKL, d) 20 ng/ml M-CSF and 3 μ g/ml BSP, or e) 20 ng/ml M-CSF, 50 ng/ml RANKL and 3 μ g/ml BSP for additional 14 days. Supernatants were collected at every media change and analysed for calcium concentration in the sample (nmole/ μ l). Data are mean $\pm$ standard deviation of two separate experiments ($n=4$). Calcium concentration in the sample (nmole/ μ l) was compared to the negative control (medium) using Two-way ANOVA with Dunnett's multiple comparison test. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$ vs. negative control (medium). BSP, bone sialoprotein; Cf, final calcium concentration; M-CSF, macrophage colony-stimulating factor; PBMC, peripheral blood mononuclear cell; RANKL, receptor activator of nuclear factor kappa-B ligand.

In summary, there was a trend for BSP to stimulate the differentiation of primary human osteoclast precursor-like cells into mature human osteoclasts after 17 days and 24 days *in vitro*. BSP may synergise with RANKL to further enhance osteoclastogenesis after 24 days. In addition, there was a trend for a dose-dependent increase in osteoclast formation with increasing BSP concentrations after 17 days and 24 days, with a potential plateau in osteoclastogenesis after 17 days at 3.33 μ g/ml BSP. Mouse anti-human BSP FP-21 antibody had a tendency to suppress BSP-stimulated osteoclast formation after 24 days. BSP and RANKL, but not BSP alone, stimulate the resorption of CaP discs by primary human osteoclasts *in vitro*. BSP potentially synergises with RANKL to enhance osteoclast activity.

3.4 Gene expression of bone sialoprotein in bone metastases of cancer patients

3.4.1 Bone sialoprotein is highly expressed at the mRNA level in bone metastatic tissue from cancer patients

BSP is synthesised by a range of primary tumours and its mRNA expression has been linked to clinical parameters such as TNM staging, RFS, and OS [330, 331, 341, 359]. However, far less is known about BSP synthesis in metastases, particularly skeletal metastases. One study reported considerably higher BSP mRNA expression in bone metastases from breast cancer compared to metastases in other tissues [347].

To address this gap, we performed RT-qPCR to investigate BSP gene expression levels in secondary bone cancers. Tables 10 and 11 present the patients' characteristics. The study included four cases with bone metastases: two patients with breast carcinoma and two with lung carcinoma. The lung carcinoma patients were, on average, 16 years older than the breast cancer patients. One patient was neither affected by bone metastasis nor cancer. There was a lack of information regarding histological subtype, disease stage and treatment intervention.

Alias	Age	Sex	Primary tumour	Site of bone metastasis
08_21	48	M	Breast carcinoma	Spinal column, L4-L6
38_23	48	F	Breast carcinoma, left sided	Proximal femur, right
17_22	60	M	Lung carcinoma	Tibia and femur, right
23_22	68	F	Lung carcinoma	Hallux, right

Table 10. Clinical and pathological characteristics of the cancer patients investigated.

M, male; F, female.

Alias	Age	Sex	Site of surgery
4	65	F	Shoulder

Table 11. Clinical and pathological characteristics of the non-cancer patient investigated.

F, female.

BSP exhibited strong mRNA expression in bone metastases from both breast and lung carcinoma (figure 27). Skeletal metastases tended to demonstrate higher transcript levels of BSP, OPN, RANKL and PTHrP compared to the control non-cancer patient.

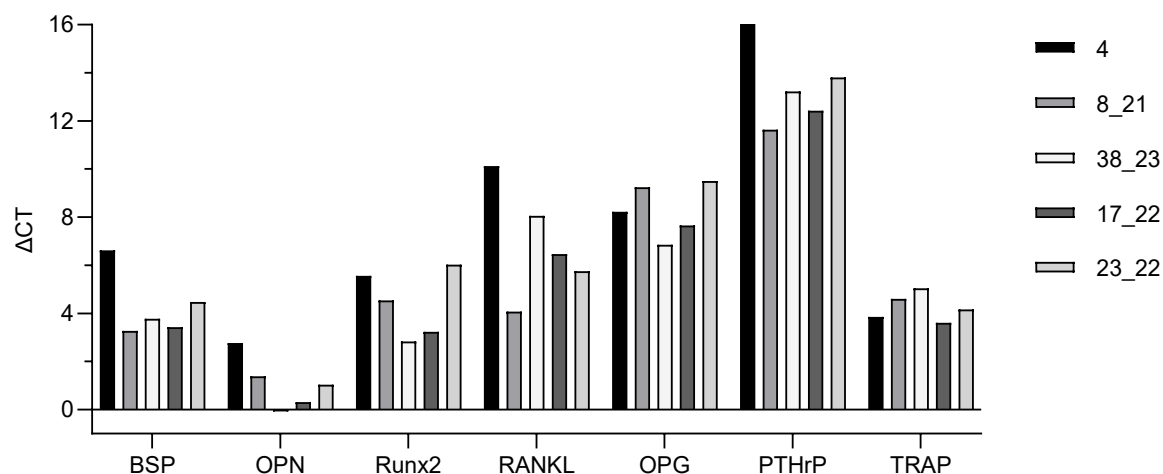


Figure 27. Elevated BSP and GOI levels in bone metastatic lesions.

Transcript levels of BSP and other GOIs show a trend toward higher expression in bone metastatic lesions from breast carcinoma (patients 8_21, 38_23) and lung carcinoma (patients 17_22, 23_22) compared to a surgical specimen from a non-cancer patient (4). Gene expression was normalised relative to the housekeeping gene GAPDH. BSP, bone sialoprotein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GOI, gene of interest; OPN, osteopontin; Runx2, runt-related transcription factor 2; RANKL, receptor activator of nuclear factor kappa-B ligand; OPG, osteoprotegerin; PTHrP, parathyroid hormone-related protein; TRAP, tartrate-resistant acid phosphatase.

4. Discussion

4.1 Cell adhesion assays

Metastases, resulting from tumour growth and dissemination, frequently involve the skeleton [235-237] and are a leading cause of cancer-related mortality [160]. Numerous stages of cancer progression, like angiogenesis [362], complement evasion [392] and cancer cell adhesion [395], are governed by bone sialoprotein (BSP). The attachment of cancer cells to the bone extracellular matrix (ECM) is essential in the metastatic process. Evidence suggests that the arginine–glycine–aspartic acid (RGD)-binding motif at the C-terminal domain of BSP interacts with integrins $\alpha\beta 3$ and $\alpha\beta 5$ expressed on the cell surface of cancer cells, promoting adhesion [381, 395]. Furthermore, endogenous BSP expression by cancer cells may regulate cell-matrix adhesion at both primary and secondary tumour sites [299]. Clarifying BSP's role and mechanisms in cancer cell adhesion may enhance our understanding of the pathophysiology of cancer metastasis and provide a basis for developing more effective therapeutic strategies.

To investigate the mechanism facilitating cancer cell adhesion and driving the homing of human breast, prostate, and lung cancer cells to bone, along with the behaviour of bone cancer (osteosarcoma) cells themselves, we conducted a series of *in vitro* adhesion assays. We theorised that human breast, prostate, lung and bone cancer cell lines exhibit adhesion to BSP, a process thought to be modulated by the RGD-sequence of BSP in conjunction with the $\alpha\beta 3$ -integrin and/or $\alpha\beta 5$ -integrin receptors on the cell surface. This investigation showed for the first time that BSP induced the adhesion of large-cell lung carcinoma NCI-H460 and osteosarcoma MG-63 cells. Moreover, in alignment with existing literature [299, 381], our findings demonstrated that both breast MDA-MB-231 and prostate PC-3 adenocarcinoma attached to BSP.

Potential for cell adhesion. Interestingly, MCF-10A cells adhered stronger to PBS-coated surfaces compared to MDA-MB-231 cells. BSP also induced adhesion of MCF-10A cells more than of MDA-MB-231 cells. MCF-10A cells are non-tumorigenic mammary epithelial cells and the greater inherent adhesive capacity may be due to their epithelial origin. Strong natural adhesion of normal epithelial cells via E-cadherin cell adhesion system is important for tissue integrity and function [421]. MDA-MB-231 were established from a female patient with metastatic breast adenocarcinoma. Both MDA-MB-231 and MCF-10A cells express the BSP protein, however, the expression of BSP is greater in MDA-MB-231 cells [346]. Hence, the lower natural adhesion of MDA-MB-231 cells was not due to a lack of BSP expression. The differences in the effects of BSP on the adhesion of normal and cancerous breast cells may reflect changes in the mechanistic interactions of cancerous cells with ECM proteins, such as BSP, in cell adhesion, as well as cell migration [422]. The influence of BSP on cancer cell chemotaxis was investigated in a separate experiment using *in vitro* cell migration assays and the results will be discussed later on. NCI-H460 cells, derived from the pleural fluid of a male patient with large cell lung carcinoma, and A-549 cells, a lung adenocarcinoma model

resembling type II alveolar epithelial cells, displayed stronger adhesion compared to MDA-MB-231 and PC-3 cells. MDA-MB-231, a highly aggressive TNBC cell line, originates from pleural effusion, while PC-3 cells were established from a bone metastasis of a prostate adenocarcinoma patient. The differences in inherent adhesion strength may reflect the heterogeneity of cancer cell adhesion and their potential for metastasis, allowing weakly adherent cells to detach from the primary site, facilitating cancer cell migration and intravasation [423]. Indeed, bone metastases are more prevalent in patients with advanced breast and prostate cancer compared to lung cancer patients [238]. Regarding the human bone cancer (osteosarcoma) cell line MG-63, these cells may possess a high inherent adhesive capacity, allowing them to adhere to the ECM of bone, perhaps in an osteoblastic manner [164].

RGD sequence of bone sialoprotein. BSP possesses the capability to activate multiple signalling cascades implicated in oncogenic proliferation and dissemination [299, 344]. Adhesion of PC-3 and MDA-MB-231 cells to rat BSP (1 h) stimulates focal adhesion kinase (FAK)-ERK signalling [299]. Notably, an intact RGD-integrin binding sequence within BSP is essential for the activation of FAK and mitogen-activated protein kinase (MAPK), since phosphorylation of FAK and ERK is diminished in MDA-MB-231 cells transfected with mutated BSP [BSP-Lys-Ala-Glu (BSP-KAE)] [299]. As with the adhesion of MDA-MB-231 and PC-3 cells driven by BSP, (BSP-RGD)-integrin coupling in NCI-H460 and MG-63 may trigger FAK and ERK signalling pathways. The results in the current study showed that the RGD sequence significantly facilitated the adhesion of all four cancer cell lines (MDA-MB-231, PC-3, NCI-H460, and MG-63) to BSP, supporting our hypothesis. Similar findings have been reported in human osteoblasts [326], human bone fibroblasts [424], human skin fibroblasts [424], murine cementoblasts [425], and murine osteoblastic cells [426]. Conversely, one study [300] observed that an exogenous commercially available GRGDS peptide ($\leq 300 \mu\text{M}$) had no notable influence on MDA-MB-231 cell adhesion to the ECM of human bone cells. However, cyclic synthetic BSP peptides characterised by the EPRGDNYR sequence were potent inhibitors of cell adhesion, with concentrations as minimal as $2 \mu\text{M}$ significantly blocking the attachment of MDA-MB-231 cells to human ECM [300]. The authors proposed that the RGD sequence in BSP does not completely regulate the adhesion of MDA-MB-231 cells to bone ECM. Our results showed that fibronectin (FN)-derived GRGDSP peptide was markedly effective in blocking the adhesion of MDA-MB-231 cells to BSP (-97% decrease). Mintz *et al* [424] additionally documented that synthetic GRGDS peptide (0.4 mM) entirely prevented rat BSP-induced adhesion of human bone cells and skin fibroblasts. The tyrosine (Tyr)-rich repeats near the RGD motif of BSP appeared to play a minimal role in the adhesion of MDA-MB-231, PC-3, and NCI-H460 cells, as cell adhesion was significantly reduced by the GRGDSP peptide [427]. Tyr-independent cell adhesion has also been documented in the SIBLING protein family member osteopontin (OPN) [428]. In contrast, the less potent effect of GRGDSP on MG-63 cell adhesion could have been due to the influence of Tyr-rich repeats.

RGD-binding integrins $\alpha\beta3$ and $\alpha\beta5$. Integrins are large transmembrane heterodimers that promote focal adhesion formation and cell adhesion through outside-in (ligand binding) and inside-out signalling [188, 243, 417, 429, 430]. We hypothesised that integrins $\alpha\beta3$ and/or $\alpha\beta5$ regulate the adhesion of cancer cells to BSP. This study provides the first evidence that BSP-induced adhesion in the PC-3 cell line primarily relied on $\alpha\beta3$ integrins, while BSP adhesion in the NCI-H460 cell line was essentially mediated by $\alpha\beta5$ integrins. BSP-stimulated adhesion in breast cancer cells was found to primarily involve $\alpha\beta5$ receptors. According to the data, $\alpha\beta3$ integrins were also partially involved in the adhesion of NCI-H460 cells to BSP. Conversely, PC-3 cells also employed $\alpha\beta5$ integrins to a lesser, yet significant, degree for the same purpose. Similarly, the adhesion of colon adenocarcinoma SW480 cells to OPN occurred via binding to $\alpha\beta3$ and $\alpha\beta5$ integrins [428]. The transmembrane integrin receptors $\alpha\beta3$ and $\alpha\beta5$ may preferentially interact with BSP, based on the specific cancer cell line. For instance, the attachment of NCI-H460 cells to BSP was significantly reduced when exposed to the $\alpha\beta5$ -integrin antibody. Nevertheless, integrin antibodies anti- $\alpha\beta3$ and anti- $\alpha\beta5$ were not sufficient to fully block the adhesion of NCI-H460, PC-3, and MDA-MB-231 cells to BSP. Notably, no effect of the integrin antibodies $\alpha\beta3$ and $\alpha\beta5$ on the adhesion of MG-63 cells was observed.

The application of exogenous agonists, such as phorbol 12-myristate 13-acetate (PMA) or adenosine diphosphate (ADP), to cancer cells subsequent to antibody incubation, may enhance integrin receptor binding to BSP [431, 432]. Nevertheless, the findings of this study indicated that integrin activation was not a prerequisite for cancer cell adhesion to BSP. Prospective research efforts should entail the pre-treatment of cells with anti- $\alpha\beta3$ and anti- $\alpha\beta5$ integrin antibodies, succeeded by exposure to PMA (200 nM or 200 ng/ml), or varying concentrations of ADP (2-2000 μ M), in order to assess the impact on BSP-induced attachment of MDA-MB-231, PC-3, NCI-H460, and MG-63 cells. BSP has also the potential to modulate the expression levels of integrins within cancer cells, thereby influencing their adhesion properties. Infection of MDA-MB-231 and PC-3 cell lines with BSP leads to significantly greater concentrations of the integrin subunits α , $\beta3$, and $\beta5$, hereby facilitating enhanced focal adhesion formation relative to non-infected cells or those infected with a mutated variant of BSP (BSP-KAE) [299]. The expression of BSP by cancer cells may amplify their metastatic capabilities. Subsequent investigations should involve the treatment or infection of cells with BSP ahead of their seeding.

As previously noted, cells utilise integrin receptors for adhesion and motility in distinct ways, which could help explain why integrin antibodies did not entirely inhibit cell adhesion to BSP. A prior study [381] found that MDA-MB-231 cell adhesion to BSP relied on the $\alpha\beta5$ integrin receptors, while invasion toward BSP-derived RGD peptides was facilitated by $\alpha\beta3$ integrins. The current study's findings indicated that $\alpha\beta3$ and $\alpha\beta5$ integrins played different roles in cell adhesion depending on the specific cell type expressing them. The study by Sung *et al* [381] did not explore other integrin receptors in their inhibition tests. Similarly, this study

focused exclusively on the $\alpha\beta3$ and $\alpha\beta5$ integrin receptors without measuring their expression levels on the cell surface. However, $\alpha\beta5$ integrin receptors are highly expressed in both the MDA-MB-231 and PC-3 cancer cell lines [381, 419, 433]. The limited information available on $\alpha\beta5$ expression in NCI-H460 cells suggests only weak levels of $\alpha\beta5$ expression in these cells [434]. There is also a lack of direct evidence of $\alpha\beta5$ expression in MG-63 cells. MG-63 cells exhibit low expression levels of the $\beta5$ subunit [435]. Expression of the $\alpha\beta3$ integrin receptor, however, differs across cancer cell types, with MG-63 cells expressing low levels [436], MDA-MB-231 cells displaying low to moderate levels [381, 419], and NCI-H460 and PC-3 cells exhibiting moderate to very high expression [433, 437-439]. It is likely that pre-treatment of MDA-MB-231 cells with an anti- $\alpha\beta5$ integrin antibody resulted in a more significant decrease in cell attachment to BSP than treatment with the $\alpha\beta3$ antibody. In contrast, the comparable levels of $\alpha\beta5$ and $\alpha\beta3$ integrin expression in PC-3 cells do not account for the observations made in this study. The findings related to NCI-H460 and MG-63 cells should be interpreted carefully due to the limited or even lack of data on $\alpha\beta5$ integrin expression in the two cell lines. Even with $\alpha\beta3$ being expressed at much greater levels than $\alpha\beta5$ in NCI-H460 cells, this receptor may not be the main driver of NSCLC cell attachment to BSP. This distinction further supports the idea that integrins play different roles in adhesion and motility. Conducting a flow cytometric analysis of $\alpha\beta5$ expression in NCI-H460 and MG-63 cells would provide valuable insights into integrin-driven adhesion to BSP in NSCLC and osteosarcoma cells.

Other RGD-binding integrins. Additional RGD-binding integrin receptors may have played a role in the cell adhesion assays. The MDA-MB-231 breast adenocarcinoma cell line expresses several RGD-binding integrin receptors, including $\alpha\beta5$, $\alpha\beta3$, and $\alpha\beta6$ [419], as well as the $\beta1$ subunit associated with RGD binding [440]. Meanwhile, the PC-3 prostate adenocarcinoma cell line exhibits a range of RGD-binding integrin receptors, including $\alpha\beta5$, $\alpha\beta3$, $\alpha\beta6$, $\alpha5\beta1$, and $\alpha\beta1$ [433]. NCI-H460 large-cell lung cancer cells express the $\alpha5\beta1$ integrin receptor, which recognises RGD, along with several RGD-binding integrin subunits, including $\alpha5$, $\alpha\upsilon$, $\beta1$, and $\beta6$ [441]. MG-63 osteosarcoma cells express various different kinds of RGD-binding integrin subunits and receptors such as $\alpha\upsilon$, $\alpha3$, $\alpha5$, $\beta1$, $\beta5$, and the RGD-binding integrin receptors $\alpha\beta3$ and $\alpha5\beta1$ [435, 436, 442-444]. Variations in integrin receptor expression specific to each cell type could have accounted for the differing concentrations of GRGDSP peptide required to effectively reduce or completely block the attachment of the cancer cell lines to BSP. The GRGDSP peptide could have also bound with greater affinity to integrin receptors on MDA-MB-231 cells compared to PC-3, NCI-H460 and MG-63 cells [445, 446]. Additional experiments will clarify if other integrin receptors like $\alpha5\beta1$, $\alpha\beta1$, or $\alpha\beta6$, are involved in the regulation of cancer cell adhesion to BSP. Notably, $\alpha\beta6$ stands out as a strong candidate since it can bind to the RGD sequence present in OPN [428].

Furthermore, integrin receptors might have competitively bound to BSP. For instance, in breast carcinoma MCF-7 cells, expression of $\alpha\beta3$ inhibits the adhesive functions of $\alpha2\beta1$

to type I collagen (COL1) [243]. In the current study, blocking specific RGD-integrin binding sites with an anti-integrin $\alpha\beta 3/\alpha\beta 5$ antibody could have actually increased the RGD-binding rate to integrin $\alpha\beta 3/\alpha\beta 5$ or potentially other RGD-binding integrins and strengthened BSP-induced cell adhesion. In pan-integrin deficient (pKO)- αv and $\beta 1$ fibroblasts, blocking of αv integrins with the cyclic RGD peptide cilengitide [RGDf-N(Me)V] enhances cell adhesion [447]. In addition, integrin crosstalk between $\alpha\beta 3$ and $\alpha\beta 5$ or other RGD-binding integrin subunits and receptors may have compensated the blocked integrin binding sites. For example, in fibroblasts, αv integrin subunits communicate with $\alpha 5\beta 1$ to set up further attachment points for focal contact formation with FN [447]. Integrin-cross talk in MG-63 cells could explain the less potent inhibition of BSP-induced cell adhesion with 300 μ M exogenous GRGDSP peptide compared to blocking experiments with other human cancer cell lines.

Heparin-binding motif of bone sialoprotein. Additional protein sequences could have impacted the results of BSP-regulated cancer cell attachment. The heparin-binding region of FN has been shown to stimulate melanoma cell adhesion in an RGD-independent manner [448, 449]. To investigate a similar mechanism, we examined the role of the heparin-binding sequence, LHRRVKI (Lys-His-Arg-Arg-Lys-Val-Ile), in the N-terminal domain of BSP in mediating the adhesion of MDA-MB-231 and PC-3 cancer cells. We hypothesised that this motif has the potential to bind to heparin and heparan sulphate on cancer cells, thereby stimulating cancer cell adhesion to BSP. Our results showed for the first time that pre-incubation of cells with a custom-made LHRRVKI peptide increased MDA-MB-231 adhesion, while decreasing PC-3 adhesion to BSP. These interesting opposing effects may have been influenced by the chemical properties of the peptide [450, 451] and glycosaminoglycan (GAG) variability between the cell lines. For instance, multimerisation of human recombinant von Willebrand factor (vWF) enhances its ability to bind to COL and aggregate platelets [452]. vWF contains two heparin-binding regions [452] and can bind to GAGs on platelets [453]. Importantly, platelets are primarily known to bind to GAGs themselves [454, 455]. Given the dose-dependent effects observed in our assay, peptide chemistry alone is unlikely to fully explain the results.

Differences in GAG expression, such as heparin and heparan sulphate, and function between the two human cancer cell lines might have played a role in BSP-induced cell adhesion. For instance, Glypican-1 (GPC-1), a member of the glypican family of proteoglycans, inhibits the migration of prostate cancer DU-145 cells *in vitro*, but stimulates the migration of PC-3 cells [456], suggesting a complex role in cancer cell motility. However, knockdown of GPC-1 in PC-3 cells does not affect adhesion [456], indicating that other proteoglycans or receptors may play a role. The synthetic peptide LHRRVKI has an overall positive charge, and could have strongly interacted with the negatively charged cell membrane molecules such as heparan sulphate proteoglycans on cancer cells. The binding of the exogenous peptide LHRRVKI to heparin on the cell surface could explain the decrease in PC-3 cell adhesion to BSP with 100 μ g/ml exogenous peptide. While we lack direct evidence for the expression of heparin in PC-3 cells, PC-3 cells express heparan sulphate proteoglycan at mRNA level [457]. Furthermore,

heparin has been shown to suppress the proliferation of PC-3M cells, a variant of PC-3 cells with high metastatic potential (125 and 25 $\mu\text{g/ml}$, 24 h) [458]. The role of heparin in MDA-MB-231 cell adhesion is questionable. Studies show that MDA-MB-231 cells express hyaluronic acid and heparan sulphate [459]. Whether MDA-MB-231 cells express heparin is unclear.

The heparin-binding motif may have interacted with the RGD sequence of BSP, leading to a change in the protein conformation to facilitate cell binding. A previous study on the heparin-binding domain of rodent BSP (FHRRKA) found that surfaces functionalised with the FHRRKA peptide led to significantly higher rat osteoblast-like cell attachment when combined with the RGD sequence, compared to FHRRKA-surfaces alone [292]. This suggests that the adhesive properties of the heparin-binding motif are RGD-mediated and may involve similar interactions with integrins [460]. In MDA-MB-231 cells, to stimulate adhesion, the RGD sequence of BSP may first bind to integrins, followed by secondary binding to heparin via the LHRRKVI sequence. This could explain why exogenous LHRRKVI peptide did not result in decreased MDA-MB-231 adhesion, but increased cell adhesion.

Our current results of the heparin-blocking experiments bear some resemblance to the previous findings on integrin $\alpha\beta 3$ - and $\alpha\beta 5$ -mediated cancer cell adhesion to BSP. The interaction between heparins and BSP, as well as their contribution to BSP-induced cell adhesion, might have differed across various cancer cell lines. We did not examine the effect of co-treatment of cells with exogenous LHRRKVI and GRGDSP peptides on human cancer cell adhesion. Future studies should further explore whether the heparin-binding domain serves as a co-factor of the RGD motif in mediating cancer cell attachment to BSP. Additionally, it would be interesting to test whether cancer cells adhere to surfaces coated exclusively with LHRRKVI peptide.

Bone sialoprotein antibodies. We demonstrated that BSP promoted the adhesion of human cancer cell lines. Through blocking experiments using exogenous GRGDSP peptide and anti-integrin antibodies $\alpha\beta 3$ and $\alpha\beta 5$, we characterised the critical roles of the RGD sequence and integrins in cancer cell attachment to BSP. We then evaluated the effectiveness of nine anti-BSP antibodies (IDK-1, FP-21, YU831-A05, YU828-D09, YU828-A01, YU828-H06, YU829-H06, YU828-D07, and YU828-A06) in blocking BSP-mediated MDA-MB-231 cell adhesion at various concentrations ranging from 300 $\mu\text{g/ml}$ to 0 $\mu\text{g/ml}$ *in vitro*. We hypothesised that the antibodies would effectively inhibit breast cancer cell adhesion to BSP, with differences in inhibitory efficacy across the various antibodies.

The mouse anti-human BSP FP-21 antibody was identified as a potent inhibitor of BSP-mediated MDA-MB-231 cell adhesion. Similarly, newer anti-BSP antibodies (YU828-H06, YU829-H06, YU828-D07, and YU828-A06) significantly inhibited MDA-MB-231 cell adhesion to BSP at concentrations above 30 $\mu\text{g/ml}$, with YU829-H06 showing nearly complete inhibition at 100 $\mu\text{g/ml}$. Additionally, YU828-A06 and YU829-H06 exhibited high potency, as indicated by their low IC_{50} values. In contrast, our results indicated that anti-BSP antibody YU828-A01 is not

a suitable antibody for blocking BSP-mediated adhesion due to its high IC₅₀, its low inhibitory activity at high concentrations and its apparent ability to provide an adhesive surface for MDA-MB-231 cells. MDA-MB-231 cells adhered to surfaces coated only with YU828-A01, unlike the minimal or absent adhesion with other anti-BSP antibody-only coated surfaces. This suggests that YU828-A01 may possess intrinsic properties or interact with cell surface receptors to facilitate cell attachment, making it unsuitable as an anti-BSP antibody for blocking adhesion.

Due to their strong inhibitory effects, anti-BSP antibodies FP-21, YU829-H06, and YU828-A06 emerge as promising candidates for further *in vitro* and *in vivo* studies in BSP-mediated processes. Their IC₅₀ and/or maximum inhibitory concentrations may also be explored in BSP-mediated cancer cell migration. Further *in vivo* studies, including tumour xenograft models, will be essential to assess the therapeutic potential of these antibodies in targeting BSP to prevent breast cancer bone metastases.

Limitations. One major drawback of this study is the reliance on the alamarBlue® assay, which is traditionally used to evaluate cytotoxicity and cell viability, to measure cell adherence. Another method involves fixing and staining adherent cells with crystal violet (CV), which binds to cellular DNA and proteins, enabling quantification of attached cells by eluting the CV dye and measuring absorbance with a spectrophotometer [409, 410]. Despite the availability of alternative methods, prior studies have also applied only the alamarBlue® assay for cell adhesion quantification [461-463], and it has been validated for evaluating cell migration and invasion in choriocarcinoma cells [464]. Accordingly, we do not believe that the exclusive use of the alamarBlue® assay for measuring cell adhesion affected our findings or final conclusions. While our data provided insight into anti-BSP antibody efficacy *in vitro*, we lack information on the production and purification processes of the antibodies. We do not know the host species and reactivity of the anti-BSP antibodies YU831-A05, YU828-D09, YU828-A01, YU828-H06, YU829-H06, YU828-D07, and YU828-A06. IDK-1, a rat anti-human BSP antibody, and FP-21, a mouse anti-human BSP antibody, were tested, but no controls using only the antibodies (antibody-only coated surface) were included in these experiments. Therefore, we cannot exclude the possibility of intrinsic adhesive properties of the antibodies, which should be investigated in future studies to ensure specificity.

Conclusions and implications. New pharmacological interventions, such as anti-BSP antibodies, in cancer treatment are needed. Integrin targeted therapeutics in cancer are non-existent, attributable to multiple factors, including the shared subunits among integrins and the intricacies inherent in the integrin signalling cascade. To date, merely seven integrin inhibitors have received approval from the U.S. Food and Drug Administration (FDA). Presently, none of the available drugs on the market specifically target the integrins $\alpha\beta 3$ or $\alpha\beta 5$, nor are they employed in cancer treatment [420]. This underscores the necessity for the development of new cancer therapeutics to disrupt integrin-ECM interactions and inhibit tumour progression and cancer metastasis. Animal research has demonstrated that BSP gene-silencing in human breast and lung cancer cell lines significantly impedes the progression of

osseous metastases *in vivo* [344, 379, 465] and this effect may be attributed to a reduction in the expression levels of $\alpha\beta 3$ [465] and matrix metalloproteinase 14 (MMP-14) [344]. Therefore, FP-21, YU829-H06 and YU828-A06 may be promising candidates as potent anti-BSP antibodies and BSP-targeted agents for cancer therapy.

In summary, our findings showed that BSP-driven adhesion of human breast and prostate adenocarcinoma, as well as NSCLC, was strongly dependent on the RGD sequence. Cancer cells adhered to BSP via binding interactions with $\alpha\beta 3$ and $\alpha\beta 5$ integrins. While osteosarcoma cells also bound to BSP's RGD sequence, this adhesion was independent of $\alpha\beta 3$ and $\alpha\beta 5$ integrins. The heparin-binding motif variably influenced cancer cell adhesion, both enhancing and suppressing cell attachment. The interaction between RGD and integrins offers a mechanistic pathway facilitating cancer cell metastasis to bone. Interrupting this cell-ECM adhesion through the use of anti-BSP antibodies or RGD-based peptides may represent a novel strategy for preventing and treating skeletal metastases.

4.2 Cell migration (Boyden chamber) assays

Cancer cell migration at the primary or secondary site is a critical step in the metastatic cascade, facilitating the spread and colonisation to distant organs, including the bone. Skeletal metastases are commonly detected in advanced stages of breast cancer (70-100%) and prostate cancer (68-100%) [240]. The success of cancer cell migration depends on the intricate interactions between cancer cells and their surrounding tissue, the ECM. Recombinant human BSP, a component of the bone ECM, has been shown to enhance the invasion of melanoma, thyroid cancer and NSCLC cells [398]. However, the impact of exogenous human BSP on cancer cell migration, particularly in prostate cancer, remains underexplored. Building on our previous findings that BSP induced the adhesion of breast, prostate, lung, and bone cancer cells *in vitro*, we aimed to determine whether BSP also facilitates the migration of cancer cells *in vitro* using cell migration (Boyden chamber) assays.

We hypothesised that BSP promotes the migration of breast and prostate cancer cells. In the current study, BSP regulated the migration of breast adenocarcinoma MDA-MB-231 and prostate adenocarcinoma PC-3 cells. Interestingly, the potential for cell migration varied between cancer cell lines and BSP concentrations. 30 $\mu\text{g/ml}$ BSP induced MDA-MB-231 cell migration, which is in agreement with a previous study [299]. No effect on MDA-MB-231 cell migration with 3 $\mu\text{g/ml}$ BSP was observed. In contrast, Sung *et al* [381] reported that 1 μM ($\approx 6.6 \mu\text{g/ml}$) recombinant human BSP fragment induced the invasion of MDA-MB-231. Our results showed that 30 $\mu\text{g/ml}$ BSP inhibited, while 3 $\mu\text{g/ml}$ BSP stimulated PC-3 cell migration.

Dual role of bone sialoprotein. BSP might have functioned as both a chemoattractant and a chemorepellent, depending on the cancer cell line and the concentration. In the current study, MDA-MB-231 and PC-3 cells responded differently to BSP concentrations to navigate cell migration. 30 $\mu\text{g/ml}$ BSP significantly decreased PC-3 cell migration, while 30 $\mu\text{g/ml}$ BSP acted as a chemoattractant for MDA-MB-231 cells. In contrast, Gordon *et al* [299] showed that

20 µg/ml BSP induced the invasion of both MDA-MB-231 and PC-3 cells. However, the authors did not include a negative control in the study. While the present results suggested that BSP acted as a chemorepellent for PC-3 cells at high concentrations, BSP might have acted as a chemoattractant for PC-3 cells at low concentrations. As discovered in the adhesion assay, PC-3 cells are sensitive to concentrations of BSP as low as 0.123 µg/ml. It seemed reasonable to assume that low BSP concentrations also stimulate the migration of PC-3 cells. Indeed, 3 µg/ml BSP enhanced PC-3 cell migration. Interestingly, 3 µg/ml BSP had no effect on MDA-MB-231 cell migration, even though 3 µg/ml BSP had significantly promoted MDA-MB-231 cell adhesion. In lung cancer cell lines, 0.03 µg/ml BSP is sufficient to significantly induce the migration of A-549 and CL1-5 cells [344]. Recombinant human BSP also dose-dependently promotes the migration of human umbilical vein endothelial cells (HUVECs) [50-1000 nM ($\approx$ 4-80 µg/ml)] [361]. In the context of our findings, it is possible that BSP acts as a chemoattractant, promoting the migration of both MDA-MB-231 and PC-3 cancer cells at concentrations below 3 µg/ml (or even below 1 µg/ml) or within the range of 3 µg/ml to 30 µg/ml. Further cell migration assays are needed to explore this potential in detail.

Receptor dynamics and migration. In cancer metastasis, cell receptor desensitisation may be important for cell navigation, particularly when competing chemoattractants are present [466]. Prolonged exposure to high BSP concentrations may have caused receptor desensitisation or saturation in PC-3 cells, reducing their responsiveness to BSP gradients. Similar effects have been observed in leukocytes exposed to chemoattractants such as formylpeptide (fMLP) and complement fragment C5a [467]. In our assay, the static chemical gradient and the 24-hour migration period potentially exacerbated receptor desensitisation in PC-3 cells, diminishing their ability to detect concentration differences and resulting in random or reduced cell motility [468].

Receptor internalisation and recycling may have further contributed to the observed effects. These processes which can occur 2-4 times within an hour [469, 470], regulate cell adhesion, polarisation, and cytoskeletal organisation. Recycling of integrins, particularly $\alpha\beta$ 3, is mediated by Rab11 GTPase and PKB/GSK-3 β (protein kinase B/glycogen synthase kinase-3 β) [469, 470], which could potentially have affected PC-3 cell migration [471]. Receptor recycling is particularly important in cells with low integrin expression [472]. PC-3 cells, which express high levels of $\alpha\beta$ 5 and moderate to high levels of $\alpha\beta$ 3 [433, 438], may be less affected. In contrast, MDA-MB-231 cells exhibit lower expression levels of integrin $\alpha\beta$ 3 and fewer integrin receptors overall [381, 419, 440], which could increase their susceptibility to receptor internalisation.

BSP-mediated migration may have involved RGD-binding integrins [299], particularly $\alpha\beta$ 3. BSP-derived RGD peptides have been shown to promote $\alpha\beta$ 3-mediated migration and $\alpha\beta$ 5-mediated adhesion in MDA-MB-231 cells [381]. Although the anti- $\alpha\beta$ 3 antibody had no effect on MDA-MB-231 adhesion to BSP in our study, $\alpha\beta$ 3 integrins may mediate the migration of MDA-MB-231 cells at 30 µg/ml BSP. Similar RGD- $\alpha\beta$ 3 interactions regulate BSP-

induced migration and invasion in HUVECs [361], human bone marrow stromal cells (BMSCs) [473] and human thyroid SW-579 cancer cells [398]. These RGD-integrin interactions activate integrin outside-in signalling pathways such as PI3K/Akt/activator protein 1 (AP-1) signalling [101, 344, 474], which regulate BSP-induced migration in lung cancer cells [344]. Similar mechanisms may explain BSP-induced migration of MDA-MB-231 and PC-3 cells at 30 µg/ml and 3 µg/ml BSP, respectively.

Conversely, the inhibitory or unchanged effects of BSP on cell migration (30 µg/ml BSP on PC-3 cells and 3 µg/ml BSP on MDA-MB-231 cells) could result from alterations in cytoskeletal dynamics or integrin crosstalk. Overexpression of cytoskeletal proteins like Nischarin disrupts actin organisation and suppress cancer cell migration by inhibiting Rac GTPase activity [475] and downstream MEK/ERK signalling [475]. MEK/ERK signalling is a well-known promoter of cancer cell migration and invasion [476, 477]. Additionally, integrin crosstalk could have disrupted integrin signalling and inhibited migration. For instance, activation of $\alpha\beta3$ integrins can suppress $\alpha5\beta1$ signalling in transfected human leukaemia cells and inhibit phagocytosis [478].

Overall, the interplay between receptor desensitisation, internalisation, cytoskeletal dynamics, and integrin crosstalk may have influenced the migration of breast and prostate cancer cells.

Integrin-bone sialoprotein interactions in cancer migration. Given that $\alpha\beta3$ and BSP are co-expressed in prostate cancer cell lines [348] and in primary breast invasive ductal carcinoma samples [349], it is crucial to investigate the role of the $\alpha\beta3$ -BSP complex and the associated signalling pathways in tumour progression and cancer metastasis. Other RGD-binding integrin receptors may have also interacted with BSP to regulate cancer cell migration, including integrins $\alpha\beta5$, $\alpha\beta6$, $\alpha5\beta1$ and $\alpha\beta1$ on PC-3 cells [433] and integrins $\alpha\beta5$ and $\alpha\beta6$ on MDA-MB-231 cells [419, 440]. Previous work from our lab demonstrated that integrin $\alpha\beta5$ mediated the adhesion of both MDA-MB-231 and PC-3 cells to BSP. A prior study investigated the role of $\alpha\beta5$ in BSP-mediated MDA-MB-231 cell migration but observed a negligible inhibitory effect of the anti- $\alpha\beta5$ antibody on migration toward human BSP-derived RGD peptide [381]. Similar findings were reported using HUVECs [361], suggesting a migration mechanism independent of integrin $\alpha\beta5$. There is in general a lack of data on BSP-integrin interactions and the migration of PC-3 cells. BSP requires the RGD sequence to stimulate the invasion of PC-3 cells [299]. The RGD sequence likely controls PC-3 cell migration as well. As mentioned earlier, OPN, a member of the SIBLING family of proteins, has been shown to bind to integrin $\alpha\beta6$ to induce SW480 human colon adenocarcinoma cell adhesion [428]. $\alpha\beta6$ also promotes the migration and invasion of oral squamous carcinoma cells [479]. Therefore, $\alpha\beta6$ may serve as an additional BSP receptor to mediate the migration of cancer cells.

To define the role of RGD-integrin interactions in BSP-mediated cancer cell migration, investigators performing *in vitro* cell migration (Boyden chamber) assays should incubate cancer cells with GRGDSP peptide (200 µM), anti- $\alpha\beta3$ (5-20 µg/ml), anti- $\alpha\beta5$ (5-20 µg/ml),

and perhaps anti- $\alpha\beta 6$ integrin (5-20 $\mu\text{g/ml}$) antibodies for 30 min to 2 h prior to cell seeding [299, 398]. Cell migration assays may also be carried out with Lys-Ala-Glu (KAE)-mutated BSP (BSP-KAE), where the RGD sequence of BSP has been replaced.

Matrix metalloproteinases as regulators of bone sialoprotein-mediated cancer migration. MMPs play an important role in cancer progression and tumour metastasis. MMPs degrade the surrounding matrix and basement membrane to promote cancer cell migration and invasion, regulate angiogenesis, cell proliferation, inflammation and stimulate epithelial-to-mesenchymal transition (EMT) [480]. EMT, a process where epithelial cells acquire mesenchymal phenotypes and traits, facilitates cell motility and promotes cancer cell migration. Interestingly, MMPs and BSP are co-expressed in various cancers. For example, MMP-2 and BSP are co-localised in prostate cancer cell lines (PC-3 and LNCaP) and in prostate neoplasm [337], with significant associations in breast and colon cancers [400]. BSP is also strongly correlated with MMP-3 in oral squamous cell carcinoma [334]. Furthermore, BSP, $\alpha\beta 3$, and MMP-2 are co-expressed in papillary thyroid carcinoma [398]. Noteworthy, MDA-MB-231 cells express 26 different MMPs [481].

BSP might have influenced cancer cell migration through interactions with integrins and MMPs, as observed in previous studies. For instance, BSP has been shown to form RGD-dependent complexes with $\alpha\beta 3$ and MMP-2 in thyroid cancer cells, promoting invasion [398]. In the current investigation, $\alpha\beta 3$ /BSP/MMP-2 complex formation may have stimulated the migration of MDA-MB-231 and PC-3 cells. BSP binding to $\alpha\beta 3/\alpha\beta 5$ integrins may also stimulate FAK/ERK signalling in MDA-MB-231 and PC-3 cells, upregulating MMP expression (e.g., MMP-2, MMP-9, MMP-14) [299, 344] and activity, thereby enhancing migration. In turn, interferences in FAK/ERK signalling or PI3K/AKT/AP-1 might have inhibited BSP-induced cancer cell migration [299, 344]. For instance, BSP stimulated invasion of MDA-MB-231 cells is blocked when cells are treated with inhibitors of FAK or ERK activity [344], providing a possible explanation as to why BSP acted as both a chemoattractant and a chemorepellent in the current study.

We hypothesise that a feedback loop between BSP and MMPs exists. Research suggests that direct or indirect (integrin) binding between BSP and MMPs stimulates the translocation of MMPs to the cell surface and promotes cancer cell migration [398] [482]. To define the role of MMPs such as MMP-2, MMP-14, or MMP-9 in BSP-mediated cancer cell migration, investigators performing *in vitro* cell migration (Boyden chamber) assays should incubate cancer cells with MMP inhibitors (5-10 μM) or antibodies (5 $\mu\text{g/ml}$) for 30 min prior to cell seeding [344, 398].

CD44 as a potential mediator of bone sialoprotein migration. In addition, other non-integrin receptors such as the hyaluronan receptor CD44 may be involved in BSP-mediated cancer cell migration. CD44 is expressed on almost all PC-3 cells [483, 484]. CD44 primarily binds hyaluronic acid [485], but can also bind to other ECM proteins such as type IV COL (COLIV) [486], FN [487] and OPN [488]. CD44 plays a role in cytoskeletal dynamics and cell

motility and has been shown to induce cancer cell migration by interacting with OPN [489]. OPN also stimulates the adhesion and migration of fibroblasts and osteoclasts by binding to CD44 [488, 490]. In contrast, the invasion of BMSCs induced by BSP is not blocked with a CD44 antibody [473]. While BSP does not regulate the invasion of BMSCs by binding to CD44, BSP may bind to CD44 on cancer cells to mediate cell migration.

Enhanced migration capacity of PC-3 cells. Of note, PC-3 cells exhibited greater inherent migratory activity compared to MDA-MB-231 cells. PC-3 is a cell line derived from a bone marrow metastasis of a male patient with grade IV prostate adenocarcinoma, while MDA-MB-231 is a highly aggressive, invasive, metastatic triple-negative breast adenocarcinoma cell line established from the pleural effusion of a breast cancer patient. Metastatic cancer cells are more motile and can invade surrounding tissue compared to primary tumour cells [491]. Interestingly, PC-3 cells also display greater capacity for migration than LNCaP cells [492], a prostate carcinoma cell line derived from lymph node metastasis. PC-3 cells express high levels of both integrins and MMPs [337, 493]. The interaction between integrins and MMPs may have led to enhanced cancer cell migration in PC-3 cells compared to MDA-MB-231 cells. Some MMPs, for instance, are capable of binding directly to integrins [398]. Furthermore, PC-3 cells may have partially undergone EMT. Sun *et al* [494] performed wound-healing assays and showed increased PC-3 cell migration compared to DU145, a human cell line derived from the brain metastasis of prostate adenocarcinoma, and the human prostate epithelial cells RWPE1. PC-3 cells exhibited low levels of E-cadherin, an epithelial cell-cell adhesion molecule, and strong levels of the mesenchymal markers, vimentin and N-cadherin [494]. The greater ability for cancer cell migration in PC-3 cells than MDA-MB-231 in general, could therefore have been due to their bone metastatic origin, their greater integrin expression, MMP activity and EMT features.

BSP expression and migration in 3D spheroids vs. 2D cultures. In the current study, we examined the effect of BSP on cancer cell migration *in vitro* using cells grown in 2D monolayers. Cancer cell migration has also been investigated using cells grown in a 3D environment. A recent study by Huang *et al* [495] employed 3D spheroid cultures of MDA-MB-231 cells alongside transwell assays to investigate cancer cell migration. Both 2D cultures and 3D spheroid cultures of MDA-MB-231 migrated towards 10% FCS; however, the migration potential was higher in the spheroids, that had been transferred to 2D cultures for cell seeding onto the upper chamber, than in 2D grown MDA-MB-231 cells [495]. Interestingly, the expression of hypo-glycosylated BSP and glycosylated (mature) BSP is elevated in MDA-MB-231 spheroids compared to 2D MDA-MB-231 cells [346]. It remains to be determined whether the increased expression of BSP observed in 3D spheroids of MDA-MB-231 is associated with cancer cell migration and whether exogenous BSP induces the migration of cancer cells cultured in 3D.

Limitations. The transwell cell migration (Boyden chamber) assay is a simple, cost-effective model that provides a controlled environment for studying cell migration. However,

as a 2D model, it lacks the 3D architecture and complexity of *in vivo* tissue environments. In our study, we focused solely on cell migration and did not coat the inserts with a synthetic ECM to investigate cell invasion. Researchers may enhance the assay's physiological relevance by using coatings made from COL or FN, or by employing commercially available Matrigel®, which is a reconstituted basement membrane composed of ECM proteins such as laminin, COLIV, heparan sulphate proteoglycans, entactin, along with various growth factors. Thus, while the transwell migration assay can serve as a valuable tool for initial investigations into cancer cell migration due to its controlled conditions and reproducibility, it ultimately has limited physiological relevance and does not fully capture the complexity of *in vivo* cell migration processes.

Conclusions and implications. Cancer cell migration facilitates tumour progression and metastatic colonisation. Using transwell migration assays, we demonstrated that BSP regulated the migration of MDA-MB-231 breast and PC-3 prostate adenocarcinoma cells. Interestingly, BSP appeared to function as both a chemoattractant and a chemorepellent in cancer cell migration, potentially depending on the cellular context or signalling environment. The mechanisms underlying BSP-regulated migration remain unclear, but interactions with integrins $\alpha\beta3/\alpha\beta5$ and MMPs may play a role in this dual function. Additionally, BSP may engage other receptors, such as integrin $\alpha\beta6$, to influence cancer cell migration. Integrin internalisation and changes in signalling pathways could allow cancer cells to modulate their adhesion and migration, ultimately controlling cell motility. Further studies using migration and invasion assay, are needed to elucidate the integrin/BSP/MMP axis and associated signalling pathways, which could inform strategies to inhibit tumour dissemination. These findings underscore the complexity of cancer cell migration and contribute to growing evidence identifying BSP as an important mediator of tumour progression and metastasis. Ultimately, understanding BSP's role in these processes may provide novel opportunities to disrupt metastatic spread, addressing a critical challenge in cancer treatment

4.3 *In vitro* evaluation of osteoclast differentiation and activation

The significance of BSP in bone remodelling has been extensively documented in murine *in vivo* models. Alterations in BSP expression, whether through knockout or overexpression, influence bone growth patterns, mineral density, trabecular bone volume, and bone resorption of mice [364, 365]. For example, 4-month-old BSP deficient mice display reduced osteoclast surfaces relative to bone surface (OC.S/BS) compared to wild-type mice [365], while 8-month-old cytomegalovirus (CMV)-BSP transgenic (TG) mice show increased osteoclast numbers and greater osteoclast surface to bone surface [364]. Despite these findings, the involvement of BSP in osteoclast formation and activity in humans is poorly defined.

To the best of our knowledge, this is the first study to explore the effect of BSP on human osteoclastogenesis and osteoclast activity *in vitro*, using primary human monocytes

from buffy coats. We performed osteoclast differentiation assays and seeded macrophages – serving as a model for osteoclast precursors – on calcium-phosphate (CaP) discs to evaluate osteoclast activity through calcium colorimetric assays. We hypothesised that BSP, both alone and in combination with receptor activator of NF- κ B ligand (RANKL) treatment, would synergistically stimulate osteoclast differentiation and activity.

Our findings indicated a trend where BSP enhanced the differentiation of human osteoclast precursors into multinucleated mature osteoclasts after 17 and 24 days. In contrast, several animal studies found no effect [291, 344, 367] or even inhibition of osteoclast differentiation with BSP treatment alone [291]. In the current study, a trend for BSP and RANKL to synergistically induce the formation of human osteoclasts after 24 days, compared to BSP alone treated cultures, was observed. Greater osteoclast differentiation has also been reported in murine monocyte/macrophage-like RAW264.7 cells and murine bone marrow-derived macrophages (BMMs) in the presence of BSP and RANKL, than in RANKL alone and BSP alone treated cultures [344, 367]. Our results suggested that BSP potentially dose-dependently stimulated osteoclastogenesis after 17 days. Similarly, a previous study showed that BSP promoted osteoclast formation of RAW264.7 cells in a dose-responsive manner after 3 days, with increasing BSP concentrations (20 and 100 ng/ml) leading to progressively higher levels of osteoclast formation [367].

The current findings provide the first direct evidence in human models that BSP alone did not induce bone resorption *in vitro* by primary human osteoclasts. In contrast, RAW264.7 cells [367] and rabbit osteoclasts [291] treated with BSP exhibited enhanced bone resorption, as evidenced by the increased number and size of resorption pits on various substrates. Treatment of human osteoclast precursor cultures with BSP and RANKL enhanced osteoclast activity, however, this was not significantly different compared to RANKL alone treated cultures. A study investigating murine bone resorption *in vitro* reported synergistic effects of BSP and RANKL on osteoclast activity of RAW264.7 cells and murine BMMs compared to cultures treated with BSP alone and RANKL alone [367]. Since we seeded primary human osteoclast precursor-like cells, rather than mature osteoclasts, on synthetic substrates, several factors may explain the results observed, including potential influences on the differentiation, activity, and survival of osteoclast precursors and mature osteoclasts.

Calcium signalling in osteoclastogenesis. BSP may have stimulated intracellular calcium signalling to induce the transcription of nuclear factor of activated T cells 1 (NFATc1) and upregulate the expression of osteoclastogenesis related genes. For instance, treatment of human osteoclast-like cells (GCT23 cells) with an 18-amino-acid-long RGD-containing BSP peptide stimulates intracellular calcium release [371]. Intracellular calcium signalling activates calcineurin [67], which dephosphorylates NFATc1 and stimulates the translocation of active NFATc1 into the nucleus [67, 496]. NFATc1 is key transcription factor of osteoclastogenesis [496]. BSP has been shown to upregulate NFATc1 protein expression in nuclear-enriched fractions and to promote the transcription of NFATc1, tartrate-resistant acid phosphatase

(TRAP) and cathepsin K (CTSK) in RAW264.7 cells [367]. TRAP is a hallmark marker of osteoclast activity, while CTSK is a cysteine protease highly expressed by mature osteoclasts. Together, TRAP and CTSK enable osteoclasts to efficiently degrade both the organic and inorganic components of the bone matrix [13]. Treatment of RAW264.7 cells with both BSP and RANKL also stimulates intracellular calcium signalling and calcineurin [367]. We did not observe a trend for a synergistic effect of BSP and RANKL on the differentiation of primary human osteoclast precursor-like cells, compared to treatment with RANKL alone. In contrast, Valverde *et al* [367] reported greater NFATc1 translocation and transcription of osteoclastogenesis related genes when RAW264.7 cells were treated with both BSP and RANKL, compared to BSP alone or RANKL alone treated cells.

The mechanism of BSP-regulated calcium signalling in osteoclast precursors remains unclear. BSP has a high affinity for calcium and can bind to calcium [290], but extracellular calcium is typically absent in osteoclast precursor culture supernatants [497]. Early osteoclast precursors lack Integrin $\alpha\text{v}\beta\text{3}$ but express integrins $\alpha\text{2}\beta\text{1}$ [498] and $\alpha\text{v}\beta\text{5}$ [499]. Osteoclastogenesis may have involved a calcium-driven, integrin-independent pathway, as evidenced by a previous study in which an Src kinase inhibitor did not affect osteoclast differentiation in RAW264.7 cells [367].

CD44, highly expressed on osteoclast precursors, may regulate the differentiation and fusion of osteoclasts into mature, multinucleated osteoclasts [500, 501]. OPN, another SIBLING protein, binds CD44 and protects valve interstitial cells against calcium deposition via Akt phosphorylation [502]. OPN is known to inhibit matrix mineralisation [503], while BSP promotes mineral deposition [290, 365]. If BSP can bind to CD44 on osteoclast precursors, it could stimulate FAK signalling, stimulate intracellular calcium concentrations and signalling [504], and influence the differentiation, migration and activity of osteoclasts. Interestingly, BSP deficient murine osteoclast precursors undergoing osteoclastogenesis express lower mRNA levels of CD44 compared to wildtype osteoclast precursor cultures [368], suggesting a potential mechanism.

Additionally, the heparin-binding motif of BSP (LHRRKVI) may facilitate calcium-driven, integrin-independent osteoclastogenesis, as research indicates that OPN's interaction with CD44 requires prior heparin binding to induce a conformational change [505].

RANK and M-CSF in the differentiation and survival of osteoclasts. BSP may have promoted osteoclast differentiation via RANKL-induced calcium signalling [497, 506] or other RANKL-induced signalling cascades, such as Src, IKK, and the MAPK pathways JNK, p38, and ERK, which induce NFATc1, c-Fos, and DC-STAMP (dendrocyte expressed seven transmembrane protein) expression. c-Fos is an important transcription factor of osteoclast differentiation and activity [507, 508], while DC-STAMP mediates osteoclast fusion [76]. RANK/RANKL signalling promotes the differentiation and survival of osteoclast precursors and mature osteoclasts [369, 370, 509, 510]. BSP may have regulated RANK expression (and RANK/RANKL signalling), as RANK is reduced in BSP-deficient mouse osteoclast precursors

compared to wildtype [368]. Treatment with macrophage colony-stimulating factor (M-CSF) likely increased RANK expression on osteoclast precursor-like cells as well [58].

Additionally, BSP may have stimulated Akt activation, JNK activation and JNK-dependent phosphorylation of c-Jun in osteoclast precursor-like cells, as observed in BSP-treated RAW264.7 cells [367]. PI3K/Akt and JNK are signalling pathways downstream of RANK [63, 64] and play crucial roles in osteoclast differentiation [369, 370]. Akt signalling also activates anti-apoptotic factors like Bcl-xL and stimulates NF- κ B, supporting cell survival [511]. BSP may have promoted the differentiation and survival of osteoclast precursor-like cells and mature osteoclasts by activating RANK associated signalling pathways and regulating the expression of c-Fms [368]. c-Fms is the receptor of M-CSF on haematopoietic precursor cells/osteoclast precursor cells [61].

M-CSF also facilitates osteoclast precursor proliferation, survival [512, 513], and RANKL-induced differentiation by activating c-Fos and ERK pathways [57, 58], which enhance osteoclast precursor migration and cell-cell fusion [510]. Therefore, BSP-induced osteoclastogenesis may have occurred via regulating the expression of the receptors RANK and c-Fms.

Osteoclast activity. We showed a trend for BSP alone to induce osteoclast differentiation, however, our results demonstrated that BSP alone did not stimulate osteoclast activity and required RANKL to induce bone resorption. This lack of effect with BSP treatment alone was likely not due to reduced cell survival or increased apoptosis, as previously noted. In RAW264.7 cells, combined RANKL (50 ng/ml) and BSP (1–100 ng/ml) treatment increases resorption pit formation compared to BSP alone [367]. Similarly, in murine BMMs, supplementation with both RANKL (50 ng/ml) and BSP (50 ng/ml) significantly enhances resorption area on osteologic or dentin discs compared to either treatment alone [367]. This synergistic effect appears RANKL-specific, as BSP combined with parathyroid hormone (PTH) reduces calvarial bone resorption compared to PTH alone [372]. PTH regulates RANKL and OPG expression to stimulate osteoclast differentiation and activity [514], while also inducing BSP expression in human osteoblast-like cells [308]. These findings align with our data that BSP and RANKL synergistically enhance osteoclast activity.

RANK signalling likely enhanced both osteoclast differentiation and the activity of mature osteoclasts [368]. Treatment of osteoclast precursor-like cell cultures with RANKL likely increased RANKL binding to RANK, activated RANK/RANKL signalling pathways, and stimulated osteoclast resorption activity. PI3K/Akt regulates bone resorption by influencing the gene and protein expression of osteoclast associated markers [56, 515]. NFATc1 and c-Fos, upregulated by RANK/RANKL signalling, induce the expression of key resorption genes such as CTSK, c-Src, integrin α v/ β 3, MMP-9, RANKL, and TRAP [96, 516, 517]. MMP-9 facilitates degradation of denatured COL and proteoglycans while interacting with other MMPs such as MMP-14 to support osteoclast migration [94]. Additionally, M-CSF enhances RANKL-induced activity in mature osteoclasts by increasing resorption pits and F-actin sealing zone formation

[57]. In the current study, RANKL signalling likely mediated bone resorption through c-Src, which we discuss further below.

RGD sequence and integrin signalling. The RGD sequence of BSP may have bound to integrin $\alpha v \beta 3$ on osteoclasts to induce integrin signalling and stimulate bone resorption [291, 518, 519]. Mature osteoclasts highly express integrin $\alpha v \beta 3$ [499] and we have shown that BSP promoted the adhesion of human NSCLC and prostate adenocarcinoma cell lines through RGD-integrin $\alpha v \beta 3$ binding. In the current study, RGD-integrin binding potentially stimulated integrin signalling and activated c-Src kinase activity [101, 367]. RANKL or BSP treatment of RAW264.7 cells has been demonstrated to activate c-Src-dependent phosphorylation of c-Cbl at the 731 position [367]. c-Src is a member of the Src family of non-receptor tyrosine kinases. Src is essential for cell adhesion and migration, as osteoclasts lacking Src exhibit a more adherent phenotype, decreased lamellipodia movement, and diminished migratory ability [101]. RGD-integrin binding may also have enhanced bone resorption by increasing TRAP activity [372]. The RGD sequence generally plays an important role in osteoclastic resorption, whether this is BSP-specific requires further investigation. Some studies reported no effect of RGD-containing proteins like OPN or VN on osteoclast-mediated bone resorption [291], while others found that OPN pre-treatment significantly reduced osteoclast adhesion and rat bone resorption [519]. These discrepancies may stem from variations in cell types (rabbit vs. chicken osteoclasts) and substrates (ivory slices vs. rat bone). In the current study, BSP-induced integrin signalling and integrin-mediated osteoclast migration and function likely required the presence of RANKL. In humans, BSP may not directly regulate bone resorption but instead play a supportive role in RANKL-mediated osteoclast activity.

Bone sialoprotein modifications and osteoclast biology. Other BSP-specific sequences and post-translational modifications could have influenced osteoclast adhesion and activity. BSP-induced osteoclast adhesion is not solely dependent on the RGD sequence [372]. For instance, BSP contains polyglutamic acid (poly-E)-rich domains near the N-terminal [304], and a decaglutamylpeptide has been shown to stimulate ivory resorption by rabbit osteoclasts [291]. Phosphorylation of specific threonine residues in BSP has also been implicated in regulating its function. Results from blocking studies using an O-phosphorylated peptide [Pro-Arg-Gly-Asp-Thr(O-phosphoryl)-Tyr] suggest that phosphorylation of threonine residues in BSP reduces its cell-binding function [303]. Furthermore, N-linked and O-linked glycosylation of BSP may modulate cell attachment indirectly [285, 520, 521], potentially reducing the adhesion of osteoclast precursors or mature osteoclasts. While glycosylation might not directly affect osteoclast activity [291], both glycosylation and phosphorylation of BSP could influence bone resorption by altering the adhesive properties of osteoclasts. In the current study, these modifications may have impaired osteoclast detachment from CaP discs and hindered migration to new resorption sites. As a result, treatment of osteoclast precursor-like cells with BSP alone did not promote osteoclast activity.

Anti-bone sialoprotein antibody. Results from our case report indicated that BSP antibody FP-21 reduced BSP-mediated osteoclast differentiation after 24 days of treatment. Similarly, Chen *et al* [344] reported that treating RAW264.7 cells with a BSP antibody significantly decreased osteoclast differentiation, compared to treatment with conditioned medium containing BSP from A-549 cells. Neutralising BSP with antibodies may represent a promising strategy to regulate BSP-driven and RANKL-driven osteoclastogenesis. Diseases characterised by excessive bone resorption such as osteoporosis and breast cancer bone metastases may benefit from the development of new therapeutic drugs. More data needs to be generated for statistical analyses.

Limitations. A significant limitation of this study was the reliance on manual quantification of osteoclast differentiation. One investigator manually counted cells and calculated the percentage of osteoclasts from five images per well, a subjective approach prone to variability. The absence of a second investigator for independent counting further introduces the potential for bias, which may have impacted the reliability of results. To address this, future studies should incorporate repeat counts or the blinding of investigators to the treatment conditions to improve accuracy.

We are currently developing an automated image segmentation technique to quantify osteoclast differentiation across entire wells. This process involves scanning the entire well with a fluorescence microscope, stitching individual images together to form a composite, and defining regions of interest (ROI) for analysis. Osteoclasts are then quantified based on criteria such as nuclear number and size. However, progress has been slowed by issues like the autofluorescence of cell culture plates, suboptimal image resolution, and the inability to accurately analyse cells near the plate walls. Additionally, vitronectin receptor (VNR)-positive cells could have been quantified by measuring fluorescence at 488/528 nm using a spectrophotometer [376], which would have complemented our manual counting and provided more comprehensive data. Overcoming these challenges to develop a fully automated method will significantly improve the accuracy and reliability of osteoclast quantification, leading to more robust and conclusive results.

The use of synthetic substrates, such as CaP-coated discs, provides a controlled but simplified representation of bone. In contrast, mineralised substrates like bone or dentin slices more closely resemble the natural *in vivo* environment, containing ECM components and SIBLING proteins, like OPN, which support osteoclast activity. Osteoclasts produce higher levels of bone degradation enzymes, such as TRAP and CTSK, when cultured on natural substrates compared to synthetic ones [377]. However, Valverde *et al* [367] reported no significant difference in resorption pit area between osteologic or dentin discs when murine BMMs were cultured with BSP and/or RANKL alone, suggesting similar overall resorption across substrates despite mechanistic differences.

The synthetic CaP discs used in this study, primarily composed of α -TCP (α -tricalcium phosphate) and CDHA (calcium deficient HA), may have influenced osteoclastogenesis and

resorption. While the expression of osteoclastic genes [e.g., TRAP, carbonic anhydrase isoenzyme II (CAII), and CTSK] is similar in RAW264.7 cells cultured on 2D or 3D β -TCP (β -triphasic calcium phosphate) and BCP (biphasic calcium phosphate, HA/ β -TCP) scaffolds [522], osteoclasts are larger and found in greater numbers on 2D scaffolds compared to 3D scaffolds [522]. CAII, in particular, is associated with osteoclast activity and pit formation on CaP bone cements [377]. Additionally, resorption pits form on 2D and 3D HA and BCP scaffolds but not on β -TCP, with lacunae formation being greater on 2D scaffolds [522]. Future research should investigate BSP's effects on osteoclast activity using different synthetic substrates, including self-printed 3D CaP-coated discs.

Conclusions and implications. This study explored the role of BSP in human osteoclast differentiation and activity *in vitro*, using isolated monocytes from buffy coats and commercially available CaP discs. We present the first direct evidence that BSP can induce the differentiation of mature human osteoclasts, potentially in a dose-dependent manner. Automating image analysis will enhance the quantification and strengthen the robustness of these findings. Our results also demonstrated that BSP and RANKL synergistically promoted human osteoclast activity compared to BSP alone. However, further research is needed to assess the impact of anti-BSP antibodies, such as FP-21, on osteoclastogenesis and bone resorption. Additionally, future studies should evaluate how the early addition of BSP (treatment of isolated monocytes with M-CSF and BSP) influences osteoclast differentiation, as well as the activity of mature osteoclasts on mineralised substrates in the presence of BSP. This approach will better define BSP's role in mature osteoclasts versus osteoclast precursor-like cells.

Understanding the role and mechanisms of BSP in human osteoclast differentiation and activity *in vitro* provides a foundation for translating these findings into *in vivo* experiments. Animal studies, such as those modelling post-menopausal osteoporosis (ovariectomised rat model) or skeletal metastases (xenograft model), could elucidate BSP's role in osteolysis and the tumour microenvironment. Clinicians may find these results beneficial in the long term, as current treatments like bisphosphonates—commonly used for osteoporosis and skeletal metastases—can have persistent effects even after discontinuation. Although we are only beginning to understand BSP-mediated human osteoclast biology, these findings establish a basis for considering BSP as a potential therapeutic target in bone disease or osteotropic cancer.

4.4 Gene expression of bone sialoprotein in secondary bone cancer

The occurrence of bone metastases in cancer patients reflects cancer progression and has a serious impact on a patient's quality of life (QOL), leading to cancer-induced bone pain [270], skeletal related events (SREs) such as pathological fractures [267] and hypercalcaemia [268], and ultimately death. Median overall survival (OS) following the diagnosis of skeletal metastases is six months [254]. Accumulating evidence supports the role of BSP in secondary

bone cancer development, potentially by facilitating various steps in the metastatic cascade like cancer cell proliferation [381], survival [389], complement evasion [392], and angiogenesis [362]. We demonstrated that BSP stimulated cancer cell adhesion and regulated migration *in vitro*, highlighting its role in cancer cell motility. Positive BSP expression has been identified as a risk factor for bone metastases in breast and lung cancer patients [523, 524], suggesting its role as a driver of tumour metastasis. Whilst primary tumours express BSP at both the protein and mRNA levels [330, 341, 344], little is known about BSP expression in skeletal metastases, particularly at the mRNA level. BSP expression by secondary bone cancers may facilitate tumour colonisation and tumour-induced bone remodelling.

In the current study, we examined the relative gene expression of BSP in bone metastatic lesions from breast and lung carcinoma patients and in the bone specimen of a non-cancer patient using qPCR. We hypothesised that BSP gene expression would be elevated in secondary bone cancer tissue. Our findings confirmed this hypothesis, demonstrating high BSP mRNA expression in skeletal metastases from breast and lung carcinomas. Gene expression levels of BSP, OPN, RANKL and parathyroid hormone-related protein (PTHrP) in bone metastatic lesions tended to be higher than in the control. Bone metastases may synthesise these proteins to promote tumour cell adhesion, invasion, survival, and growth within bone, and may also stimulate tumour-induced bone remodelling.

Mechanisms of bone sialoprotein expression. BSP expression may be stimulated through the cyclic AMP (cAMP)/protein kinase A (PKA) pathway in metastatic breast and lung cancer cells. In bone, BSP is synthesised by osteoblasts, osteoclasts, osteocytes [148, 305] and BMSCs [306]. Ductal carcinoma cells within bone metastases also express BSP at the mRNA level [347]. Cancer cells may induce BSP expression via the cAMP/PKA pathway [281, 345, 525], potentially explaining the elevated BSP levels in bone metastases. However, we did not differentiate between tumour-derived and non-tumour-derived BSP expression. This distinction is crucial, as no study has investigated the complete cellular composition of primary or metastatic tumour tissue. Earlier research revealed that malignant epithelial cells constitute approximately half of the of primary breast tumors and 70-75% of lymph node metastases [526]. In general, metastatic solid tumours contain approximately 76% tumour cells and contain a lower proportion of lymphocytes (ca. 5%) compared to primary tumours (ca. 13.5%) [527]. While cAMP/PKA-mediated BSP expression has been reported in primary breast and prostate cancer cells, its role in bone metastases warrants further exploration. Analysis of transcription factors involved in the cAMP/PKA pathway, such as CREB (cAMP response element-binding protein), may provide valuable insights. Additionally, oestrogen receptor (ER) α can induce BSP expression in osteoblastic cells [311], and osteoblast formation may have been upregulated in bone metastases, as indicated by strong Runx2 (runt-related transcription factor 2) gene expression.

Gene expression trends in metastases. There was a trend for increased transcription of OPN, RANKL, and PTHrP genes in breast and lung carcinoma bone metastatic lesions

compared to non-tumour tissue. Linforth *et al* [528] detected PTHrP mRNA expression in 81% of breast cancer bone metastases. Data on OPN and RANKL synthesis by bone metastases remains inconclusive. For example, RANKL protein expression has been reported as upregulated [529], non-uniform [530], or even absent in metastatic bone tumour cells [531]. One study reported lower RANKL mRNA expression in a bone metastasis from breast cancer compared to healthy breast tissue [532], contrasting our observed results. Similarly, while OPN is moderately to strongly expressed in the majority of bone metastases and in 83% of tumour cells of breast cancer bone metastases at protein level [347, 533], its mRNA is weakly detected [347]. In our study, OPN transcription was strongly upregulated in bone metastatic lesions, potentially due to synthesis by osteoclasts rather than tumour cells. Ibrahim *et al* [347] observed high OPN mRNA expression in osteoclasts of bone metastases, supporting this hypothesis.

BSP, OPN, RANKL and PTHrP are involved in bone remodelling and tumour progression. Their expression in primary tumour tissue and tumour cells is associated with disease progression, metastasis and poor survival [341, 528, 529, 534]. OPN and PTHrP regulate cancer cell proliferation and survival [244, 535, 536], while RANKL promotes primary tumour growth [537] and cancer cell migration [538, 539]. *In vivo*, BSP and OPN contribute to the development of bone metastases of breast [382] and lung [344] cancer, while RANKL supports tumour growth at bone sites [540].

PTHrP also plays a critical role in regulating cancer cell motility, as seen in giant cell tumour of bone (GCTB), where it modulates genes associated with adhesion and migration, such as C-X-C motif chemokine ligand 10 (CXCL10), vascular cell adhesion protein 1 (VCAM-1), and ITGB3 (integrin β 3) [541]. Similarly, in bone metastases, stromal cell-derived PTHrP may have influenced cancer cell adhesion and migration [542, 543]. The elevated expression of PTHrP in breast and lung cancer bone metastases highlights its role in facilitating tumour colonisation within the bone microenvironment. This is supported by evidence showing that PTHrP mRNA is more frequently and abundantly expressed in bone metastases compared to primary breast cancer tissue [528]. In contrast, Bryden *et al* [544] detected lower PTHrP expression in poorly differentiated prostate cancer bone metastases, relative to primary prostate tumours, which may reflect cancer-specific differences in tumour-induced bone remodelling.

Cancer cell-bone interactions. Cancer cell adhesion in bone metastatic lesions may have been influenced by BSP, OPN, and PTHrP. Our lab showed that breast, lung and prostate carcinoma cells adhere to BSP via integrins α v β 3 and α v β 5. Integrin α v β 3 and α v β 5 expression correlates with cancer progression [418], and BSP at mRNA expression in secondary bone tumours may facilitate cancer cell binding to the bone ECM. OPN, as an anchoring protein, may also contribute to this adhesion [410]. Additionally, BSP and OPN may enhance osteoclast binding to bone via α v β 3 integrins, promoting osteoclast-mediated resorption [291, 519].

BSP and OPN may have also activated pro MMPs (proMMPs) such as proMMP-2 [400, 482], proMMP-3 [400] and proMMP-9 [545], thereby regulating matrix degradation [546], bone resorption and cancer cell invasion [480, 547]. While we demonstrated that high exogenous BSP concentration promoted breast cancer cell migration *in vitro*, BSP's *in vivo* significance remains unclear. The co-expression of BSP and MMPs (e.g., MMP-2, MMP-9, and MMP-14) in primary tumour tissue [398, 400] and potentially metastatic lesions may establish a potential feedback loop in which these proteins mutually enhance each other's expression [299, 322] and activity [322, 344, 362], ultimately driving bone degradation and tumour progression.

Bone sialoprotein in bone remodelling. BSP influences both bone formation and bone resorption. It stimulates osteoblast differentiation [149], matrix mineralisation [149, 365], osteoclastogenesis [365, 368], and bone resorption [291, 367]. Our lab showed a trend suggesting that BSP alone promoted human osteoclastogenesis and demonstrated that co-treatment with BSP and RANKL enhanced human osteoclast activity. Elevated BSP expression in bone metastases likely disrupted bone remodelling, as evidenced by osteopenia, an increased number of giant osteoclasts, and altered osteoblast and osteoclast differentiation markers in CMV-BSP-overexpressing mice [364]. BSP and OPN can bind to hydroxyapatite (HA) [294], influencing HA nucleation and formation [19, 366].

Runx2 in bone metastasis. There was a trend towards higher Runx2 gene expression in the majority of secondary bone cancer tissue (75%) compared to non-cancerous bone tissue, consistent with prior reports [548]. Runx2 may promote tumour growth, motility [549, 550], and bone remodelling. Runx2 is a key osteoblastic transcription factor [551] and regulates bone matrix synthesis [552]. Its expression correlates with the expression of OPN, MMP-9, MMP-13, and vascular endothelial growth factor (VEGF) [553], potentially contributing to tumour progression. In our study, Runx2 expression may have played a role in increasing OPN transcription and promoting metastatic development.

TRAP expression in metastasis. Transcript levels of TRAP tended to be lower in the majority of secondary bone cancer tissue (75%) compared to non-cancerous bone tissue. Interestingly, the 42 kDa variant of the TRAP 5a isoform is only detected in secondary bone tumours from breast, prostate, kidney, and lung cancer and not found in healthy bone [554]. In bone, TRAP is synthesised by mature osteoclasts and tissue expression of TRAP is associated with the presence of active osteoclasts [555]. TRAP regulates bone resorption by dephosphorylating matrix proteins like OPN and BSP, facilitating osteoclast detachment and migration [95]. TRAP contributes to tumour progression, regulating the survival [556] and proliferation [556, 557] of cancer cells and promoting distal metastases [558]. TRAP expression by secondary bone tumours may have stimulated tumour growth and tumour-induced osteolysis.

The strong yet lower TRAP mRNA levels in bone metastases compared to non-tumour tissue cannot be explained solely by the RANKL/OPG ratio, which regulates bone remodelling.

RANKL/RANK interactions initiate signalling cascades and promote osteoclast differentiation and bone resorption [65], while OPG, a decoy receptor, inhibits these processes by preventing RANKL/RANK binding [559, 560]. The RANKL/OPG ratio governs bone remodelling, but the low RANKL/OPG ratio in non-cancer bone tissue did not correspond to reduced TRAP expression. Other RANKL/RANK-independent signalling pathways may have contributed to the observed patient results. Notch signalling can either promote or suppress osteoclast formation, potentially depending on the subtype and developmental stage of osteoclast precursors [561]. For example, Notch1 reduces RANK expression and osteoclastogenesis [562], while Notch2 enhances osteoclast activity [563]. In this investigation, Notch signalling may have reduced RANK positive (RANK⁺) osteoclast precursors in bone metastases, although osteoclast precursors are typically increased in advanced breast cancer [564], potentially due to enhanced expression of inflammatory protein-1 delta (MIP-1 delta) [565]. Noteworthy, Notch signalling has a dual role in cancer [566-568].

Lower TRAP mRNA levels in bone metastases than non-tumour tissue, despite strong expression, may reflect a mixed phenotype of osteolytic and osteosclerotic characteristics in breast and lung cancer metastases [258, 569]. The elevated transcript levels of BSP, OPN and Runx2 support this hypothesis. Moreover, bone metastatic lesions demonstrated moderate to strong expression of PTHrP. PTHrP may have regulated key genes like RANKL, OPG, and BSP, influencing bone remodelling [570, 571] and phenotype of bone metastases. PTHrP suppresses BSP in cementoblasts [310], while inducing BSP expression in cancer cells, potentially via IL-8 stimulation [312, 541, 572]. The heterogeneous OPG and Runx2 transcript levels in bone metastases may have resulted from PTHrP's gene regulation and its role in tumour growth and metastasis progression [573].

Distribution of bone metastases in lung and breast cancer. Our data showed that lung cancer patients had bone metastatic lesions in the tibia, femur, and hallux. The distal extremities are less commonly targets of lung cancer bone metastases [250, 253, 574], likely due to reduced blood flow and decreased bone marrow activity in that area. In contrast, long bones are commonly affected by adenocarcinoma of the lung and small cell lung cancer [250, 253]. Lung adenocarcinoma is also significantly correlated with a greater risk of developing bone metastases [575]. Breast cancer bone metastases were found in the lumbar vertebrae and proximal femur, anatomical locations frequently invaded by breast carcinoma [251, 576-578]. The spread of bone metastasis is significantly associated with the tumour grade, with high-grade invasive breast cancer strongly correlated with the development of skeletal metastases in the vertebrae and ribs [579].

Limitations. Potential confounders, such as patient demographics and medical history, were not controlled. Data on neoadjuvant therapy were unavailable at the time of the study. Although age may have played a role in the gene expression levels observed in bone metastases from cancer patients [580-582], this influence seems limited, given that the non-cancer patient was only one year older than the lung carcinoma patients. Moreover, aging

cannot account for the mRNA transcript levels in the younger breast carcinoma patients. A recent literature review by Nisha *et al* [583] found that chemotherapy decreases bone mineral density (BMD) in pre-menopausal women with breast cancer. Patients with progressive breast cancer receiving neoadjuvant chemotherapy have vitamin D deficiency, decreased serum concentrations of calcium and RANKL and increased levels of OPG in the sera, resulting in a decreased RANKL/OPG ratio [584]. As described earlier, the RANKL/OPG ratio is an important determinant osteoclast differentiation and bone remodelling [559]. In the current study, bone metastatic lesions from breast and lung cancer strongly expressed both RANKL and OPG. Interestingly, one breast cancer bone metastasis displayed greater expression of OPG compared to RANKL. It is unknown whether the decreased RANKL/OPG ratio was due to neoadjuvant chemotherapy. Chemotherapy also increases the risk of SREs [585]. In contrast, radiotherapy increases bone density in irradiated bone metastases of breast cancer patients [586]. Radiotherapy may also control local bone metastatic lesions in patients with lung cancer [587], although the response seems to depend on the histological subtype of the cancer [588]. Additionally, radiotherapy alone or with hormone therapy increases plasma OPN levels in prostate cancer patients [589], potentially due to a radiation-induced inflammatory response [590]. In this study, high OPN expression was observed in bone metastases, though it's unclear if prior treatments contributed to these levels. Bone metastases from breast and lung cancer may have produced OPN to suppress immunosurveillance and promote metastasis development. Klement *et al* [591] demonstrated that OPN inhibits human CD8⁺ T cell activation and proliferation *in vitro*, reducing T-cell mediated cytotoxicity and cancer cell death. Additionally, tumour-cell expressed OPN promotes immune evasion by stimulating extramedullary myelopoiesis and increasing immunosuppressive cells like myeloid-derived suppressor cells (MDSCs) *in vivo* [592], which suppress T-cell and natural killer (NK) cell activity [593, 594]. Furthermore, OPN is expressed by tumour-infiltrating macrophages (TIMs) [534], which are key players in cancer progression [595]. Bone metastases may have also expressed BSP to aid immune escape by protecting cancer cells from complement-mediated attack through factor H association [392]. Collectively, these mechanisms may have contributed to tumour immune escape and facilitated the establishment of bone metastases.

In the current study, the histological type of cancer, the cancer grade and the cancer stage were unknown. Whilst oestrogen and progesterone receptor (PgR) status do not appear to affect BSP immunostaining in primary human breast cancer cells [329], the expression of BSP is significantly higher in infiltrating ductal carcinoma compared to infiltrating lobular carcinoma [329]. OPN mRNA level is also significantly higher in ductal breast carcinoma tissue compared to lobular breast carcinoma tissue [341]. Interestingly, the expression of RANKL is associated with PgR expression in primary breast tumours [596]. RANKL expression is higher in small, well-differentiated tumours and luminal A-like breast carcinoma compared to small, poorly differentiated tumours and other breast cancer subtypes [596]. Noteworthy, the mRNA expression of BSP in primary lung tumour tissue increases with increasing TNM staging [341,

344]. Gene expression levels of BSP and RANKL were likely influenced by cancer subtype. However, it is unclear whether the strong expressions of BSP and RANKL observed in breast cancer bone metastatic lesions in this study were due to high expression in the metastatic breast cancer cells themselves or in other cells, such as osteoblasts, T cells, or osteocytes.

Conclusions and implications. In summary, our findings demonstrated that bone metastases from breast and lung carcinoma patients exhibited strong BSP expression at the mRNA level. We observed a trend of increased gene expression levels for BSP, OPN, RANKL, and PTHrP in metastatic lesions compared to normal bone tissue. The production of these proteins by metastatic cells or other cells in the tumour microenvironment may contribute to tumour cell adhesion, survival, and facilitate tumour-driven bone remodelling. The trend for BSP expression to be stronger in skeletal metastases compared to non-cancerous bone tissue provides further evidence of its role in cancer metastasis to the bone.

Future studies employing immunohistochemistry or *in situ* hybridisation could elucidate the specific cellular sources of BSP within bone metastases. Biomarkers of human breast and lung cancer include p53 and Ki67 [597, 598]. Given that biomarker discordance between primary and metastatic tumour sites has been reported [599], careful validation of BSP and related proteins as prognostic markers is warranted. While our sample size precluded statistical analysis, ongoing data collection will enable a more robust assessment of BSP's role in bone metastases, potentially extending to prostate cancer bone metastases and osteosarcoma. A refined molecular characterisation of BSP in skeletal metastases could enhance its potential utility as a biomarker or therapeutic target in metastatic bone disease.

5. Summary

5.1 English summary

Around 90% of cancer deaths are attributed to metastasis rather than the primary tumour. Bone is a frequent site of cancer metastasis, where it disrupts the bone remodelling process - osteolytic, osteoblastic or mixed – resulting in skeletal related events (SREs) such as microfractures, that significantly impair patients' quality of life (QOL). Advancing our fundamental knowledge of cancer and bone biology is crucial for driving translational research and developing refined or novel strategies for preventing, detecting, and treating cancer and bone diseases. Investigating how certain proteins mediate physiological and pathological bone remodelling, particularly in the context of skeletal metastases, provides valuable insights into the intricate mechanisms of cancer cell homing to bone.

Bone sialoprotein (BSP) has emerged as a potential key regulator in the skeletal metastasis cascade. The protein BSP is a member of the small integrin-binding ligand, N-linked glycoprotein (SIBLING) family and is a component of mineralised tissue. It is well-recognised for its role in bone formation, particularly in promoting late-stage osteoblast differentiation and matrix mineralisation. However, BSP has also been implicated in processes critical to tumour progression, including angiogenesis, cancer cell proliferation, survival, adhesion, migration, and invasion. BSP's contains an RGD (Arg-Gly-Asp) motif as well as a heparin-binding site LHRRVKI (Lys-His-Arg-Arg-Lys-Val-Ile), which may facilitate cellular adhesion. Both adhesion and migration are critical processes for tumour cell dissemination and colonisation of distant organs.

Animal studies propose that BSP also regulates osteoclast differentiation and activity, but its effects on human osteoclastogenesis and bone resorption are unknown.

The goals of this project were to investigate the role of BSP in various biological processes, including its function as an anchor protein for tumour cells via RGD-integrin $\alpha v \beta 3 / \alpha v \beta 5$ and heparin-binding interactions; its influence as a chemoattractant in cancer cell migration; its contribution to bone resorption as an osteoclastogenic and osteoclast-activating protein; and its gene expression in skeletal metastases.

In vitro cancer cell adhesion assays using BSP, supplemented with blocking experiments (e.g., RGD and LHRRVKI peptides, and anti-integrin and anti-BSP antibodies), showed that BSP acts as an anchor protein facilitating adhesion of breast (MDA-MB-231), prostate (PC-3), lung (NCI-H460, A-549), and osteosarcoma (MG-63) cancer cells. Adhesion of MG-63 cells was not inhibited by anti-integrin antibodies, suggesting additional binding mechanisms or compensatory integrins. Blocking experiments with LHRRVKI peptide showed mixed effects, enhancing or suppressing adhesion depending on the cancer cell type. The role of the heparin-binding motif in cancer cell attachment may have depended on glycosaminoglycan (GAG) availability and involved interactions with the RGD sequence. BSP's influence on MDA-MB-231 and PC-3 migration (Boyden chamber) enhanced, suppressed, or had no effect depending on the cellular context and potentially signalling environment. BSP

alone induced the differentiation of human primary osteoclast progenitors into osteoclasts, but required the presence of RANKL (receptor activator of NF- κ B ligand) to stimulate osteoclast activity on calcium phosphate (CaP) discs. BSP appeared to synergise with RANKL to promote osteoclast differentiation and activity over 24 days. BSP gene expression, quantified using qPCR, was elevated in skeletal metastases from breast and lung cancers, with a trend for higher expression compared to healthy bone tissue. Anti-BSP antibodies effectively inhibited breast cancer cell adhesion to BSP, with FP-21 showing efficacy at low concentrations and YU829-H06 exhibiting the greatest inhibitory potency. FP-21 also showed a potential trend toward suppressing BSP-induced osteoclastogenesis after 24 days.

Thus, the results of this project suggest that BSP contributes to tumour progression, metastasis, and pathological bone remodelling, including tumour-mediated osteolysis. BSP's roles in processes such as cancer cell adhesion and migration underscore its potential as a driver of malignancy. Targeting BSP, particularly its RGD motif, integrin interactions, or other functional domains, offers a promising approach for suppressing tumour progression and pathological bone remodelling. Future investigations should explore BSP's interactions with other integrins (e.g., $\alpha\beta$ 6), hyaluronan receptor CD44, matrix metalloproteinases (MMPs MMP-2, MMP-9, and MMP-14), and potential integrin-independent pathways, such as calcium signalling. Defining BSP gene expression in prostate skeletal metastases and osteosarcoma tissue is another key area for future research. Given the limitations of current therapies targeting cancer and bone remodelling—such as the lack of FDA (food and drug administration)-approved drugs for integrins $\alpha\beta$ 3 and $\alpha\beta$ 5, and the prolonged effects of bisphosphonates even after discontinuation—this study underscores the importance of BSP as a target for developing novel therapies to improve QOL and survival in patients with cancer and bone disorders.

Keywords: Bone sialoprotein, cancer metastases, RGD, integrins, adhesion, migration, osteoclastogenesis, bone resorption

5.2 German summary

Rund 90% der krebsbedingten Todesfälle sind auf Metastasen und nicht auf den Primärtumor zurückzuführen. Ein von Metastasen häufig betroffenes Organ ist der Knochen, wo Metastasen den Knochenumbau – osteolytisch, osteoblastisch oder gemischt – beeinflussen und zu skelettassoziierten Ereignissen (SREs) wie Mikrofrakturen führen, die die Lebensqualität (QOL) der Patienten erheblich beeinträchtigen. Ein fundiertes Verständnis der Krebs- und Knochenbiologie ist essenziell, um translationale Forschung voranzutreiben und Strategien zur Prävention, Früherkennung und Behandlung von Knochenmetastasen und -erkrankungen weiter- oder neu zu entwickeln. Die Erforschung spezifischer Proteine, die den physiologischen und pathologischen Knochenumbau regulieren, insbesondere im Kontext von Knochenmetastasen, liefert wertvolle Einblicke in die komplexen Mechanismen der ossären Metastasenbildung.

Bone Sialoprotein (BSP) hat sich als potenzieller Schlüsselfaktor in der Kaskade von Skelettmetastasen hervorgehoben. BSP gehört zur SIBLING-Familie (small integrin binding ligand N-linked glycoprotein) und kommt im mineralisierten Gewebe vor. Es reguliert die Knochenbildung, indem es sowohl die späte Differenzierung von Osteoblasten als auch die Mineralisierung der Knochenmatrix unterstützt. Zugleich ist BSP an Prozessen beteiligt, die für die Tumorprogression wesentlich sind, darunter Angiogenese, Zellproliferation, Adhäsion, Migration, Invasion und Überleben von Tumorzellen. BSP besitzt sowohl eine RGD (Arg-Gly-Asp)-Sequenz als auch eine Heparin-Bindungsstelle LHRRVKI (Lys-His-Arg-Arg-Lys-Val-Ile), die zur Zelladhäsion beitragen könnte. Adhäsion und Migration von Krebszellen sind zentrale Prozesse bei der Ausbreitung von Tumoren und der Besiedlung entfernter Organe.

Tierstudien deuten darauf hin, dass BSP die Differenzierung und Aktivität von Osteoklasten beeinflusst. Die Wirkung von BSP auf die humane Osteoklastenbildung und den Knochenabbau ist jedoch unbekannt.

Die Ziele des Projekts waren, die Rolle von BSP in verschiedenen biologischen Prozessen zu untersuchen. Dies umfasste seine Funktion als Ankerprotein für Krebszellen über RGD-Integrine ($\alpha\beta3/\alpha\beta5$) und Heparin-Bindungsinteraktionen, seine Wirkung als Chemoattraktant bei der Zellmigration, seine Beteiligung als osteoklastogenes und osteoklastenaktivierendes Protein sowie die Untersuchung der Genexpression von BSP in Skelettmetastasen.

Zelladhäsionsassays zeigten, dass BSP als Ankerprotein wirkte und die Adhäsion von Brust- (MDA-MB-231), Prostata- (PC-3), Lungen- (NCI-H460, A-549), und Osteosarkom- (MG-63) Krebszellen förderte. Blockierungsexperimente mit RGD-Peptiden und Anti-Integrin-Antikörpern bestätigten, dass die RGD-Sequenz und Integrinrezeptoren $\alpha\beta3$ und $\alpha\beta5$ die Adhäsion von Brust-, Prostata- und Lungenkrebszellen förderten, während die Adhäsion der MG-63-Zellen unabhängig von Anti-Integrin-Antikörpern war. Dies deutet auf alternative Bindungsmechanismen oder eine Kompensation durch andere Integrine hin. Blockierungsexperimente mit dem LHRRVKI-Peptid führten zu variierenden Ergebnissen, die

abhängig von der getesteten Zelllinie (MDA-MB-231 und PC-3) entweder eine Förderung oder Hemmung der Adhäsion zeigten. Der Einfluss der Heparin-Bindungsstelle auf die Zelladhäsion war möglicherweise auf die Verfügbarkeit von Glykosaminoglykanen (GAGs) und der Interaktion mit der RGD-Sequenz angewiesen. BSP förderte, hemmte oder zeigte keine Effekte auf die Migration von Krebszellen (MDA-MB-231 und PC-3). Der Einfluss von BSP auf die Migration von Krebszellen (Boyden-Kammer-Assays) war vermutlich abhängig vom zellularen Kontext und Signalen der Mikroumwelt. BSP allein induzierte die Differenzierung humaner primärer osteoklastischer Vorläuferzellen zu Osteoklasten. Die Aktivierung der Osteoklasten auf Kalziumphosphat (CaP)-Platten war jedoch nur in Kombination mit RANKL (receptor activator of NF- κ B ligand) möglich. Über einen Zeitraum von 24 Tagen zeigten BSP und RANKL synergistische Effekte. Die qPCR-Analyse ergab, dass Skelettmetastasen von Brust- und Lungenkrebs eine erhöhte BSP-Genexpression aufwiesen und einen Trend zu einer stärkeren Expression im Vergleich zu gesundem Knochengewebe andeuteten. Experimente mit Anti-BSP-Antikörpern zeigten, dass der FP-21-Antikörper bereits bei niedrigen Konzentrationen die Adhäsion von Brustkrebszellen zu BSP effektiv hemmte, während der YU829-H06-Antikörper die stärkste Hemmwirkung aufwies. Der FP-21-Antikörper zeigte zudem einen Trend zu einer teilweisen Hemmung der BSP-induzierten Osteoklastogenese nach 24 Tagen.

Die Resultate des Projektes zeigen, dass BSP eine zentrale Rolle in der Tumorprogression, Metastasierung und im pathologischen Knochenumbau, einschließlich tumorinduzierter Osteolyse, spielt. Die Rolle von BSP in Prozessen wie der Adhäsion und Migration von Krebszellen unterstreichen BSP als potenziellen Wachstumstreiber von malignen Tumoren. Die gezielte Blockade von BSP, durch Inhibitoren oder Antikörper könnte eine vielversprechende Strategie zur Hemmung der Tumorprogression und des pathologischen Knochenumbaus sein. Zukünftige Studien sollten die Interaktionen von BSP mit anderen Integrinen (z.B. $\alpha\beta 6$), dem Hyaluronatrezeptor CD44, Matrix-Metalloproteinasen (MMP-2, MMP-9, MMP-14) und potenziell integrinunabhängigen Signalwegen wie der Kalzium-Signalgebung untersuchen. Ein weiterer Faktor ist die Bestimmung der Genexpression von BSP in Prostatakrebsmetastasen und Osteosarkomen. Die begrenzte Verfügbarkeit von Therapien, die gegen Integrine $\alpha\beta 3$ und $\alpha\beta 5$ gerichtet sind, sowie die langanhaltenden Effekte von Bisphosphonaten nach deren Absetzen unterstreichen die Notwendigkeit neuer therapeutischer Ansätze. Das gezielte Hemmen von BSP (und damit die Funktionsweise des Proteins) ist ein vielversprechender Ansatz, um die Lebensqualität und das Überleben von Patienten mit Krebs oder Knochenerkrankungen zu verbessern.

Schlüsselwörter: Bone sialoprotein, Krebsmetastasen, RGD, Integrine, Adhäsion, Migration, Osteoklastogenese, Knochenabbau

Appendix

2.1 Materials

2.1.1 Equipment

Product	Specification	Manufacturer
Autoclave	Systec DX-45	Systec GmbH, Linden, Germany
Automated cell counter	LUNA™	Logos Biosystems, Villeneuve d'Ascq, France
Automated cell counter	LUNA-FX7™	Logos Biosystems, Villeneuve d'Ascq, France
Centrifuge	5804 R	Eppendorf AG, Hamburg, Germany
Centrifuge	5810 R	Eppendorf AG, Hamburg, Germany
Centrifuge	Heraeus Fresco 17	Thermo Electron LED GmbH, Osterode, Germany
Centrifuge	5415C	Eppendorf AG, Hamburg, Germany
Circulating water bath	Lauda E100	IKA®-Werke GmbH & Co. KG, Staufen, Germany
CO ₂ Incubator	HERAcell™ 240	Thermo Scientific™, Waltham, USA
Counting chamber, Neubauer improved	BLAUBRAND®	BRAND GmbH + Co. KG, Wertheim, Germany
Cryogenic storage tank (-180°C)	Arpege 110	Cryopal, Bussy-Saint-Georges, France
Drying chamber	WTB binder	Binder GmbH, Tuttlingen, Germany
Electronic multichannel pipette (1250 µl)	E1-ClipTip™	Thermo Scientific™, Waltham, USA
Electronic pipette (200 µl)	ErgoOne® E Single-Channel	STARLAB International GmbH, Hamburg, Germany
Fluorescence microscope	Thermo AMG EVOS® FL Cell Imaging System	Lifetechnologies Corporation, Carlsbad, USA
Fluorescence microscope	BZ-X800	KEYENCE CORPORATION OF AMERICA, Itasca, USA
Freezer (-20°C)	LGex 3410 23B / 001	Liebherr-International Deutschland GmbH, Biberach an der Riß, Germany
Freezing container	CoolCell® LX	BioCision, California, USA
Fridge-Freezer (4°C, -20°C)	KGV33VW31	Robert-Bosch GmbH, Gerlingen-Schillerhöhe, Germany
Fridge-Freezer (4°C, -20°C)	GCv 4010-20E 001	Liebherr-International Deutschland GmbH, Biberach an der Riß, Germany
Fridge-Freezer (4°C, -20°C)	CUN 3923	Liebherr-International Deutschland GmbH, Biberach an der Riß, Germany
Heating chamber	WTB binder	Binder GmbH, Tuttlingen, Germany
Heating plate	13800	Medax GmbH & Co. KG, Neumünster, Germany
Inverted microscope	Wilovert S	Helmut Hund GmbH, Wetzlar, Germany
Laboratory shaker	Polymax 1040	Heidolph Instruments GmbH & Co. KG, Schwabach, Germany
MACS® MultiStand	130-042-303	Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany
Magnetic stirrer with heating	RET basic	IKA®-Werke GmbH & Co. KG, Staufen, Germany
Microbiological safety cabinet	Safe 2020 1.8	Thermo Electron LED GmbH, Langenselbold, Germany
MiniMACS™ Separator	130-042-102	Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany

Multimode reader	GloMax® Multi+ Detection System	Promega, Walldorf, Germany
pH-meter	Sartorius Basic Meter PB-11	Sartorius AG, Göttingen, Germany
Pipetboy	Pipetus®	Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany
Pipetboy	BRAND® accu-jet® <i>pro</i>	Brand GmbH + Co KG, Wertheim, Germany
Pipetboy	BRAND® accu-jet® S	Brand GmbH + Co KG, Wertheim, Germany
Pipettes (10, 100, 1000)		Thermo Scientific™, Waltham, USA
Pipettes (2.5, 10, 100, 1000)	Eppendorf Eppendorf Reference Eppendorf Research Eppendorf Research Plus	Eppendorf AG, Hamburg, Germany
Real-time PCR thermal cycler	qTOWER ³ G	Analytik Jena GmbH, Jena, Germany
Scale	SI-234	Denver Instrument
Scientific spectrophotometer	NanoDrop One	Thermo Scientific™, Waltham, USA
Surgical scissors		Aesculap, Inc., Pennsylvania, USA
Thermocycler	Peqlab peqSTAR 2X gradient	VWR International GmbH, Darmstadt, Germany
Tissue homogeniser	Precellys® 24	Bertin Technologies, Montigny-le-Bretonneux, France
Tweezers		Burchardt & Zimmermann Medizin-Technik OHG, Mainz, Germany
Ultra-low temperature freezer (-80°C)	U570	New Brunswick Scientific GmbH, Nürtingen, Germany
Vacuum pump	BVC professional	Vacuubrand GmbH + Co. KG, Wertheim, Germany
Vortex mixer (ZK)	PV-1	Grant-bio, Grant Instruments Ltd, Cambs, England

Table A 1. List of equipment used in experiments.

2.1.2 Software

Product	Manufacturer
GloMax® Discover System	Promega Corporation, Madison, USA
GraphPad Prism 9.2.0	GraphPad Software, San Diego, USA
qPCRsoft 3.4.6.0	Analytik Jena AG, Jena, Germany

Table A 2. Software used for data analysis and experimental procedures.

2.1.3 Consumables

Product	Specification	Manufacturer	Catalogue number
2D cell culture inserts		Innotere GmbH, Radebeul, Germany	111CC2
Cell counting slides	LUNA™	Logos Biosystems, Villeneuve d'Ascq, France	L12001
Cell culture flasks (T175)	Cellstar®	Greiner Bio-One GmbH, Frickenhausen, Germany	660175
Cell culture flasks (T25)	Cellstar®	Greiner Bio-One GmbH, Frickenhausen, Germany	690175

Cell culture flasks (T75)	Cell+	Sarstedt AG & Co. KG, Nümbrecht, Germany	83.3911.302
Cell culture flasks (T75)	Cellstar®	Greiner Bio-One GmbH, Frickenhausen, Germany	658175
Cell culture inserts (24-well plate, 8 µm)	FALCON® Permeable Support	Corning Incorporated, Corning, New York, USA	353097
Cell scraper (28 cm length)		Greiner Bio-One GmbH, Frickenhausen, Germany	541 070
Cryogenic vials	Nalgene® Cryoware	Thermo Fisher Scientific, Rochester, USA	5000-0020
Culture plate (24-well)	Cellstar®	Greiner Bio-One GmbH, Frickenhausen, Germany	662 160
Culture plate (24-well)	Falcon® 24-well permeable support companion plate	Corning Incorporated, Corning, New York, USA	353504
Culture plate (96-well)	Cellstar®	Greiner Bio-One GmbH, Frickenhausen, Germany	655180
Disposable hemocytometer	C-CHIP, Neubauer Improved, DHC-N01	NanoEntek, Inc., Hwaseong-si, Korea	2N18741
Eppis (0.5 ml)	Eppendorf Safe-Lock® Tubes	Eppendorf AG, Hamburg, Germany	0030 121.023
Eppis (1.5 ml)	Eppendorf Safe-Lock® Tubes	Eppendorf AG, Hamburg, Germany	0030 120.986
Eppis (2 ml)	Eppendorf Safe-Lock® Tubes	Eppendorf AG, Hamburg, Germany	0030 120.094
Eppis (5 ml)	Eppendorf Safe-Lock® Tubes	Eppendorf AG, Hamburg, Germany	0030 119.401
FACS tubes (5 ml)		Greiner Bio-One GmbH, Frickenhausen, Germany	115101
Falcons (15 ml)	Cellstar®	Greiner Bio-One GmbH, Frickenhausen, Germany	188271
Falcons (50 ml)	Cellstar®	Greiner Bio-One GmbH, Frickenhausen, Germany	227261
Filtered pipette tips (10 µl)	TipOne®	STARLAB International GmbH, Hamburg, Germany	S1121-2710
Filtered pipette tips (1000 µl)	TipOne®	STARLAB International GmbH, Hamburg, Germany	S1122-1730
Filtered pipette tips (200 µl)	TipOne®	STARLAB International GmbH, Hamburg, Germany	S1120-8710

Gloves (S)	VWR® latex powder free gloves	VWR International bvba, Leuven, Belgium	112-2750
Leukosilk® S		BSN medical GmbH, Hamburg, Germany	46834-00 AP
LS columns		Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany	130-042-401
Microplates (96-well)		Greiner Bio-One GmbH, Frickenhausen, Germany	655101
Microscope cover glasses	20x26x0,4mm	Paul Marlenfeld GmbH & Co. KG, Lauda-Königshofen, Germany	0350000
MS columns		Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany	130-042-201
Multichannel pipette tips (1250 µl)	ClipTip™	Thermo Fisher Scientific Oy, Vantaa, Finland	94410818
Multichannel pipette tips (300 µl)	ClipTip™	Thermo Fisher Scientific Oy, Vantaa, Finland	944020513
Nitrile gloves (M)		VWR International bvba, Leuven, Belgium	112-2372
Optical adhesive films		Life Technologies Corporation, Carlsbad, USA	201806 084
Pasteur pipettes (230 mm)		DWK Life Sciences GmbH, Wertheim / Main, Germany	91704024
PCR Eppis (0.5 ml)	Eppendorf Safe-Lock® Tubes	Eppendorf AG, Hamburg, Germany	0030 123.301
PCR Eppis (1.5 ml)	Eppendorf Safe-Lock® Tubes	Eppendorf AG, Hamburg, Germany	0030 123.328
PCR Eppis (2 ml)	Reaction tubes	Greiner Bio-One GmbH, Frickenhausen, Germany	623 201
PCR plates (96-well)		Biozym Scientific GmbH, Hess. Oldendorf, Germany	710893
PCR strips (0.2 ml) with domed cap strips		Peqlab, Erlangen, Germany	82-0266-A
Pipette tips (10/20 µl)	TipOne®	STARLAB International GmbH, Hamburg, Germany	S1110-3700
Pipette tips (1000 µl)	TipOne®	STARLAB International GmbH, Hamburg, Germany	S1111-6701
Pipette tips (200 µl)	TipOne®	STARLAB International GmbH, Hamburg, Germany	S1111-0706
Precellys® tubes (2 ml)	MK28-R	Bertin Technologies, Montigny-le-Bretonneux, France	P000917-LYSKO-A
Precision wipes (30x30 cm)		Kimberley-Clark Europe Limited/Professional Sector, Surrey, UK	75512
Pre-separation filters (30 µm)		Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany	130-041-407

Serological pipettes (10 ml)	Corning® Costar® Stripette®	Corning Incorporated, Corning, New York, USA	4488
Serological pipettes (2 ml)	Cellstar®	Greiner Bio-One GmbH, Frickenhausen, Germany	710 180
Serological pipettes (25 ml)	Cellstar®	Greiner Bio-One GmbH, Frickenhausen, Germany	760 180
Serological pipettes (5 ml)	Corning® Costar® Stripette®	Corning Incorporated, Corning, New York, USA	4487
Serological pipettes 5 ml)	Cellstar®	Greiner Bio-One GmbH, Frickenhausen, Germany	606 180
Sterile applicators		Paul Boettger GmbH & Co. KG, Bodenmais, Germany	1102241
Sterile reagent reservoirs (25 ml)		VWR International, West Chester, USA	613-1175
Suspension plates (24-well)	Cellstar®	Greiner Bio-One GmbH, Frickenhausen, Germany	662160
Suspension plates (96-well)	Cellstar®	Greiner Bio-One GmbH, Frickenhausen, Germany	655 185
Suspension plates (24-well)		Sarstedt AG & Co. KG, Nümbrecht, Germany	83.3922500
Terralin® liquid		Schülke & Mayr GmbH, Norderstedt, Germany	102001
Tube, 5 ml, PP, sterile		Greiner Bio-one GmbH, Frickenhausen, Germany	115261

Table A 3. Consumables used in experiments.

2.1.4 Reagents

Product	Specification	Manufacturer	Catalogue number
Acridine Orange/Propidium Iodide Stain		Logos Biosystems, Villeneuve d'Ascq, France	F23001
Accutase® solution		Sigma-Aldrich, Co., St. Louis, USA	A6964-100 ml
Acetic acid	ROTIPURAN®, 100%	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	3738.1
AlamarBlue™ cell viability reagent		Invitrogen by Thermo Fisher Scientific, Life Technologies Corporation, Eugene, USA	DAL1100
Aqua ad injectabilia	Ampuwa® Spüllösung 1000 ml Plastipur®	Fresenius Kabi, Sèvres, France	1088811
Aqua ad injectabilia Braun		Braun Melsungen AG, Melsungen, Germany	6724123.00.00
Blue S'Green qPCR 2x Mix		Biozym Scientific GmbH, Oldendorf, Germany	331416
BSA solution	30%	PAA Laboratories GmbH, Pasching, Austria	K11-013

BSP CR-1	280 µg/ml	Immundiagnostik AG, Bensheim, Germany (gift)	Not applicable
BSP CR-3	420 µg/ml	Immundiagnostik AG, Bensheim, Germany (gift)	Not applicable
Crystal violet for microscopy		Sigma-Aldrich, Co., St. Louis, USA	61135-25g
Deionised water		Universitätsmedizin Mainz, Mainz, Germany	
dNTPs, 4you4 dNTP Mix		BIORON GmbH, Römerberg, Germany	110002
Dulbecco's PBS		Sigma-Aldrich, Co., St. Louis, USA	D8537-500 ml
Ethanol	Min. 99.8%	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	K928.5
Ethanol absolute for molecular biology	Min. 99.8%	AppliChem GmbH, Darmstadt, Germany	A3678,0250
Erythrosin B Stain		Logos Biosystems, Villeneuve d'Ascq, France	L13002
FN	Human plasma FN purified protein, 1 mg/ml	Merck Millipore, Temecula, USA	FC010
Formaldehyde	≥37%	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	CP10.1
Formaldehyde, 4.5%	ROTI®Histofix, 4.5%	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	2213.5
GRGDSP	Fibronectin-derived	Sigma-Aldrich, Co., St. Louis, USA	SCP0157-5 mg
Heparin binding peptide (LHRRVKI)	1 mg	BioCat GmbH, Heidelberg, Germany	
HEPES buffer	Gibco™ HEPES, 1 M, pH 7.2-7.5	Life Technologies Limited, Paisley, UK	15630-049
Histopaque®-1077	1.077 g/ml	Sigma-Aldrich, Co., St. Louis, USA	10771-500 ml
huBSP2 (TAL)	2640 mg/ml	Immundiagnostik AG, Bensheim, Germany (gift)	Not applicable
Isopropanol	Min. 99.8%	AppliChem GmbH, Darmstadt, Germany	UN1219
M-MuLV Reverse Transcriptase		New England BioLabs Inc., Ipswich, USA	M0253L
10x M-MuLV Reverse Transcriptase Reaction Buffer		New England BioLabs Inc., Ipswich, USA	B0253S
NaCl for molecular biology	Min. 99.5%	AppliChem GmbH, Darmstadt, Germany	A2942,0500
NaOH pellets for analysis	EMSURE®, 40 g/mol	Merck KGaA, Darmstadt, Germany	1310-73-2
Random primers		Promega, Madison, USA	CC118A
rhBSP11	50 µg/mL	R&D Systems, Inc., Minneapolis, USA	4014-SP-050
rhRANKL	10 ng/µl	BioLegend, San Diego, USA	717502
RNase-free H ₂ O		Qiagen GmbH, Hilden, Germany	1020506
Sequence specific primers		Eurofins Genomics, Ebersberg, Germany	

Soluble collagen type I solution, bovine	5 mg/ml	Viscofan BioEngineering, Weinheim, Germany	500060635
TRizol® Reagent		ambion® by life technologies, Carlsbad, USA	15596026
Trypan blue stain	0.4%	Gibco by Life Technologies, NY, USA	15250-061
Trypsin/EDTA	Trypsin 0.05%/EDTA 0.02% in PBS	PAN-Biotech GmbH, Aidenbach, Germany	P10-023100
VN	0.5 mg/ml	Advanced BioMatrix, Carlsbad, USA	5051-0.1 mg
VN ACF (human recombinant)	0.5 mg/ml	Sartorius, Göttingen, Germany	05-754-0002
β-Mercaptoethanol		Sigma-Aldrich, Co., St. Louis, USA	63689-25 ml-F

Table A 4. Reagents used in experiments.

2.1.5 Cell culture

Product	Manufacturer	Catalogue number
A-549	Kindly provided by Dr. Eva Jüngel (gift)	Not applicable
Buffy coat	Universitätsmedizin Mainz, Mainz, Germany	
DMEM/F-12 (1:1) (1X) + GlutaMAX™	Life Technologies Limited, Paisley, UK	31331-028
DMSO	Sigma-Aldrich, Co., St. Louis, USA	D2650-100 ml
DU-145	Kindly provided by Dr. Eva Jüngel (gift)	
FCS	Biochrom AG, Berlin, Germany	S 0115
Iscove's Basal Medium	Bio&SELL GmbH, Feucht, Germany	BS.FG0465
Iscove's Basal Medium without L-Glutamine	Bio&SELL GmbH, Feucht, Germany	BS.F 0465
L-Alanyl-L-Glutamine, 200 mM	Sigma-Aldrich, Co., St. Louis, USA	G8541
LNcaP	Kindly provided by Dr. Eva Jüngel (gift)	
MCF-10A	ATCC, Manassas, USA	ATCC CRL-10317
MDA-MB-231	DSMZ, Braunschweig, Germany	ACC 732 (RRID:CVCL 0062)
MEBM	Lonza, Köln, Germany	CC-3151
MEGM SingleQuots Kit	Lonza, Köln, Germany	CC-4136
MEM Alpha Medium (1X) + GlutaMAX™-I	Life Technologies Limited, Paisley, UK	32561-029
MEM NEAA 100X	Sigma-Aldrich, Co., St. Louis, USA	M7145-100 ml
MG-63	ATCC, Manassas, USA	ATCC CRL-1427
NCI-H460	ATCC, Manassas, USA	ACC 737
P/S solution, Hybri-MAX™	Sigma-Aldrich, Co., St. Louis, USA	P7539-100 ml
PC-3	Kindly provided by Dr. Eva Jüngel (gift)	
rhM-CSF	PeproTech, Rocky Hill, USA	300-25
RPMI 1640 medium (1X)	Life Technologies Limited, Paisley, UK	11875-093
Sodium pyruvate	Sigma-Aldrich, Co., St. Louis, USA	S8636-100 ml

Table A 5. Cell culture-specific materials.

2.1.6 Kit systems

Product	Specification	Manufacturer	Catalogue number
Calcium Colorimetric Assay Kit		Sigma-Aldrich, St. Louis, USA	MAK022

RNeasy Midi Kit	Qiagen GmbH, Hilden, Germany	75144
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Table A 6. Kit systems used in experiments.

2.1.1.7 Antibodies

Antigen	Antibody type and species	Dilution	Manufacturer	Catalogue number
Anti-integrin $\alpha\beta 3$	Mouse monoclonal IgG1	1:100	Abcam plc, Cambridge, UK	ab78289
Alexa Fluor™ 488 anti-mouse IgG (H+L)	Goat anti- mouse polyclonal IgG (H+L)	1:1000	Invitrogen by Thermo Fisher Scientific, Rockford, USA	A-11001
Hoechst 33342		1:250	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany	14533
Anti- $\alpha\beta 3$, clone LM609	Mouse anti- human monoclonal IgG1	10 $\mu\text{g/ml}$	Millipore, Merck Chemicals GmbH, an affiliate of Merck KGaA, Darmstadt, Germany	MAB1976Z
Anti- $\alpha\beta 5$, clone P1F6	Mouse anti- human monoclonal IgG1	10 $\mu\text{g/ml}$	Millipore, Merck Chemicals GmbH, an affiliate of Merck KGaA, Darmstadt, Germany	MAB1961Z
Isotype control: Anti-mouse IgG1	Mouse IgG1	10 $\mu\text{g/ml}$	Thermo Fisher Scientific, Rockford, USA	02-6100
CD14 MicroBeads, human		20 $\mu\text{l}/10^7$ total cells	Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany	130-050-201
Anti-BSP (IDK-1)	Rat anti-human IgG2A		Immundiagnostik AG, Bensheim, Germany (gift)	Not applicable
Anti-BSP (FP-21)	Mouse anti- human IgG1		Immundiagnostik AG, Bensheim, Germany (gift)	Not applicable
Anti-BSP (YU828-A01)			Immundiagnostik AG, Bensheim, Germany (gift)	Not applicable
Anti-BSP (YU828-H06)			Immundiagnostik AG, Bensheim, Germany (gift)	Not applicable
Anti-BSP (YU829-H06)			Immundiagnostik AG, Bensheim, Germany (gift)	Not applicable
Anti-BSP (YU828-D07)			Immundiagnostik AG, Bensheim, Germany (gift)	Not applicable
Anti-BSP (YU828-A06)			Immundiagnostik AG, Bensheim, Germany (gift)	Not applicable
Anti-BSP (YU831-A05)			Immundiagnostik AG, Bensheim, Germany (gift)	Not applicable
Anti-BSP (YU828-D09)			Immundiagnostik AG, Bensheim, Germany (gift)	Not applicable

Table A 7. Antibodies used in experiments.

2.1.8 Buffers and solutions

Buffer	Composition
CD14 ⁺ isolation buffer	2 mM EDTA, pH 8.0 0.5% BSA in PBS, pH 7.2
Crystal violet (0.5%)	0.5 g Crystal violet 20 ml EtOH 80 ml dH ₂ O
10% formalin, neutral buffer	10 ml 37% formaldehyde solution 0.4 g NaH ₂ PO ₄ (monobasic/anhydrous) 0.65 g Na ₂ HPO ₄ (dibasic/anhydrous) 90 ml dH ₂ O
5 M NaOH	10 g NaOH 50 ml dH ₂ O
0.1 N (0.1 M) NaOH	0.2 g NaOH 50 ml dH ₂ O
33% acetic acid	33 ml 100% acetic acid 67 ml ddH ₂ O

Table A 8. Preparation of buffers and solutions.

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