β1 Integrin Regulates PC3 Prostate Cancer Cell Phenotypes in part via Regulation of Matricellular SPARC

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A thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the MSc degree in Biochemistry

Biochemistry, Microbiology and Immunology

Faculty of Medicine

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ABSTRACT

We have shown herein that β1 integrin stably depleted PC3 sub-clonal cells confer a trend towards increased survival of mice compared to β1 integrin expressing counterparts when tested in an intracardial bone metastasis model. Therefore, we sought to investigate novel factors that mediate β1 integrin-dependent cellular migration and three dimensional growth of prostate cancer PC3 cells *in vitro*. We show herein that depletion of β1 integrin using siRNA directed techniques results in increased SPARC protein expression. We further show that suppression of SPARC by β1 integrin appears to occur through a JNK dependent mechanism. Moreover, siRNA mediated depletion of β1 integrin results in impaired sphere formation in 3D BME assays. This was mediated in part by the increased production of SPARC. β1 integrin-depleted cells also diminished the enhanced migration of cells on the predominant bone matrix, collagen I. Concomitant SPARC depletion in β1 integrin-depleted cells did not rescue this enhanced migration. These findings suggests that the role of β1 integrin in mediating 3D growth of PC3 cells occurs at least in part through the suppression of SPARC protein expression.

ACKNOWLEDGEMENTS

Thank you first and foremost to Dr. Christina Addison for granting me the opportunity to conduct scientific research in her lab in pursuit of a Master's degree. I am grateful for her guidance and insight during this process.

A special thank you to my committee members, Dr. Doug Gray and Dr. Luc Sabourin whose scientific minds have kept me on my toes and whose office doors were always open.
My thanks to the University of Ottawa, the Ottawa Hospital Research Institute and the Ottawa Hospital Cancer Center for providing the education, training and facilities to investigate basic research questions.

A sincere thank you to my lab mates both in Dr. Addison's lab and throughout the 3rd floor cancer center for endless discussions, aid, encouragement, troubleshooting, pondering of new ideas and for making this experience an enjoyable one. To my friend and colleague Roshan Sriram for our many scientific discussions from biology to physics and beyond.

Throughout this degree my parents, three cousins, friends and family have been incredibly supportive both through action and discussion. With their love and support this path was far more direct, predictable and efficient. A special thank you to my girlfriend, Roxanne for her understanding of busy schedules and for her contributions throughout this process. Thanks also to my best friend Eric for always keeping up to date with my progress.

Last but certainly not least, a great thank you to my Oma. Her encouragement and genuine interest in whatever it was I was pursuing has always been a driving force throughout my life. Especially pursuing a master's degree, her sincere intent during an explanation and genuine awe of biology, something we both shared, was inspiring and would ignite me to pursue my passion.

CONTENTS

ABBREVIATIONS

BM	Basement Membrane
BMDC	Bone Marrow-Derived Cells
BME	Basement Membrane Extract
BMEC	Bone Marrow Endothelial Cell
BMP	Bone Morphogenic Protein
BSA	Bovine Serum Albumin
bZIP	Basic Region-Leucine Zipper
CAF	Cancer-Associated Fibroblast
cAMP	Cyclic AMP
CAT	Chloramphenicol Acetyltransferase
CDK	Cyclin-Dependent Kinase
CSC	Cancer Stem Cell
DDR	Discoidin Domain Receptor
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EMT	Epithelial-Mesenchymal Transition
ERK	Extracellular signal-Regulated Kinase
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FGF	Fibrobast Growth Factor
FN	Fibronectin
GPCR	G-Protein Coupled Receptors
GSK3β	Glycogen Synthase Kinase 3 Beta
HSC	Hematopoietic Stem Cells
IHC	Immunohistochemistry
ILK	Integrin-Linked Kinase
JNK	c-jun-N-Terminal Kinase
LLC	Lewis Lung Carcinoma
LOX	Lysyl Oxidase
MMP	Matrix Metalloproteinase
MRI	Magnetic Resonance Imaging
MSC	Mesenchymal Stem Cell
NT2	Non-targeting 2
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-Derived Growth Factor
PDL	Poly-D-Lysine
PMSF	Phenylmethanesulphonylfluoride

PTK	Protein Tyrosine Kinase
rhSPARC	Recombinant Human SPARC
SCLC	Small Cell Lung Carcinoma
SFK	Src Family Kinase
SH2	Src-Homology 2
SMC	Smooth Muscle Cell
siRNA	Small Interfering RNA
SPARC	Secreted Protein Acidic and Rich in Cystein
TGFβ	Transforming Growth Factor β
TIMP-2	Tissue Inhibitor of Metalloproteinase-2
TNC	Tenascin C
TPA	12-*O*-tetradecanoyl-phorbol-13-acetate
TRAMP	Transgenic Adenocarnicoma of Mouse Prostate
VEGF	Vascular Endothelial Growth Factor

TABLE OF FIGURES

1.0 INTRODUCTION

1.1. Preamble

Over a century ago, a surgeon by the name of Stephen Paget recognized the non-random nature of established secondary sites of tumor growth from analyses of breast cancer morbidities. From the records of 735 fatal cases, he noticed 241 cases presented with metastasis to the liver while only 17 cases presented with metastasis in the spleen, 30 in the kidneys and 70 in the lungs. Interestingly, he noted the nature of metastasis "cannot be due to chance." This observation was in part because other explanations of metastasis, such as embolism, did not explain why the spleen, which has an artery much greater than that of the liver, does not even remotely present with the same level of metastasis (1). Furthering the theory of discrete sites of metastasis given a particular cancer type, he reported that when reviewing 20 cases of cancer of the thyroid, at least 10 patients presented with secondary growths in the bone, sometimes the cancer attacking several different bones at once or even becoming greater than the primary tumor. By heavy contrast, in 903 necropsies of cancer of the stomach and pylorus, not a single case could conclusively be described as attaining bone metastasis. He was capable of clearly outlining evidence for a preferred secondary site of metastasis depending on the primary tumor which today is known as the seed and soil hypothesis of cancer metastasis (1). Recent literature reports predominant metastatic sites in cancers such as breast (2), lung (3) and prostate (4) and since the development of the seed and soil hypothesis, a large portion of research has been centered on the dynamic processes involving both the characteristics of tissues and cancer cells to mediate metastasis. Herein is an overview of the current knowledge of how certain cancers metastasize to particular organs with emphasis on the extracellular matrix (ECM), integrins, matricellular proteins

and the development of a metastatic niche. Finally there will be particular focus on what is currently known of prostate cancer metastasis highlighting the role of the β1 integrin subunit and the matricellular protein, secreted protein acidic and rich in cysteine (SPARC).

1.2. Extracellular matrix

1.2.1. Normal ECM composition and function

The extracellular matrix can be divided into two major components: the basement membrane (BM) and interstitial complex. The basement membrane acts to maintain apicobasal polarity and differentiation of cells (5) and is found as a 50-100 nm layer of protein basolateral to all epithelial and endothelial cell layers in the body and is composed primarily of non-fibrillar collagen IV, laminin, nidogen/entactin and perlecan (6). There is heterogeneity in the composition of basement membrane depending on the tissue type, which leads to a unique and specific biological function (6). The heterogeneity of BM between tissues largely depends on the heterogeneity of Collagen IV protomers and laminin subtypes as well as the minor constituents of BM such as agrin, fibulin, various other collagens (type XV and XVIII) and non-structural matricellular proteins (6). Type IV collagen constitutes 50% of the BM and it consists of three α-chains organized into a triple helical structure each with a C-terminal globular non-collagenous domain and an N-terminal 7S domain which are crucial for collagen IV network formation (6). There are six distinct α-chains (α1-6) encoded by six distinct genes, COL4A1, COL4A2, COL4A3, COL4A4, COL4A5 and COL4A6 (7), that combine in sets of three to form the triple helical structure of collagen IV protomers. The triple-helical GFOGER residues mediate binding of integrin α2β1 (8) while the binding of the other major integrin receptor for collagen IV, α1β1 is mediated through Arginine (461) and Aspartic acid (461) on α2 and α1 chains of the collagen IV triple helix, respectively (9).

Laminin is the most abundantly expressed non-collagenous protein in the basement membrane. Eleven genes encode for eleven chains of the laminin family that contribute in different combinations to form 15 distinct laminin subtypes. Moreover, these different laminin subtypes are differentially distributed in BM in a tissue-dependent manner (6) and have a diverse array of integrin heterodimers that constitute the major cellular receptors such as α1β1, α2β1, α3β1, α7β1, α9β1 as well as αvβ3 and α6β4 integrins (10). Collagen IV and laminin self-assemble into networks in the BM whereas the other major constituents, nidogen and perlecan are secreted as single molecules that do not self-assemble but instead bind laminin and type IV collagen to bridge these two networks (6). In addition to providing structural support to endothelial and epithelial monolayers in tissue, the BM exists as a reservoir for growth factors including vascular endothelial growth factor (VEGF), fibroblast growth factors (FGF), transforming growth factor β (TGFβ) -1,2, bone morphogenic proteins (BMPs) and platelet-derived growth factor (PDGF) (11).

The second component of the ECM is the fibrous interstitial matrix which comprises the stroma of tissues, provides structural support, elasticity and mediates cell attachment (12). It is primarily made of proteins such as fibrillar collagens (collagen type I, III, V, VI, VII, XII) (13), elastins and fibronectin (FN). Resident tissue fibroblasts are responsible for the secretion and organization of collagens, elastin, fibronectin as well as proteoglycans in the interstitial ECM (12). The proteoglycans can be further divided into chondroitin sulfate, heparan sulfate and keratin sulfate classes (12, 13) and occupy the majority of the remaining extracellular space in the form of a hydrated gel to aid in hydration, binding of growth factors and force resistance of the tissue (12). Collagen I is the most abundant member of the collagen family in stroma and has a central role as a scaffold and driver of cellular migration

and adhesion through activation of membrane proteinases and signals to define cell shape and function (14). Collagen I is a triple helical protein consisting of two COLIA1 and one COLIA2 α-chains which are encoded by two genes (7). Collagen I in mammalian cells is synthesized as pre-procollagen which undergoes post-translational modifications (hydroxylation of proline residues and glycosylation of lysine residues) and triple helix formation resulting in procollagen before it is secreted in to extracellular space. Once secreted, cleavage of the C- and N- terminal domains of the procollagen triple helix preceeds the self-assembly of collagen into fibrils. The collagen fibrils are strengthened by covalent cross-linking by the fibroblast-secreted enzyme hypoxia-induced lysyl oxidase (LOX) (12).

Within the collagen I fibril, like collagen IV, residues GFOGER that present together in the triple helix mediate integrin binding to this molecule (8), particularly integrins α1β1, α2β1 and α11β1 (15, 16). The integrin heterodimer αvβ3 also interacts with collagen I although this occurs through RGD motifs in the collagen fibril (16). FN on the other hand is secreted as a dimer which upon binding cell-surface receptors is assembled into longer fibrils, which occurs through the exposure of cryptic binding sites in FN that allow FN-fibril assembly (12). FN contains RGD domains that are primarily bound by integrins, particularly integrin α5β1, αvβ1, αvβ3, αvβ5, αvβ6, αIIbβ3 and α8β1 to promote cellular adhesion and spreading (10).

The extracellular matrix has an instrumental role in embryogenesis and development particularly in the context of mediating migration, organization and expansion. A very interesting review written by Song & Ott (17) describes the temporal requirement of ECM deposition for embryogenesis and organogenesis. Of note, laminin is deposited intracellularly as early as the eight-cell stage embryo and assists in migration and adhesion

during gastrulation. Moreover, collagen IV and heparin sulfate glycosaminoglycans appear later and are required for basement membrane construction to aid in forming specialized tissue like secondary epithelium and primary mesenchyme. Lastly, FN guides branching morphogenesis in organ development and is essential for formation of the heart lung and kidney (17). The importance of the ECM has been described in mediating organo-genesis, particularly in the context of tissue engineering using renal organs of the Rhesus Monkey. Decellularization of tissue to provide the ECM scaffold induces $PAX2^{+}/WT1^{+}/Vimentin^{+}$ mesenchymal cells of an explant overlay to migrate into the scaffold and begin developing a new organ recapitulating what occurs during organogenesis in normal development (18). Many studies have highlighted the importance of particular ECM components in development through loss of function or mutations in ECM constituents in both mouse and zebrafish models, resulting most predominantly in death and severe abnormalities of embryos. Miner and colleagues (19) had discovered that germ-line knock out of laminin α5 resulted in death of embryos in late embryogenesis as well as failed neural tube closure and digit septation (19). Later studies found that laminin α5 functions to mediate directed migration of neural crest cells during embryogenesis and thus contributes to normal neural crest development (20). In a zebrafish model of heart tube formation, it was recognized that mutations in the *natter* (*nat*) locus or the cardia bifida locus, which encodes the ECM component FN, resulted in myocardial precursors remaining as two distinct populations separate from the midline of the heart tube whereas in the wildtype zebrafish, two populations of myocardial precursors had fused at the midline. FN was required for maturation of the myocardial epithelium through mediation of adherens junctions clustering which was ultimately critical for migration during development (21).

In addition to a critical role in development, the ECM also contributes to the maintenance of stem cell populations as outlined by Lai et al. (22). Extracellular matrix derived from cultured human bone mononuclear cells contains typical ECM proteins in abundance such as collagen I, III, FN, biglycan, decorin, perlican and laminin. Interestingly, ECM derived from bone marrow mononuclear cells was capable of enhancing colony growth of bone marrow-derived cells as well as specialized colony growth in response to adipocyte and osteoblast differentiation media compared to uncoated, FN or collagen I coated plates. Moreover, the same cultured ECM was able to retain 70-82% of stage-specific Embryonic Antigen-4 (SSEA-4^{+}) cells, representing bone marrow mesenchymal stem cells (MSCs) within the bone marrow cell population, following 14 days in culture compared to a 7-10 fold reduction in these cells when cultured on plastic, FN or collagen I alone. Interestingly, there was also an increased sensitivity to BMP-2-induced expression of osteoblastic markers of these MSC when cultured on bone marrow derived ECM compared to uncoated dishes (22).

The extracellular matrix induces cellular phenotypes such as migration, differentiation and stemness critical for normal embryo- and organo-genesis. Highly structured and tissue specific composition of the ECM maintain normal functioning of cells. It comes as no surprise that aberrant ECM may contribute to the progression of cancer through dysregulation of cellular phenotypes.

1.2.2. ECM in cancer

The extracellular matrix has been shown to mediate tumorigenesis either in the primary or secondary sites of cancer. In the primary tumor , interactions with ECM constituents such as collagen I aid in progressing a subset of cells through epithelial-mesenchymal-transition

(EMT), a process that is coordinated by gene expression changes that occur through signaling events such as the nuclear translocation of β-catenin (23). EMT is a process described as a precursor to tumor cell dissemination and is marked by reduced cell-cell junctions with a decrease in E-cadherin expression and upregulation of N-cadherin conveying a motile phenotype (23). EMT allows detachment from the primary site and invasion through the tissue and is thus very important when considering cancer metastasis. Many ECM proteins are known to aid in this process including hyaluronan, collagen I, periostin and tenascin C (TNC), all of which are capable of inducing the EMT phenotype, as reviewed by Venning et al. (24). At secondary sites, endogenous ECM may aid in cellular attachment and cell cycle regulation to potentiate metastatic disease. Basement membrane components such as laminin and hyaluronic acid serve as ligands for tumor cells to establish in secondary organs. When present in the basement membrane, laminin facilitates the adherence of particular tumor cells such as breast and colon which often express re-distributed and amplified laminin receptors prior to invasion (25). The proteoglycan hyaluronic acid serves as a ligand for CD44 which has been shown to enhance tumor cell binding and metastasis (25). In pulmonary metastasis of the human fibrosarcoma cell line HT1080, exposed patches of basement membrane of pulmonary vasculature allows binding of laminin-5 mediated by the integrin receptor α3β1 which leads to cellular arrest during metastasis, a requirement for tumor metastasis in distant organs (26). In addition to tissue-specific ECM, tumor cells themselves contribute to their own progression by remodeling the ECM (5). Cancer cells may generate their own ECM (27, 28) or secrete matrix metalloproteinases (MMPs) to function in matrix remodeling (5). Following are some

examples of what is known about ECM remodeling in the primary and secondary tumor sites dictated by cancer.

ECM remodeling in primary tumor

The ECM has been shown to change quite drastically in the microenvironment of a tumor compared to the normal stroma. One major difference is enhanced tissue stiffness surrounding a tumor attributed to the increase in deposition (5) and realignment (29) of major constituents of the interstitial matrix such as collagen I (5, 29). In breast cancer, stromal collagen I becomes oriented adjacent to the epithelium or projecting into the tissue with highly linearized and rigid fibrils compared to the relaxed non-oriented collagen I fibrils in normal tissue stroma (29). Thus, mammary tissue and stroma are 5-20 times harder in tumors compared to normal mammary glands (30) and realigned collagen fibers have been shown to aid tumor cells in the process of intravasation into the bloodsteam and invasion through tissues (5). Another hallmark of ECM remodeling in the primary tumor is degradation of the BM (5). Considering BM composition and structure is necessary for the normal apicobasal polarity and differentiation status of cells, disruption of the BM may lead to changes in cellular behavior which can potentiate tumorigenesis (5).

Fibroblasts are important contributors to the constituents of the normal interstitial ECM as they are responsible for the synthesis and secretion of fibrillar collagens type I, III, V as well as FN (31). At tumor sites, fibroblasts are activated displaying enhanced ECM deposition and proliferation. These fibroblasts are considered cancer associated fibroblasts (CAFs) (31) and are abundant in many cancer types such as breast, prostate and pancreatic cancer (32). CAFs contribute to tissue remodeling in cancer by modifying the constituents of the ECM through enhanced deposition of ECM proteins and secretion of ECM degrading

enzymes such as MMPs. As a result, many cancers are associated with the accumulation of FN and fibrillar collagens in addition to the degradation of BM components such as collagen IV (32).

In addition to ECM remodeling by resident CAFs, the primary tumor cells themselves may remodel their microenvironment to enhance survival (5). This remodelling may occur through modulation of local ECM. Small cell lung cancer (SCLC) from human pathology files were analyzed by Sethi et al. (27) for the ECM constituents, FN, laminin, collagen IV and TNC. Unsurprisingly, laminin and collagen IV staining was localized to basement membranes and FN was diffusely stained throughout the stroma with no expression of TNC in normal lung tissue. By contrast, 56% of tumors showed positive staining for FN, 87% for collagen IV and 61% for TNC in areas that covered >50% of the section area in each case. Of particular interest, 26% and 16% of tumor cells were stained intracellularly for FN and laminin, respectively. Also of interest, matching lymph node metastases showed FN, collagen IV, and TNC immunoreactivity at levels that were as great as the primary tumor. The majority of staining is likely due to CAFs; however, the intracellular staining of FN and laminin suggests tumor cells as a source of this matrix as well (27).

Pancreatic cancer stroma expresses high levels of collagen IV and circulating collagen IV levels associate with poor prognosis (33). The pancreatic cancer cells themselves were capable of producing this ECM protein *in vitro* suggesting the protein was potentially derived from tumor cells themselves *in vivo* (33). The authors followed up on this paper in 2013 and were able to show that collagen IV is produced by the tumor cell compartment in pancreatic tumor tissue and further *in vitro* characterization by use of siRNA proved that cell-derived collagen IV is capable of enhancing migration, inhibiting apoptosis and

promoting growth in pancreatic cancer cells primarily through interactions with integrins (28).

Another group, Sherman-Baust et al. (34) had determined that collagen VI which is expressed by ovarian cancer cell lines *in vitro* and is absent in the stromal compartment of ovarian cancers but present in the tumor cell compartment *in vivo* conveys cisplatin resistance (34). Therefore, these recent studies have shown that tumor cells produce their own ECM to generate a favorable tumor microenvironment conveying enhanced growth, migration and survival in multiple cancer types.

ECM remodeling in the primary tissue may also occur due to secretion of proteases such as MMPs that cleave ECM proteins mediating an invasive phenotype. For example, hyaluronan, has been shown to activate MMP-2 secretion and mediate enhanced invasiveness in the human SCLC cell line, QG90, (35). The ovarian cancer cell line, NOM1 (mucinous adenocarcinoma) secretes MMP-9 which acts to degrade collagen IV in response to FN engagement of integrins (36). Immunohistochemistry (IHC) from human patients with breast cancer showed that MMP-13 is expressed in breast cancer cells as well as surrounding stromal cells (37). MMP-13, also known as collagenase-3 is known to degrade extracellular collagen, particularly collagen I, II and III and is associated with more aggressive cancer phenotypes (37, 38). Lastly, in human prostate cancer samples, of 187 patients biopsies analyzed by IHC, 75.9% of the cases showed MMP-2 expression in the stroma and 70% of cases displayed MMP-2 expression in the cancer cells themselves (39).

Metastatic niche

"… the ECM composition of metastases to liver resembles more the ECM of primary colorectal tumors than that of normal liver (40)."

ECM remodeling is important in the primary tumor to initiate dissemination, but it is also instrumental in secondary sites of metastasis to establish growth of disseminated cancer cells. The above quote resonates with the notion that the ECM is a primary driver of tumor metastasis. Thus, understanding the ECM remodeling that occurs in secondary tissues is necessary to unveil potential therapeutic approaches for treatment of metastatic disease.

Although the normal ECM in distant metastatic sites contributes to facilitating the establishment of tumors, recent literature describes a change in the extracellular matrix in secondary regions either preceding or occurring after the infiltration of tumor cells as the major driver of site-directed metastasis. Naba et al. (40), by means of a proteomic screen, determined that ECM components such as collagens and an array of glycoproteins that differed between normal and metastatic liver were the same between primary colon tumors and their corresponding liver metastasis. This study captured how the endogenous ECM of the secondary site may change to more closely resemble that of the primary tumor. Also, it provides evidence of the active remodeling of a niche to provide a favorable microenvironment for metastasis (40).

The importance of FN in mediating metastasis has been highlighted by Kaplan et al. (41). FN is normally expressed in the lung as detected by quantitative RT-PCR. This group; however, was able to show that after the intradermal implantation of lewis lung carcinoma (LLC) cells, which metastasize to lungs and liver, there was a marked increase in FN expression in the metastatic niche of lung tissue preceding the infiltration of tumor cells. In these mice, tumor cell metastasis was less frequent when mice were injected with antibodies against the alpha-4 integrin subunit of the FN receptor α4β1 integrin (VLA-4) compared to mice treated with an IgG control illuminating the importance of this matrix and its receptor

in mediating the binding and establishment of these cells (41). Interestingly, there was an upregulation of FN in multiple tissue types such as lungs, intestines and oviduct when B16 melanoma conditioned media was injected into mice compared to LLC-conditioned media or media alone and this is consistent with the more diverse metastatic nature of melanoma (41). As an example of the importance of tumor-influenced FN deposition in dictating site of metastasis, melanoma conditioned media injected to mice was capable of redirecting the site of metastasis for implanted LLC cells towards the pattern normally expressed by melanoma which included the kidney, spleen, intestine and oviduct (41).

In another example of how tumor influence on the ECM impacts the pre-metastatic niche, Erler et al. (42) have suggested an interplay between factors secreted by primary tumor and the effect on ECM components of distant, non-malignant tissue to establish a microenvironment fitting for the growth of disseminated tumor cells. This study had assessed the capacity of breast cancer cells to prime the lungs for impending colonization. The MDA-MB-231 breast cancer cell line is known to secrete LOX. Collagen IV already present in the lungs undergoes cross-linking as a consequence of LOX secreted by tumor cells in the primary site (42). Enhanced rigidity of collagen IV increased the pulmonary accumulation of CD11b+ myeloid cells. Interestingly, biopsies of human lung metastasis show a co-localization of LOX with CD11b+ bone marrow derived cells (BMDCs). In mice injected with shLOX treated cancer cells, CD11b+ BMDCs and tumor cells did not infiltrate the lungs compared to wildtype tumor mice. Moreover, in mice inoculated with shLOX cells supplemented with LOX via injection, this phenotype was rescued and BMDCs and cancer cell infiltration back to wildtype levels. Interestingly when shLOX cancer bearing mice were supplemented with LOX in the presence of clodronate – a drug used to ablate myeloid cells,

a significant reduction in breast cancer lung metastasis was evident alongside decreasing numbers of myeloid cells in the lungs. Lung metastasis in this model was explained whereby LOX-dependentincreased rigidity of collagen IV enhances BMDC infiltration and subsequent release of MMP-2; thus, establishing a pre-metastatic niche wherein MMP-2 activity facilitates the invasion of metastatic breast cancer cells and binding to rigid collagen IV enhances their growth (42).

Burnier et al. (43) have demonstrated a role of acquired collagen IV expression in M-27 murine lung carcinoma cells to mediate liver metastasis. Overexpression of collagen IV α1 and α2 chain enhanced liver metastasis of these cells via rescue from anoikis. On the other hand, siRNA against collagen IV α1 chain reduced the ability of injected M-27 cells to form liver metastasis via enhanced anoikis. Very interestingly, 65 human liver metastatic tissue samples from an array of different primary tumor types all showed high levels of collagen IV staining, while primary tumors show very little to no collagen IV expression (43). Using a primary melanoma tumor sample and samples from bone, skin, liver and lung metastatic melanoma, only the liver metastasis sample showed extremely high levels of collagen IV staining. Finally, in colorectal cancer liver metastasis, normal liver tissue adjacent to the tumor does not express collagen IV; however, the stroma surrounding the colorectal metastasis and the cancerous cells themselves do express collagen IV. This paper demonstrates an intriguing example whereby, tumor-associated ECM that is different from endogenous ECM is a major factor in maintaining survival and growth of metastatic cancer cells in the liver (43).

1.3. Matricellular proteins in Cancer

Although ECM constituents such as collagens and FN receive most of the attention when describing the role of ECM in tumorigenesis, matricellular proteins that do not primarily serve a structural role but instead modulate cell-cell and cell-ECM interactions are also important to consider in the context of cancer progression.

Matricellular proteins are secreted into the ECM space but do not serve a primary structural role. Instead, these proteins modulate cell-ECM interactions, cell-surface receptor interactions and protease activity (44). One way which these proteins serve a functional role in normal tissue is evident in hematopoetic stem cell (HSC) regeneration (45). For example, in an adult mouse, the ECM of the bone marrow primarily consists of typical collagens I/IV, FN and the less typical matricellular protein TNC. TNC has been shown to be a major contributor in the re-establishment of HSC populations marked by an increase in TNC expression and increased detection of $Lin^{-}Sca1^{+}c\text{-}Kit^{+}$ (LSK) stem cells following myeloablation in wildtype mice compared to a significant reduction in repopulated LSK cells in $TNC^{-/-}$ mice (45).

In cancer, matricellular proteins have recently been shown to drive particular oncogenic pathways (46), regulate growth and migration of cancer cells (47, 48) as well as to serve as a factor in the maintenance of cancer stem cells (CSCs) (49), all important factors for metastasis. As described in the previous section, it is well understood that tumor cells modulate their microenvironment to provide a suitable niche for growth and survival. Recent literature has also expanded this idea to the role of matricellular proteins in establishing a favorable microenvironment for the growth and survival of cancer.

One such example exists in the cellular modulation of the matricellular protein, SPARC, in ovarian cancer (50). SPARC is expressed strongly in the surface epithelial cells of normal

ovaries and strong immunoreactivity is also observed in benign ovarian tumors (50). However, a reduction of SPARC staining is observed in the cytoplasm of borderline and invasive serous carcinoma cells. In addition, grade I, II and III invasive carcinoma tissue showed overall reduced SPARC staining with only the occasional positive epithelial cell (50). Ovarian cancer cell lines also show substantial decrease in SPARC expression and secretion by western blot compared to normal ovarian cell lines. Interestingly, SPARC was shown to induce apoptosis solely in SKOV3 ovarian cancer cells but not in the normal ovarian epithelial cell line HOSE (50). This is direct evidence for transformed ovarian cells contributing a favourable microenvironment for growth and survival through suppression of SPARC which in this case acts as an inducer of apoptosis.

Oskarsson et al. (48) have recently shown that the matricellular protein TNC is associated with shorter lung-metastasis free survival in breast cancer patients and that TNC staining in lung tissue was evident in the invasive front of the cancer. Interestingly in a mouse model of lung-metastatic breast cancer, TNC was only expressed by micrometastatic breast cancer cells at day 10 but produced by both stromal and cancer cells by day 36 post-injection (48). TNC knockdown prior to injection of two lung-metastatic cell lines, MDA231-LM2 and CN34-LM1 significantly reduced (90% reduction) their lung colonization *in vivo.* Moreover, TNC knockdown inhibited migration of these cells *in vitro* and enhanced the apoptosis marker, cleaved caspase 3 *in vivo.* Doxycycline-induced knockdown of TNC in the breast cancer cells specifically administered at day 2 and day 6 was capable of inhibiting the outgrowth of metastasis whereas treatment with doxycycline on day 21 had no effect on metastatic load (48). This shows that initially, breast cancer cells express their own TNC which inhibits apoptosis and promotes invasion and at later time

points when metastatic nodules have become large, TNC becomes less relevant as loss of TNC does not affect increases in metastatic load (48).

Finally, in an example whereby matricellular proteins contribute to CSCs, periostin, was found to be necessary for lung metastasis in MMTV-PyMT transgenic mice. It has been shown in this model that the tumor cells at the metastatic site induce expression of periostin by the stromal cells which in turn potentiate wnt signalling to promote cancer stem cell expansion (49).

It is clear that tumor cells themselves establish a favorable microenvironment at least in part by generating their own matrix with the presence of new pro-tumoral proteins or the absence of anti-tumoral proteins to promote growth, survival and migration (Figure 1). Taken together, basement membrane, interstitial, and matricellular components contribute significantly to cancer progression. Tumor cells utilize the ECM by dysregulating tissue homeostasis in a manner that establishes a favorable microenvironment rich in ECM that promotes malignancy and is devoid of ECM that impinges on progression. Although establishing a favorable microenvironment is critical, an understanding of tumor growth and metastasis in response to ECM is incomplete without considering the key mediators of ECM induced phenotypes, the heterogeneic family of integrin receptors.

1.4. Integrins

Integrin receptors function to link the extracellular matrix to the cytoskeleton. In humans there are 18 α and 8 β subunits combining together to form 24 distinct integrin heterodimers with unique ligand and signaling properties (51). Integrin clustering via interactions with the ECM leads to formation of focal adhesions (52). These focal adhesions are characterized by the recruitment of integrin-associated proteins that mediate downstream signaling (52). Focal

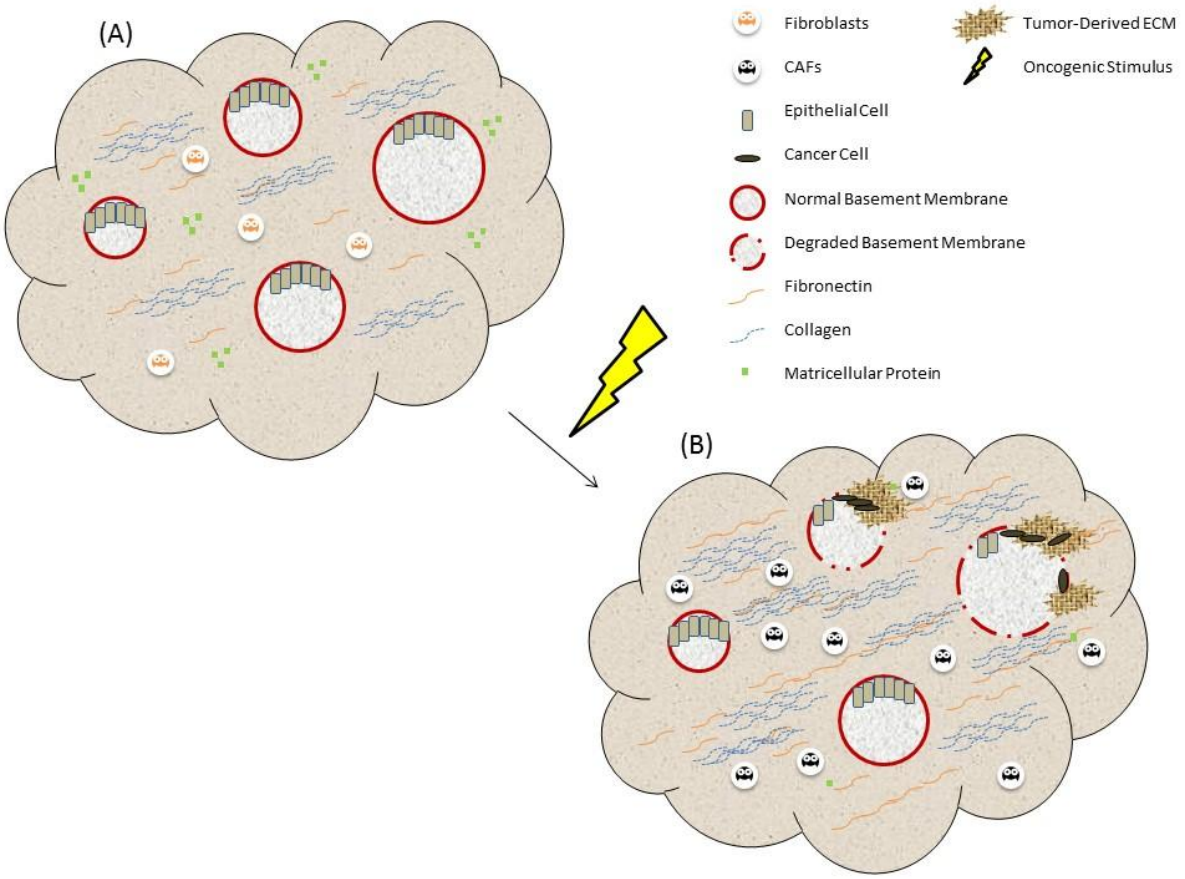

(A)
(B)
Fibroblasts
CAFs
Epithelial Cell
Cancer Cell
Normal Basement Membrane
Degraded Basement Membrane
Fibronectin
Collagen
Matricellular Protein
Tumor-Derived ECM
Oncogenic Stimulus

Figure 1. Changes in the ECM during tumorigenesis.
Changes in the ECM surrounding cancer cells established by both tumor cells themselves and the influence of CAFs. (A) Normal stroma and epithelial cells in glandular tissue consisting of fibronectin, collagen fibrils and intact basement membrane. (B) Changes in the ECM in response to tumor involving degraded basement membrane, laying of new matrix by cancer cells, enhanced fibronectin and collagen deposition by CAFs and suppression of particular matricellular proteins in the microenvironment.

adhesions are enormously complex consisting of many different signaling and adaptor proteins (53). Given the complexity of focal adhesions and the vast heterogeneity of integrins and their ligands, activation of integrins mediates a diverse range of cellular phenotypes. For instance, integrins are known to mediate epithelial cell polarity through engagement of basement membrane ECM in normal tissue (54). Moreover, activation of integrins influences the regulation of ECM constituents (55), cell shape (56), cell survival (43), as well as proliferation and migration (57).

Structure and Ligands

Both the α and β subunits of integrins contain a relatively complex extracellular domain with a single helix transmembrane domain and a short intracellular cytoplasmic tail. The extracellular domain between α subunits differ slightly in one aspect, nine out of eighteen of these subunits contain an extra α-I domain, which exists the utmost distal to the membrane and mediates ligand binding for these integrins. Otherwise the α subunits from membrane proximal to distal contain two calf domains (calf1-2), a thigh domain and a seven blade β-propeller, within which the extra α-I domain resides between propellers 2 and 3. The α subunit likely retains flexibility in the extracellular domain via flexible linkers between the β-propeller and thigh domain as well as between the thigh and calf-1 domain. The extracellular region of the β subunit contains seven distinct domains. From proximal of the membrane towards the extracellular space there are the β-tail domain, which confers flexibility at the "ankle" of the subunit, four cysteine-rich epidermal growth factor (EGF) modules (E4-1) connected to a PSI domain, and a hybrid domain. The α-*I*-homologous, β-*I* domain, most distal to the membrane inserts into the hybrid domain and is responsible for extrinsic ligand binding. The crystalized structures of αIIbβ3 integrin transmembrane

domains show the existence of a perpendicular helix with respect to the membrane in the α subunit whereas the helix corresponding to the β subunit is slightly tilted, although an interface between the two tails exists at a resting state mediated by electrostatic interactions. The cytoplasmic tails of each subunit are short and appear to be flexible and not in any distinct conformation (58).

Different integrin heterodimers confer different ligand specificities. For instance, all integrins containing αV as well as integrins α5, α8/β1 and αIIbβ3 bind ligands that consist of an RGD tripeptide site. This RGD site binds at an interface between the α subunit β propeller and the β subunit β-*I* domain. Another active site, LDV which is functionally related to RGD and present in the ECM protein, fibronectin, is bound by integrin heterodimers, α4β1, α4β7, α9β1, all four members of the β2 subunit integrins and integrin αEβ7. Important differences are noted for distinct binding to collagen and laminin ligands. For instance, integrins expressing the unique α-*I* domain, α1, 2, 10 and 11 combine with β1 integrin to bind the collagenous motif GFOGER (16). β1 combines with α3, 6 and 7 confer selectivity for laminin receptors (58).

Activation and downstream signaling

Integrins express bi-directional signaling, namely "inside-out" and "outside-in" signaling. Inside-out signaling results from other signaling pathways such as G protein-coupled receptors (GPCRs) that affect the activation state of integrins mediated by intracellular signals (59). Out-side in signaling involves binding of integrins to extracellular ligands leading to the propagation of an intracellular signal that results in cellular responses such as growth, survival and migration (60).

Talin is an important molecule in mediating "inside-out" integrin activation. As modeled by integrin αIIbβ3, in an inactive state, the transmembrane domains of the α and β subunits exist in association due to a salt bridge in the cytoplasmic tails of αIIb R995 and β3 D723. Upon talin binding to the membrane-proximal NPxY motif on β3 cytoplasmic tail, residue K324 competes for electrostatic association on the β subunit thus disrupting the salt bridge between the α and β subunits physically separating their association with one another leading to an active state (61). Another important protein in mediating inside-out signaling is kindlin, a protein with a FERM domain with sequence homology to that of talin although it binds a distinct NxxY motif in the membrane distal portion of the β cytoplasmic tail and functions to facilitate talin activity (62).

Following ligand binding (outside-in signaling), a number of signaling cascades result from the recruitment of intracellular protein-tyrosine kinases (PTKs) such as focal adhesion kinase (FAK), integrin-linked kinase (ILK) and Src family kinases (SFK) (57). The subsequent signaling cascades promote phenotypes such as growth, survival and migration. FAK is recruited to activated integrins through interaction with proteins such as talin or paxillin (63) associated with the cytoplasmic tails of the β integrin subunit. Once recruited, FAK auto-phosphorylates at Y397 and creates a recruitment site for Src-homology 2 (SH2) domains of SRC family kinases. Following binding, Src further phosphorylates FAK at residues Y576 and Y577 within the activation loop and residues Y861 and Y925 within the C-terminal domain (64). FAK association with Src and p130cas in association with Crk downstream of integrin signaling has been shown to mediate activation of c-Jun NH2-terminal kinase (JNK) through upstream kinases such as MKK4/7 (65) thereby promoting progression through the G1 phase of the cell cycle (57). Similarly FAK has been shown to

mediate downstream extracellular signal-regulated kinase (ERK) activation (66). ILK, another kinase functioning as an immediate effector molecule downstream of integrin engagement is also responsible for actin rearrangement, cell polarization, migration, proliferation and survival (67). ILK contains three domains, which from N- to C- terminal include five ankyrin repeats, a pleckstrin homology-like domain, and finally a kinase-like domain although recent evidence suggests ILK may function more as a scaffold than a kinase (67). ILK interacts directly with integrin cytoplasmic tails and its recruitment is dependent on kindlin, α-parvin or paxillin. In cancer, ILK has been reported to promote anchorage-independent growth, proliferation and migration through influencing many known oncogenic proteins including protein kinase B (aka AKT) as well as glycogen synthase kinase-3 beta (GSK3β) which leads to enhanced stability of β-catenin (67). The structure of integrins and some of the key mediators of downstream signaling are outlined in Figure 2.

Responding to the environment and regulation of matricellular proteins

It is rare to come across a study involving the extracellular matrix without the mention of integrins. Direct evidence of integrins mediating cellular phenotypes in cancer induced by ECM proteins is presented from studies already mentioned in previous sections. In the study by Ohlund et al. (28) wherein collagen IV secretion by pancreatic cells mediated enhanced growth, blocking of integrin receptors was capable of inhibiting this phenotype. Growth inhibition in cells depleted for collagen IV expression was rescued when plated on collagen IV; however, the same experiment in the presence of a β1 integrin blocking antibody did not rescue growth, showing the requirement for β1 integrin binding in the collagen IV feedback loop for pancreatic cancer growth (28). In the study by Burnier et al. (43) it was shown that

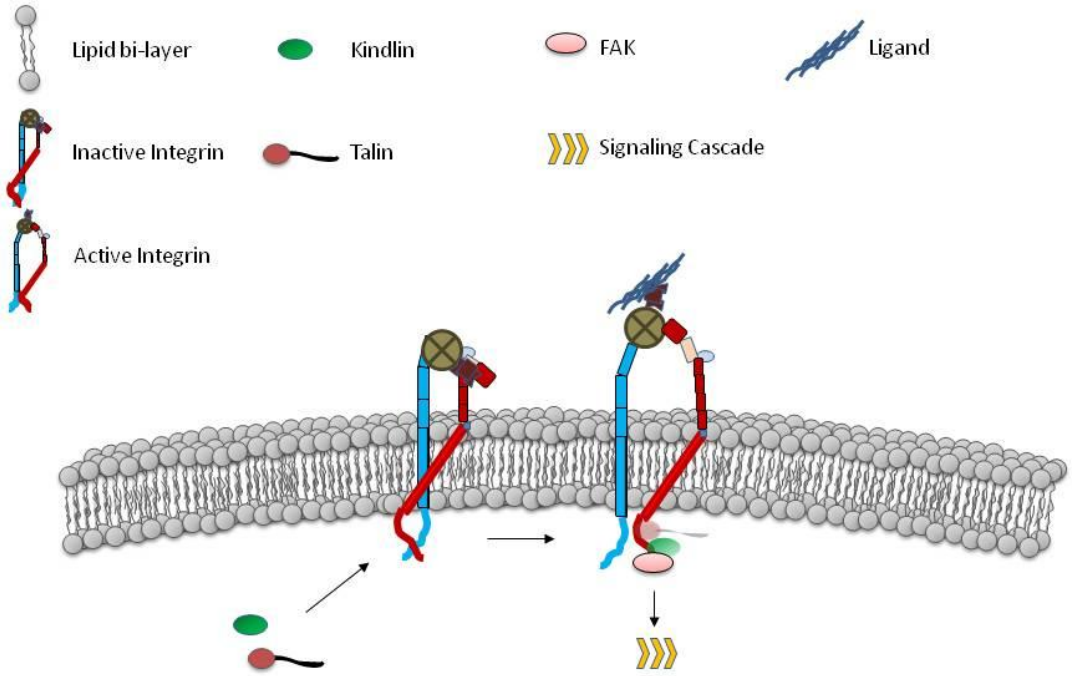

Lipid bi-layer
Kindlin
FAK
Ligand
Inactive Integrin
Talin
Signaling Cascade
Active Integrin

Figure 2. The switch-blade model of integrin signaling.
Integrin signaling displaying the “switch-blade” model with focus on key regulators such as kindlin, talin and FAK.

α2 integrin-FAK signaling was responsible for collagen IV conveying a growth advantage in the liver and a subsequent increase in anoikis and decrease in liver metastasis was observed when FAK was depleted using siRNA (43). Moreover, the metastasis that occurred as a result of a developed metastatic niche characterized by an increase in FN was abrogated by treatment with an antibody to the FN receptor, integrin α4β1 (41). Thus, integrins are direct mediators of ECM-induced growth, metastasis and survival in cancer.

Integrins are central in mediating the effects of ECM in cancer. Interestingly integrins also regulate the expression of ECM proteins that ultimately contribute to the tumor stroma or metastatic niche. Expression of the β1(C) splice variant which is normally downregulated in prostate cancer has been shown to increase levels of the matricellular protein, thrombospondin 1 (TSP1), which acts as an angiogenesis inhibitor (68). In breast cancer cells, integrin β4 was shown to enhance the expression of SPARC protein to promote invasion (69). Moreover in immortalized keratinocytes, α3β1 integrin was shown to positively regulate the ECM proteins fibulin-2 and SPARC, and negatively regulate thrombospondin-2 (70). Thus, in addition to engaging the ECM to modulate cellular phenotypes, integrins also play a role in modulating the ECM through either positively or negatively regulating its constituents.

Integrin-JNK mediated phenotypes

JNK is an important mediator of integrin signaling which occurs downstream of FAK activation (57). Many cellular responses downstream of integrin activation are mediated through integrin-JNK signaling (57, 71, 72). The effects elicited by JNK occur through phosphorylation of the transcription factor c-jun (57) which by induction of its activity dimerizes with other basic region-leucine zipper (bZIP) proteins belonging to the Jun, Fos or

ATF families (73). These dimers form the AP-1 transcription factor that recognizes 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) or cyclic AMP (cAMP) DNA response elements and regulates genes important for proliferation, (57) survival and migration (73). Interestingly, c-jun has also been shown to regulate the expression of matricellular proteins (74), another phenotype elicited by integrins (68-70) that is important in establishing a pro-tumoral microenvironment.

1.5. Prostate cancer bone metastasis: β1 integrin and SPARC

Upon autopsy, up to 90% of patients who pass from prostate cancer show bone metastasis (4). Unfortunately, bone metastasis is an incurable phenotype associated with skeletal fracture, spinal cord compression, intense pain, hypercalcemia and impaired mobility. The treatment of these patients is primarily palliative with the use of drugs or radiotherapy for pain, and the use of bisphosphonates to impede osteoclast activity to effectively reduce bone degradation (75). Thus furthering our understanding of the mechanisms behind prostate tumor cell establishment in this site is pressing.

Integrin β1 is known to play an instrumental role in the progression of prostate cancer and has been shown to play a role in bone metastasis. Firstly, β1 integrin is more highly expressed in poorly differentiated tumors(76). β1 integrin forms a heterodimer with α2 integrin to grant ligand specificity to collagen I, the most abundantly expressed protein in the bone (77). The α2β1 heterodimer is therefore strongly associated with site directed metastasis showing upregulation in bone lesions compared to other soft tissue metastasis in humans and α2β1 integrin has been attributed to the formation of tumors following intratibial injections in an *in vivo* mouse model (78, 79). Furthering β1 integrin as a major player in bone metastasis of prostate cancer, it has also been shown to increase migration of

prostate cancer cells towards collagen I *in vitro* (79). In aggressive prostate cancer cell lines, namely PC3-mm2, inhibition of β1 integrin using the neutralizing antibody mAb 33B6 inhibited the phosphorylation of FAK and AKT leading to a 3-fold increase in apoptosis and concurrently mAb 33B6 inhibited migration of these cells on both collagen and fibronectin substrates (80). Systemic delivery of mAb 33B6 *in vivo* substantially reduced tumor burden in femurs following intracardiac injections (80). Previous work by Schooley et al. (81) has shown further that β1 integrin mediates sphere formation of prostate cancer cells in soft agarose through a mechanism that involves ligation to FN (81). Moreover, it was also shown that loss of β1 integrin resulted in loss of invasion through basement membrane extract (BME) which was attributed to loss of MMP9 expression and increases in tissue inhibitor of metalloproteinase-2 (TIMP-2) (81).

SPARC, also known as Osteonectin, is the most abundant non-collagenous protein in the bone and is otherwise restricted to areas of consistent turnover or injury (82, 83). It has been suggested to contribute to the organization of matrix in connective tissue and basement membrane considering it has the capacity to bind several extracellular proteins including thrombospondin 1, vitronectin, entactin/nidogen, fibrillar (types I, II, III and V) and non-fibrillar (type IV) collagens (83). SPARC exhibits counter-adhesive effects as indicated by the induction of cell rounding and disruption of cell-matrix interactions; moreover, SPARC-null cells may proliferate at an accelerated rate compared to SPARC-wildtype counterparts (84). In cancer however, the effects of SPARC are context dependent with endogenous expression of SPARC eliciting inhibition of 3D growth that is not recapitulated following exogenous treatment with recombinant SPARC (85).

Interestingly, the effects of SPARC in cancer remain controversial. Although some reports suggest a pro-tumoral role in breast cancer (69, 86), SPARC has also received a lot of attention as an inhibitory protein in breast cancer (87),(88) as well as multiple other cancer types including ovarian cancer (50) and pancreatic cancer (89). Some attention however, has been attributed to its modulation of prostate tumor growth and metastasis specifically. In a transgenic adenocarcinoma of mouse prostate (TRAMP) model (T^+) of prostate cancer in mice either wildtype ($SP^{+/+}$) or knocked out ($SP^{-/-}$) for SPARC it was reported that primary tumors developed in each mouse but elicited a worsened tumor burden in $T^+SP^{-/-}$ mice. Tumor burden was defined as the differentiation status of the tumor (i.e. well differentiated versus poorly differentiated) using histological analysis. Moreover, metastasis occurred in 4/12 mice in the SPARC null group with secondary sites including the lymph nodes, lungs and liver displaying metastatic lesions. Only 1/10 mice in the wildtype group presented with metastasis although this single mouse showed metastasis in all of the aforementioned organs. Interestingly, more poorly differentiated areas of the primary tumor and metastatic tumors in the $SP^{+/+}$ mouse showed a progressive decrease in SPARC levels relative to normal prostatic tissue (47). This is consistent with reports from human prostate tumor samples wherein SPARC expression levels are elevated in adjacent normal tissue but decrease in tumors with a higher Gleason score (90). Thus it appears SPARC may function as an inhibitory protein in prostate cancer and *in vivo* data coincides with human data suggesting a loss of SPARC protein in poorly differentiated prostate tumors.

Aside from the well-known fact that SPARC expression is increased in response to TGFβ (91), very little is known about the mechanisms by which this protein is regulated. There are few publications describing signaling pathways that modulate SPARC expression;

however, the role of viral-jun (v-jun) in mediating tumorigenesis in chick embryonic fibroblasts with suppression of SPARC as tumorigenic readout (92) and a potentially indirect role for c-jun in rat embryonic fibroblasts to mediate suppression of SPARC through a putative secreted factor has been investigated (74). The findings that c-jun may regulate SPARC expression is intriguing considering the known role of c-jun as a transcription factor downstream of JNK signaling that occurs via activation of integrins (57).

1.6. Summary, Rationale and Hypothesis

Prostate cancers are found to metastasize to the bone in up to 90% of patients upon autopsy (4). Site-directed metastasis is directly influenced by the metastatic niche (41). One of the main contributing factors that defines the metastatic niche is the ECM which either lacking in anti-tumoral proteins or rich in pro-tumorigenic proteins defines a microenvironment fitting for the growth and survival of cancer (28, 33, 34, 41, 43, 48-50). Integrins have been shown to not only mediate the response to ECM (28, 41, 43) but to also regulate ECM proteins known to contribute to tumorigenesis (68-70). β1 integrin is overexpressed in prostate cancer and is the primary integrin receptor for collagen I (76, 79), the predominant ECM constituent of bone (77). Previous work in the literature as well as our lab designates a role for β1 integrin in promoting growth, migration, invasion (81) as well as bone colonization (80) of prostate cancer cells. However, the mechanisms by which these processes occur are not well understood. The matricellular protein SPARC is shown to be downregulated in advanced prostate tumors (90) and elicits anti-proliferative, anti-migratory effects in prostate cancer (47). Considering a putative link between SPARC suppression and integrin signaling via JNK (74) ***we hypothesize that activation of β1 integrin by collagen I***

mediates prostate cancer growth and metastasis to the bone through suppression of matricellular SPARC.

1.8. Approach

Objectives:

1) Assess incidence of bone colonization in an intracardiac injection mouse model of human prostate cancer using cells either expressing or depleted of β1 integrin.
2) Investigate the relationship between β1 integrin and SPARC in the context of collagen I.
3) Confirm role for β1 integrin in 3D growth and migration and assess a role for SPARC in these β1 integrin-mediated phenotypes.

2. MATERIALS AND METHODS

2.1 Cell culture

PC3 human prostate carcinoma cells are derived from a bone metastasis of a grade IV adenocarcinoma (ATCC CRL-1435) (Manassas, VA, USA) and were maintained in high glucose Dulbecco's modified eagle medium (DMEM) (Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS) (Seradigm, Radnor, PA, USA). Cells were grown in a humidified incubator at 37°C, and 5% CO_2. Cells were passaged every three days for maintenance by first washing twice with phosphate buffered saline (PBS) (Corning, Manassas, VA, USA) and incubating at 37°C for 5 minutes with 1x citric saline to detach cells which were subsequently reseeded at 1/3 the previous cell density into fresh DMEM + 10% FBS. PC3 subclones stably depleted of β1 integrin were constructed previously in the lab (81). Briefly, a HuSH 29-mer shRNA plasmid against β1 integrin: GAGGATATTACTCAGATCCAACCACAGCA in a pRS plasmid backbone and an empty pRS vector plasmid were obtained from Origene (Rockville, MD, USA). β1 integrin depleted Sh 2-10 and vector control RSC5 cells were generated whereby PC3 cells were transfected at 90% confluency with each plasmid using Lipofectamine (Invitrogen, Carlsbadm, CA, USA) and selected using puromycin (Sigma, Oakville, ON, CND) followed by expansion of resistant cell colonies. Following analysis of β1 integrin status, multiple clones from each group were selected for further study (81). Sh 2-10 and RSC5 cells were maintained and grown in the same conditions as the parental cell line, PC3, as mentioned above.

2.2 Intracardiac model of bone colonization using CD1 nude mice

Thirty-two male CD1 nude mice (Charles River Laboratories, Wilmington, MA) were obtained and housed in specific pathogen free conditions. These mice were split equally into two groups of 16 to be injected with β1 expressing and depleted cells, RSC5 and Sh 2-10, respectively. One hour prior to injections mice were treated with buprenorphine for pain. While under anesthesia, each mouse received $1.0x10^6$ cells/100µl in sterile PBS of either RSC5 or Sh 2-10 cells depending to which group the mouse belonged. To prepare cells for injections, cells were lifted from culture dishes using citric saline as previously described and resuspended in PBS at the appropriate concentration for subsequent injection and then kept on ice. These cells were loaded into a sterile, UV treated and ethanol washed, 1ml syringe that was modified to connect to a piece of PE-10 tubing (Instech, Plymouth Meeting, PA, USA) at the end of which was inserted a 30G1/2 needle head. The injections were conducted by inserting the needle into the left ventricle of the mouse heart by orienting the mouse such that the anterior side was up and the needle was to the right of the sternum directly underneath and on the same plane as the elbow. Left ventricle insertion was characterized by the appearance of bright red blood in the PE-10 tubing which is distinct from darker red blood characteristic of accidental insertion into the right ventricle (93). For each injection, cells were mixed with the blood by administering and retracting the blood-cell mixture a few times during the entire 100µl delivery. One mouse in the RSC5 group died immediately following injections. Four other mice were removed from this group as they displayed lung colonization at endpoint, a phenotype characteristic of spillover into the chest cavity during injections. Moreover, two mice were removed from the Sh 2-10 group as they too displayed lung colonization at endpoint. All other mice were monitored regularly

and were either collected at time of death or sacrificed by the use of CO_2 due to observable endpoints suggested by the *Canadian Council on Animal Care* such as measurable clinical signs (change in heart rate or respiratory rate), changes in unprovoked behavior, weight loss exceeding 20% of normal body weight and tumor mass causing pain or distress due to its location. Deceased animals were collected for magnetic resonance imaging (MRI) or dissected for limb bones, spine, lungs and liver. Tissue was maintained in 50ml falcon tubes containing 10% formalin (Sigma, St. Louis, MO, USA) for at least one week, following which only the limb bone/spines were moved into 10% EDTA, pH 7.4 that was changed once a week for several weeks for decalcification. Once decalcified, the bones were subjected to paraffin embedding for future analysis. MRI was performed post-mortem by Gregory Cron at the University of Ottawa pre-clinical imaging core using a 7 Tesla GE/Agilent MR 901. Common parameters for all imaging sequences were: axial (transverse) slice orientation, slice thickness = 350 µm, in-plane field of view = 3.5 cm, matrix size = 256 x 256 x 74, voxel spatial resolution = 137 x 137 x 350 µm3. Each mouse was imaged with three fast spin echo sequences and one gradient echo sequence (Table 1). The total imaging time per mouse was approximately 1.5 hours.

2.3 Matrix coating

PureCol® Bovine Hide Collagen I (Advanced Biomatrix Inc, Carlsbad, CA, USA) stock was used at 30µg/ml in a volume of sterile H_2O enough to coat the plate at 5µg/cm^2 and allowed to incubate at room temperature for 2 hours, following which plates were washed twice with sterile PBS and stored in a cool, dry place or used immediately. Poly-D-lysine (PDL) (Sigma, St. Louis, MO, USA) was used at 3.2µg/cm^2 and incubated for 5 minutes, at which

Table 1. MRI sequence parameters.

Sequence	Echo Time (ms)	Repetition Time (ms)	ETL or flip angle	Bandwidth (kHz)	# of averages
T2w with FatSat	25	2000	ETL=8	16	2
T2w no FatSat	25	2000	ETL=8	83	2
T1w no FatSat	7	300	ETL=2	63	4
Fast Gradient Echo	2	15	Flip = 10 deg	63	1

point plates were washed 2x with sterile H_2O and allowed to dry uncovered for approximately 2 hours and coated plates were then stored in a cool, dry place or used immediately. Recombinant human SPARC (rhSPARC) was purchased from R&D Systems (Minneapolis, MN, USA) and was used to co-coat cell culture plates at 60µg/ml in sterile H_2O containing 30µg/ml collagen I and allowed to sit for approximately 2 hours before aspirating excess liquid and washing with PBS prior to use.

2.4 siRNA transfections

Small interfering RNA (siRNA) oligonucleotides for β1 integrin were purchased from Dharmacon (Chicago, IL, USA). Four duplexes were used throughout this work: Siβ1#1 (D-004506-01) GAACAGAUCUGAUGAAUGAUU (Sense), UCAUUCAUCAGAUCUGUUCUU (Antisense), Siβ1#2 (D-004506-02) CaaGAGAGCUGAAGACUAUUU (Sense), AUAGUCUUCAGCUCUCUUGUU (Antisense), Siβ1#3 (D-004506-03) GAAGGGAGUUUGCUAAAUUUU (Sense), AAUUUAGCAAACUCCCUUCUU (Antisense), and Siβ1#4 (D-004506-04) CCACAGACAUUUACAUUAAUU (Sense), UUAAUGUAAAUGUCUGUGGUU (Antisense). Moreover, siGENOME human SPARC (6678) siRNA - SMART pool (M-003710-02-0005) was purchased from Dharmacon (Chicago, IL, USA) which consisted of siRNA directed towards four separate target sequences, siSPARC (D-003710-01) UGAAGAAGAUCCAUGGAA, (D-003710-03) ACUCUGAGCUGACCGAAUU, (D-003710-04) GCAGAAGGAUAUCGACAAG, and (D-003710-05) CAACAAGACCUUCGACUCU. Lastly, si*CONROL* Non-Targeting siRNA #2 (NT2) (D-001210-02-05) (Dharmacon, Chicago, IL, USA); sense, UAAGGCUAUGAAGAGAUAC was utilized as an siRNA control.

Prior to transfection, cells were seeded in 10cm plates at a density of $1.0x10^6$ cells/dish in 10% FBS DMEM for 24 hours. For conducting transfections, oligofectamine (Invitrogen. Carlsbad, CA, USA) was combined with 1X Opti-MEM (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturers protocol and incubated at room temperature for 8 minutes. During this time, siRNA (concentrations described in figure legends) was combined with 1X Opti-MEM at the desired final working concentration. Following the 8 minute incubation, the oligofectamine mixture was added to each siRNA preparation and allowed to incubate for an additional 24 minutes, following which the oligofectamine-siRNA mixture was added drop-wise to each treatment dish containing the cells and serum free Opti-MEM. The transfection mixture was allowed to incubate with the cells for 4 hours at 37°C, 5% CO_2, after which, 30% FBS DMEM was added to the mixture and cells underwent a media change the next day with 10% FBS DMEM.

2.5 Western Blotting

In preparation for western blotting, cells were washed twice with ice cold PBS and lysed in the dish with ice cold radioimmunoprecipitation (RIPA) buffer [20mM Tris-HCl pH 7.2, 150mM NaCl, 1% NP40 Alternative (Calbiochem, San Diego, CA, USA), 1% Triton-X100 (Sigma, St. Louis, MO, USA), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1mM ethylenediaminetetraacetic acid (EDTA)]. RIPA buffer was supplemented with phosphatase inhibitors, including sodium fluoride (4mM), sodium pyrophosphate (4mM), ammonium vanadate (1mM) and the protease inhibitors, phenylmethanesulphonylfluoride (PMSF) (0.4mM), aprotinin (4µg/ml) and leupeptin (10µg/ml). Lysed cells were scraped, collected and vortexed every 5-10 minutes for 30 minutes to stimulate lysis prior to centrifugation at maximum speed at 4°C for 30 minutes. Using a BioMate3 (ThermoFisher

Scientific, Waltham, MA, USA) spectrophotometer, lysates were quantified for protein concentration using the Bio-Rad protein assay to compare measured absorbance at 595nm of treatment samples to a standard curve generated using samples of known protein concentrations. Typically, protein lysates were diluted in RIPA plus inhibitors such that the final concentration (50-100µg of total protein) and volume was equal between all samples and 5X sample buffer (312.5mM Tris-HCl pH 6.8, 10% SDS, 5% 2-mercaptoethanol, 50% glycerol and 7.5% bromophenol blue), was added such that the sample buffer was at a 1X working dilution. This mixture was boiled for 5 minutes at 100°C and separated on a 4% stacking, 10% resolving polyacrylamide gel in running buffer (24mM Tris-base, 190mM glycine, 0.1% SDS). Following separation, an Immobilon-P transfer membrane (Millipore, Billerica, MA, USA) that was previously incubated in methanol then equilibrated in transfer buffer (12mM Tris base, 95mM glycine, 20% methanol), was used to transfer protein from the gel in an electrophoretic transfer cassette containing transfer buffer set for 90 minutes at 100V. Membranes were blocked in 5% milk in TBS-T (50mM Tris-base, 150mM NaCl, 0.05% tween 20, pH 7.6) or, for detection of phosphorylated proteins, 5% bovine serum albumin (BSA) in TBS-T following which membranes were incubated with specified antibodies overnight at 4°C. Membranes were washed 3 times for 5 minutes each with TBS-T and incubated for 1 hour at room temperature with horseradish peroxidase (HRP)-labeled secondary antibody specific to the primary antibody host. Following secondary antibody incubation, membranes were washed 5 times for 5 minutes each prior to illuminating protein bands which involved applying Clarity Western ECL Substrate (1:1 Peroxide solution:Luminol/enhancer solution) (Bio-Rad, Hercules, CA, USA) to the membrane and imaging with the Chemiluminescent Imaging System: GeneGnome (Syngene, Frederick,

MD, USA). Western blots were analyzed using GeneSnap software (Syngene, Frederick, MD, USA).

2.6. Reagents and Antibodies

The pharmacological inhibitor of JNK activity (SP600125) was used at 10μM and was purchased from Cell Signaling (Danvers, MA, USA). Antibodies and their dilutions were used as follows: Mouse anti-β1 integrin (610468) was from BD Transduction Laboratories (San Jose, CA, USA) and was used at a 1:1000 dilution. Polyclonal goat anti-human SPARC was from R&D Systems (Minneapolis, MN, USA) and was used at a dilution of 1:300. Rabbit polyclonal SAPK/JNK (#9252) and Phospho-SAPK/JNK (Thr183/Tyr185) (#9251) antibodies were from Cell Signaling (Danvers, MA, USA) and were each used at a 1:400 dilution. Antibodies directed against p21 (ab18209) (Abcam, Cambridge, MA, USA) and PCNA (PC10) (sc-56) (Santa Cruz, Dallas, TX, USA) were gifts from Dr. Stanford (Ottawa Hospital Research Institute) and were used at a dilution of 1:1000. Cyclin D1 (#2922) (Cell Signaling, Danvers, MA, USA) was a gift from Dr. Dimitroulakos (Ottawa Hospital Research Institute) and was also used at a 1:1000 dilution. Mouse monoclonal GAPDH (ab8245) antibodies were obtained from Abcam (Cambridge, MA, USA) and were used at a 1:10, 000 dilution. Donkey anti-goat IgG HRP conjugated secondary antibody (A15999) was from Thermo Fisher Scientific (Waltham, MA, USA) and was used at a 1:4000 dilution. Goat anti-mouse IgG (DC02L) and goat anti-rabbit IgG (DC03L) HRP conjugated secondary antibodies were from Calbiochem (San Diego, CA, USA) and were also used at a 1:4000 dilution.

2.7. BME 3D Growth Assay

Two chambers per siRNA treatment of Four-well Nunc Lab-Tek II Chamber Slides (154526) obtained from Thermo Scientific (Waltham, MA, USA) were coated with Cultrex 3D Culture Matrix Reduced Growth Factor Basement Membrane Extract (BME), Pathclear (3433-005-01) obtained from Trevigen (Gaithersburg, MD, USA). To coat the wells, the BME was thawed overnight at 4°C and next day, 100µl was administered to each well of the chamber slides. Slides were allowed to solidify at 37°C for 15-30 minutes prior to use and seeding of cells. Transfected cells were treated with citric saline as stated above and suspended in serum free DMEM at 10,000 cells/ml. Equal volumes of DMEM supplemented with 20% horse serum (ThermoFisher Scientific, Waltham, MA, USA), 5% growth factor reduced BME and 10ng/ml EGF (ThermoFisher Scientific, Waltham, MA, USA) were mixed 1:1 with cell suspension and 1ml of this mixture was added to each BME coated chamber. Cells were allowed to grow into spheres for 4 days before the chamber was sectioned into 8 equal parts. Each section was photographed at 10x magnification and quantified for number and area of spheres using ImageJ (94).

2.8. Scratch Wound Assays

Culture plates were coated according to the directions above with either PDL, collagen I or rhSPARC in combination with collagen I depending on the assay. PC3 cells were transfected as indicated in the figure legends and at time of seeding were treated with citric saline, resuspended in serum free DMEM and plated into 4 replicate wells of a 96 well plate per treatment at a cell density of $1.0x10^5$. Fully confluent monolayers were scratched with a p200 tip the next day and washed once with PBS before adding 150µl of fresh serum free DMEM/well. One image of a single scratch was taken per well for four wells per treatment

at time 0 and time 24. Wound fronts were detected and outlined by ImageJ software and percent wound closure was calculated for each of the four wells per treatment using the area (arbitrary units) of the wounds in the following equation: [(Area time 0 - Area Time 24) / Area time 0] x 100%. Percent wound closure from each of four wells per treatment were averaged to provide the mean and standard deviation.

2.9. Soft Agarose 3D Growth Assay

A 1% Low Melting Point Agarose (ThermoFisher Scientific, Waltham, MA, USA) suspension mixed 1:1 with 10% FBS DMEM was added to each treatment well of a 24-well plate and allowed to solidify at room temperature for approximately twenty minutes. Treated cells were lifted with citric saline and resuspended in a 5% agarose 10% DMEM mixture at 10,000 cells/ml and seeded into each of 2 replicate wells of a 24-well plate at 500µl/well. Spheres were allowed to grow for 4 days and were photographed and quantified with ImageJ.

2.10. Statistical Analysis

When presented with two sets of means to asses for statistical significance, an unpaired students *t*-test with a confidence interval of 95% was implemented using GraphPad Prism version 3.00 (GraphPad Software, San Diego, CA, USA). When three or more sets of means were to be compared to one another for significance a one-way ANOVA with the Tukey post-test was used with a 95% confidence interval, also from GraphPad. Finally, for the Kaplan-Meier curve a Logrank Test of significance was used in GraphPad 3.0 to assess the significance between groups. Means were considered significant when $P<0.05$ denoted by an asterisk (*).

3. RESULTS

Prostate cancer has been shown to readily metastasize to bone, liver and lung in patients (4). Mouse models utilizing intracardiac injection into the left ventricle as a means to establish cancer metastasis have been conducted by many groups as described by Park et al. (93). Given previous work in our lab identifying a role for β1-integrin in modulation of the colony growth capacity of PC3 prostate cancer cells in either soft agarose or BME as well as migration of PC3 cells, we hypothesized that β1 integrin could mediate prostate cancer metastasis. We thus utilized an *in vivo* intracardiac injection model of metastasis that results predominantly in tumor growth in the bone, given that this is a primary site of metastasis for prostate cancer patients (4, 93). In addition, we sought to determine potential mechanisms by which the loss of β1 integrin resulted in impaired prostate tumor cell migration and 3D colony formation. For long term *in vivo* studies assessing the effect of β1 integrin on the ability of prostate tumor cell lines to form bone metastases we used PC3 subclones expressing (RSC5) or depleted of (Sh 2-10) β1 integrin which were previously generated in the lab following transfection of plasmids containing β1 integrin-targeted shRNA under control of the constitutively active U6 promoter. *In vitro* work additionally utilized transient transfection systems using β1 integrin-targeted siRNA or control scrambled siRNA to assess the potential role of β1 integrin in the modulation of cell migration and 3D growth and to elucidate the potential mechanisms responsible for the observed phenotypes.

3.1. Loss of β1 integrin in an intracardiac model of prostate cancer

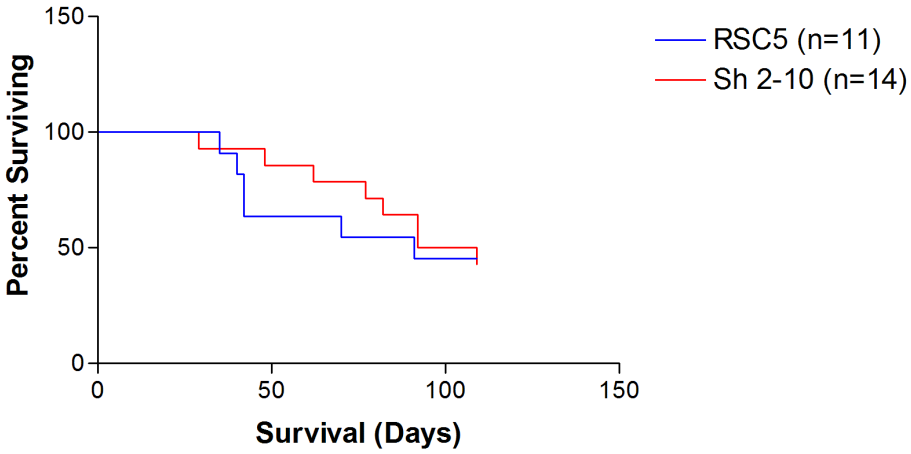
Prostate cancer frequently metastasizes to the bone, lungs and liver (4). PC3 cells can metastasize to both lungs and bone *in vivo* using either orthotopic injection or intra-cardiac injections, respectively (93, 95, 96). β1 integrin has been implicated in this process through

modulation of growth and migratory phenotypes (80) as well as interacting with ECM proteins that exist in the secondary sites of metastasis including bones and lung (41, 43, 97). Given our interest in signaling downstream of β1 integrin, a known mediator of bone matrix-induced phenotypes (79, 97) and the fact that currently, treatments for bone metastasis are palliative and there is no cure (75) we chose the intracardiac model to assess bone colonization of prostate cancer cells. To assess the role of β1 integrin in this process, RSC5 and Sh2-10 cells, derived from the parental cell line PC3, were intracardially injected into 16 CD1 nude mice for each cell type at a concentration of $1.0x10^6$ cells/100µl. These two cell lines were selected as previous work in the lab had shown Sh2-10 cells displayed a significant decrease in β1 integrin expression along with a significant reduction in invasive capabilities on BME and adherence to collagen I and plastic while maintaining the same growth characteristics as RSC5 control cells in monolayer.

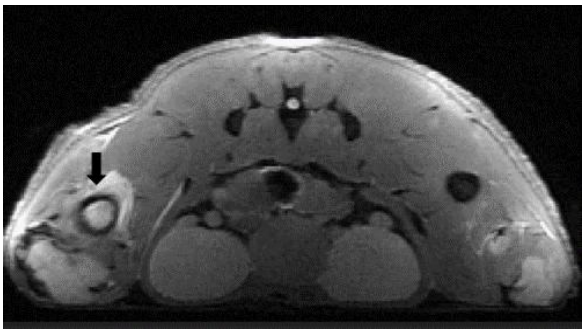
One mouse immediately died following injections in the RSC5 group and was removed from the study. Moreover, at the time of analysis, 4 additional mice in the RSC5 group had developed lung tumors and thus were also removed from the study as the presence of lung tumors assumes mice were improperly injected (93). In the Sh2-10 group, 2 mice were removed from the study as they had also developed lung tumors. Therefore, the study was refined to 11 mice in the RSC5 group and 14 mice in the Sh 2-10 group. The first endpoint characterized by death came at week 5 and 6 for mice injected with Sh2-10 or RSC5 cells, respectively and the first MRI analysis was conducted at week 7 following the third mouse to die in the RSC5 group. Every subsequent endpoint following week 7 in either group was retrieved for MRI analysis for up to 15 weeks with the exception of two mice (one in each group due to reasons out of our control such as inaccessibility to MRI, and

improper disposal by animal care technicians). Within 15 weeks of the study due to death or euthanization at clinical endpoints, 6/11 mice in the RSC5 group had passed away with 3 mice undergoing MRI and 8/14 mice had passed away in the Sh 2-10 group with 5 mice undergoing MRI. Following image analysis, animals were necropsied and lungs were also assessed macroscopically for tumor lesions as a control for appropriate intracardiac injections. Long bones and spines were also collected for future histological analysis. Signs of clinical endpoints were first observed within 5-6 weeks post-injection. Figure 3A shows the survival curve for each group of injected animals with the Sh 2-10 group showing slightly longer survival than animals injected with vector control RSC5 cells, however this difference was not significant as determined by the logrank statistical test (Figure 3A). Liver metastasis was not visible on a macroscopic level in any mice. MRI was conducted on several mice in each group and seems to depict that these cells are capable of forming bone metastasis (Figure 3B); however, assessing tumor burden by MRI requires radiologist assessment. Given the data, depletion of β1 integrin does not confer a significant increase in overall survival.

A


150
100
50
0
Percent Surviving
0
50
100
150
Survival (Days)
RSC5 (n=11)
Sh 2-10 (n=14)

B



C

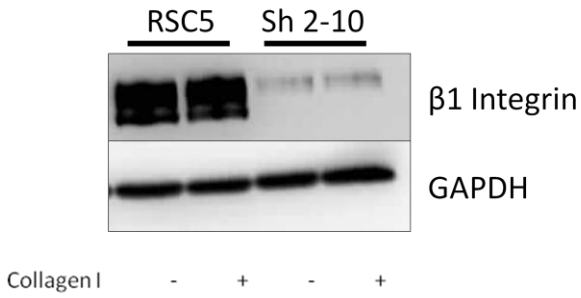

RSC5
Sh 2-10
β1 Integrin
GAPDH
Collagen I
-
+
-
+

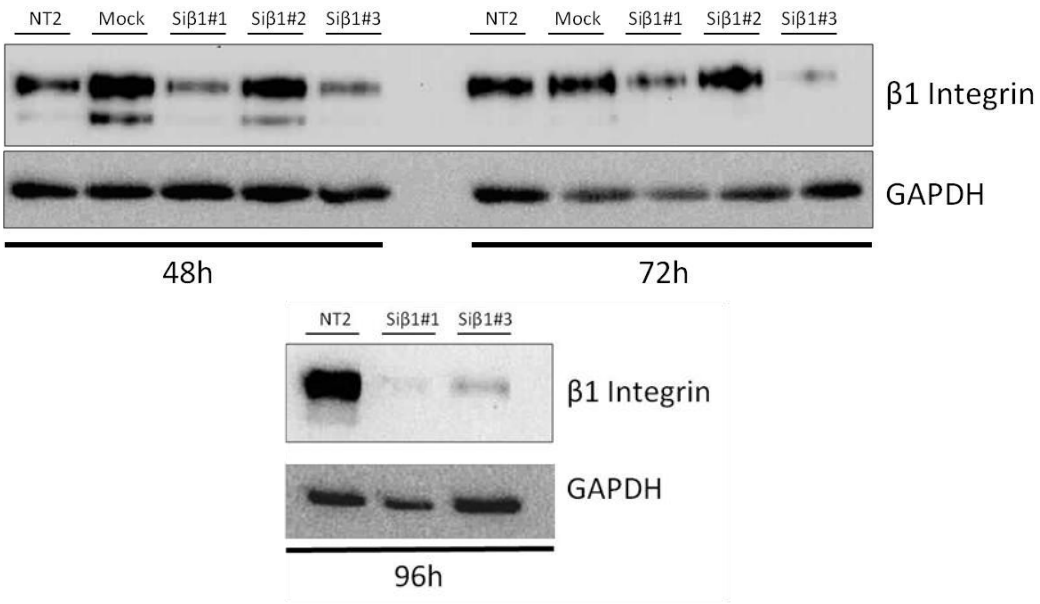
Figure 3. Nude mice injected with β1 integrin depleted cells (Sh 2-10) does not display a greater overall survival at 11 weeks relative to mice injected with β1 integrin expressing (RSC5) controls.
(A) Kaplan-Meier curve showing endpoint (death or predetermined clinical endpoint) for the two groups of mice, RSC5 and Sh2-10. (B) An example of what presents as a bone metastasis via MRI in the hind limb of an RSC5 cell-bearing mouse (depicted by arrow). (C) β1 integrin expression levels measured on plastic and collagen I in both RSC5 and Sh 2-10 subclones at the time of intracardiac injection.

3.2. Loss of β1 integrin may result in increased SPARC protein expression

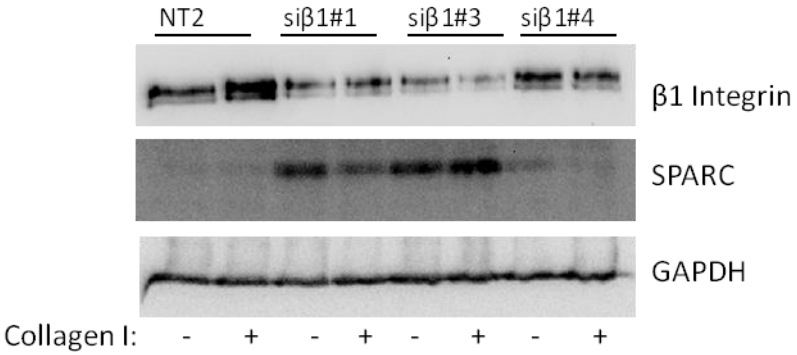
Increased β1 integrin expression has been observed in poorly differentiated prostate cancers (76) and β1 integrin is an established mediator of prostate cancer tumorigenesis *in vivo* (68, 79) and of 3D growth and migration *in vitro* (68, 81, 98). One way in which β1 integrin may modulate tumor phenotypes is through regulating the expression of ECM proteins known to modulate tumorigenesis (70, 99). Moreover, β1 integrin is known to mediate cellular signaling through activation of the transcription factor c-jun (100) and interestingly, c-jun is a known repressor of the matricellular protein SPARC (74). It has recently been shown that the matricellular protein SPARC acts as an anti-tumor protein (47, 90) and displays absent or reduced expression in aggressive or metastatic tumor cells in both ovarian and prostate cancer (50, 90). Also, phenotypes associated with increases in SPARC expression have been shown to correlate with phenotypes that are also obtained with loss of β1 integrin (81, 87). Based on our previous work showing that loss of β1 integrin resulted in decreased 3D colony growth in soft agar and invasion in BME (81) and the potential inhibitory role of SPARC in these same processes, we sought to assess whether there was an association between β1 integrin and SPARC protein expression or activity. Given that β1 integrin is a collagen I receptor (79), and collagen I deposition increases in tumors (32) and it is the most abundant ECM protein in bone (77), we also investigated the association between β1 integrin and SPARC expression in the context of collagen I.

First we assessed the knockdown efficiency of different siRNA targeting β1 integrin. Three separate siRNAs (siβ1#1, 2, and 3) were used at 50nM and β1 integrin levels were assessed 48 and 72 hours post-transfection (Figure 4A: top). Siβ1 #1 and #3 displayed optimal knockdown at 72 hours post-transfection compared to mock and NT2 so #1 and #3

A


NT2
Mock
Siβ1#1
Siβ1#2
Siβ1#3
NT2
Mock
Siβ1#1
Siβ1#2
Siβ1#3
β1 Integrin
GAPDH
48h
72h
NT2
Siβ1#1
Siβ1#3
β1 Integrin
GAPDH
96h

B


NT2
siβ1#1
siβ1#3
siβ1#4
β1 Integrin
SPARC
GAPDH
Collagen I: - + - + - + - +

C

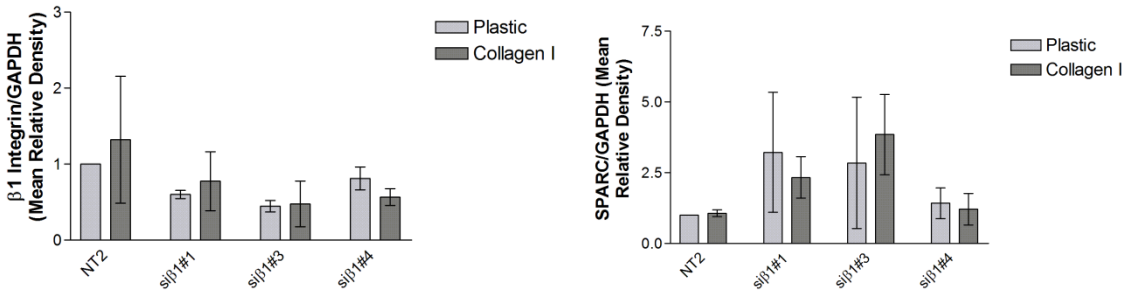

β1 Integrin/GAPDH (Mean Relative Density)
Plastic
Collagen I
NT2
siβ1#1
siβ1#3
siβ1#4
SPARC/GAPDH (Mean Relative Density)
Plastic
Collagen I
NT2
siβ1#1
siβ1#3
siβ1#4

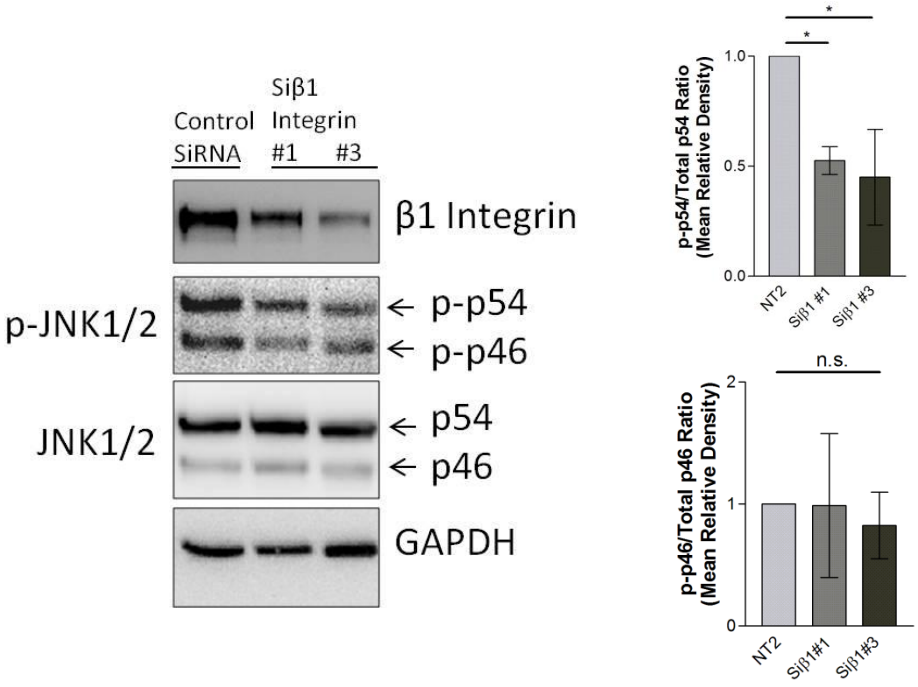
Figure 4. β1 integrin depletion in PC3 cells results in increased SPARC expression. PC3 cells were transfected with 50nM NT2, oligofectamine alone (mock) or siβ1 #1, #2 and #3 for 48 and 72 hours (A: top). Further, 50nM of siβ1 #1 and #3 were used to transfect PC3 cells and levels of β1 integrin were determined compared to 50nM NT2 following 96 hours post-transfection (A: bottom). PC3 cells were transfected with 50nM of three different sequences of siRNA directed against β1 integrin (#1, #3, #4) as well as 50nM of NT2 on both plastic and collagen I for 96h, following which cell lysates were collected for western blot analysis and probed using antibodies against β1 integrin, SPARC and GAPDH (B) and quantified in (C).Values are plotted as the mean of three independent replicates $\pm$ SD. A one-way ANOVA was used to determine significance between mean values (*=$p<0.05$).
These are representative blots of n-3 biological replicates.

were used in future experiments. These two siβ1 siRNA's were further assessed at 96 hours post-transfection and at this time point, β1 integrin remained depleted in these cells compared to NT2 (Figure 4A: bottom). A fourth siRNA targeted against β1 integrin (siβ1#4) that had not been previously assessed for knockdown efficiency, was also available and thus was used as well in Figure 4B to assess the regulation of SPARC by β1 integrin. PC3 cells plated on either plastic or collagen I coated plates were transfected with each of siβ1#1,3,4 and NT2 for 96 hours as this was within the optimal range for β1 integrin knockdown (Figure 4A bottom). Protein lysates were then collected and SPARC protein levels were examined. Reduction of β1 integrin protein on both plastic and collagen I using three siβ1 did not reach significance. There was a substantial increase in SPARC protein expression following treatment with siβ1 (#1 and #3); however, this was not significant. Lack of induction of SPARC following use of siβ1#4 was likely due to the incomplete knockdown of β1 integrin observed when using this siRNA. Taken together with data obtained from similar assays using siβ1#3 (Figure 7B) where increased expression levels of SPARC was statistically significant in β1 integrin-depleted versus control cells, our results suggest that an inverse correlation exists between β1 integrin and SPARC expression. The data in Figure 4B may not resemble Figure 7B as fresh batches of siRNA were used in later experiments and PC3 cells were used at lower passage number in later experiments potentially resulting in more reliable data. It should also be noted that increases in SPARC levels were observed regardless of whether cells were in the context of collagen I or not, which is expected given the enforced depletion of its primary receptor in our experiments (Figure 4B). Although expression levels of SPARC were not detectable in control NT2-treated cells, SPARC expression in cells in which β1 integrin has been partially depleted using siβ1#4 appeared to

be slightly lower when cells were on collagen I, suggesting that perhaps β1 integrin-ligand activity plays an active role in SPARC suppression.

Integrin signaling is known to activate JNK (100) which is a critical regulator of c-jun (101). Interestingly, c-jun is known to repress SPARC expression (74). Therefore we assessed if JNK-c-jun mediates the regulation of SPARC by β1 integrin. First we assessed the activity of JNK1/2 (p54/p46) downstream of β1 integrin in PC3 cells. Consistent with the literature, seventy-two hours following transfection with siβ1 (within optimal time-frame for β1 integrin knockdown) there is a significant reduction in JNK1/2 (p54) activity as evidenced by decreased Thr183/Tyr185 phosphorylation compared to NT2 (Figure 5A) although there was no significant change in the less predominant splice variant of JNK1/2 (p46). Next we assessed the regulation of SPARC by JNK1/2. Treatment with 10μM of the pharmacological inhibitor SP600125 resulted in an increase in SPARC protein expression following complete inhibition of JNK1/2 phosphorylation on residues Thr183/Tyr185 at 48 hours compared to dimethyl sulfoxide (DMSO) controls (Figure 5B). This data provides evidence whereby the suppression of SPARC by β1 integrin may occur through JNK; although, we have not yet established a direct link.

A


Siβ1
Control
SiRNA
Integrin
#1
#3
β1 Integrin
p-JNK1/2
← p-p54
← p-p46
JNK1/2
← p54
← p46
GAPDH
*
*
p-p54/Total p54 Ratio
(Mean Relative Density)
1.0
0.5
0.0
NT2
Siβ1 #1
Siβ1 #3
n.s.
p-p46/Total p46 Ratio
(Mean Relative Density)
2
1
0
NT2
Siβ1#1
Siβ1#3

B

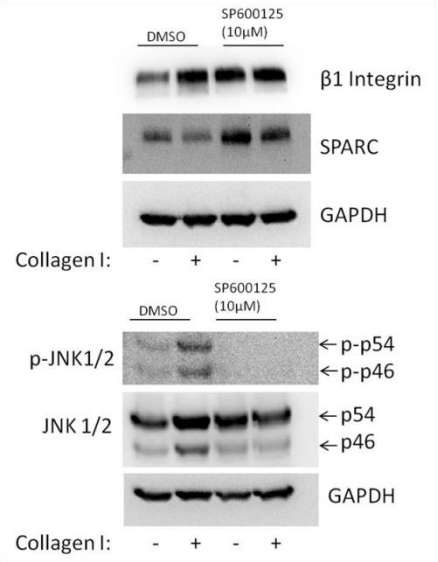
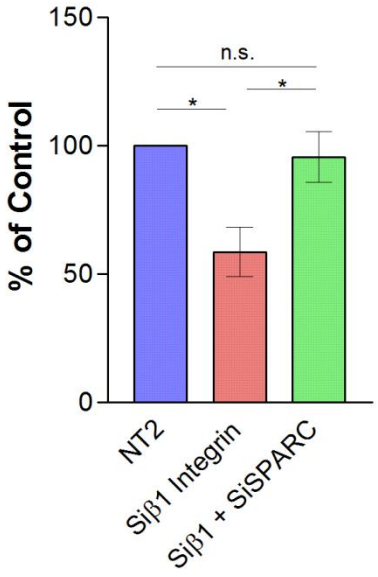

DMSO
SP600125
(10μM)
β1 Integrin
SPARC
GAPDH
Collagen I:
- + - +
DMSO
SP600125
(10μM)
p-JNK1/2
← p-p54
← p-p46
JNK 1/2
← p54
← p46
GAPDH
Collagen I:
- + - +

Figure 5. β1 integrin regulates SPARC through a putative JNK signaling mechanism. (A) PC3 cells were transfected with two different sequences of SiRNA directed against β1 integrin (#1, #3) for 72h, following which cell lysates were collected for western blot analysis and probed using antibodies against β1 integrin, Phospho- and Total JNK and GAPDH. There is a decrease in JNK1/2 (p54) phosphorylation when β1 integrin protein levels are low relative to control (NT2) transfected cells. Densitometry was conducted for total p46/p54 on blots using optimal, non-saturated, exposures for each. (B) PC3 cells were treated with either DMSO (Control) or the JNK inhibitor (SP600125) on both plastic and collagen I for 48 hours and protein lysates were collected for western blot analysis. Inhibition of JNK phosphorylation results in a substantial increase in SPARC expression. Blots are representatives of n-3 biological replicates. Values are plotted as the mean of three independent replicates $\pm$ SD. A one-way ANOVA was used to determine significance between mean values (*=$p<0.05$).

3.3. Loss of β1 integrin inhibits sphere formation in BME and this phenotype is rescued by simultaneous knockdown of SPARC

SPARC is known to be anti-proliferative (88) as endogenous expression of SPARC in breast cancer cells MDA-MB-231 through adenoviral infection inhibited the growth of these cells in 3D (87). Therefore, we sought to determine whether the endogenous increase in SPARC expression as a function of β1 integrin knockdown we observed in prostate tumor cells growing in 2D, could mediate the previously described role for loss of β1 integrin in inhibiting prostate cancer growth in BME (81). BME coated wells were seeded with an overlay of cells previously transfected for 72 hours with NT2, or siβ1#1, or a combination of siβ1#1 and siSPARC. A separate aliquot of cells were obtained on the day of seeding to check the protein expression levels of β1 integrin and SPARC in each of the treatment samples and to confirm depletion of specific target proteins (Figure 6C: β1 integrin and SPARC levels are also shown in Figure 7B using the same transfection protocol). Cells were imaged on day 4 post-seeding (Figure 6B) and quantified using imageJ software to determine the number and surface area of cell spheres. Cells depleted for β1 integrin displayed a significant reduction in sphere numbers on day four, confirming results previously observed in our lab. Interestingly, co-transfecting with siβ1#1 and siSPARC simultaneously, resulted in a recovery of sphere formation by β1-depleted transfected cells in the BME assay, with sphere number almost reaching control levels (Figure 6A).

A


150
100
50
0
% of Control
n.s.
*
*
NT2
Siβ1 Integrin
Siβ1 + SiSPARC

B


NT2
Siβ1 Integrin
Siβ1 Integrin + SiSPARC

C

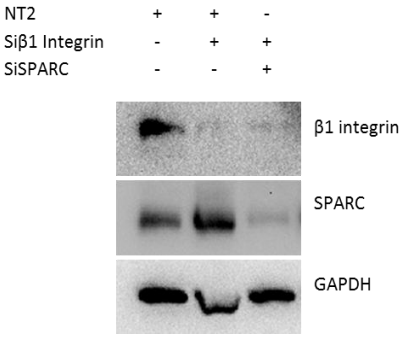

NT2 + + -
Siβ1 Integrin - + +
SiSPARC - - +
β1 integrin
SPARC
GAPDH

Figure 6. β1 integrin mediates PC3 cell sphere formation in a BME sphere-forming assay via suppression of SPARC.
PC3 cells were transfected with NT2, SiRNA directed against β1 integrin or co-transfected with SiRNA directed against β1 integrin and SPARC for 72h. (A) Sphere size was assessed on day 4 and spheres measuring between 1500 and 3000μm^2 were quantified using imageJ software. B) Photographs of spheres in BME coated wells and on Day 4. (C) Following 72 hours post-transfection of siRNA, cell lysates were collected for western blot analysis and probed using antibodies against β1 integrin, SPARC and GAPDH to confirm specific target protein depletion. There was a significant decrease in sphere formation when cells were depleted for β1 integrin and this phenotype was rescued by the dual knockdown of β1 integrin and SPARC together. Values are plotted as the mean of three independent replicates $\pm$ SD. A one-way ANOVA was used to determine significance between mean values (*=$p<0.05$).

3.4. Increased SPARC expression with loss of β1 integrin does not modulate the expression of proliferation and quiescence markers

SPARC and β1 integrin have both been shown to modulate the cell cycle (100, 102). Therefore, we sought to assess if the cell cycle was regulation by the coordinated expression levels of these two proteins and may in part contribute to the growth differences observed in BME between NT2, siβ1 and siβ1-siSPARC transfected cells. We thus examined the levels of p21, PCNA and cyclin D1 as indicators of cell cycle regulation. A well-known cell cycle inhibitor, p21 promotes cell cycle arrest through inhibiting the kinase activity of cyclin-dependent kinases (CDKs), primarily CKD2 (103). Proliferating cell nuclear antigen (PCNA) is a cell-proliferation marker and is critical in the initiation of DNA replication. Accordingly, PCNA shows increased expression in late G1 and during the S-phase of the cell cycle but is minimally expressed in the G0, early G1, G2 and M phase (104). Cyclin D1 is a marker of proliferation as its expression level increases in the G1 phase of the cell cycle to initiate DNA synthesis and neutralization of cyclin D1 ceases progression through the cell cycle. Interestingly, expression of cyclin D1 has also been shown to increase upon exit of quiescence and entering the cell cycle (105). Therefore, cells were transfected similarly to the previous section with NT2, siβ1 or co-transfected with siβ1 integrin and siSPARC together, and protein lysates were generated. There was a significant decrease in β1 integrin expression and a significant increase in SPARC expression consistent with previous data using siRNA-mediated depletion of β1 integrin (Figure 7B). SPARC expression in β1 integrin depleted cells was significantly inhibited by co-transfection with siRNA specific to SPARC compared to solely siβ1 transfected cells

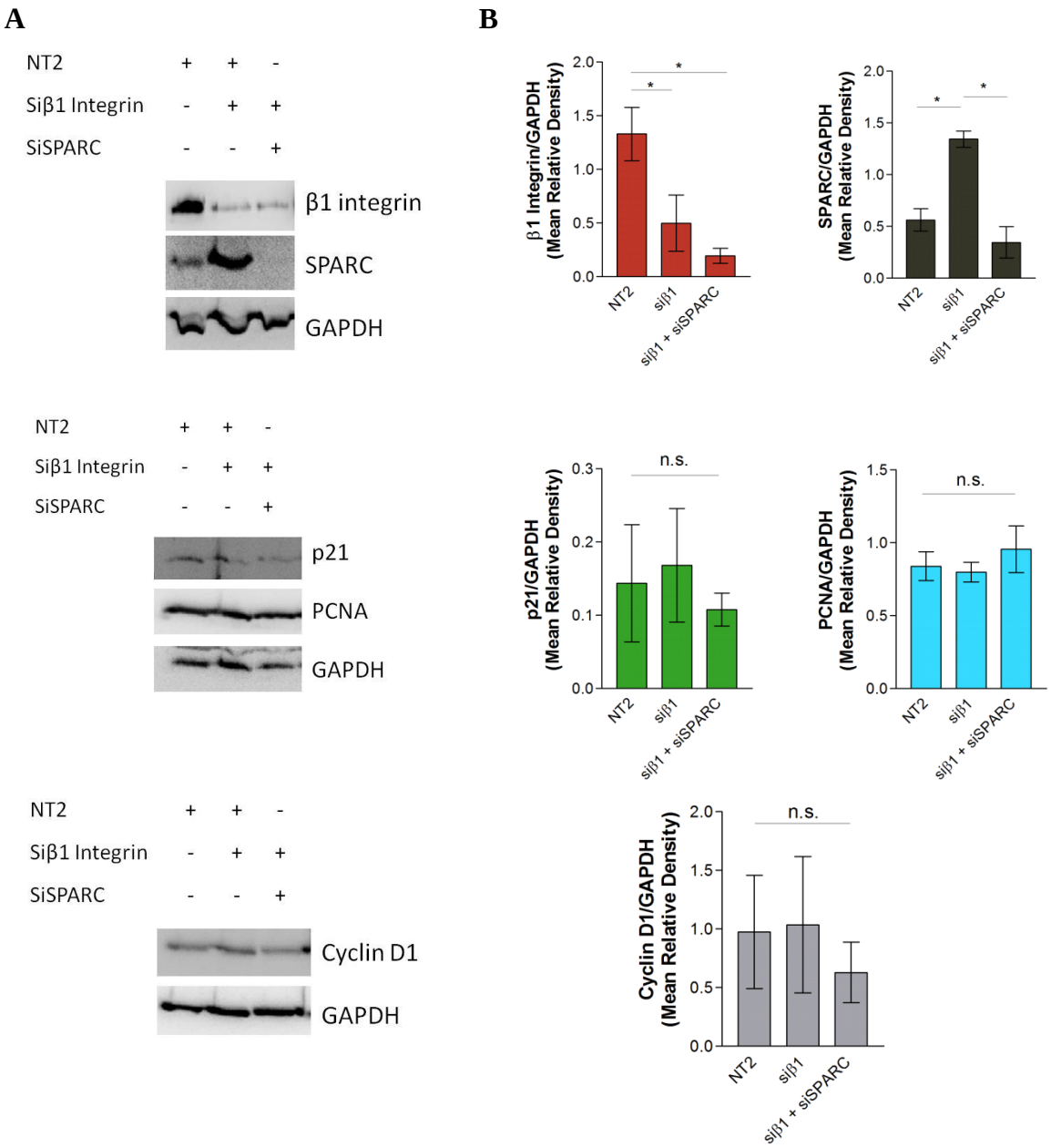

A
NT2 + + -
Siβ1 Integrin - + +
SiSPARC - - +
β1 integrin
SPARC
GAPDH
NT2 + + -
Siβ1 Integrin - + +
SiSPARC - - +
p21
PCNA
GAPDH
NT2 + + -
Siβ1 Integrin - + +
SiSPARC - - +
Cyclin D1
GAPDH
B
β1 Integrin/GAPDH (Mean Relative Density)
SPARC/GAPDH (Mean Relative Density)
p21/GAPDH (Mean Relative Density)
PCNA/GAPDH (Mean Relative Density)
Cyclin D1/GAPDH (Mean Relative Density)
n.s.
NT2
siβ1
siβ1 + siSPARC

Figure 7. Neither β1 integrin nor SPARC in β1 integrin-depleted PC3 cells modulate proliferation markers in 2D cultures.
PC3 cells were seeded in monolayer onto plastic plates and transfected with 100nM of NT2 or 50nM siβ1 + 50nM NT2 or 50nM siβ1 + 50nM siSPARC. Seventy-two hours following transfection, cell lysates were collected and analyzed by western blot for β1 integrin, SPARC, p21, PCNA, cyclin D1 or GAPDH as an endogenous loading control (A). (B) Densitometry conducted on each western blot with protein of interest expressed at a ratio of GAPDH. Values are plotted as the mean of three independent replicates $\pm$ SD. A one-way ANOVA was used to determine significance between mean values (*=$p<0.05$).

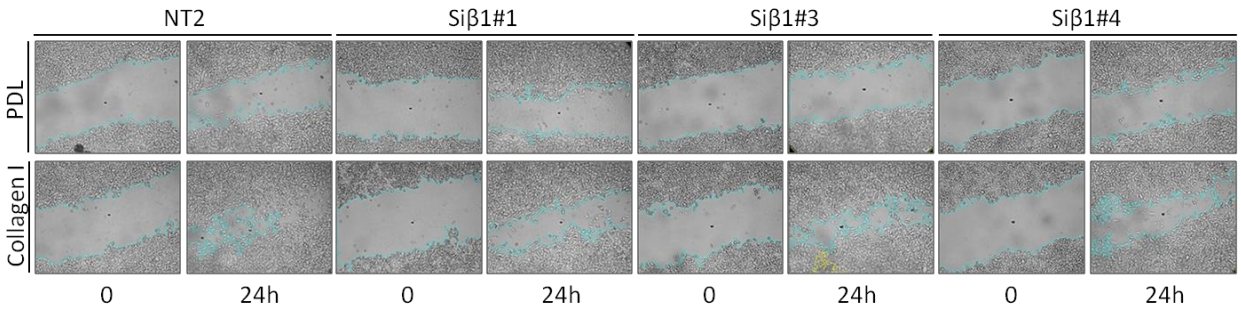
(Figure 7B). There was no change in the expression levels of p21, PCNA or cyclin D1 with any of the treatment groups in 2D cultures (Figure 7A).

Thus far the measurement of proliferation associated proteins has been conducted on 2D culture plates. In order to obtain data that is more relevant to colony formation, these same experiments must be conducted with 3D cultures.

3.5. Loss of β1 integrin reduced collagenI-mediated enhanced migration; although, this phenotype is not mediated by SPARC

As part of the current study, in parallel we wished to confirm that the use of siRNA resulted in phenotypes similar to those we had previously observed using shRNA-mediated depletion of β1 integrin. Given the increase in collagen I expression in tumoral stroma and the influence of collagen I in prostate cancer phenotypes involved in metastasis including migration (97), we sought to assess the role of β1 integrin in mediating migration on collagen I. NT2 treated PC3 cells were plated on either PDL, used as a non-integrin engaging polymer control, or collagen I, scratched and allowed to migrate for 24h. PC3 cells showed enhanced wound closure on collagen I-coated plates versus PDL with 75% and 25% wound closure, respectively. While loss of β1 integrin using multiple siRNAs did not result in a significant change in wound closure on PDL compared to NT2 there was a significant decrease, about 25%, in wound closure on collagen I between each of the siβ1 treatments and NT2. Thus, collagen-I dependent migration is in part mediated by β1 integrin (Figure 8).

It has been established herein that knockdown of β1 integrin results in an increase in SPARC expression. In the literature, SPARC has been demonstrated to decrease prostate cancer migration (90); therefore, we assessed the role of increased SPARC in mediating the observed decrease in migration following β1 integrin depletion. PC3 cells were transfected


NT2
Siβ1#1
Siβ1#3
Siβ1#4
PDL
Collagen I
0
24h
0
24h
0
24h
0
24h

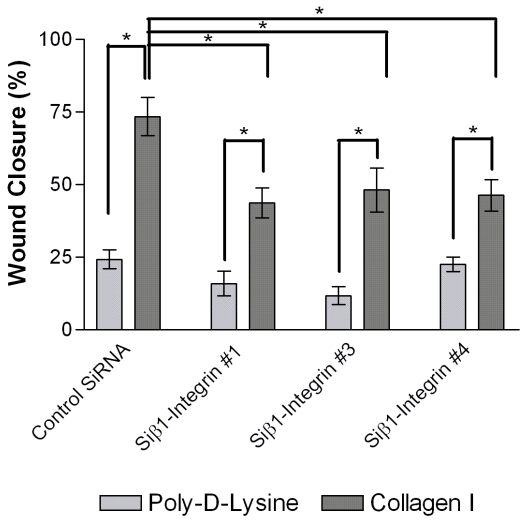

Wound Closure (%)
100
75
50
25
0
*
Control SiRNA
Siβ1-Integrin #1
Siβ1-Integrin #3
Siβ1-Integrin #4
Poly-D-Lysine
Collagen I

Figure 8. β1 integrin partially mediates collagen I enhanced migration of PC3 cells. PC3 cells were transfected with NT2 or three different siRNA directed against β1 integrin (#1, 3 and 4) for 72h, following which cells were seeded onto PDL or collagen I coated plates, scratched to generate a wound front and photographed at time 0 and 24 hours later. Knockdown of β1 integrin did not modulate basal migration on PDL but did partially inhibit enhanced migration on collagen I. Values are plotted as the mean of three independent replicates $\pm$ SD. A one-way ANOVA was used to determine significance between mean values (*=$p<0.05$).

with either 50nM siβ1 + 50nM NT2 or 50nM siβ1+ 50nM siSPARC and seeded onto both PDL and collagen I coated plates, scratched and allowed to migrate for 24h. On both PDL and collagen I coated plates, there was no significant difference in the percentage wound closure between cells transfected with siβ1 + NT2 or siβ1+ siSPARC on either PDL or collagen I (Figure 9). This suggests that the inhibition of migration observed with β1 integrin depletion is not affected by SPARC levels.

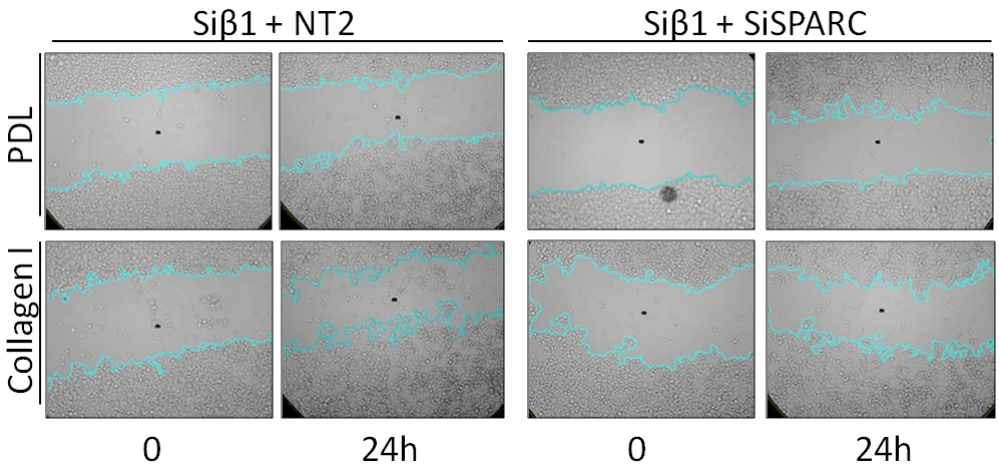
3.6. Exogenous SPARC does not modulate PC3 cell migration on collagen I

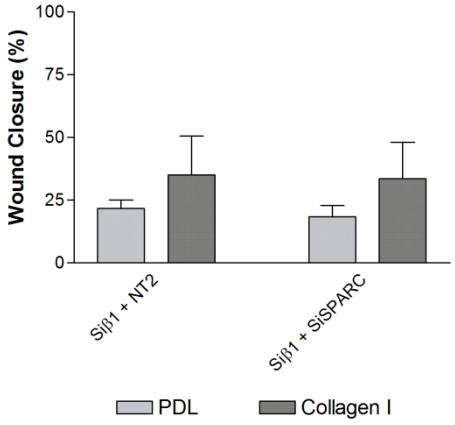
Although increases in endogenous levels of SPARC does not modulate migration in siβ1 treated cells, a recent publication has shown that the inhibition of prostate cancer cell migration by exogenous SPARC occurs through binding β1 integrin and inhibiting integrin signaling (90). Therefore a different approach using wildtype PC3 cells was employed to assess the regulation of migration on a collagen I substrate with the use of exogenously administrated SPARC. Plates were coated with 30μg/ml collagen I alone or 30μg/ml collagen I + rhSPARC at a 1:2 ratio as determined by a dose-response curve using increasing amounts of rhSPARC. Plates coated with both collagen I and rhSPARC show a modest reduction in % wound closure relative to collagen I alone although these results were not significant (Figure 10).

3.7. Summary

The work conducted herein *in vivo* could not conclusively establish the role for β1 integrin in mediating prostate cancer cell colonization of the bone following intracardiac injection although we observed a slight increase in survival with β1 integrin depleted cells. However, we confirmed that defects in BME colony formation in β1 integrin depleted cells was

A

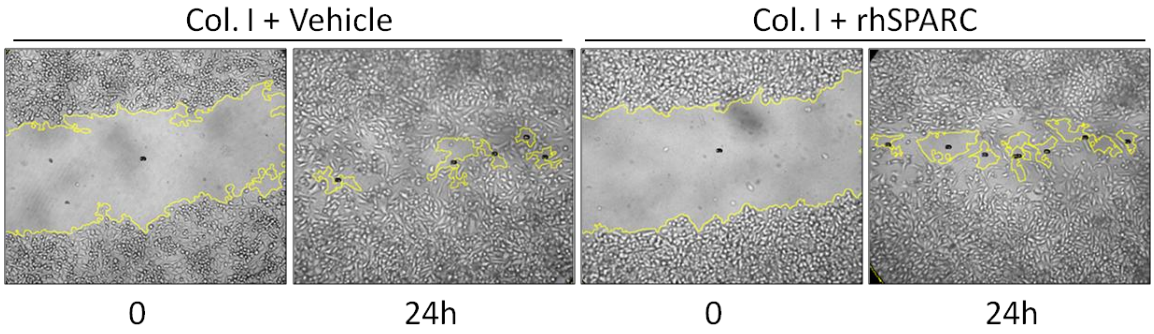

Siβ1 + NT2
Siβ1 + SiSPARC
PDL
Collagen I
0
24h
0
24h


Wound Closure (%)
100
75
50
25
0
Siβ1 + NT2
Siβ1 + SiSPARC
PDL
Collagen I

B


Siβ1 Integrin
Siβ1 Integrin + SiSPARC
β1 Integrin
SPARC
GAPDH

Figure 9. SPARC expression does not mediate loss of collagen I enhanced migration in β1 integrin depleted PC3 cells.
PC3 cells were transfected with 50nM siRNA directed against β1 integrin + 50nM NT2 or co-transfected with 50nM siRNA each directed against β1 integrin and SPARC for 72h, following which each group of transfected cells were (A) seeded onto PDL or CI coated wells, scratched next day and photographed at time 0 and time 24 and quantified for % wound closure or (B) cell lysates were collected for western blot analysis and probed using antibodies against β1 integrin, SPARC and GAPDH. Percent wound closure is expressed as the mean of two independent experiments $\pm$ SD. A one-way ANOVA was used to determine significance between mean values (*=$p<0.05$).


Col. I + Vehicle
Col. I + rhSPARC
0
24h
0
24h

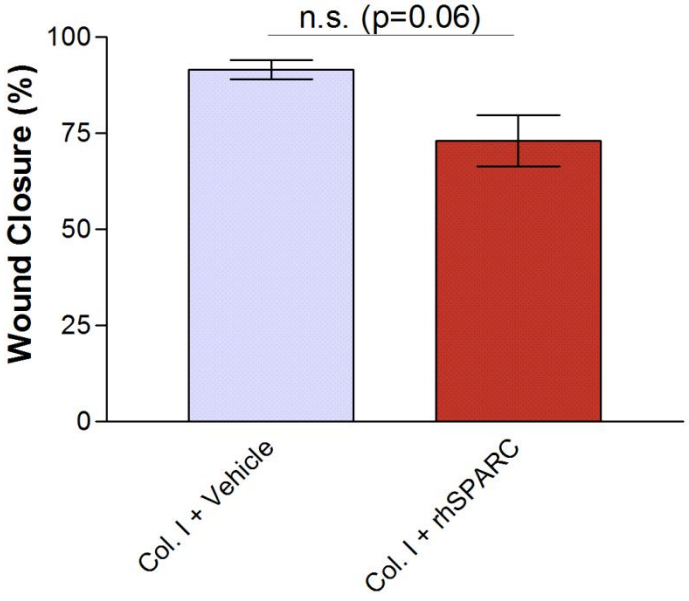

n.s. (p=0.06)
Wound Closure (%)
100
75
50
25
0
Col. I + Vehicle
Col. I + rhSPARC

Figure 10. Recombinant human SPARC does not modulate the % wound closure of PC3 cells expressing normal β1 integrin levels on a collagen I substrate.
PC3 cells were seeded onto plates coated with 30µg/ml Collagen I or on plates coated with 30µg/ml Collagen I together with 60µg/ml rhSPARC. Cells were scratched next day, photographed at time 0 and following 24h cells were photographed again and analyzed using imageJ software. Percent wound closure is presented as the mean of two independent experiments $\pm$ SD. Students *t*-test was used to determine significance of the means (p=0.06).

attributed in part to enhanced SPARC expression. Moreover, we've shown that the predominant JNK isoform (p54) activity is diminished following loss of β1 integrin and that in an independent experiment, inhibition of JNK1/2 activity using the pharmacological inhibitor SP600125 resulted in enhanced SPARC protein expression, suggesting a link between β1 integrin, JNK signaling and SPARC. We were unable to establish a role for either β1 integrin or SPARC to modulate the expression of proliferation associated proteins p21, PCNA and cyclin D1 in 2D cultures although we must assess the role of these proteins in 3D cultures. Lastly, we have confirmed a role for β1 integrin in mediating enhanced migration of PC3 cells on collagen I. We were unable to confirm however that SPARC expression in these cells with reduced β1 integrin expression was responsible for the observed decrease in wound closure. Moreover we were unable to confirm a role for recombinant human SPARC in blocking migration on a collagen I substrate in PC3 cells expressing normal levels of β1 integrin.

4. DISCUSSION

4.1. Loss of β1 integrin in an intracardiac model of prostate cancer

There are many ways to model cancer cell metastasis *in vivo* either using transgenics, such as the TRAMP mouse model to study prostate cancer (106), or through injecting mice with cells grown in culture via routes such as orthotopic (107, 108), subcutaneous (108), tail vein (109), or intracardiac injections (109, 110). Each injection model in combination with particular cancer types or cancer cell lines provides its own discrepancies as to which secondary site is preferred for colonization and therefore it is critical to choose the cell line and model that best represents the research question (109, 110). For example, in a mouse model of human prostate cancer metastasis using orthotopic transplantation, the prostate cancer cells lines Du145 and PC3 derived from brain and bone metastasis, respectively, have shown to colonize predominantly in retroperitoneal lymph nodes with adrenal metastasis also predominant in PC3 cells (111). This pattern of colonization occurs within 5-11 weeks of orthotopic transplantation following euthanization protocols based on tumor size and clinical status (111). Thus, this model may be suitable for studying lymph node metastasis in both Du145 and PC3 cells or adrenal gland metastasis in PC3 cells; however, in this model it is exceptionally rare to observe bone metastasis following transplantation of prostate cancer cells, therefore particular preferred sites of prostate cancer metastasis such as the bone should be investigated using other models of metastasis (4, 107, 111).

Our lab is interested in prostate cancer metastasis to the bone for several reasons: 1) its predominance in patients of upwards of 90% at autopsy (4), 2) our interest in signaling downstream of a known mediator of bone matrix-induced phenotypes, namely β1 integrin (79, 97) and 3) the fact that current treatments for bone metastasis are palliative and there is

no cure (75). Thus, an intracardiac injection model was chosen for this study as this model predominantly provides bone colonization of prostate cancer cells relative to other methods such as orthotopic transplantation or tail vein injection and it relies on injecting cells already grown in culture (93, 111, 112). A limitation to using intracardiac injections however is that it is not a true depiction of the metastatic process as it bypasses the early stages of metastasis such as intravasation through the vasculature and invasion through the primary tissue; thus, this method instead assesses the capacity of cancer cells to extravasate into secondary tissue and colonize particular organ sites after systemic delivery (113). Finally, using this approach comes with technical challenges that must be overcome. Ensuring injection into the left ventricle of the heart is difficult but ensures direct systemic delivery whereas accidentally injecting the right ventricle allows passage through the lungs. Moreover, missing the heart completely would result in spillover in the chest cavity. Analysis by bioluminescence to ensure a signal throughout the body following injections may be employed to overcome these issues to ensure proper, systemic delivery of the cells. Otherwise a bioluminescent signal solely at the site of injection indicates improper technique and these mice would be removed from the study (93).

In our intracardiac injection model, we injected sub-clones of the PC3 parental cell line either expressing (RSC5) or depleted of (Sh 2-10) β1 integrin at 10^6cells/100µl/mouse and we observed colonization in bone within both sets of mice. Mice displaying lung colonization were assumed to have been injected improperly and were removed from the study. Mice showing lung colonization did not display bone colonization and the reverse was also true, with the exception of one mouse out of thirty-two. Thus, mice presenting solely with lung lesions were deemed to have been improperly injected given the concomitant

absence of bone colonization which is known to occur using intracardiac injections (112). There are reports in the literature wherein injecting PC3 cells at the same cell density as our lab not only formed extensive bone metastasis but also other tissue metastasis (96), although we cannot find any reports of lung colonization with intracardiac injection of PC3 cells in the literature, again supporting our conclusions that the development of lung metastases in these mice was a result of inappropriate injection techniques. We hypothesized that Sh 2-10 cells with loss of β1 integrin expression would result in fewer metastatic lesions in the bone relative to β1 integrin expressing RSC5 control cells. This hypothesis was based on reports in the literature wherein β1 integrin has proven to be a major contributor of prostate cancer bone metastasis *in vivo* (78, 80). Considering the MRI data has not been assessed by a radiologist, and we have not assessed bone by immunohistochemistry (IHC) we cannot draw any conclusions about the role of β1 integrin in establishing bone metastasis at this time. Our mouse survival data between RSC5 and Sh2-10 cells was not significant. This result is surprising in that it is inconsistent with what is reported in the literature (78-80, 114). One such report has shown that the PC3-mm2 cell line derived from serial passage of PC3 cells *in vivo* are significantly reduced in the ability to form colonies in male SCID mouse femurs if injected with 30μg/ml anti-β1 integrin Ab (mAb 33B6) for 30 minutes prior to intracardiac injection of $1x10^6$cells/mouse compared to IgG controls (80). Another report suggests that β1 integrin is important in the initiation of extravasation of prostate cancer cells into the bone such that PC3 cells show an approximate 60% reduction in binding to human bone marrow endothelial cells (BMECs) when pre-incubated with monoclonal antibodies against β1 integrin relative to IgG controls although there are confounding results in the literature as another work reports β1 integrin has no effect in this process (114, 115). Although our lab

has previously shown there is a depletion of β1 integrin in sh 2-10 cells compared to control RSC5 cells and these cells show differential 3D growth and invasion through BME, the clonal nature of these cells may still modulate their inherent capacity to colonize the bone. Otherwise, the results in hand are likely due to technicalities in performing intracardiac injections.

In addition to integrins, there are several other surface molecules present on PC3 cells which may also be involved in extravasation and colonization of the bone by cancer cells that if differentially expressed may contribute to the bone colonizing capacity of these sub-clonally derived cells. The binding of PC3 cells to human BMECs has been shown to be significantly disrupted by pre-incubation of PC3 cells with antibodies to an array of surface molecules including Gelactin 3, VCAM, CD11a, CD18 and LFA 1 (115). Any significant differential expression in these surface proteins on the clonally derived cell lines may impact their efficiency to initiate extravasation into the bone given their ability to modulate cell binding to the endothelium. Traversing the basement membrane after invading through the endothelium is a subsequent step in the process of extravasation into tissues (116). The chemokine SDF-1, expressed by human osteoblasts, has been shown to enhance invasion through BME via binding with the SDF-1 receptor, CXCR4 expressed by both PC3 and the bone metastatic LNCaP-derived, C4-2B prostate cancer cell lines (117). Therefore differential expression of particular chemokine receptors may be another factor mediating differential bone colonization observed between the two cell lines, RSC5 and Sh 2-10 as mechanisms that drive invasion may be important in establishing cancer cell growth in secondary sites (118). Finally, there are other collagen I receptors that may modulate the growth of these cells in the bone that may be variably expressed between these two clonally

derived cells lines. The expression of receptors such as discoidin domain receptors (DDRs) 1 and 2 which have been shown to play a role in establishing bone metastasis in prostate cancer (119), if variably expressed between subclones, may explain the lack of significant difference between observed bone colonization. The expression of other collagen I as well as adhesion and chemokine receptors requires investigation to ensure the differences observed between these cell lines are attributable solely to the status of β1 integrin expression and not due to expression patterns of other receptors that mediate bone metastasis.

It is also possible that the sensitivity of technical precision during intracardiac injection may have led to accidental inoculation of cells to the lungs instead of administration systemically resulting in colonization of the lung and the subsequent removal of these mice from the study. A quality control approach to overcome these technical variables may be to use bioluminescent labeled PC3 cells for imaging immediately after injection to ensure systemic delivery. This approach will confidently ensure the phenotypes observed are the result of biological differences between cell lines and not due to technical shortcomings. Given the data, it is impossible to draw conclusions about the function of β1 integrin to mediate bone colonization of prostate cancer cells following intracardiac injection without the suggested quality assurance.

4.2 Loss of β1 integrin may induce SPARC protein expression

Given the role of β1 integrin to modulate prostate cancer phenotypes in the literature (68, 79, 80, 97) and in previous work conducted in our lab (81), we sought to investigate new mechanisms underlying the role of β1 integrin to modulate 3D growth and migration *in vitro.* β1 integrin expression is increased in poorly differentiated prostate cancers (76) and has an established role as a mediator of cell migration, growth and survival of prostate cancer cell

lines *in vitro* (68, 79-81). In cancer cells derived from pancreatic and head and neck carcinomas, β1 integrin has particularly been shown to elicit phenotypes such as migration and resistance to radiation particularly through the JNK pathway (71, 72, 120, 121). In poorly differentiated prostate cancers there has been an observed decrease in the matricellular protein SPARC (90). Given its downregulation in prostate cancer it comes to no surprise that SPARC has been shown to mediate inhibition of migratory and growth phenotypes in prostate cancer cells lines *in vitro* (47, 90). Interestingly, SPARC has previously been shown to be repressed by c-jun, a key transcription factor in the JNK pathway (74). As such, we sought to evaluate the potential role of β1 integrin in suppressing SPARC protein expression through the activity of JNK. Our lab had chosen to use the prostate cancer cell line PC3 *in vitro* as it was derived from a human bone metastasis and is known to colonize the bone efficiently in *in vivo* (111) and is thus a good model cell line to investigate potential mechanisms by which bone colonization occurs in prostate cancer. Additionally, this cell line expresses high levels of β1 integrin in the constitutively active conformation as depicted by binding of the conformation sensitive mAb 9EG7 and high basal FAK activity (80) and also expresses exceptionally low basal levels of SPARC protein (90). PC3 cells also represent a poorly differentiated cancer cell line characterized by a less organized and mesenchymal phenotype (122, 123). Therefore it follows that the expression pattern of β1 integrin and SPARC in PC3 cells represents the expression pattern of these two proteins independently observed in more poorly differentiated tumors (76, 90). Using an siRNA mediated approach, our lab has uncovered a novel role for the β1 integrin subunit in PC3 cells; that is, loss of this protein results in a substantial increase of SPARC protein

expression suggesting that β1 integrin may function to suppress matricellular SPARC in this cell line.

Regulation of the ECM protein SPARC is consistent with reports in the literature that β1 integrin also regulates the expression of other ECM proteins such as collagens (99) and other matricellular proteins (68, 70). Moreover, one work in the literature particularly supports the regulation of SPARC by integrins although this work identified β4 integrin as a promoter of SPARC expression (69) whereas our findings suggest β1 integrin as a repressor. Using MDA-MB-435 breast cancer cells that endogenously lack β4 integrin, it was shown that upon transfecting this subunit into the cell line, there was a significant increase in SPARC protein expression. The mechanism by which β4 integrin induces SPARC protein in these breast cancer cells was attributed to the resultant decrease in miR-29a expression, a microRNA that targets SPARC mRNA for degradation (69). Although this work identified β4 integrin as a promoter of SPARC protein expression, their findings in combination with the findings in our lab contributes to a greater perspective wherein integrins across multiple cancer types may function to regulate SPARC protein expression.

As previously mentioned, β1 integrin has been shown to mediate cancer cell phenotypes through the activity of JNK (71, 72, 120, 121). In our work we have provided evidence that PC3 cells depleted of β1 integrin show enhanced SPARC expression. Considering the suggested role for c-jun in the process of SPARC suppression (74), it was intuitive to assess the potential role of c-jun and its activator JNK downstream of β1 integrin in mediating the suppression of SPARC in our cell line. JNK proteins arise from three genes, *jnk1, jnk2* and *jnk3*. *Jnk1* and *jnk2* are ubiquitously expressed whereas *jnk3* is selectively expressed in the brain and testis (124). *Jnk1* and *jnk2* both contribute to p54 and

p46 isoforms as a product of differential splicing and may elicit different functions (124). Firstly, p54 phosphorylation, an indication of its kinase activity, relative to total JNK1/2 (p54) levels decreased with siRNA mediated depletion of β1 integrin. Conversely, p46 did not show a significant decrease in phosphorylation following β1 integrin depletion. However, we observed that although p46 phosphorylation appeared to decrease in a similar manner upon β1 integrin depletion as p54, analysis by densitometry was confounded by issues surrounding low levels of total p46. Whether these blots show true representations of the total p46/p54 protein levels, or are confounded by the specific antibody to p46/54 used perhaps having stronger affinity for p54 remain to be determined. Other antibodies clones should be employed to conclusively decipher the role of p46 in this model. In wildtype β1 integrin expressing PC3 cells, treatment with the pharmacological inhibitor of JNK (SP600125) reduced JNK phosphorylation levels relative to total JNK and SPARC protein levels were substantially increased compared to DMSO controls. Thus we have begun to characterize the signaling cascade that mediates suppression of SPARC by β1 integrin in PC3 cells by demonstrating that the activity of JNK decreases following depletion of β1 integrin and that inhibition of JNK phosphorylation by SP600125 results in increased SPARC expression. This data establishes an indirect link whereby JNK activity downstream of β1 integrin may mediate suppression of SPARC in PC3 cells. To our knowledge, there are no reports in the literature whereby loss of JNK activity increases SPARC protein expression.

To further characterize the suppression of SPARC by β1 integrin with regards to the signaling pathway downstream of JNK, we must consider the work by Mettouchi et al. (74). In line with our observations, it has been shown that direct activation of c-jun represses

SPARC expression in rat embryonic fibroblasts (74). Treatment with TPA, a reagent that directly activates c-jun, took between 2 to 6 hours to elicit a decrease in SPARC mRNA expression and a maximal suppression occurred at 24 hours. Additionally, when the SPARC promoter was transfected into FR3T3 cells or a sub-clone stably overexpressing c-jun, FRcJ-4 there was no change in SPARC promoter activity depicted by a chloramphenicol acetyltransferase (CAT) reporter although FRcJ-4 exhibited a significant decrease in basal SPARC mRNA expression relative to FR3T3 (74). Moreover, wildtype FR3T3 cells display a decrease in SPARC mRNA expression when co-cultured with c-jun-overexpressing FRcJ-4 cells (74). These findings taken together suggest that the suppression of SPARC in rat embryonic fibroblasts overexpressing c-jun occurs through a post-transcriptional modification and may be attributable to a secreted factor and we have not ruled out this possibility in our current work. Recreating their experiments with an assessment of SPARC protein expression whereby wildtype PC3 cells are treated with conditioned media from β1 integrin depleted cells would address the potential role of a secreted factor in our analysis. Interestingly, miR-29a, the same microRNA shown to be repressed by β4 integrin in breast cancer cells and which inhibits SPARC expression (69) is highly expressed in PC3 cells relative to other prostate cancer cell lines (125). Coincidently, microRNA are small, non-coding RNA that function intracellularly but have also been shown to be secreted and elicit a suppressive effect on protein expression through post-transcriptional degradation of target mRNA (126). Cancer cells have been shown to secrete MicroRNA's (including miR-29a) within a couple of hours following stimulation *in vitro* (127). Very interestingly, the repressive effects of c-jun on SPARC mRNA expression occurs between 2 and 6 hours following c-jun activation (74). Thus, the highly expressed mir-29a in PC3 cells follows the

kinetics by which c-jun suppresses SPARC in rat embryonic fibroblasts and this putative mechanism bypasses the SPARC promoter and may represent the elusive secreted factor attributed to the repressive function of c-jun (74). Therefore, mir-29a is a promising candidate in characterizing the key players downstream of β1 integrin and JNK in mediating suppression of SPARC in PC3 cells and warrants further investigation.

There are confounding data in the literature for the role of jun proteins in SPARC regulation (86, 92, 128). In MCF7 breast cancer cells, c-jun overexpression led to an increase in SPARC mRNA expression, although direct binding to the promoter was dispensable as evaluated by direct mutagenesis of the SPARC promoter to elicit more or less binding of AP-1 with no subsequent change in promoter activity (86). Instead overexpression of c-jun elicited an increase in SPARC promoter activity indirectly through a mechanism involving SP1/3 transcription factor binding (86). This is contrary to the work of Mettouchi et al. (74) wherein c-jun overexpressing cells did not show any modulation in SPARC promoter activity. Moreover, v-jun, the viral counterpart of c-jun similarly expresses a transactivating domain at its NH2 terminus and a DNA-binding and dimerization bZip domain in the C-terminal domain, and also forms homo- and heterodimers with other proteins of the AP-1 or ATF families (129). V-jun has also been shown to repress SPARC activity involving interactions with SP1/3 transcription factors (92, 128). Finally JunB may be an attractive target because it has been shown to repress transcription as part of the AP-1 complex (130). Considering the aforementioned confounding data of c-jun in either promoting or repressing SPARC expression, and the opposing effects of c-jun and v-jun, c-jun must be directly targeted in PC3 cells to delineate the involvement of this transcription factor when further characterizing the signaling cascade accountable for our observations.

Moreover, given the conflicting evidence regarding c-jun activity at the SPARC promoter, SPARC promoter activity in response to β1 integrin depletion must also be assessed when characterizing the downstream signaling responsible for SPARC suppression in PC3 cells. To test the promoter activity of SPARC in β1 integrin depleted cells, a luciferase reporter construct with the SPARC promoter sequence may be employed and the luciferase activity compared to control cells that also express the luciferase reporter construct. Moreover these results may be used in tandem with mRNA analysis via RT-qPCR to assess if post-transcriptional regulation is affecting SPARC mRNA levels following activity at the promoter level. If necessary, DNA affinity chromatography-pulldown with subsequent proteomic techniques such as mass spectometry to analyze the DNA binding proteins may be employed to assess which DNA-binding proteins contribute to the suppression of SPARC at the promoter level (131).

4.3. Loss of β1 integrin impedes growth in BME by induction of SPARC protein

In vitro 3D growth has been shown to recapitulate corresponding tumor associated phenotypes observed *in vivo* such as cellular growth and organization as well as invasive capacity and is thus a good model to use when investigating the role of particular proteins in mediating tumorigenesis (132, 133). Reports in the literature have demonstrated that β1 integrin plays an intricate role in mediating 3D growth in both prostate (68) and head and neck squamous cell carcinoma (71) cells. Additionally, previous work in our lab using stably transfected cells has demonstrated a role for β1 integrin in supporting the growth of PC3 sub-clones in BME (81). In the current work, we have established that SPARC protein is overexpressed in PC3 cells with forced depletion of β1 integrin. Therefore, we sought to assess the role of SPARC expression as a mediator of diminished 3D growth in β1 integrin

depleted PC3 cells. Using an siRNA mediated approach, we confirmed the previously established role of β1 integrin in mediating 3D growth in BME as loss of β1 integrin resulted in fewer colonies. Moreover, using a combined siRNA approach whereby β1 integrin and SPARC were concomitantly depleted, we observed a rescue in the 3D growth capacity of PC3 cells compared to β1 integrin depletion alone. Therefore, this data suggests a functional link whereby one role of β1 integrin is to suppress matricellular SPARC protein in order to promote 3D growth of PC3 cells in BME.

In support of our data, MDA-MB-231 cells with adenoviral-mediated expression of SPARC show a significant and dose-dependent reduction in stellate colony formation when grown on BME compared to control counterparts (87). Moreover, in human hepatocellular carcinoma cells HepG2, SPARC overexpression decreased the growth of 3D spheroids *in vitro* (85). The effects of SPARC seem to be context dependent; however, as with HepG2 cells, exogenous treatment with recombinant human SPARC did not decrease the 3D growth of spheroids and thus the function of SPARC in 3D growth relied on endogenous expression (85).

Interestingly, it appears that the function of SPARC in β1 integrin depleted PC3 cells is also context dependent. Concomitant knockdown of both β1 integrin and SPARC did not rescue the loss of colony growth in a preliminary soft agarose assay attained with β1 integrin depletion alone (Supplemental Fig. 1). BME and soft agarose assays are similar in that they both determine the capacity of cells to form colonies from a single cell origin; however, they differ in that soft agarose only contains ECM components from supplemented serum such as fibronectin (134) whereas BME is a gelatinous basement membrane composed of many ECM components organized into a structural conformation such as laminin, collagen IV and

entactin (135). ECM components such as collagen and laminin are known to interact with SPARC and SPARC has been shown to influence ECM assembly, cellular-ECM interactions and tumorigenesis via these interactions (136). Moreover, SPARC is known to bind collagen at sites that are also bound by distinct integrin heterodimers (136) suggesting SPARC may inhibit the adhesion of surface receptors to the surrounding ECM. Thus, the basement membrane components of BME are likely important in the context-dependent manner of SPARC mediated growth inhibition in PC3 cells with diminished β1 integrin expression.

Given that SPARC may act as an anti-proliferative molecule (47), loss of β1 integrin and subsequent induction of SPARC in modulating the cell cycle was assessed in PC3 cells. None of the cell cycle regulators assessed, cyclin D1, PCNA or p21 were modulated in the absence of β1 integrin or the absence of both β1 integrin and SPARC. Of course, since the assessment of cell cycle regulation was conducted in 2D monolayer cultures, these experiments must be repeated in the context of 3D growth in BME as 3D cultures have been shown to elicit different, context-dependent signaling (137).

Another potential route of study may include an assessment of anoikis in SPARC-mediated inhibition of 3D growth in β1 integrin depleted PC3 cells. Given that concomitant depletion of β1 integrin and SPARC rescues 3D growth in BME, the presence of SPARC in β1 integrin depleted cells may impede ECM-cellular interactions that may otherwise rescue the cell from anoikis in the absence of β1 integrin. A tentative description of potential mechanisms whereby β1 integrin depletion mediates increased SPARC expression with subsequent inhibition of 3D growth is depicted in Figure 11.

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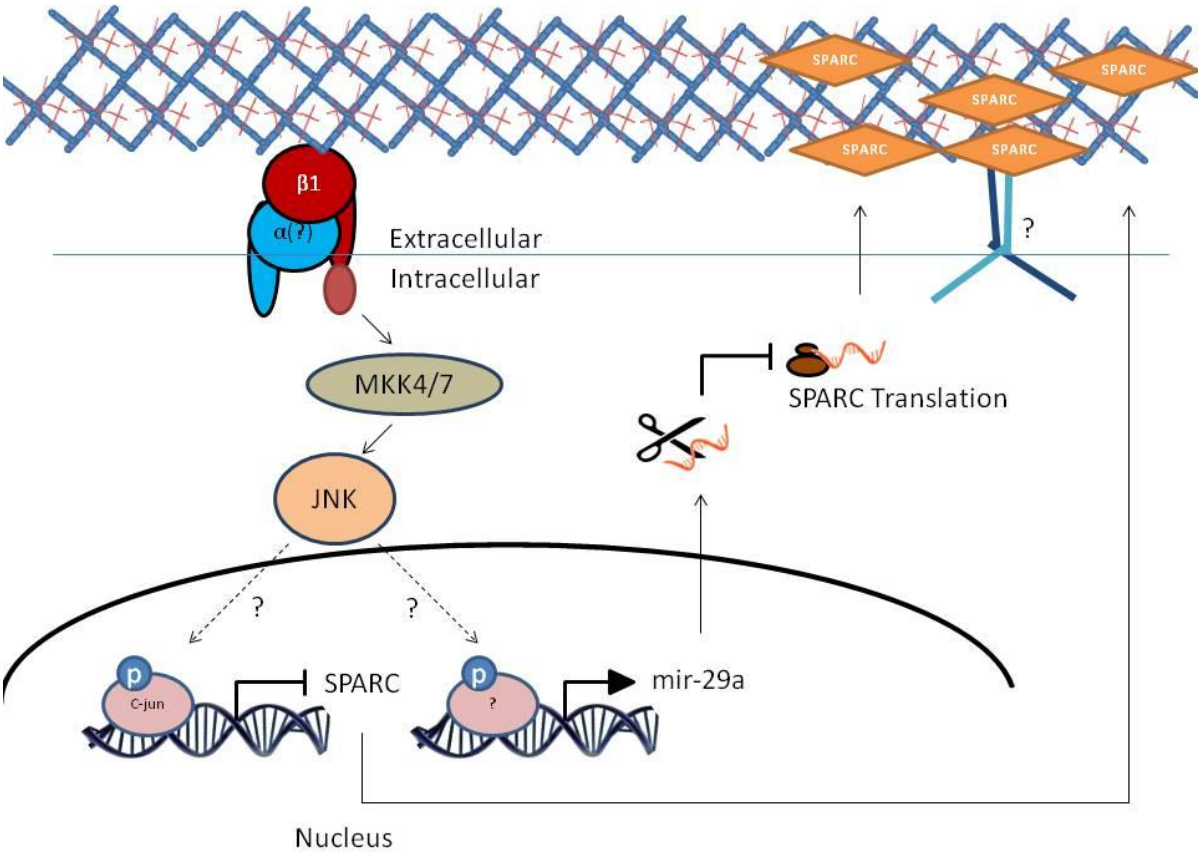
SPARC
SPARC
SPARC
SPARC
SPARC
β1
α(?)
Extracellular
Intracellular
?
MKK4/7
SPARC Translation
JNK
?
?
p
C-jun
SPARC
p
?
mir-29a
Nucleus

Figure 11. Tentative mechanism outlining the potential downstream mediators of β1 integrin directed suppression of SPARC through JNK.
The literature suggests c-jun represses SPARC (74); however, it is unknown in PC3 cells if c-jun acts to directly repress SPARC at the promoter level. Secondly, mir-29a is known to be highly expressed in PC3 cells and is also known to target SPARC mRNA for degradation (69, 125); however, it is unknown whether or not JNK activity promotes mir-29a expression facilitating suppression of SPARC at the mRNA level. These candidate mechanisms may play a role in our observation that β1 integrin expression ultimately leads to the suppression of SPARC protein levels. Phenotypically, in the absence of β1 integrin, we have shown that SPARC expression is increased and mediates loss of 3D growth in BME. Therefore, since SPARC is known to bind ECM proteins at regions also bound by their surface receptors (136), it is possible that SPARC expression in β1 integrin-depleted PC3 cells impedes ligation of an unknown surface receptor to the ECM that would otherwise rescue a loss of 3D growth with concomitant depletion of SPARC expression.

4.4. Loss of β1 integrin decreases collagen I enhanced migration although increased SPARC expression does not mediate this effect

Collagen I is the major ligand for β1 integrin and it has been shown in the literature that β1 integrin mediates migratory phenotypes induced by collagen I in prostate cancer. For instance, Hall et al. (79) have shown that collagen I is capable of enhancing migration *in vitro* in a β1 integrin dependent manner (79). In our work, collagen I was capable of enhancing migration of PC3 cells compared to PDL coated plates. Moreover, knockdown of β1 integrin was capable of inhibiting this enhanced migration as migration on collagen I was decreased but basal migration on PDL was unaffected. This is consistent with reports in the literature whereby β1 integrin mediates migration on collagen I in human pancreatic cancer (120) and human osteosarcoma (138) cells. The potential limitation of using a scratch-wound assay is the potential for treatment to affect the proliferation rates of cells. Given that our monolayer cultures showed no difference in proliferation markers with knockdown of β1 integrin and SPARC and that our assays were measured prior to the doubling time of these cells (33 hours) (139), we are confident these limitations do not confound our data. Given SPARC is a known inhibitor of prostate cancer cell migration *in vitro* (47, 90), we sought to address whether the loss of migration in β1 integrin depleted cells was mediated by resultant SPARC expression.

Utilizing β1 integrin depleted PC3 cells by means of β1 integrin targeted siRNA, concomitant transfection with either NT2 or siSPARC did not modulate migration on either PDL or collagen I. If SPARC expression was responsible for inhibition of migration on collagen I in β1 integrin depleted PC3 cells, the treatment group with siSPARC would have displayed enhanced migration relative to control NT2 treated-β1 integrin-depleted cells.

Although surprising, these reports are consistent with the literature in that SPARC has been shown to inhibit prostate cancer cell migration through direct binding to β1 integrin; therefore, SPARC would have no effect in β1 integrin-depleted cells (90). Moreover, establishment and subsequent turnover of integrin-mediated focal adhesion complexes are key processes during cellular migration(53). Focal adhesions are highly complex and consist of about 150 different molecules including integrins, proteins linked to actin (talin, vinculin), integrin mediated signaling proteins (paxillin, FAK, ILK) and other signaling molecules such as the Rho family of GTPases (53). For example, FAK downstream of integrin signaling (57), is critical for focal adhesion turnover (140) and thus play a central role in migration (141). Thus it is likely that a mechanism responsible for directing focal adhesion dynamics mediates the observation that β1 integrin depleted cells show reduced collagen I-dependent migration.

Recombinant human SPARC was used to explore the inhibitory potential of SPARC on PC3 cells expressing β1 integrin on a collagen I substrate. Consistent with our previous findings, rhSPARC does not significantly inhibit migration of PC3 cells on collagen I. Interestingly, this observation is consistent with the endogenous versus exogenous dependent nature of SPARC-based phenotypes. One such report occurs wherein endogenous expression of SPARC inhibits 3D growth of hepatocellular carcinoma cells but this is not recapitulated with the exogenous administration of recombinant SPARC (85). On the other hand it has been demonstrated that exogenous SPARC is capable of inhibiting migration of prostate cancer cell lines *in vitro* through direct ligation of β1 integrin on non-ECM coated culture dishes (90). The context of collagen I-enhanced migration however must be considered in the current work. DDRs 1 and 2 are both expressed in PC3 cells and constitute two non-

integrin receptors for collagen I (142). DDR1 has been shown to mediate collagen-I enhanced migration of smooth muscle cells (143) and DDR2 has been shown to mediate invasion of cells within collagen I gels (144). One explanation may be that these receptors are compensating for SPARC inhibition of β1 integrin mediated migration on collagen I. However, SPARC has been shown to reduce DDR1 binding to collagen I (145) and employs the same binding motif as DDR2 in collagens I-III (146). With this data it seems more likely that the presence of SPARC would additionally inhibit these potential 'compensatory' receptors. Therefore, considering SPARC may modulate the interactions between collagen I and three separate collagen I receptors (β1 integrin, DDR1 and 2), co-plating rhSPARC with collagen I displays a trend towards inhibition of PC3 cell migration compared to collagen I + vehicle (where p=0.06) (Figure 10). Slight tweaks in technical procedures may elicit a more intuitive result. Some optimization steps may include pre-incubating the cells with rhSPARC or using more rhSPARC in the current system to account for the abundance of binding targets (Collagen I, Integrins, DDRs) prior to plating.

4.5 Summary

In this work we sought to uncover novel findings by which β1 integrin mediates 3D growth and migration *in vitro* as well as tumorigenicity *in vivo*. Although the functional role of β1 integrin in mediating bone colonization *in vivo* remains inconclusive, slight refinements to our experimental procedures will undoubtedly provide additional insight into the role of β1 integrin in our model. *In vitro*, we have discovered herein that β1 integrin plays a primary role in mediating 3D growth in BME by suppressing the protein expression of matricellular SPARC. Moreover we have provided an indirect link whereby β1 integrin mediated suppression of SPARC may occur through a JNK-dependent mechanism. Given refinement

to the technical procedure of intracardiac injections, a tumorigenic role for β1 integrin mediated by SPARC suppression may be unearthed *in vivo*. Consistencies between our observations *in vitro* and those seen in the literature present SPARC as a strong candidate for mediating anti-tumoral effects observed with loss of β1 integrin and the signaling mechanisms responsible for its expression warrant further investigation.

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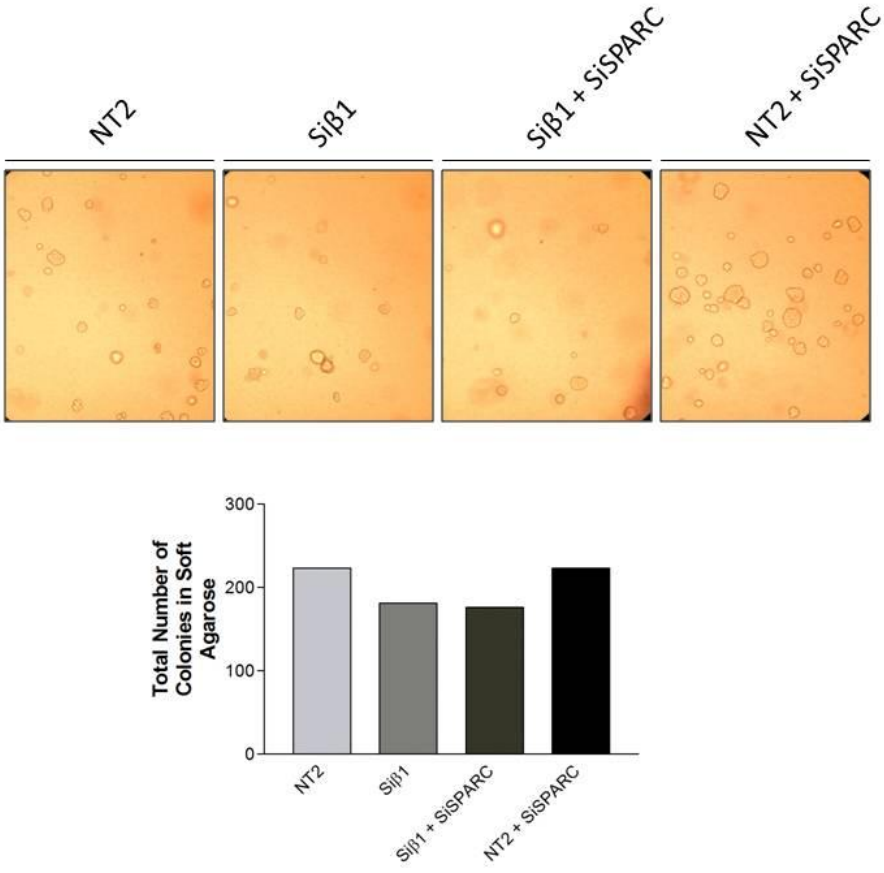
APPENDIX A


NT2
Siβ1
Siβ1 + SiSPARC
NT2 + SiSPARC
Total Number of Colonies in Soft Agarose
300
200
100
0
NT2
Siβ1
Siβ1 + SiSPARC
NT2 + SiSPARC

Suppl. Fig. 1. Loss of β1 integrin results in loss of 3D growth in soft agarose but this phenotype was not rescued with concomitant loss of SPARC in preliminary experiments.
PC3 cells were transfected for 72h to allow for sufficient knockdown of both β1 integrin and SPARC protein levels with 100nM NT2, 50nM NT2 + 50nM Siβ1, 50nM Siβ1 + 50nM siSPARC or 50nM NT2 + 50nM siSPARC. Following 72h, cells were seeded into a 1% soft agarose base in a 0.5% soft agarose overlay. Colonies were photographed and quantified on day 4. This data is graphed from one biological replicate.