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B_1 Integrin Modulates the Anchorage Independent Growth, Invasion and Migration of Prostate Cancer Cell Line PC3

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β_1 INTEGRIN MODULATES THE ANCHORAGE INDEPENDENT GROWTH, INVASION AND MIGRATION OF PROSTATE CANCER CELL LINE PC3

By

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ABSTRACT

Integrins provide mechanical continuity between the extra- and intracellular environments. Upon binding to extracellular matrix (ECM), integrins interact with downstream effectors to promote cell adhesion, proliferation and migration. In contrast to its well-known role in adhesion, we have found that β_1 integrin is also involved in the anchorage independent (AI) growth of prostate tumor cells. In concurrence with our previous findings, stable depletion of β_1 integrin in PC3 cells using an shRNA approach resulted in the complete inability of cells to form colonies in soft agarose, while adherent monolayer growth remained unaffected. In order to address the mechanism for β_1 integrin dependent AI growth, we examined the expression and localization of β-catenin and E-cadherin, both of which are known to modulate AI growth. Migration and invasion through matrigel were found to be impeded by depletion of β_1 integrin. A qPCR array identified potential downstream targets that could be altered as a result of β_1 integrin depletion, these were further examined in all cell lines and it was found that TGFβ1 is reduced in shITGB1 expressing cells. Taken together, these findings suggest a role for both β_1 integrin in driving anchorage independent growth and invasion of cancer cells.

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ABBREVIATIONS:

α-MEM – α-Modified Eagle Medium
AI – Anchorage Independent
Amp – Ampicilin
bFGF – basic Fibroblast Growth Factor
BM – Basement Membrane
BSA – Bovine Serum Albumin
CDH1 – E-cadherin
DMEM – Dulbecco's Modified Eagle Medium
ECM – Extracellular Matrix
EGF – Epidermal Growth Factor
EGFR – Epidermal Growth Factor Receptor
EMT – Epithelial to Mesenchymal Transition
FBN – Fibronectin
FBS – Fetal Bovine Serum
FAK – Focal Adhesion Kinase
GFP – Green Fluorescent Protein
GFR – Growth Factor Receptor
GSKβ – Glycogen Synthase Kinase β
H & E – Hematoxylin and Eosin
HBSS – Hank's Buffered Saline Solution
HRP – Horseradish Peroxidase
HS – Horse Serum
IB – Immunoblotting (or Immunoblot)
IGF – Insulin-like Growth Factor
IGFR – Insulin-like Growth Factor Receptor
ILK – Integrin Linked Kinase
IP - Immunoprecipitation
ITGB1 – Integrin β_1
LB – Luria Bertani [Medium]
LMP – Low Melting Point
MAPK – Mitogen Activated Protein Kinase
MMP – Matrix Metalloproteinase
NFκB – Nuclear Factor κB
nSCLC – non-Small Cell Lung Cancer
PBS – Phosphate Buffered Saline
PDGF – Platelet Derived Growth Factor
PI3K – Phosphatidylinositol 3 Kinase
PIP2 – Phosphatidylinositol 4,5-biphosphate
PIP3 – Phosphatidylinositol 3,4,5-triphosphate
PTEN – Phosphatase and Tensin Homolog
RTK – Receptor Tyrosine Kinase
shRNA – short hairpin RNA
siRNA – small interfering RNA
Ten-C – Tenascin-C

TBST – Tris-buffered Saline with Tween
TGFβ1 – Transforming Growth Factor β1
TIMP2 – Tissue Inhibitor of Metalloproteinases 2
TNF-α – Tumor Necrosis Factor α
VTN – Vitronectin
wtPC3 – wild-type PC3

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1. INTRODUCTION

1.1 - Preamble

This year, nearly 24,600 men will be diagnosed with prostate cancer in Canada, of which it is estimated 4,300 will succumb to the disease. Prostate cancer remains the most commonly diagnosed cancer in men representing about 27% of all cancer diagnoses. (1) The majority of deaths from prostate cancer occur as a result of invasive and metastatic disease: the dissemination of the primary tumor to secondary sites elsewhere in the body. One of the primary sites of prostate cancer metastasis is to the bone, with skeletal metastasis occurring in approximately 90% of patients with metastatic prostate carcinoma. (2) Bone metastases are one of the most clinically relevant aspects of prostate cancer progression and are indicative of poor prognosis as they are difficult to abolish. (3) It is believed that the specialized collagen rich microenvironment of the bone contributes to the preferential location of prostate tumor cells within this organ. The complement of integrin cell surface receptors, which bind extracellular matrix (ECM) proteins such as collagen, that are expressed by cancer cells is believed to aid in this process. β_1 integrin is upregulated in prostate cancer and although β_1 integrin expression has been examined in primary prostate tumors, the role it plays in the growth and metastatic progression of the disease has not been thoroughly examined. (4, 5) Through various *in vitro* experiments, we assessed the contribution of β_1 integrin in the anchorage independent, metastatic and invasive properties of prostate cancer cells.

1.2 The Extracellular Matrix in Cancer

1.2.1 The Normal Extracellular Matrix: Composition and Function

The extracellular matrix can be defined as the proteinaceous stromal and basal components that surround cells and tissues. Comprised mostly of interstitial matrix and basement membrane, it is a dynamic scaffold structure that acts not only as a structural barrier but also as a ligand for cellular receptors. (6, 7) The ECM has been implicated in such processes as development and growth, differentiation, modulating cell viability and the maintenance of tissue architecture. (8-10) The ECM plays a vital role in normal tissue homeostasis and resistance to injury, however it also has an established role in the advancement of a number of diseases. For example, alteration of the expression of ECM components often occurs in disease states such as atherosclerosis and cardiac dysfunction. (11-13) The extracellular matrix can also sequester growth factors that become released upon matrix cleavage. Bone ECM remodeling, for example, can release mitogens such as insulin-like growth factor (IGF) and basic fibroblast growth factor (bFGF). (14) The ECM itself also becomes biologically active when cleaved as ECM fragments partake in signaling events that their full-length counterparts do not. One such example is endostatin, an angiogenesis inhibitor that is a c-terminal fragment of collagen XVIII. (15)

The primary component of the extracellular matrix is the collagen scaffold. Normal ECM also includes laminin and tenascin-C as well as proteoglycans that adhere to the matrix. (6) Signaling through the ECM occurs primarily through integrins, the most important family of matrix receptors. Integrins have the ability to use most ECM proteins as

ligands and integrin signaling can lead to the remodeling of the ECM and therefore, both have an established role in the progression of cancer. (8, 16)

1.2.2 Changes in the Extracellular Matrix in Prostate Carcinogenesis

The ECM that surrounds cancer cells is markedly different than that of normal healthy tissue. Changes in the composition of the ECM, including extensive breakdown and remodeling of the matrix, have been shown to play an important role in the progression of cancer, including prostate cancer. (6, 7, 17) Altered ECM has been shown to contribute to cancer progression by enhancing pro-survival and anti-apoptotic signals in cancer cells and multi-drug resistance in both cancer and endothelial cells. (8, 18, 19)

The ECM components of the basement membrane (BM) act as a structural barrier, maintaining the architecture and defining the tissue of the prostate. The BM is the final barrier that cancer cellsmust cross to breach the confines of the prostate during the invasive progression of the disease. (20) The major constituents of the BM in the tissues of the prostate, as with most epithelial sheets, are: laminin, tenascin-c, collagen type IV (as well as minor amounts of other collagen subtypes), entactin, perlecan and nidogen. (3, 6, 20-23) Stromal expression of vitronectin and fibronectin has also been reported in normal prostate tissues, although these ECM molecules are not often detected in the epithelium. (22, 24)

During the advancement of prostate cancer, the expression patterns of these basal lamina components and ECM proteins often become dysregulated; some become increased or decreased, while others may be lost all together. BM matrices are broken down in favor

of matrices that promote angiogenesis, invasion and anchorage independent cell growth. *In vivo* studies on prostate cancer have shown a decreased expression of collagen VII and collagen IV subchains α5 and α6, while expression of subchains α1, α2 and α3 persisted, during tumor progression. (20, 22, 23, 25) Periglandular expression of laminin-γ2 (or laminin 5) and tenascin-C also become reduced with the progression of prostate cancer and discontinuities in their expression patterns indicate interruptions in the composition of the BM. (25, 26) This was accompanied by a reported decrease in the expression of laminin integrin receptors $\alpha_6\beta_4$ and $\alpha_6\beta_1$. (22, 25) Interestingly, although expression of laminin subchain 1/3 is also decreased with the malignant progression of prostate cancer, expression of subchain 10/11 persists. (20, 27) Laminin 10/11 is the major ligand for integrins $\alpha_3\beta_1$ and $\alpha_6\beta_1$, the laminin binding integrins whose expression is not lost in prostate cancer, suggesting that basal lamina surrounding cancer is laminin 10 rich. (20, 27) Fibronectin is expressed in prostatic stroma in both normal tissues and malignancies, (24) and *in vitro* studies have shown that prostate cancer cells which are derived from metastatic bone lesions, secrete high levels of fibronectin and laminin. (24, 28, 29) Certain fibronectin isoforms, (e.g. the ED-B segment) are overexpressed in prostate cancer compared to normal prostate tissue. (24) Furthermore, the structure of fibronectin has also been shown to change during tumorigenesis, becoming fragmented and increasingly glycosylated in prostate cancer compared to benign prostate hyperplasia. (30)

The ability of tumor cells to respond to altered ECM microenvironments likely contributes to their metastatic capabilities. While the ECM of metastatic sites such as the bone is primarily composed of collagen I (95%), it also consists of many non-collagenous molecules such as fibronectin, vitronectin, thrombospondin and bone specific proteins such

as osteocalcin and osteonectin. (14) It is the composition of this microenvironment, along with the complement of ECM receptors on the tumor cells themselves that can make the bone an enticing location for prostate cancer cells to metastasize. (3)

1.2.3 Extracellular Matrix Remodeling in Cancer

Tumor cells are self-sufficient with regards to growth signals, stimulating surrounding stromal cells to produce matrices that aid in the development of neoplastic lesions. In concert, tumors cells direct the remodeling of the ECM which can increase integrin receptor signaling. (8, 31, 32) In the early and latent stages of metastatic progression, cancer cells secrete matrix metalloproteinases (MMPs), these are secreted proteins that have the specific role of cleavage and degradation of ECM components, including basement membranes. There are at least 20 known MMP family members, (33) and all are zinc-dependent endopeptidases able to cleave ECM substrates including several collagens, fibronectin, laminin, gelatin, elastin and proteoglycans. (34, 35) The main categories of MMPs are collagenases, membrane-type MMPs and gelatinases. Although a detailed description of theirstructure, function and specificity is beyond the scope of this project, *Das et al.* provides a comprehensive review. (36)

MMPs are widely expressed in human tissues and are involved in many normal processes including wound repair; however they also play a significant role in pathological processes including chronic ulcers and hematological disorders, among others. (37, 38) Moreover, MMPs are known contributors to metastatic progression of cancer and are often

upregulated with increased tumorigenicity. They have been implicated in normal bone remodeling but also in prostate cancer metastasis to the bone. (39, 40) In prostate cancer, malignant progression is marked by increased protein expression of MMP9 whereas tissue inhibitor of MMP1 (TIMP1), an MMP antagonist, has been shown to be downregulated, indicating a proteolytic imbalance. (3, 20, 41) Studies in primary cultures suggest that an increased expression of transforming growth factor β (TGFβ) contributes to this increased expression of MMP9. (42) Interestingly, MMP9 is transcriptionally regulated by ILK via the AP-1 transcription factor and thus establishes a link between integrins and MMP expression. (43) Although MMP2 and MMP9 are considered the major players in prostate cancer progression, it has been shown that several additional MMPs are also upregulated. (44, 45) Membrane type 1 MMP (MT1-MMP or MMP14), for example, which activates pro-MMP2, is expressed in many prostate cancer cell lines including PC3 and has been linked to increased invasion *in vitro*. (46, 47)

From a physiological perspective, cancer cells utilize MMPs to breakdown their surrounding ECM environment, and infiltrate blood vessels to disseminate throughout the body. (3) These cells, however, must theoretically survive in circulation, away from the adherent growth of the primary tumor. This process in itself is crucial to the malignant progression of the disease.

1.3 An Introduction to Integrins

1.3.1 – Integrin receptors: Welcome to the Family

An essential aspect of cellular regulation is the process by which signals are relayed from the plasma membrane into the cell and furthermore to specific intracellular sites. Integrins are the most important family of cell adhesion and extracellular matrix (ECM) receptors. (6, 48) Integrins are heterodimeric transmembrane receptors that provide mechanical continuity between the intra- and extracellular environments. Intracellularly, they bind a cluster of intracellular effector proteins that then link the actin cytoskeleton to the cellular membrane, while simultaneously mediating cell-ECM interactions extracellularly. (49-51) All integrins are non-covalently associated heterodimers and are comprised of one α subunit and one β subunit. Currently, 8 β subunits and 18 α subunits have been identified in humans and together, can assemble into 24 distinct integrins. (Reviewed in (48, 51)) These integrin receptor combinations have different binding specificities for various ECM proteins including collagen, fibronectin, laminin and vitronectin. Through integrin-mediated assembly of cytoskeletal effector proteins, ECM-integrin interactions regulate such cellular processes as proliferation, cell survival and apoptosis, angiogenesis, cell shape and polarity, motility and differentiation. (51-54)

1.3.1.1 – β_1 Integrin Binding Pairs and Ligands

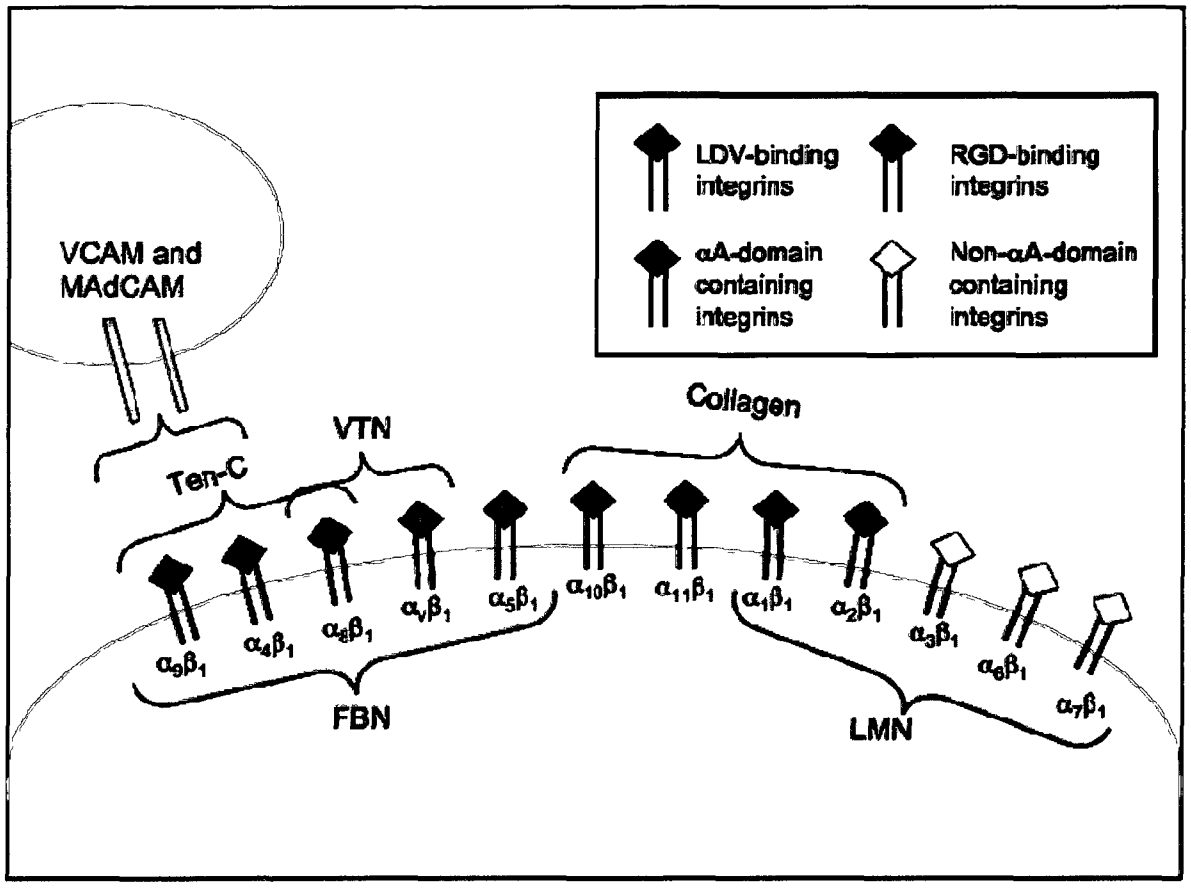
Integrins are the most prevalent ECM receptors and there is a wide range of specific ECM targets that integrin heterodimers can recognize. Each matrix protein is often bound by multiple different integrin receptor pairs. (49) Of the various β subunits, β_1 integrin is by far

the most diverse creating up to 12 different α subunit pairings that act as specific receptors for a broad range of ECM ligands, including some cell surface counter receptors. (51, 55) (Figure 1) β_1 integrin is universally expressed in human tissues with the exception of mature erythrocytes. (52) Of the β_1 containing integrin receptors, one subgroup recognizes fibronectin, namely $\alpha_5\beta_1$, $\alpha_8\beta_1$, and $\alpha_v\beta_1$, the latter two of which also bind vitronectin. These integrins are able to recognize the tripeptide RGD sequence that occurs in fibronectin, which can also be recognized by the α_v subunit in combination with β_3 and β_6 subunits. (48) Related integrin pairs$\alpha_4\beta_1$ and $\alpha_9\beta_1$ also recognize fibronectin, however they additionally bind tenascin-C and the Ig-superfamily of cell surface receptors including VCAM-1 and MAdCAM-1 via the acidic motif LDV domain (which is functionally related to the RGD domain). (48, 51, 55) The laminin binding β_1 subunit-containing receptor family includes integrins $\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_7\beta_1$. This family also includes integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$, which in addition to laminin can also specifically recognize collagen via anαA-domain. They share this domain in common with other collagen binding specific integrins, namely $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$. (48)

1.3.1.2 – β_1 Integrin: Structure and Splice Variants

Integrin subunits each have a large, extracellular domain, a short transmembrane domain and a small intracellular domain that is non-enzymatic but required for proper signaling. (49, 51, 52)β_1 integrin is highly conserved at the amino acid level throughout metazoans – from sponges to humans – especially with regards to the cytoplasmic and

Figure 1: β_1 integrin Ligand Binding Receptor Family. Schematic diagram showing β_1 subunit-containing integrin receptors and their ligand affinities based on distinct interaction motifs. Matrices bound by β_1 integrin-containing receptors include fibronectin (FBN), laminin (LMN), collagen, vitronectin (VTN), tenascin-C (Ten-C) and cellular receptors (VCAM) and (MAdCAM).


LDV-binding integrins
RGD-binding integrins
αA-domain containing integrins
Non-αA-domain containing integrins
VCAM and MAdCAM
Ten-C
VTN
Collagen
α9β1
α4β1
α8β1
αvβ1
α5β1
α10β1
α11β1
α1β1
α2β1
α3β1
α6β1
α7β1
FBN
LMN

transmembrane domains. (56) There are 5 known cytoplasmic variants of the β_1 integrin subunit: β_{1A}, β_{1B}, β_{1C}, β_{1C-2} and β_{1D}. (Reviewed in (52, 57)) Structurally, they all share a common extracellular N-terminal domain and thus have the same ligand specificity,however, they differ in the cytoplasmic domain, where the sequence and length of the domain are modified. (52) This cytoplasmic divergence results in alteration of ligand affinity, integrin receptor localization, downstream activation of signaling pathways and ultimately cellular responses. (52, 57, 58))

Splice variant β_{1A} is commonly referred to as simply β_1 integrin and is essentially the wild-type fully functional variant. In PC3 prostate cancer cells, whereas both β_{1A} and β_{1C} have decreased mRNA levels, β_{1A} has been found to be overexpressed at the protein level due to an approximately 2 fold increase in translation. Upregulated levels of β_{1A} integrin have been linked to increased cell proliferation and contribution to anchorage independent growth of PC3 cells. (57, 59)

In comparison to β_{1A}, the integrin splice variant β_{1B} has a truncated c-terminus, lacking 9 amino acids found in β_{1A}. In addition, the last 12 amino acids are derived from an intronic sequence and are therefore different from β_{1A}. β_{1B} has only been identified in humans and its expression at the protein level shows some tissue specificity as it has only been detected in keratinocytes and hepatocytes. (52, 60) The β_{1B} variant acts as a dominant negative subunit by competing for available α subunits but without localizing to focal adhesions or activating intracellular signaling. (61)

Compared to the wild-type integrin, the cytoplasmic domain of the β_{1C} splice variant has an extended c-terminus. Of the two β_{1C} variants (1 and 2) the difference lies in an inserted 6 amino acid sequence. β_{1C} has been shown to be expressed in non-proliferative,

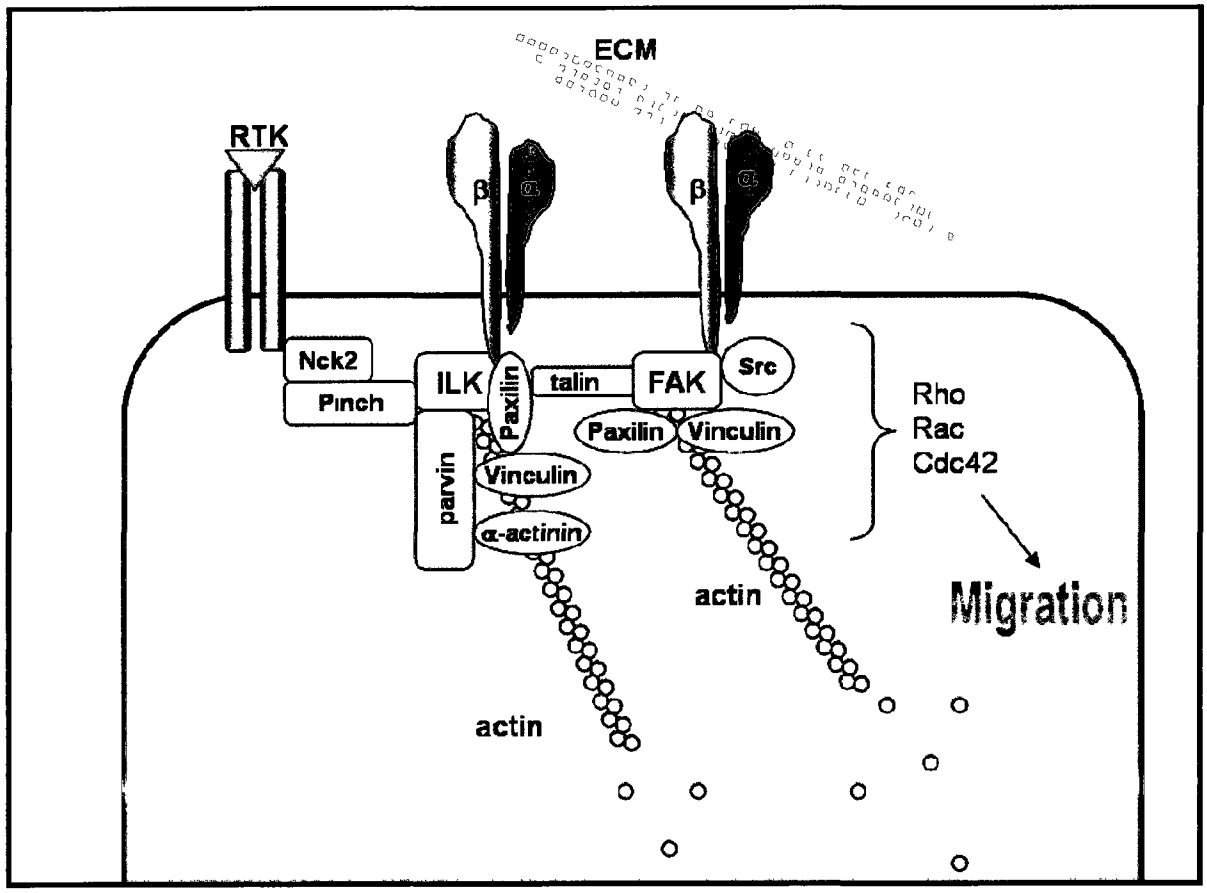
differentiated prostate epithelium *in vivo*. (52, 57, 62)β_{1A} and β_{1C} have been shown to act as a stimulator and repressor of cell proliferation respectively, and β_{1C} prevents proliferation by increasing levels of cell cycle inhibitor $p27^{kip1}$ and $p21^{Cip1}$ as well as the association between $p27^{kip1}$ and cyclin A. (62) While β_{1A} integrin has been shown to be upregulated, β_{1C} is selectively downregulated in prostate cancer and these changes are correlated with poor prognosis. (4, 57, 62)

For its part, β_{1D} shows significant homology to the wild-type β_1 integrin with only two residues that are not conserved. It has restricted expression however, and is found exclusively in cardiac and skeletal muscle in humans. β_{1D} maintains some physiological function in these cell types, although reports detailing that function are conflicting. (52)

1.3.2 – Overview of Integrin Signaling

Integrins are primarily known as the cornerstone of focal adhesions and therefore have important roles in cell adhesion and motility. The β_1 and β_3 integrin subunits are known to localize in focal contacts and to mediate spreading and cytoskeletal rearrangement in normal cells. (58) In focal adhesions, β_1 integrin links to the actin cytoskeleton through a variety of linker proteins, including talin, α-actinin, vinculin and paxilin. (6) (Figure 2) Focal adhesions are crucial in maintaining cell adhesion and the cooperative and reciprocal communication between the components of these adhesions have important roles in cell polarity, proliferation and migration. (49)β_1 integrin also plays a crucial role in development as knock-out mice are embryonic lethal, dying shortly after implantation

Figure 2: β_1 integrin links the extracellular environment to the actin cytoskeleton of cells. Schematic illustration of the structural role of β_1 subunit-containing integrins. Integrins can mediate cell rigidity, adhesion and migration through their interaction with the actin cytoskeleton. Integrin linked kinase (ILK) links integrins to growth factor receptors (GFRs) through its interaction with PINCH and Nck2 while also mediating interactions with the actin cytoskeleton via parvin, paxilin, vinculin and α-actinin. β_1 integrin mediated adhesion to the extracellular matrix (ECM) often relies on the formation of focal adhesion contacts. These contacts contain focal adhesion kinase (FAK) which is believed to interact with the cytoplasmic tail of β_1 integrin through talin and paxilin. Along with vinculin, this complex can regulate changes in the actin cytoskeleton, and through activation of such downstream effectors as Rho, Rac and Cdc42, alters cell migration.


ECM
RTK
β
α
β
α
Nck2
Pinch
ILK
Paxillin
talin
FAK
Src
Paxilin
Vinculin
parvin
Vinculin
α-actinin
Rho
Rac
Cdc42
actin
Migration
actin

during development which is not surprising considering the ubiquitous expression of β_1 integrin. (51, 63) Signaling through integrins is highly complex, yet has been well characterized.

1.3.2.1 – From Ligand Binding to Signal Transmission and Vice-Versa

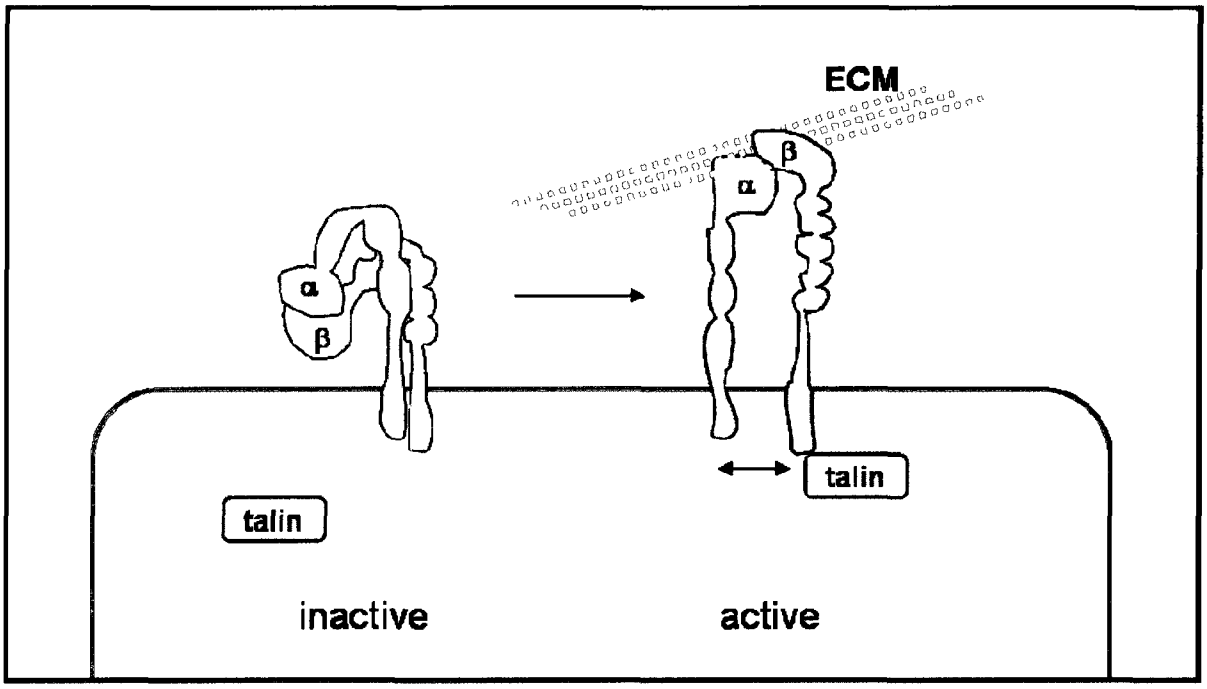
Most signaling and adaptor proteins rely on phosphorylation of target molecules to transmit signals. (64) Integrins however, lack both an actin binding domain and catalytic activity and thus rely on conformational changes to mediate signaling.(51) Integrins are anchored within their microenvironment on the cytoplasmic side of the membrane by effector proteins and extracellularly by matrix proteins or counter-receptors on adjacent cells. Both environments contribute to integrin signaling. Integrins depend on associated intracellular effector proteins to elicit and mediate signals transmitted through the plasma membrane. (55, 65) For outside-in signaling, matrix binding transmits cues intracellularly for cell survival, proliferation and migration. (48) Integrin linked kinase (ILK) and focal adhesion kinase (FAK) are the major integrin binding kinases for signal transmission, they are also involved in bidirectional linkages between the integrin and the cytoskeleton. Their signaling is quite complex and since there is no integrin-specific signaling mechanism, the relative contribution of integrins to the activation of their downstream pathways, like AKT for example, also depends on the activation status of all other signaling pathways regulating those molecules.

Integrins can also partake in a dynamic signaling process referred to as inside-out signaling, whereby intracellularly generated integrin conformational changes regulate ECM ligand binding affinity. (66, 67) These alterations in integrin activation can be mediated by receptor tyrosine kinases (RTKs) and growth factor receptors (GFRs) and integrin RTK/GFR cross-talk plays an important role in the dynamics of integrin signaling as modulating ECM affinity alters cell adhesion dynamics. It has been found that the cytoplasmic domains of the α and β integrin subunits can interact to direct integrin activation states and are required for cell type specific affinity modulation. (51, 68) Studies in platelet integrin $\alpha_{IIb}\beta_3$ have shown that mutation of a highly conserved α-subunit GFFKR domain results in a constitutively activated integrin receptor. (68) Integrin mediated cell adhesion can be modified by either altering the complement of integrin receptors that are expressed at the cell surface or by shifting the activation state, and thus the ligand affinity, of the integrin receptor. (69) Integrin activation and signal transduction both rely on important conformational changes in the receptor. (Figure 3A) It has been suggested that inactivated integrins are found in a folded "flick-knife" conformation and could be activated into an upright or high ligand-affinity conformation as a result of cytoplasmic tail modification. (51) The molecular nature of this activation is not thoroughly understood, however it is believed that focal adhesion protein talin could cause a steric disruption in the α/β chain interactions. (70) The currently favored model for integrin activation within the cell is the separation of the α and β subunit cytoplasmic tails. (51)

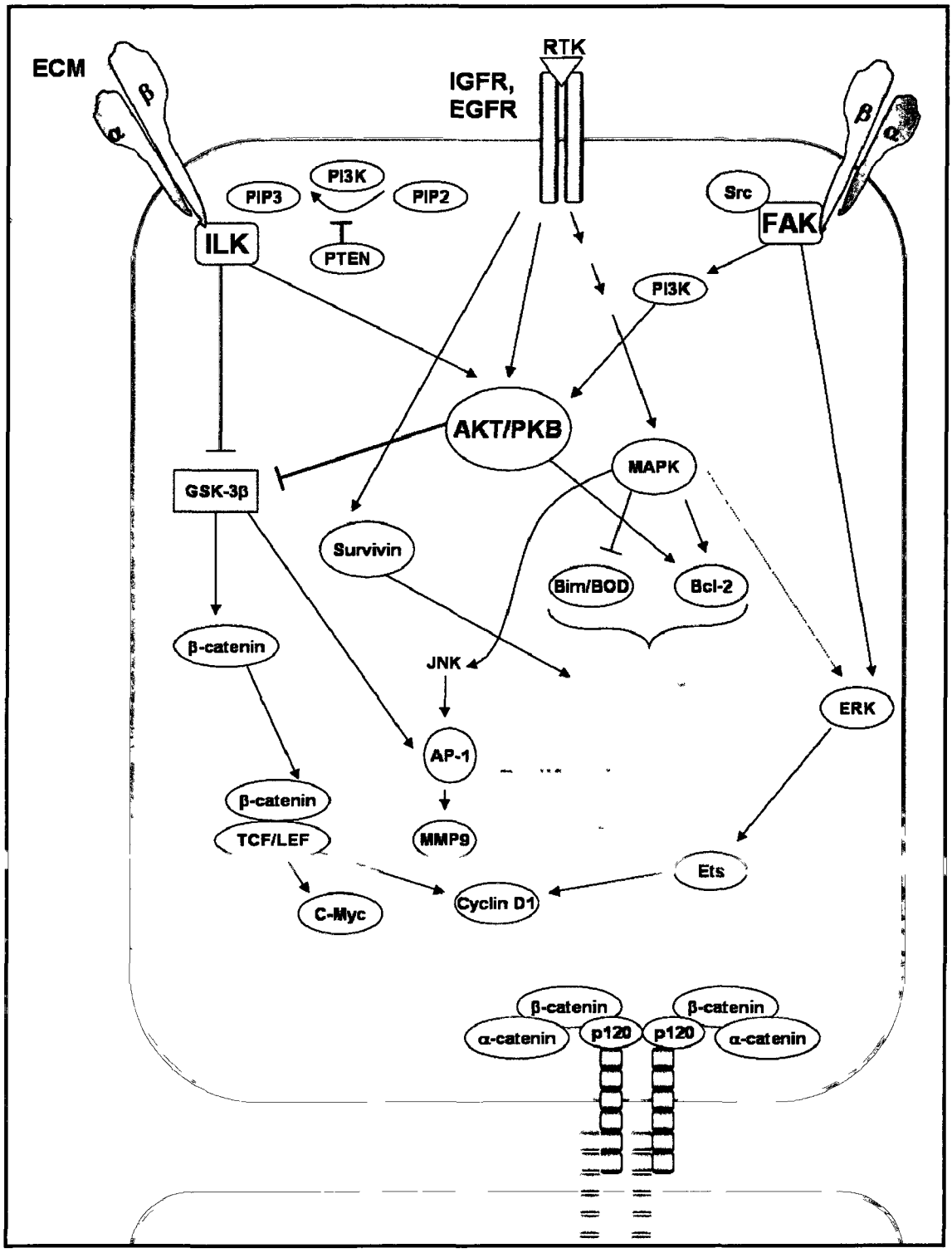
The following is a brief overview of the downstream pathways that can be activated by β_1 integrin outside-in signaling. Described are the integrin linked kinase (ILK) and focal

Figure 3: Integrin signaling. (A) Inside-out integrin activation. Intracellular signaling can alter integrin structure and modulate ligand binding affinity. Inactive integrin pairs are found in a folded, "flick-knife" position with closely linked cytoplasmic domains. The currently favored model for integrin activation involves the intracellular separation of α and β subunit cytoplasmic tails by talin, which leads to conformational changes in the integrin receptor. Upon activation, integrin receptors adopt an upright position with increased ligand affinity. **(B)** Outside-in integrin activation. Generalized schematic diagram of integrin signaling via ECM ligation and subsequent integrin activation, focusing on pathways covered in this thesis. The dynamic interaction between integrin receptors and growth factor receptors (GFRs) is also highlighted. Integrins can mediate survival, proliferation and invasion upon ligand binding through myriad downstream effectors such as ILK and FAK. These effectors modulate PI3K activity which in turn, can activate the AKT pathway. Integrins can affect survival via the AKT pathway, activating pro-survival protein Bcl-2 and inhibiting the apoptotic protein Bim/BOD. Proliferation is mediated by upregulation of Cyclin D1 by the TCF/LEF1 and by Ets transcription factors. Increased expression of MMPs, including MMP9 can be driven by β_1 integrin signaling via ILK activation and the subsequent activation the AP-1 transcription factor, and results in increased invasion.

A



B


ECM
RTK
IGFR,
EGFR
β
α
β
α
PI3K
PIP3
PIP2
PTEN
ILK
Src
FAK
PI3K
AKT/PKB
MAPK
GSK-3β
Survivin
Bim/BOD
Bcl-2
β-catenin
JNK
ERK
AP-1
β-catenin
TCF/LEF
MMP9
Ets
C-Myc
Cyclin D1
β-catenin
β-catenin
α-catenin
p120
p120
α-catenin

adhesionkinase (FAK) pathways as well as a summary of some growth factor pathways that are know to couple with integrin signaling.

1.3.2.2 Integrin linked kinase (ILK) and AKT pathway signaling

The phosphatidylinositol 3-kinase (PI3K) /ILK/AKT pathway has been extensively studied and has a documented importance in tumor-related biological processes, and additionally, it has been linked to anoikis and shown to be critical for anchorage independent cell survival and growth. (58, 71-76)

Integrin-mediated cell adhesion activates phosphatidylinositol 3-kinase (PI3K), a kinase that converts phosphatidylinositol 4,5-diphosphate (PIP_2) into phosphatidylinositol 3,4,5-triphosphate (PIP_3). (77)(Figure 3B) PIP_3 has been shown to activate ILK, a serine-threonine kinase that associates with the cytoplasmic tail of β_1 integrin. ILK is a multi-domain focal adhesion protein that mediates signals from integrins and receptor tyrosine kinases. (74) ILK expression and activity have also been linked to tumor cell invasion, migration and anchorage independent growth. (74) PI3K mediated ILK activation can be inhibited by phosphatase and tensin homolog (PTEN). PTEN is often mutated in cancer and PTEN null cells display constitutive ILK activity that is independent of anchorage or presence of serum. (58, 78-80) ILK activation by PIP_3 mediates PI3K dependent signaling of the cytoplasmic effector protein AKT, and activates it by phosphorylation on serine473. (79) In biopsies of human non-small cell lung cancer (NSCLC), β_1 integrin, ILK and AKT were found to be upregulated and this overexpression was associated with poor prognosis. (81)

ILK also inhibits glycogen synthase kinase 3β (GSK3β) that, upon phosphorylation, inhibits the normal ubiquitin mediated degradation of β-catenin. This increases the cytoplasmic pool of β-catenin and leads to its enhanced translocation into the nucleus. AKT mediated translocation of β-catenin to the nucleus activates the TCF/LEF-1 transcriptional targets c-myc, cyclin D1, MMP9, MMP7 and CD44. (82-85) ILK activation has been linked to proliferation and advancement of cell cycle, (79) likely in part as a result of the β-catenin dependent expression of cyclin D1, as inhibition of β-catenin dependent cyclin D1 transcription has been shown to inhibit cell growth in 2D and in soft agarose concomitant with a G0/G1 phase cell cycle arrest and subsequent apoptosis. (86, 87) Cyclin D1 activation by β-catenin can also occur as a result of canonical Wnt signaling (88-90) and in addition to β-catenin, cyclin D1 expression can also be induced by Ras, Neu (growth factor pathways) and Src (FAK pathway). (89) These interactions all suggest important integrin-mediated cross-talk between cell-matrix signaling, cell-cell adhesion and the Wnt signaling pathway via ILK. (85, 89) However, ILK can also act as both a scaffold and signaling molecule, creating an integrin-cytoskeletal linkage through pinch and parvin. (Figure 2) ILK, however, is not the only β_1 integrin downstream effector that has physical ties to the cytoskeleton. (91)

1.3.2.3 Focal Adhesion Kinase signaling

Focal adhesions mediate and are crucial for cell structure and function, organizing the actin cytoskeleton and contributing to the motility of the cell. FAK is a non-receptor tyrosine kinase implicated in large focal adhesion complexes and localizes to the cytoplasmic ends of integrins involved in focal adhesions, namely β_1 and β_3. (69, 92) Structurally, FAK interacts with other focal adhesion proteins such as tensin, talin, vinculin and α-actinin, also recruiting paxilin and filamin to create an adherent anchoring point for actin and the cytoskeleton. (93, 94) (Figure 2) Although FAK localizes to focal adhesions, a direct interaction between FAK and the cytoplasmic tail of β_1 integrin has not been demonstrated *in vivo*. This may be as a result of a low-affinity interaction, in keeping with the dynamic nature of actin cytoskeletal remodeling. (69) FAK has been found to be upregulated in several cancer types, including prostate cancer, where it was overexpressed in human metastatic prostate cancer cell lines and *in vivo*. (Reviewed in (92)) Integrin mediated cell adhesion is the main upstream activator of FAK, however tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine, and growth factor receptors epidermal growth factor (EGF) and platelet derived growth factor (PDGF) have also been shown to activate FAK. (92, 95) FAK has the ability to auto-phosphorylate (Y397) upon integrin ligation, creating a binding site for Src. (96, 97) Activation of the FAK-Src complex is critical to the regulation of down-stream signaling pathways that control cell spreading, cell movement and cell survival. (93) Subsequent phosphorylation of FAK on other amino acid residues is then mediated by Src and can lead to activation of the Ras/MAPK (mitogen activated protein kinase) pathway. (95) Fibronectin activation of $\alpha_5\beta_1$ integrin promotes

prostate cancer cell invasion via FAK/PI3K, inducing FAK phosphorylation and FAK association with PI3K. (98) The association of FAK with PI3K can also activate AKT, thus promoting cell survival, namely via activation of Bcl-2, an anti-apoptotic protein. (99, 100) FAK has also been implicated in anchorage independent growth (97) and can prevent apoptosis via upregulation of nuclear factor kappa B (NFκB). (101) Not unlike ILK, FAK has also been involved in cancer invasion as FAK activation resulted in the production of ECM degrading matrix metalloproteinases (MMPs) MMP2 and MMP9 via activation of the Ras/MAPK pathway. (95) Additionally, the role of FAK in focal adhesion turnover and remodeling contributes to cell migration in cancer via activation of Rac and Rho-GTPases, (102) illustrating the diverse role of FAK in cancer.

1.3.2.4 β_1 integrin signaling coupled with growth factor receptors RTKs.

Integrin signaling is complex and multi-faceted. Cross-talk between integrins and growth factor receptor tyrosine kinases is increased in prostate cancer progression and can cooperatively regulate many of the same processes that are controlled by cell-matrix interactions such as proliferation, survival, cell shape and polarity, adhesion, migration and differentiation. (49, 103) Integrin and growth factor pathways frequently converge and in several cases, the convergence has a synergistic output. (9, 104) It has been suggested that integrins and growth factor receptors act upon different points in the same signaling pathways and integrin mediated adhesion has been shown to regulate growth factor receptor signals. (104) Cellular responses to growth factors such as epidermal growth factor (EGF)

and PDGF have been shown to rely upon integrin mediated adhesion and some convergent pathways are briefly summarized in Figure 3. (51) Prostate epithelial cell survival via the epidermal growth factor receptor (EGFR) and Ras/MAPK pathway activation requires $\alpha_3\beta_1$ integrin (103) and overexpression of EGFR induces epithelial cell proliferation upon integrin engagement. (105) C-myc for example is stimulated both by integrin mediated cell adhesion, as a β-catenin target gene, and by growth factors. (104) AKT can also be activated through the insulin-like growth factor receptor (IGFR), thus stimulating survival through upregulation of Bcl-2 and GF induction of Survivin. (103, 106, 107)

1.4 – β_1 Integrins and ECM Signaling in Prostate Cancer: Bridging the Gap

Since their discovery in the 1980's integrins have been linked to cancer as they have such integral roles with regards to proper cell functioning. (108) Integrin pairs $\alpha_{(12346v)}\beta_1$ and $\alpha_6\beta_4$ have been shown to be expressed in normal prostate tissue. (20, 27) Prostate tumor progression was accompanied by a reported decrease in the expression of the laminin integrin receptor $\alpha_6\beta_4$. (20, 25) It has also been shown that α integrin subunits α_2, α_4, α_v, α_5 and α_6 as well as collagen IV receptor pair $\alpha_1\beta_1$ are lost or show decreased expression in malignant prostate tissues. (20, 27, 109) Integrin pair $\alpha_v\beta_3$ is expressed in several prostate cancer cell lines and its expression increases with cancer progression. (3, 110)$\beta_1$integrin expression has also been shown to increase with an increasing tumor grade in prostate cancer (5) and its overexpression in prostate cancer has been linked to poor prognosis. (111)

Aside from the demonstration that its expression is increased in prostate cancer, the contribution of β_1 integrin to prostate tumorigenesis or metastasis is less well described. However, β_1 integrin has been shown to play a role in other cancers, including mammary, lung and squamous cell carcinomas, among others, and has been shown to adversely affect primary tumor size, metastatic ability and prognosis as well as regulating the adhesive, proliferative and invasive properties of these cancer cells. (53, 54, 81, 112-115)The ability of cancer cells to thrive in the absence of adhesion in the body is a fundamental hallmark of cancer and is reflected *in vitro* by anchorage independent growth. (31, 104) Anchorage independent (AI) growth refers to the ability of cells to survive and proliferate without the physical and structural support of attachment to the ECM. Anoikis is the term used to describe the apoptosis that normal cells undergo upon detachment from the substratum to which they are adhered. (72, 116) Cancer cells have developed the ability to evade these signals and survive in the absence of adhesion. Anchorage independent growth of tumor cells *in vitro* is strongly correlated with tumorigenic potential *in vivo*. (49, 117)

Integrin interaction leads to the remodeling of the ECM and hence, not surprisingly, expression of β_1 integrin has been linked to disorganized three-dimensional cell growth in mammary epithelial cells. (8, 118) Along these lines, a murine mammary carcinoma that expressed fibronectin receptor $\alpha_5\beta_1$ was shown to require fibronectin for three-dimensional soft agarose growth. Similarly, cells that express $\alpha_2\beta_1$ and $\alpha_5\beta_1$ have increased colony formation in soft agarose. (119) Increased cell survival mediated by β_1 integrin may in part be regulated as a result of the fact that β_1 integrin engament by fibronectin upregulates the expression of survivin, and increases protection of PCa cells from apoptosis induced by TNF-α. (58) In addition to enhancing cell survival, β_1 integrin signaling may also modulate

tumor progression and metastasis, as it has been shown in mammary carcinoma cells,expression of $\alpha_3\beta_1$ contributed to increased invasiveness (112) while downregulation of β_1 integrin in the mammary epithelium dramatically impaired mammary tumorigenesis in mice. (120)β_1 integrin overexpression has also been found to cause larger primary tumors in mice and an increased incidence of metastasis to both lung and liver in this model. (121)It has also been suggested that cancer cells target organs whose microenvironment is compatible with the complement of ECM receptors they express. To this end, studies have implicated $\alpha_2\beta_1$ as a possible modulator of prostate cancer metastasis to the bone matrix proteins, as it is a receptor for laminin and the primary receptor for collagen I, an important component of the bone matrix. (14, 122)

Anchorage independent growth is made possible once tumor cells have gained a motile phenotype and leave the primary tumor site. (3, 91) In normaldevelopment and differentiation, a mesenchymal-to-epithelial transition occurs spatiotemporally in order to create specialized tissues. (Reviewed in (91, 123, 124) The reverse process, epithelial to mesenchymal transition (EMT) is the phenotypic reversion of epithelial cells to a motile mesenchymal cell type. (91, 124) EMT occurs naturally in wound repair and tissue remodeling in response to damage however, it is also a hallmark of cancer characterized by loss of cell-cell adherens junctions, increased cell motility and is believed to be a key player in the metastatic progression of cancer. (91, 123, 125) Transforming Growth Factor β1 (TGFβ1) is a ubiquitously expressed cytokine that has been shown not only to control many aspects of cell homeostasis but also act as a primary regulator and inducer of EMT. (123, 126) Overexpression of TGFβ1 in prostate cancer enhances tumor growth and metastasis and carries a poor prognosis. (126) TGFβ1 has been extensively implicated in epithelial to

EMT and can activate FAK and AKT, as well as other growth factor and integrin linked pathways that contribute to survival. (127) TGFβ1 can lead to increased expression of MMP9 and therefore to increased invasion in prostate cancer cells. (128, 129) Moreover, β_1 integrin expression has been shown to be regulated at the transcriptional level by cell attachment to the ECM and by TGFβ1 treatment during both differentiation and cancer progression.(57, 130, 131) Arguably, there is evidence to suggest interplay between TGFβ1 signaling and the progression of EMT in prostate cancer.

Redundancy in survival pathways have to be taken into account for successful therapies. For this reason, therapies that target molecules far upstream in a given pathway have a higher likelihood of being effective by targeting the crossroads of major survival pathways.(49)β_1 integrin is therefore an attractive target to further investigate, potentially leading to therapeutic potential in prostate cancer treatment.

1.5 Summary, Rationale and Hypothesis

Among the alterations described in prostate cancer are abnormal expression and function of integrins and of their ECM ligands. The resulting abnormal cellular interactions with the ECM promote cell proliferation, migration, and differentiation and contribute to cancer progression. (19)β_1 integrin can signal through myriad signaling pathways including coupling with growth factor receptors to promote cancer progression. Arguably, it is not one specific pathway but rather the combined effects of several that contribute to the pro-

tumorigenic effects of increased β_1 integrin expression. Although there is evidence for the role of β_1 integrin in prostate cancer, the effect of β_1 integrin in the metastatic properties of prostate cancer and the mechanism by which they do so, has not been thoroughly examined. PC3 prostate carcinoma cells are a metastatic cell line derived from osseous lesions and represent a suitable model to further examine the role of β_1 integrin in tumorigenic growth properties of prostate cancer cells. β_1 integrin is the predominant integrin expressed by PC3 cells. (132) Previous observations in the laboratory suggested that β_1 integrin modulated anchorage independent tumor cell growth, a hallmark of the tumorigenic phenotype. Several cancer cell lines, including PC3 prostate cancer cells, A2780S ovarian cancer cells and SF295 glioblastoma cells were shown to have impaired colony formation in three dimensional soft agarose culture when levels of β_1 integrin were depleted with the use of targeted small-interfering RNA (siRNA) compared to scrambled siRNA controls. It was noted that depletion of β_1 integrin using this transient approach resulted in approximately 50% of tumor cells with decreased β_1 integrin expression, consistent with the overall transfection efficiency, and suppression could be maintained for up 120hrs. (133) Although it had been established that β_1 integrin modulated soft agar colony formation, the transient siRNA approach did not provide a homogeneous population of β_1 integrin depleted cells or the potential for long-term analysis of tumorigenic properties. Thus a strategy to generate cell lines with stably depleted β_1 integrin levels was used to facilitate analysis of the mechanisms by which β_1 integrin modulates the tumorigenic and metastatic phenotypes of PC3 cells. The experiments presented herein are designed to test the hypothesis :**β_1 integrin modulates the three-dimensional tumorigenic growth properties of PC3 prostate cancer cells.**

1.6 Approach

Objective 1: Generate stable β_1 integrin short-hairpin RNA (shRNA) expressing cell lines to confirm previous results obtained using an siRNA approach. PC3 cells lines stably expressing shITGB1 constructs were generated and characterized. The three-dimensional soft agarose colony forming abilities of these cells were examined and their adhesive and growth properties in adherent and three-dimensional conditions were studied to provide insight into the potential mechanism responsible for AI growth modulation.

Objective 2: Determine the putative mechanism of β_1 integrin control of three-dimensional tumor growth. Previously reported novel interactions between β_1 integrin and β-catenin in three-dimensional culture were further examined in a bid to elucidate the mechanism responsible β_1 integrin mediated anchorage independent growth of PC3 cells. Both western blot analysis and quantitative PCR methods were implemented to examine potential downstream molecules of interest that may mediate observed phenotypes in anchorage independent growth.

Objective 3: Modulation of PC3 prostate cancer cell line invasion and migration by β_1 integrin depletion and potential effect on in vivo tumorigenicity. Both control and shITGB1 expressing stable cell lines were assayed for invasion, migration and growth over reconstituted basement membrane. Selected control and β_1 integrin depleted stable cell lines were selected to assess the effect of β_1 integrin expression on skeletal metastasis in mice using an intraventricular injection method followed by immunohistochemistry.

2. MATERIALS AND METHODS

2.1 Cell culture

PC3 Human Prostate Carcinoma cells were received from ATCC (Manassas, VA) and were cultured in High Glucose Dulbecco's Modified Eagle Medium (DMEM) (Thermo, Logan, UT) supplemented with 10 % Fetal Bovine Serum (FBS) (PAA Laboratories, Etobicoke, ON) in a humidified environment at 37°C containing 5% CO_2. Cells were passaged regularly by washing twice with warm Phosphate Buffered Saline (PBS) (Thermo, Logan, UT) and incubating at 37°C with 0.5% trypsin (Thermo, Logan, UT) in Hank's Buffered Salt Solution (HBSS) to detach cells before re-plating in fresh medium. When grown in suspension, cells were cultured over a solidified 1% low-melting point (LMP) agarose underlay (Ultra Pure, Invitrogen, Carlsbad, CA).

2.2 siRNA transfection with integrin β_1 (ITGB1) siRNA

PC3 prostate cancer cells were seeded 24 hrs prior to transfection so that they would be 30-50% confluent at time of transfection. Cells were transfected using Oligofectamine (Invitrogen, Carlsbad, CA), as per the protocol outlined by the manufacturer. siRNA concentration optimization was previously carried-out to determine the lowest effective dose in an effort to minimize off-target effects. (133) Briefly, on the day of transfection, stock ITGB1 (01, 02 or 03) siRNA (20nM) (Dharmacon, Logan, UT) was combined with OPTI-MEM (Gibco, Carlsbad, CA) for a final siRNA concentration of 5nM. During this

incubation, cells to be transfected are washed 2x with OPTI-MEM. After the incubation, the duplex mixture is added drop-wise to OPTI-MEM containing dishes to be transfected. Media was replaced with serum containing media 8-22 hrs post transfection.

2.3 Stable cell line generation

HuSH 29 mer shRNA constructs against integrin β_1 (ITGB1) were obtained from Origene (Rockville, MD). Four different expression vectors in the pRS plasmid were used in the generation of stable PC3 cell lines: TI378785 (sh ITGB1-1) GAAGGAATGCCTACTTCTGCACGATGTGA; TI378786 (sh ITGB1-2) GAGGATATTACTCAGATCCAACCACAGCA; TI378787 (sh ITGB1-3) TCACTGATTGGCTGGAGGAATGTTACACG and TI378788 (sh ITGB1-4) GTGATGCCTTACATTAGCACAACACCAGC. An empty pRS vector was used as a negative control (TR20003) and a pRS plasmid containing an expression vector against green fluorescent protein (GFP) (TR30003) was also used as a negative control (all from Origene, Rockville, MD). The pRS plasmid contains resistance genes for both ampicillin and puromycin to allow for selection in bacterial and mammalian cell systems respectively.

Bacterial transformation:

Origene HuSH ITGB1 shRNA plasmids were reconstituted according to the manufacturer's protocol and transformed into $CaCl_2$ competent DH5α bacteria with heat shocking at 42°C. Bacteria containing each plasmid were streaked onto separate Luria

Bertani broth (LB)-Agar plates containing 75ug/mL of ampicillin (Amp) (Sigma, St. Louis, MO) and allowed to incubate overnight at 37°C.

DNA extraction:

Individual colonies from each Amp selective plate were collected, grown in LB overnight and were used for plasmid extraction using Qiagen CompactPrep Plasmid Midi Kit (Mississauga, ON) in accordance with manufacturer's protocol.

Transfection and selection:

Passage 18 PC3 cells were seeded 24 hrs prior to transfection to obtain a confluency of 90-95% at the time of transfection. Cells were transfected using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, 4ug of plasmid DNA were transfected into each well of a 6-well plate. The transfection medium was replaced 24 hrs after transfection with DMEM containing 10% FBS. Forty-eight hrs later, media was replaced with DMEM containing 10% FBS and 2.5ug/mL of puromycin (Sigma, MO, USA). Twenty-one days after cells had initially been placed in selection medium, isolated colonies were collected using cloning cylinders coated with sterile grease on the bottom which then formed a sealed well around each colony into which trypsin was added to collect colonies. Each colony was grown in a separate dish, in selection media DMEM containing 10% FBS supplemented with 2.5ug/mL puromycin (Gibco, Carlsbad, CA) until there were sufficient cells for lysate collection to assess β_1 integrin expression.

2.4 Soft agarose colony formation assay

Two sterile LMP-agarose solutions (1% and 1.5%) were heated to melting point and allowed to cool to 37°C in water bath. Warm 1.5% agarose was combined in equal parts (1:1 ratio) with 2x alpha-Modified Eagle Medium (α-MEM) (Gibco, Carlsbad, CA) containing 20% FBS to produce a final agarose media mixture of 0.75% agarose, 1x media and 10% FBS. In a 24-well plate, 400uL of this mixture was added to each well and allowed to solidify at room temperature to form the agarose underlay. The 1% agarose solution was prepared in a similar manner. The final composition of the overlay was 0.5% agarose, 1x α-MEM media and 10% FBS. Cells to be seeded into agarose were washed twice with PBS and trypsinized for 5 min at 37°C. Cells were collected and counted using a Bight-Line Hemacytometer (Hausser Scientific, Horsham, PA). Following trypsinization, cells were centrifuged and resuspended at a set concentration in DMEM supplemented with 10% FBS. From the stock of resuspended cells, 28,457 cells were aliquoted into a 15mL plastic tube containing 5mL of the liquid 0.5% agarose media mixture which was then serially diluted to generate a dilution series containing 2000, 1000, 500, 250, 125 or 62.5 cells/700ul. To each well, 700uL of each cell-agarose-media dilution mixture was added to the surface of the solidified agarose underlays in triplicate. The agarose mixture was not allowed to solidify completely, and was transferred to 37°C when it became "sloppy" at room temperature. The assays were then incubated at 37°C for 14 days to maintain the consistency of the low melting temperature agarose and allow cell growth. Colonies were counted after 14 days at the 125 dilution.

2.5 Preparation of matrix coated tissue culture dishes

Monomeric Collagen I

Plates were coated with PureCol (Fremont, CA) diluted to 5ug/cm^2 in filter-sterilized 0.01N HCl. Coated tissue culture dishes were allowed to dry overnight in a sterile hood. Before use, plates were washed twice with PBS.

Fibronectin

Fibronectin (Invitrogen, Carlsbad, CA) was diluted to 1ug/cm^2 into sterile PBS (Thermo, Logan, UT). The solution was allowed to incubate in tissue culture dishes for 1 hr at room temperature. Remaining material was aspirated and wells were washed carefully once with sterile ddH_2O. Plates were stored at 4°C overnight for use the following day.

Vitronectin

Vitronectin (Sigma, St.Louis, MO) was diluted to 100ng/cm^2 into sterile PBS (Thermo, Logan, UT). Tissue culture dishes were incubated in this solution for 2 hrs at 37°C. Remaining material was aspirated and wells were washed carefully once with PBS. Plates were stored at 4°C overnight for use the following day.

2.6 Adhesion Assay

For both shITGB1-PC3 stable cell lines (and controls) and PC3 cells transfected with siRNA targeted against ITGB1, cells were washed twice with warm PBS before being incubated with warm citric saline (1.35M KCl, 0.15M sodium citrate) at 37°C. Citric saline was used in favour of trypsin to maintain the integrity of the integrin receptors. Cells were collected and counted using a ViCell Coulter counter and centrifuged at 281.7 g for 5 min. The cell pellet was resuspended in DMEM with 10% FBS and seeded in triplicate at 1 x 10^5 cells per well of a pre-coated 12-well tissue culture dish. Cells were allowed to adhere at 37°C for 1 hr 30 min. Wells were gently washed once with PBS to remove un-bound cells. Adherent cells weretrypsinized, retrieved and counted in the ViCell Coulter counter to assess the number of adhered cells per well.

2.7 Growth Experiments

For non-adherent growth, 6-well plates (Costar, Corning, NY) were coated with a 1% agarose solution (2mL/well) and allowed to solidify to room temperature. Cells to be seeded were washed twice with PBS and incubated with 0.05% trypsin in HBSS (Thermo, Logan, UT) at 37°C for 5 min. After sufficient pipetting to homogenize the mixture, samples were counted by trypan blue exclusion with a hemacytometer in triplicate per cell line. The remaining cells were centrifuged at 236.7 g for 5 min and resuspended in DMEM with 10% FBS. Cells were seeded at 2 x 10^5 cells per well in DMEM containing 10% FBS. Cells were

incubated at 37°C in 5% CO_2. Every 24 hrs following seeding up to 96 hrs, cells were counted. For cells grown in suspension, media and cells were collected in a 15mL tube and cells were centrifuged at 281.7 g for 5 min. Cells were resuspended in trypsin to disassociate cell clusters that form when PC3 cells are cultured in suspension, incubated at 37°C for about 5 min, mixed by pipetting and viable cells counted by trypan exclusion using the ViCell XR Coulter Counter (Beckman-Coulter, Brea, CA). For cells grown in adherent conditions, cells were washed once with PBS and trypsinized before being well mixed and counted using the ViCell Coulter Counter.

2.8 Western Analysis and Antibodies

Cells lysates to be analysed by western were collected by first washing cells 2x with warm PBS and scraping tissue culture dishes with a rubber cell scraper (BD Falcon, Bedford, MA) in either Frack's lysis buffer (10mM Tris-HCl pH7.4, 150mM NaCl, 5mM EDTA, 1% Triton X-100) or NP40 lysis buffer (150mM NaCl, 1% Nonidet P40 (NP40), 0.5% deoxycholic acid, 50 mM Hepes pH7.5) containing protease inhibitors leupeptin (5ug/ml), aprotinin (2ug/ml), ammonium vanadate (500 μM), sodium phosphopyrate (2 mM) , Sodium Fluoride (2 mM) and phenylmethanesulphonylfluoride (PMSF, 0.2 mM).Lysates were then transferred into a 1.5mL microcentrifuge tube, vortexed briefly at high speed or sheared with 5 passes through a 20 gauge needle before being incubated in ice for 30 min. Lysates were then centrifuged at 14,000 RPM in a microcentrifuge for 20 min at 4°C. Supernatant was collected and stored at -20°C.

The concentration of protein in each sample was quantified using the BioRad (Hercules, CA) Bradford detection system according to manufacturer's protocol and absorbances were measured at 595 nm. For loading onto gels, samples were prepared with equal total protein quantities in 4x NuPage loading buffer supplemented with 10x DTT (0.5M) to final concentrations of 1x for both solutions (as per manufacturer's protocol). Samples were heated to 70°C for 10 min and centrifuged briefly.

Up to 40 uL of sample was loaded into the wells of a gradient 4-12% Bis-Tris SDS-PAGE gel (Invitrogen, Carlsbad, CA) using the NuPAGE system (Invitrogen, Carlsbad, CA) in 1x MOPS running buffer (Invitrogen, Carlsbad, CA) and run at 200V until dye eluted (approx 1hr). Cell was transferred to a 45 micron nitrocellulose membrane (Amersham Biosciences, UK) in NuPAGE 1x Transfer Buffer with 20% methanol at 30V for 70 min in ice.

Membrane was blocked in 5% skim milk powder (5% milk) in Tris-buffered saline with Tween (TBST, containing: 10mM Tris-HCl pH 8.0, 150mM NaCl, 0,05% Tween20) or 3% bovine serum albumin in TBST (3% BSA), depending on antibody to be used, rocking overnight at 4°C. Primary antibody was diluted in either 5% milk or 3% BSA and set to rock according to optimised conditions for the antibody in question. Blots were subsequently washed by shaking in TBST for 4 min, 3 times, at room temperature. Blots were set to rock with secondary mouse or rabbit antibodies (Sigma, St. Louis, MO) at a 1:5,000 dilution in 5% milk in TBST for 1 hr at room temperature and were subsequently washed 3 times for 4 min each time. Blots were exposed using the Immobilon Western chemiluminescent horseradish peroxidase (HRP) substrate (Millipore, Billerica, MA) and the GeneGnome (SynGene Bio Imaging, Frederick, MD) gel documentation system and GeneSnap software.

If re-probing was required, blots were stripped by shaking for 20 min in Restore™ PLUS Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL) at room temperature, followed by 3 four-min washes in TBST before starting the probing process over.

The following antibodies were used according to the protocol outlined above, and any exceptions to this above described method, are referred to along with the details of the specific antibody in question. Monoclonal β_1 integrin antibody clone 18 (BD, Mississauga, ON) was used for immunoblotting (IB) at a 1:1000 dilution in 5% milk. Two β_1 integrin adhesion blocking antibodies, clone 6S6 (Chemicon, Temecula, CA) and PC410 (Chemicon, Temecula, CA), are both mouse monoclonal antibodies and were used for immunoprecipiation (IP) assays at dilutions of 1:30. Two β-catenin antibodies were used for both IB and IP. Clone 6F9 (Sigma, St. Louis, MO) is a mouse monoclonal antibody that was used at a 1:1000 concentration for IBs and at 1:30 for IPs. A rabbit polyclonal antibody (Cell Signaling, Danvers, MA) against β-catenin was used at 1:1000 for IB and at 1:30 for IPs in 5% BSA in TBST. A mouse monoclonal E-cadherin antibody (Clone 18, BD, Mississauga, ON) was used at 1:1000 dilution for IB. The rabbit polyclonal Bim/BOD antibody (Assay Designs, Anna Arbor, MI) was used at 1:1000 for IB. Antibodies against ILK (Clone 3, BD, Mississauga, ON) and p120 catenin (Clone YE372, Novus, Littleton, CO) were both used at 1:1000 for IB. Mouse monoclonal β-actin antibody (Clone AC-74, Sigma, St.Louis, MO) was used at a 1:10,000 dilution for IB. For protein expression confirmation of qPCR analysis, α-rabbit antibodies against MMP7, MMP9, MMP14 and TGFβ1 were obtained from Assay Biotechnology (Sunnyvale, CA) and used at a 1:750 dilution in 5% milk in TBST.

The Bcl-2 monoclonal hamster-anti-human antibody (Clone 6C8, BD, Mississauga, ON) required a different protocol: Primary Bcl-2 antibody was used at a concentration of 0.5ug/mL and was incubated with shaking in 5% milk in TBST for 2 hrs at room temperature. After washing with TBST as previously described, the blot was set to rock with a secondary anti-armenian hamster HRP antibody (Jackson Immunoresearch, West Grove, PA) diluted 1:10,000 in 5% milk in TBST for 1 hr at room temperature before being washed and exposed as previously outlined.

2.9 Scratch Wound Assay

PC3 cells stably depleted of β_1 integrin were seeded at approx 1.5 x 10^6 cells per well of a 6-well plate in duplicate to reach confluency in 24 hrs. The next day, wells were washed twice with PBS, the monolayer was wounded by scratching cells with a 2mm scraper and cells were washed again 2-3 times in PBS to remove dislodged cells. Pictures were taken at time of wounding (t_0 time point) and again 24 hrs later (t_{24}). Wound images were taken at 40x magnification using a Nikon Eclipse TE2000-U microscope and camera. Three pictures were taken per well, from which three measurements are taken in a blinded fashion at the same place for each time point, measuring the wound front and not the location of individual cells. Wound diameter at time t_{24} divided by the diameter at t_0 multiplied by 100 determined % wound closure and was plotted on a graph.

2.10 Immunoprecipitation

For immunoprecipitation assays, protein samples were prepared as previously described. A total of 300-400ug of protein were combined with NP40 lysis buffer and antibody (as described above) in a total volume of about 110 uL for proper mixing and the mixture was set to rotate overnight at 4°C. GammaBind G, protein G sepharose beads (GE Healthcare, Sweden) were washed twice with NP40 lysis buffer with protease inhibitors before being prepared as a 60% bead slurry in NP40 lysis buffer. Rotated IP samples were combined to 50uL of bead slurry and allowed to rotate a further 4 hrs at 4°C. IP beads were centrifuged and then washed 3 times with NP40 lysis buffer to remove un-bound proteins. NuPage sample buffer was added to the beads to a final concentration of 2x and samples were boiled for 5 min to dissociate protein from beads. Beads were briefly centrifuged and supernatant was loaded onto an SDS-PAGE gel for Western analysis as previously described.

2.11 Invasion Assay

Invasion chambers (BD BioCoat Growth Factor-Reduced Matrigel^{TM} Invasion Chamber, PET membranes, Mississauga, ON) with 8um pores and precoated with growth-factor reduced matrigel were used according to manufacturer's protocol. Briefly, thawed 24-well plate inserts were incubated with warm, serum-free DMEM at 37°C for 2 hrs to reconstitute matrigel. Cells were prepared by trypsinizing and collecting as previously

described, they were counted with a hemacytometer by trypan blue exclusion and centrifuged at 281.7g for 5 min. A cell suspension containing 5 x 10^4 cells/mL in DMEM with 0.1% BSA was prepared. Rehydration media was carefully removed without disturbing the matrigel and invasion chambers were carefully lowered into the chemoattractant (DMEM with 5% FBS) which had been placed in the bottom well. A total of 500uL of cell suspension (2.5 x 10^4 cells) were loaded into each upper chamber over the surface of the rehydrated matrigel. Cells were then allowed to invade the matrigel for 4 hrs at 37°C, passing through the pores and resting on the underside of the membrane. After incubation, matrigel and non-invaded cells were removed by scrubbing the surface of the well with a Q-tip and invaded cells remaining on the filters were stained with crystal violet, by placing membranes in ice-cold Crystal Violet (0.5% crystal violet solution in 25% methanol) followed by incubation at room temperature for 10 min. Membranes were then carefully and gently rinsed in water and allowed to dry at room temperature. Membranes were then examined under a microscope at a magnification of 200x and invaded cells were counted in 10 fields of view spanning the entire membrane for quantification of the assay. Results are expressed as mean cells invaded per field of view for three independent experiments.

2.12 Matrigel Migration

Four-chamber slides (Nalge Nunc International, Naperville, IL) were coated with growth-factor reduced basement membrane (Culturex, Gaithersburg, MD) that was prepared according to manufacturer's protocol. Briefly, the basement membrane was thawed

overnight at 4°C before use and all pipette tips were kept on ice during coating. A total of 100uL of basement membrane was added to each chamber of the slide which was spread evenly with a pipette tip, avoiding bubbles and meniscus formation. Slides were placed in a humidified environment at 37°C containing 5% CO_2 for 30 min to allow solidification of the gel. Cells were then trypsinized, collected, counted and centrifuged (281.7 g for 5 min) as previously described. Cells were resuspended in DMEM containing 20% horse serum (HS) (Gibco, New Zealand) at 11,000 cells/450uL. An equal volume of assay media containing 5% Culturex BM, 20% HS and 10ng/mL epidermal growth factor (EGF, Peprotech Inc, Rocky Hill, NJ) was added to 450uL of the cell mixture and 850uL of this final mix was spread over the BM coated chamber. Cells were allowed to migrate, grow and form colonies over 4 days before being counted from 10 random fields of view. Pictures were taken at total of 200x magnification and both colonies over 25um and colonies over 50um were counted for quantification.

2.13 In vivo Mouse Model

In our model of prostate cancer bone metastasis, 16 male CD1 nude mice (Charles River Laboratories, Wilmington, MA) were injected intra-ventricularly with 1 x 10^6 prostate cancer cells in 100uL of sterile PBS. The prostate cancer cells were injected directly into the left ventricle of the heart to ensure a systemic delivery of the cells and increased incidence of bone metastasis. Four mice were injected with each cell line and cell lines used were wtPC3, shGFP-1, sh2-10 and sh3-2. Cells were trypsinized as previously described,

resuspended in PBS and kept on ice until shortly before injections. Injections into the left-ventricle were carried out by a trained animal technician and required a small incision into the skin of the chest. One mouse (sh3-2) died during surgery and another from surgical complications the following day (shGFP-1). The remaining 14 mice were regularly monitored and all mice were sacrificed concurrently when endpoint (as defined by animal care technicians for the humane keeping of animals) was reached by one of the mice at 9 weeks post-injection. At end-point, all mice were euthanized by CO_2 and limb bones, livers and lungs (which were perfused with PBS) were collected for immunohistochemical analysis. All organs were preserved in 10% formalin (Sigma, St.Louis, MO). All bone specimens were placed in a decalcifying solution of 10% EDTA, pH 8.0 (Fisher Scientific, Fair Lawn, NJ) that was refreshed every 3 days for 2 weeks, prior to fixation. Decalcified organs were sent for paraffin embedding, sectioning, mounting and H&E staining. Unstained sections were used for immunohistochemical analysis.

2.14 Immunohistochemistry

Paraffin was removed from tissues by sequentially placing slides in the following solutions for the indicated times: Xylene (3 times, 5 min each), 100% Ethanol (1 time, 10 min), 95% Ethanol (1 time, 10 min), 80% Ethanol (1 time, 10 min), 70% Ethanol (1 time, 7 min, all reagents were from Fisher Scientific, Fair Lawn, NJ) Slides were rinsed gently under running water for at least 5 min. Slides were placed in a 10mM sodium citrate buffer, pH 6.0 (Bioshop, Burlington, ON) and microwaved until boiling for antigen heat-retrieval.

Slides were allowed to cool to room temperature before being rinsed in tap water for 5 min. To neutralize endogenous peroxidases, slides were incubated with 0.3% H_2O_2 (Sigma, St.Louis, MO) for 30 min at room temperature followed by a 5 min wash in PBS (pH7.4). Slides were subsequently incubated with 5% goat serum diluted in PBS (pH7.5) for 1 hr at room temperature as a non-specific blocking agent. Primary Cytokeratin 18 antibody (Rabbit polyclonal, AbCam, Cambridge, MA) was optimally diluted 1:250 in 5% goat serum in PBS (pH7.5) and incubated with slides overnight in a moistened environment at 4°C. Slides were rinsed once with PBS (pH7.4) and then washed in PBS for 5 min. Goat-anti-rabbit secondary antibody was diluted in PBS to 8ug/mL and allowed to incubate on slides for 30 min at room temperature. Slides were washed 2 times in PBS for 5 min. Subsequently, ABC reagent (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) was prepared according to manufacturer's instructions and placed on slides which were then incubated at room temperature for 30 min, rinsed and washed for 5 min in PBS. Sections were incubated with 3,3-Diaminobenzidine (DAB) (DAKO Corporation, Carpinteria, CA) until they turned brown. Slides were rinsed in water and hematoxylin (Fisher Scientific, Fair Lawn, NJ) was applied for 2 min followed by another rinse in water. Sections were then dipped into a 0.5% acid alcohol bath (0.5% HCL in 70% Ethanol) and washed with water, followed by two dips in an ammonia water bath (400 uL of ammonium hydroxide (Acros Organics, NJ) in 200mL tap water) and another wash with water. Sections were allowed to dry completely before being mounted using Cytoseal-60 medium (Richard-Allan Scientific, Kalamazoo, MI).

2.15 qRT-PCR Array plates, validation and primers

Cell lines to be assayed using qPCR were grown on plastic or fibronectin (1ug/cm^2, Invitrogen, Carlsbad, CA) for 48 hrs prior to RNA harvesting using the RT^2 RNA extraction kit (SA Biosciences, Frederick, MD) as per manufacturer's instructions. RNA was quantified by spectrophotometry (Eppendorf Biophotometer, Hamburg, Germany) and either 0.5 ug or 1 ug was used to create cDNA using the RT^2 first strand cDNA kit (SA Biosciences, Frederick, MD). Presence of cDNA was confirmed by PCR using actin primers (Forward: 5'-GGGCATGGGTCAGAAGGAT-3', Reverse: 5'-GTGGCCATCTCTTGCTCGA-3') (25 cycles). PCR product was run on a 1% agarose gel containing EtBr (Fisher Scientific, Fair Lawn, NJ) and visualized for the presence of a band indicating successful amplification. Validated cDNA was then diluted in 91uL sterile ddH_2O and combined with RT2 Real-time SYBR Green/Rox PCR master mix (SA Biosciences, Frederick, MD), as per manufacturer's instructions, before being loaded onto the 96-well array plate already containing primers. The "Human Extracellular Matrix and Adhesion Molecules" qRT-PCR Array kit was obtained from SA Biociences (Frederick, MD). Plates were run in the ABI7500 machine according to manufacturer's protocol. $\Delta\Delta$Ct values were compared between β_1 integrin depleted cell line sh2-10 and control cell line shGFP-1 to generate a heat map based on relative message levels.

Data generated from the arrays was then validated using custom designed primers (all from Invitrogen, Carlsbad, CA) for E-cadherin (CDH1), β_1 integrin (ITGB1), matrix metalloproteinase 7 (MMP7), matrix metalloproteinase 9 (MMP9), matrix metalloproteinase

11 (MMP11), matrix metalloproteinase 14 (MMP14), transforming growth factor βinduced (TGFBI) and tissue inhibitor of metalloproteinase 2 (TIMP2). Primer sequences were as follows:

CDH1: (Forward) 5'-CCCGGGACAACGTTTATTAC-3', (Reverse) 5'-GCTGGCTCAAGTCAAAGTCC-3', ITGB1 : (Forward) 5'-CGATGCCATCATGCAAGT-3', (Reverse) 5'-ACACCAGCAGCCGTGTAAC-3', MMP7: (Forward) 5'-GACATCATGATTGGCTTTGC-3', (Reverse) 5'-TCTCCTCCGAGACCTGTCC-3', MMP9: (Forward) 5'-GAACCAATCTCACCGACAGG- 3', (Reverse) 5'-GCCACCCGAGTGTAACCATA- 3',MMP11 : (Forward) 5'-GGTGCCCTCTGAGATCGAC-3', (Reverse) 5'-TTCACAGGGTCAAACTTCCAG-3', MMP14: (Forward) 5'-TACTTCCCAGGCCCCAAC-3', (Reverse) 5'-GCCACCAGGAAGATGTCATT-3', TGFBI: (Forward) 5'-GACACCTTTGAGACCCTTCG-3', (Reverse) 5'-CTTCAAGCATCGTGTTGAGC-3', TIPM2 : (Forward) 5'-GAAGAGCCTGAACCACAGGT-3', (Reverse) 5'-CGGGGAGGAGATGTAGCAC-3'

For validation runs, primers were diluted to 10 uM, and 1ug of isolated RNA (Qiagen RNeasy PLUS kit, Mississauga, ON) was used to generate cDNA (using MMLV and random hexamers according to manufacturer's protocol, both Invitrogen, Carlsbad, CA). SYBR-Green reagent is supplied as a 2x master-mix and manufacturer's protocol was followed for the preparation of the plates. The cDNA reactions were diluted 1:10 and 1ul was added to each well which contained a total of 10 uL of final reaction mixture (5uL SYBR-Green 2x Master Mix, 1uL 1:10 cDNA, 1uL 10uM primer pair (+/-) and 3uL

ddH_2O). qRT-PCR was then performed on the ABI7500 FAST (Applied Biosciences, Carlsbad, CA) machine on standard settings ($\Delta\Delta$Ct analysis with melt curve). Analysis of results was performed with the Applied Biosciences Software specific to the 7500 FAST machine.

2.16 Statistical Analysis

Significant differences between biological replicates were ascertained using a two-tailed unpaired t-test with a 95% confidence interval. In instances where multiple comparisons were required, a nonparametric one-way ANOVAtest with Dunnett's multiple comparison was performed with a 95% confidence interval (GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego, CA).

SECTION 3: RESULTS

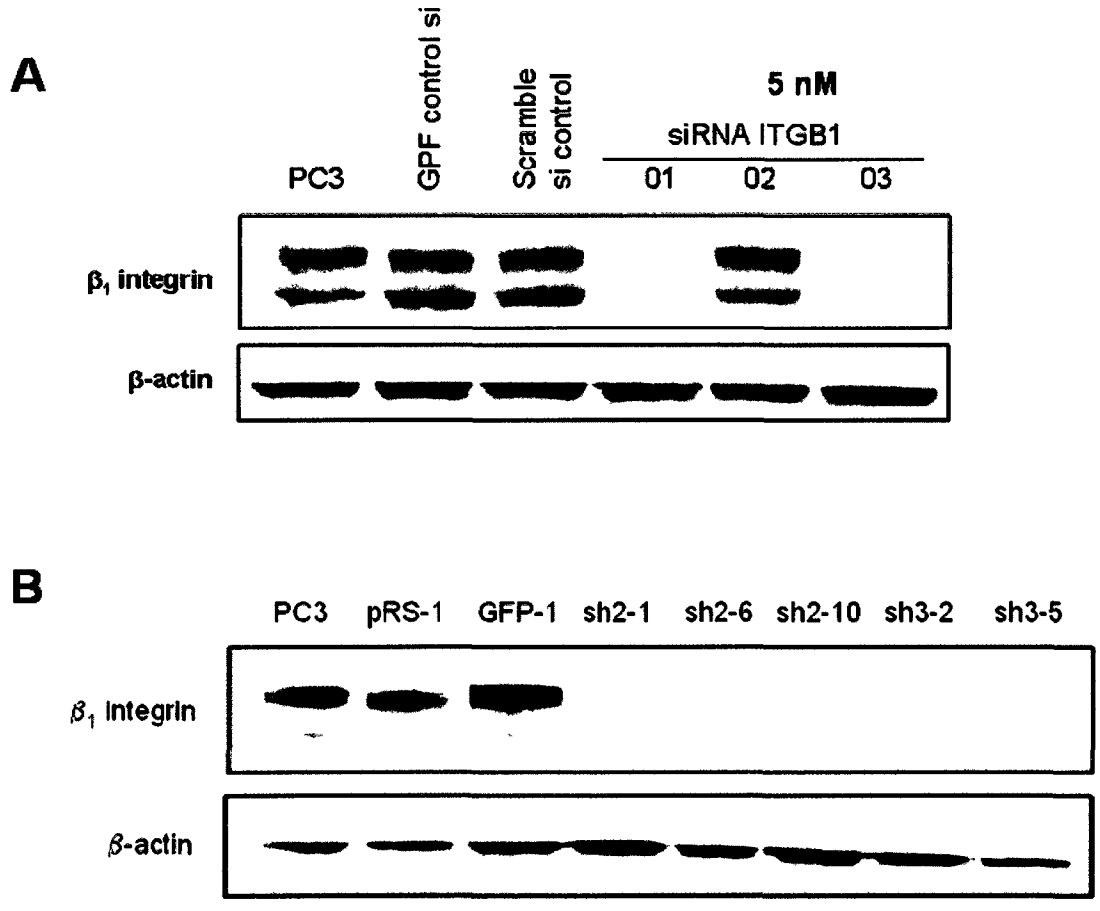
The ability of cancer cells to survive and proliferate in the absence of integrin-mediated adhesion *in vitro* strongly correlates with *in vivo* tumorigenesis and is suspected to play a role in metastasis. (134) Previous findings in our laboratory have shown that a reduction in the expression of β_1 integrin in prostate cancer cell line PC3 by small interfering RNA (siRNA) or inhibition of outside-in integrin signaling by neutralizing antibodies to β_1 integrin resulted in a decreased ability of these cells to form colonies in soft agarose. There was roughly a 50% decrease in anchorage independent colony formation by β_1 integrin depleted cells compared to controls, concurrent with an approximately 50% transfection efficiency of β_1 siRNA and corresponding reduction in β_1 integrin levels as ascertained by western blot. We wished to further characterize the role of β_1 integrin in the anchorage independent growth of PC3 cells, however the transient nature of siRNA prevented its use in certain experimental approaches. Hence we chose to generate cell lines with stably depleted levels of β_1 integrin using a shRNA approach directed against β_1 integrin. These cell lines were generated and compared to their siRNA counterparts to assess the impact of the loss of β_1 integrin, whether stably or transiently, on soft agarose colony formation, 2D adhesion on matrix and proliferation in adherent and suspension conditions. We also wished to determine the putative mechanism of β_1 integrin control on 3D *in vitro* tumor growth. This entailed the examination of molecular interactions occurring in 3D but not in 2D growth. To further elucidate the underlying mechanisms of anchorage independent growth control, a qRT-PCR microarray approach was used for high throughput determination of potential genes of interest that are altered in cell lines with a reduced

capacity to form soft agarose colonies. Finally, the mechanisms of β_1 integrin control on tumorigenic growth properties and *in vivo* tumorigenicity were assessed. A scratch wound migration assay, an invasion assay and a matrigel colony formation assay examined the effect of reduced β_1 integrin expression on established tumorigenic growth properties *in vitro* and a mouse model pilot study was performed with the stable cell lines to examine the contribution of β_1 integrin on *in vivo* tumorigenicity.

3.1 - GENERATION OF STABLE PC3 CELL LINES EXPRESSING ITGB1 shRNA

In order to assess the effect of complete β_1 integrin depletion on the anchorage independent growth of prostate cancer cells, a short hairpin RNA (shRNA) strategy was used to produce stable clones with reduced expression levels of β_1 integrin. PC3 prostate carcinoma cells were transfected with 4 distinct shRNA constructs targeting β_1 integrin (ITGB1) and selected using puromycin over several weeks. Two control constructs were also implemented; an empty vector control (pRS) and a vector control containing a shRNA targeting GFP (pRS-shGFP). Individual clonal populations of control and shITGB1 transfected cells were tested for β_1 integrin expression through western blot analysis. (Appendix A) Two of the β_1 integrin targeting shRNA constructs (#1 and #4) did not yield any selection-surviving clones. All clonal populations were compared to wild-type PC3 (wtPC3) cells with regards to the reduction in β_1 integrin levels. Compared with the level of β_1 integrin expression in the siRNA transfected cells, the stable cell lines that were generated have further depleted levels of β_1 integrin (Figure 4A and 4B). It was found that

Figure 4: Representative western blot showing reduced β_1 integrin protein levels following specific mRNA depletion by transient siRNA targeting or by stable shRNA transfection. **(A)** Western blot showing whole cell lysates from PC3 cells transfected with 5nM individual integrin β_1 siRNA (ITGB1-01, ITGB1-02, or ITGB1-03) or control siRNA (scrambled siRNA: Scr control (P1) or GFP-targeting control (GFP)). Total cellular protein lysates were collected 48hrs post-transfection, and integrin β_1 levels were assessed by western blot analysis. **(B)** β_1 integrin expression levels of PC3 prostate cancer cells stably transfected with empty control vectors pRS, shGFP targeted control or shITGB1 constructs #2 (derived clonal cell populations being sh2-1, sh2-6 and sh2-10) or #3 (derived clonal cell populations being sh3-2 and sh3-5).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Scramble si control
5 nM
siRNA ITGB1
01
02
03
β1 integrin
β-actin
B
PC3
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GFP-1
sh2-1
sh2-6
sh2-10
sh3-2
sh3-5
β1 integrin
β-actin

clones 1, 6 and 10 from shRNA construct 2, had significantly reduced levels of β_1 integrin expression. The shRNA construct 3 yielded two clones (2 and 5) with depleted β_1 integrin expression. Only one pRS-shGFP clone was produced (shGFP-1) and three pRS empty vector clones were produced (clones 1-3). (Figure 4B) The pRS-2 cell line was not used for further analysis as levels of β_1 integrin were slightly less than the wild-type PC3 (wtPC3) cells.

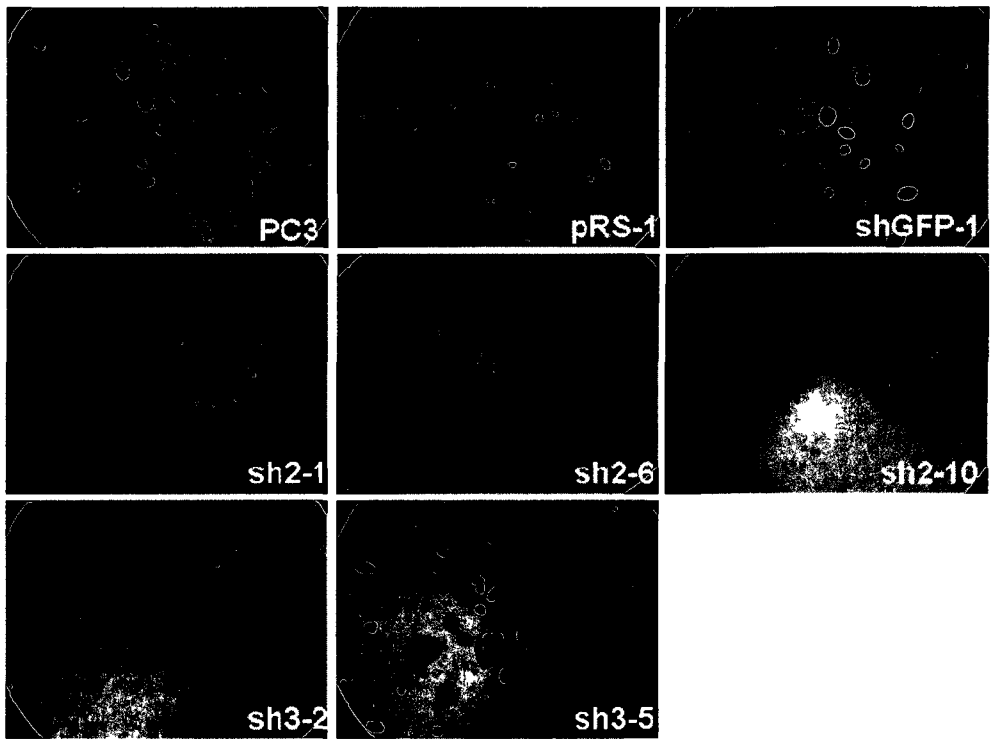
3.2 - β_1 INTEGRIN DEPLETION OF PC3 CELLS INHIBITS THEIR ANCHORAGE INDEPENDENT GROWTH IN SOFT AGAROSE

Soft agarose colony formation assays have been established as a reliable method to gauge the oncogenic capabilities of cells. (72) We hypothesize that β_1 integrin expression in PC3 cells is required for soft agarose colony formation and that in previous studies, the colonies that formed from cells transfected with β_1 integrin siRNA (siITGB1), were cells that retained β_1 integrin expression as a result of an ~50% transduction efficiency of PC3 cells with siRNA. (133) Previous attempts in the lab to confirm this were met with technical difficulties and hence were not possible, however the stably depleted β_1 integrin levels found in our shITGB1 clones would allow us to overcome these issues and confirm our hypothesis.

The stable shITGB1 cell lines, and corresponding controls, were initially assessed for their ability to produce soft agarose colonies (Figure 5). Compared to wtPC3 cells and to the pRS-1 and pRS-shGFP-1 controls, there was a significant reduction in colony formation by

Figure 5: Stable β_1 integrin depleted cell lines have reduced colony formation in soft agarose anchorage independent growth assays. shITGB1 cell lines (sh2-1, sh2-6, sh2-10, sh3-2 and sh3-5) and control cell lines (PC3, pRS-1 and pRS-shGFP-1), were cultured in 0.5% soft agarose media mixture in the presence of 10% FBS for 14 days at 37°C. Colony numbers were assessed at day 14 by a blinded count of all colonies per well (in triplicate) of an appropriate dilution using a bright-field microscope at 200x magnification. (**A**) Bright field photographs of PC3 cell colonies taken from a representative field of view on day 15 at a magnification of 40x. (B) Graphical representation of the mean and standard deviation of soft agaose colony formation by stable cell lines (individual clones) at the 125 cell/well dilution. Bars represent pooled means from triplicate wells in two independent experiments (n=6). Statistical significance was measured using a nonparametric One-way ANOVA and Dunnett's multiple comparison test, and a p-value <0.05 (*) was considered statistically significant.

A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shGFP-1
sh2-1
sh2-6
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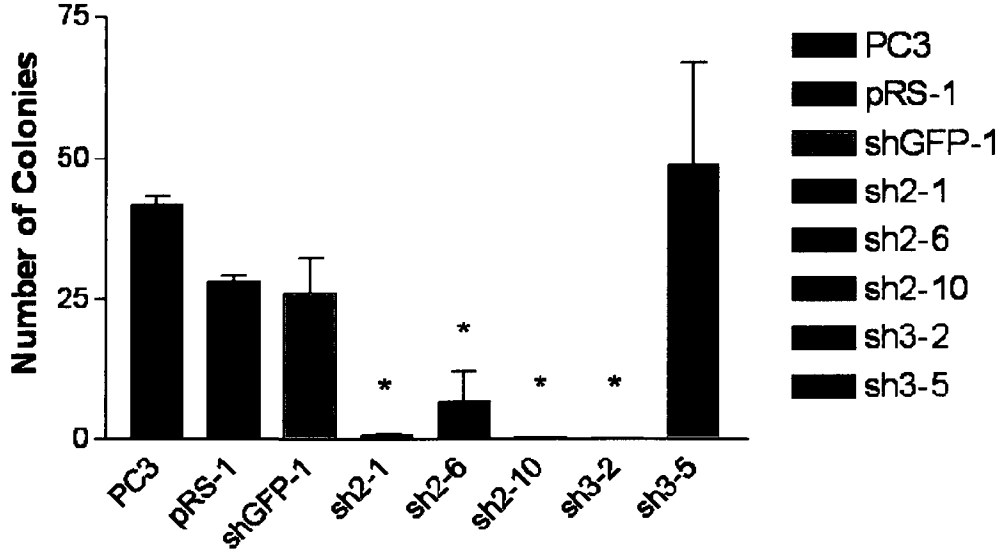
sh3-5

B


Number of Colonies
75
50
25
0
*
*
*
*
PC3
pRS-1
shGFP-1
sh2-1
sh2-6
sh2-10
sh3-2
sh3-5

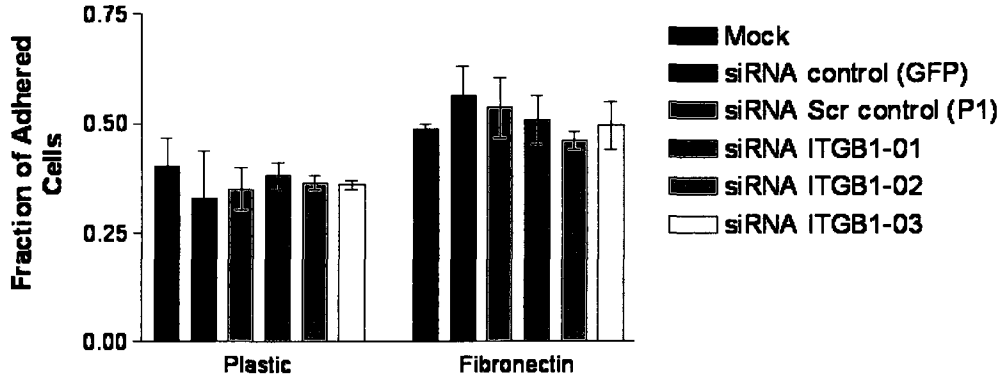
the cell lines sh2-1, sh2-6, sh2-10 and sh3-2. Clone sh3-5 did produce colonies in soft agar in similar numbers to controls. We attributed this phenomenon to clonal variation or insertional mutagenesis, and hence this clone was excluded from further experiments. The stable β_1 integrin depleted cell clones produced very few to no colonies at all in the soft agar assay, and this reduction in colony formation was considerably greater than what had previously been demonstrated with the transient siRNA depletion approach. (133) These results suggest that ablation of β_1 integrin corresponds to a significant reduction in soft agarose colony formation and confirms that β_1 integrin is required for anchorage independent (AI) growth in PC3 prostate cancer cells.

3.3 - ADHESION OF ITGB1 siRNA TRANSFECTED AND ITGB1 shRNA EXPRESSING PC3 CELLS ON VARIOUS EXTRACELLULAR MATRICES ARE NOT ALTERED BY DEPLETION OF β_1 INTEGRIN EXPRESSION.

In order to determine which underlying mechanisms may be responsible for the anchorage independent growth defect observed in β_1 integrin depleted cells, we assessed adhesion of the stable cells lines and of PC3 cells transiently transfected with siRNA-ITGB1. As we were depleting expression of a molecule known to mediate adhesion of cells to extracellular matrices, we wanted to test whether or not reduced β_1 integrin expression altered the ability of the cells to adhere to plastic along with other extracellular matrix proteins known to bind cells via β_1 integrin containing heterodimers (Figure 6). Initially, we tested the adherence of siRNA transfected cells to plastic and fibronectin as it was

Figure 6: Adhesion of β_1 integrin expressing and depleted cells on various extracellular matrices shows some clonal variation. Cells were plated in dishes precoated with fibronectin (1ug/cm^2), vitronectin (100ng/cm^2) or monomeric collagen I (5ug/cm^2) or on uncoated plastic tissue culture dishes. **(A)** Individual integrin β_1 siRNA targeted PC3 transfectants were collected with EDTA at 48 hrs post-siRNA transfection and seeded at $2x10^5$ cells per well and allowed to adhere to plastic, or fibronectin coated plates for 1.5 hrs. Adherent cells were counted by trypan exclusion and are expressed as the mean fraction of total cells plated. Statistical significance was measured using a nonparametric One-way ANOVA and Dunnett's multiple comparison test, and a p-value <0.05 (*) was considered statistically significant. **(B)** Stable shITGB1 expressing and control PC3 cell clones were seeded at $1x10^5$ cells per well and allowed to adhere to plastic, fibronectin, monomeric collagen or vitronectin-coated plates for 1.5 hrs. Adherent cells were counted by trypan exclusion and were expressed as the average cells counted. Bars represent the mean values with associated standard error from triplicate wells for three independent experiments (n = 9). Statistical significance was measured using a nonparametric One-way ANOVA and Dunnett's multiple comparison test, and a p-value <0.05 (*) was considered statistically significant.

A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0.50
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Mock
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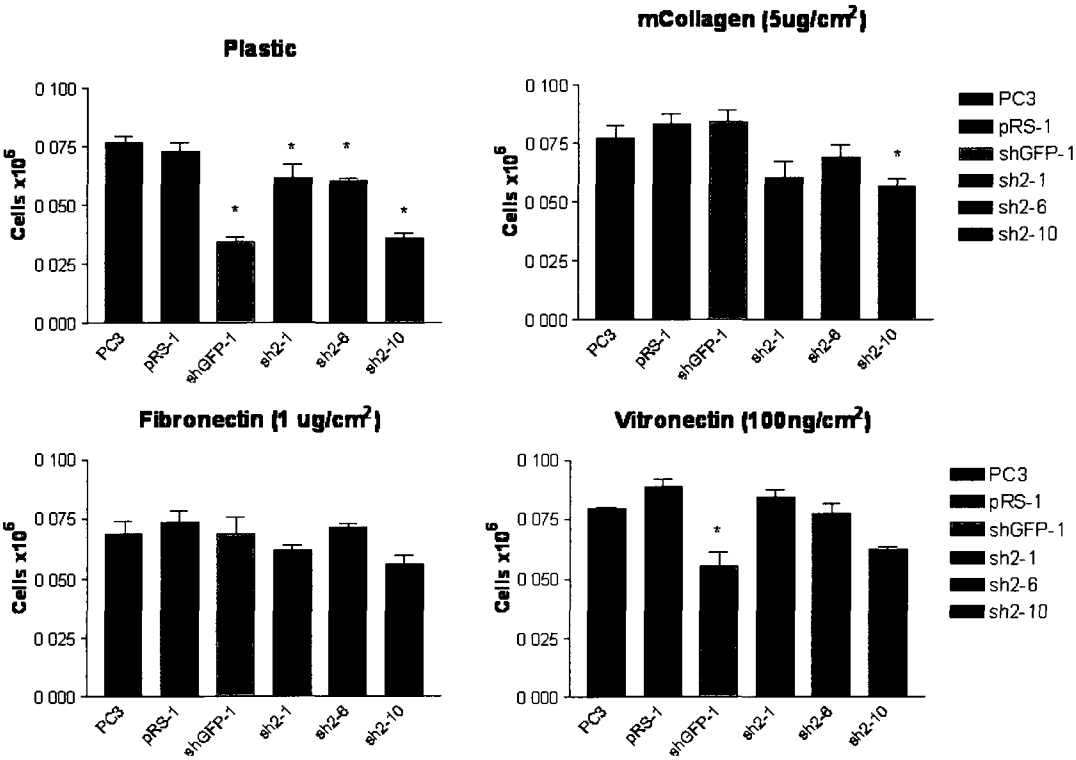
siRNA ITGB1-03

B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Fibronectin (1 ug/cm2)
Vitronectin (100ng/cm2)
Cells x10^6
0 100
0 075
0 050
0 025
0 000
PC3
pRS-1
shGFP-1
sh2-1
sh2-6
sh2-10

previously shown that soft agarose colony formation is fibronectin dependent, as well as being β_1 integrin dependant. (133) There was no significant difference in adhesion between β_1 integrin expressing and depleted cells to either plastic or fibronectin coated tissue culture plates. (Figure 6A) It is possible that other fibronectin binding integrins (such as $\alpha_v\beta_3$ and $\alpha_v\beta_6$) are compensating for the β_1 integrin deficiency in this case, or that the residual levels of β_1 integrin following siRNA depletion are sufficient to mediate cell adhesion. (48) We also tested the adherence of the shRNA stable cell lines on plastic, fibronectin, monomeric collagen I and vitronectin. (Figure 6B) On plastic, there was significant variability in adhesion with all the clones however, the noted adhesive differences would not account for the reduced anchorage independent growth that was previously observed as AI growth impairment was only found in β_1 integrin depleted cells. Although adhesion of shITGB1 expressing cells was somewhat reduced, it was in fact similar to the adhesion noted in the control shGFP-1 cell clone. On monomeric collagen, we noted a slight decrease in cell adhesion by the stable shITGB1 cell lines, however, with the exception of the sh2-10 cell line, the reduced adhesion noted with the β_1 integrin depleted clones did not reach statistical significance when compared to PC3 cells. No significant differences in adhesion were observed on fibronectin. Potential compensation from other integrins may allow β_1 integrin depleted cells to bind fibronectin effectively, although this has not been confirmed. On vitronectin, there appears to be variability between clones and once again, a significant reduction in the adhesion of the shGFP-1 cell line was observed. Clonal variation may be responsible for the observed difference in adhesion of this cell line. In all instances, with the exception of monomeric collagen, decreased adhesion in shITGB1 expressing cells is matched in at least one control clone. The observed decreased in

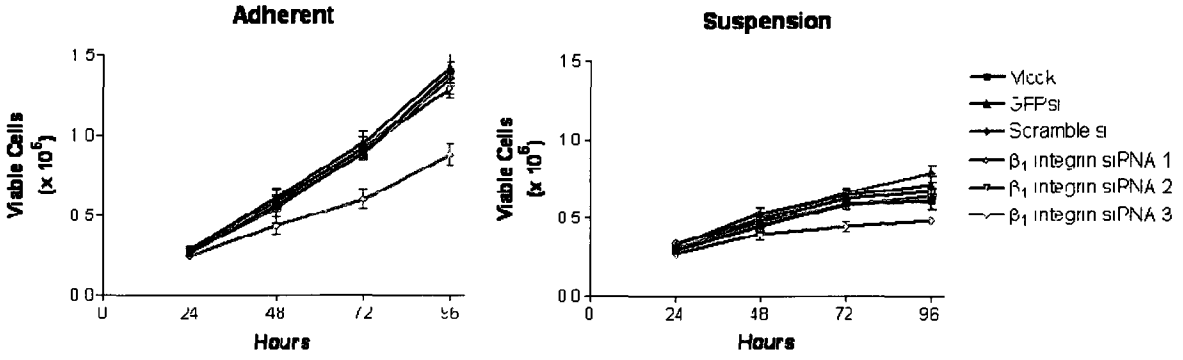
anchorage independent growth of β_1 integrin depleted PC3 cells is therefore not likely attributable to differences in adhesion.

3.4 - GROWTH RATES OF ITGB1 siRNA TRANSFECTED AND ITGB1 shRNA EXPRESSING PC3 CELLS IN ADHERENT AND SUSPENSION CONDITIONS ARE NOT ALTERED BY DEPLETION OF β_1 INTEGRIN EXPRESSION.

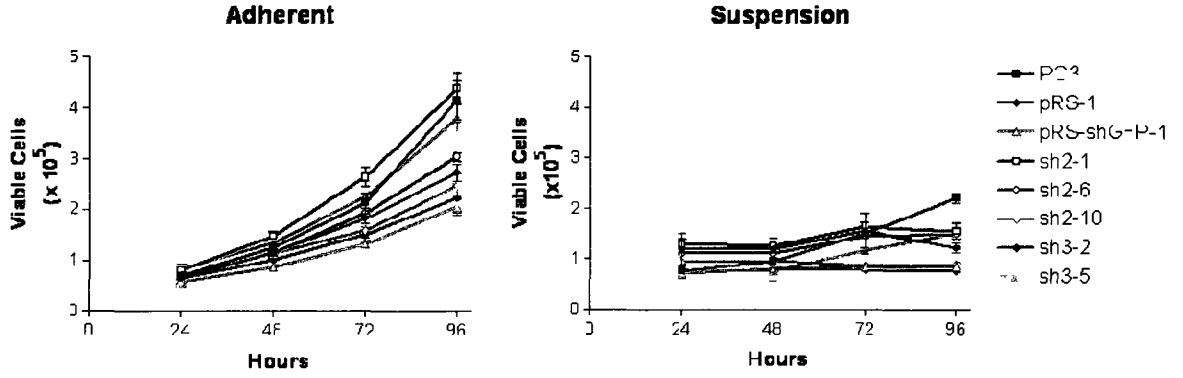
As differences in adhesion were not generally altered by reduced β_1 integrin expression, and thus were not likely to account for the anchorage independent growth phenotype, we assessed proliferation capacity in the stable cells lines and in PC3 cells transiently transfected with siRNA-ITGB1. Proliferation was examined under both adherent and suspension culture conditions. It is not possible to reliably quantify cell proliferation within agarose as cells cannot be extracted for analysis. We therefore used a model of suspension cell growth, whereby cells are grown over an agarose underlay to which adherence does not occur. Although this type of suspension growth does not exactly mimic three-dimensional culture, the cells must survive independently of adhesion to a substratum, and hence provides a reasonable means for assessing growth under suspension conditions. Previous studies have used this method to gauge the influence of a cellular adhesive state on a given signaling pathway, including proliferation. (135, 136) Wild-type PC3, control and shITGB1 stable cells lines and siRNA transfected cells were grown in adherent or in suspension conditions for 96 hours and viable cells were counted every 24 hrs with a Coulter counter using trypan blue exclusion (Figure 7). For the siRNA transfected PC3 cells, there

Figure 7: Growth rates of siRNA transfected PC3 cells or clonal shRNA transduced PC3 cell lines in adherent and suspension conditions are relatively similar. Cells were grown on plastic in adherent conditions and over a 1% agarose underlay for suspension conditions. **(A)** PC3 cells transfected with 5nM of scramble control siRNA (Scramble si), siGFP control (GFPsi) or three distinct ITGB1 targeting siRNA constructs (ITGB1-01, ITGB1-02, ITGB1-03) were seeded 24 hrs post-transfection and grown on plastic or in suspension for 96 hrs. Viable cells were quantified every 24 hrs following collection by trypan exclusion. Bars represent mean and standard deviation for duplicate wells from 3 independent experiments (n=6). (B) Stable PC3 cells expressing ITGB1 targeted shRNA (sh2-1, sh2-6, sh2-10, sh3-2 and sh3-5), a pRS empty vector control or a shGFP control were grown over plastic or agarose for 96 hrs and viable cells were collected and enumerated every 24 hrs by trypan exclusion. Bars represent mean and standard deviation for duplicate wells from 3 independent experiments (n=6). In both cases, statistical significance was measure by one-way ANOVA and multiple comparison.

A


Adherent
Suspension
Viable Cells (x 10^6)
Hours
0
24
48
72
96
Mock
GFPsi
Scramble si
β1 integrin siRNA 1
β1 integrin siRNA 2
β1 integrin siRNA 3

B

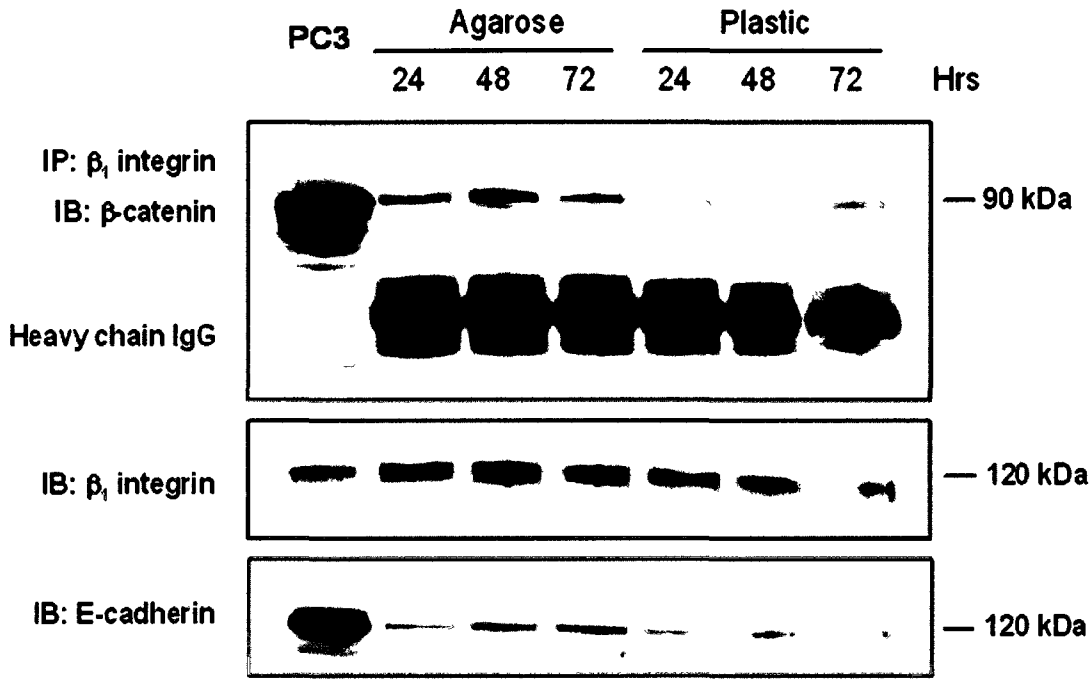

Adherent
Suspension
Viable Cells (x 10^5)
Hours
0
24
48
72
96
pRS-1
pRS-shG-P-1
sh2-1
sh2-6
sh2-10
sh3-2
sh3-5

were no significant differences in the number of viable cells over time between wild type PC3 (mock) or other control transfected cells lines and those expressing β_1 integrin siRNA (Figure 7A). The exception to this was noted with cells transfected with β_1 integrin siRNA-ITGB1-03, which proliferated at a significantly lower rate than the other siRNA transduced cells. Cell proliferation over time in suspension conditions was more limited compared to that in adherent conditions, which was exponential, however again, no differences in cell growth or viability was noted with the exception of cells transfected with siRNA-ITGB1-03. We suspect the variability observed with the siRNA-ITGB1-03 construct is an off-target effect of that particular siRNA. When we assessed cell growth over time for the stably transduced cell lines, we noted that the proliferation rates of the cell lines stably expressing shITGB1 and controls were more variable (Figure 7B). There were significant differences noted between clones with regards to proliferation in both adherent and suspended culture conditions. However, clones from both control and β_1 integrin specific shRNA treated cells can be found throughout the range of observed proliferation rates, suggesting that the differences observed are a result of clonal variation rather than a result of dependence on β_1 integrin expression. In suspension conditions, there was little proliferation with the exception of the PC3 cell line. Again there are discrepancies between cell lines, but none that can be attributed to a specific lack of β_1 integrin expression.

3.5 - DETERMINATION OF THE PUTATIVE MECHANISM OF β_1 INTEGRIN CONTROL ON 3D *IN VITRO* TUMOR GROWTH: NOVEL MOLECULAR INTERACTIONS IN SUSPENSION GROWTH CONDITIONS.

In our initial work characterizing our stably depleted cell lines, we did not see any significant differences in proliferation nor adhesion that would be specifically attributable to a lack of β_1 integrin expression. However, we did observe a significant impairment in the ability of cells to grow in anchorage independent fashion in soft agarose assays when cells were depleted of β_1 integrin and wished to determine what possible mechanisms might contribute to this phenotype. In an effort to tease out the downstream effectors of fibronectin mediated β_1 integrin dependent anchorage independent growth, we further examined a novel molecular interaction previously observed in three-dimensional but not two-dimensional culture. It has previously been shown that β_1 integrin co-immunoprecipitated with β-catenin in suspension but not in adherent culture of PC3 cells. (133) It has also been previously shown that novel molecular interactions and signaling pathways occur in anchorage independent growth that were not observed in adherent growth conditions. (137) As this novel interaction could provide insight into the mechanisms responsible for three-dimensional growth, PC3 cells were grown on plastic or in suspension over an agarose underlay and cell lysates were collected at 24, 48 and 72 hrs. Cell lysates were then subject to immunoprecipitation (IP) using a β_1 integrin specific antibody (6S6) that had been used in the previous analyses. (133) Western blot analysis of immunoprecipitated lysates demonstrated that there was enhanced association of β-catenin with β_1 integrin when cells were cultured in suspension growth versus adherent conditions suggesting that this preferential interaction is an anchorage independent phenomenon (Figure 8). E-cadherin also co-immunoprecipitated in this complex, and appeared to be at levels that were proportional to β_1 integrin. Given the known role of β-catenin and E-cadherin in anchorage independent

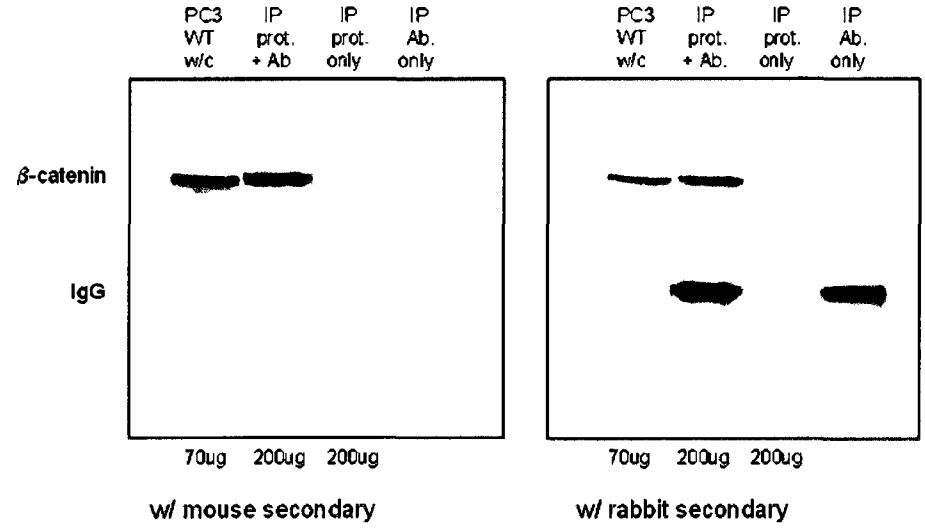
Figure 8: β-catenin co-immunoprecipitates with β1 integrin in suspension growth conditions. Whole cell lysates from PC3 cells grown adherently or induced to grow in suspension over a 1% solid agarose base for 24, 48 or 72 hrs were subjected to immunoprecipitation (IP) with an anti-β_1 integrin antibody followed by SDS-PAGE and immunoblotting (IB) with either anti-β_1 integrin, β-catenin or E-cadherin antibodies. Lysates from adherent cells were incubated in the absence of β1 integrin antibody ± mouse IgG1 to confirm the specificity of the IP.


PC3
Agarose
Plastic
24
48
72
24
48
72
Hrs
IP: β1 integrin
IB: β-catenin
Heavy chain IgG
— 90 kDa
IB: β1 integrin
— 120 kDa
IB: E-cadherin
— 120 kDa

growth (138, 139), we pursued this avenue of investigation further. To confirm these results, the reverse immunoprecipitation, namely, isolation of β-catenin by IP, and subsequent probing for β_1 integrin in the IP by western blot, was performed (Figure 9). An initial immunoprecipitation was done with PC3 cell lysates using a "protein alone" control and "antibody alone" control to confirm the effectiveness and specificity of the IP (Figure 9A). No signal was detected in the "protein only" condition, suggesting that the cell lysates do not have undesirable interactions with the sepharose beads used for immunoprecipitation. No β-catenin signal was detectable in the "antibody only" control suggesting that detection of β-catenin is not otherwise an artifact. This lane also shows an IgG control band confirming that the antibody effectively binds the bead. Following the β-catenin immunoprecipitation, β-catenin could be detected by subsequent western blot in both culture conditions (adherent and suspension) at all time points (Figure 9B). E-cadherin was detected proportionally to β-catenin indicating the success of co-immunoprecipitating out β-catenin containing complexes with this approach. However, despite this, β_1 integrin could not be detected in either culture condition. The possibility exists that the binding site for the β-catenin antibody coincides with the hypothetical interactions site of β_1 integrin therefore resulting in the IP of only integrin-unbound forms of β-catenin. Therefore, a second antibody that recognized a different epitope in β-catenin was employed to repeat the co-immunoprecipitation (Figure 10A). Co-immunoprecipitation of β-catenin with the second antibody was repeated and was also tested for IP efficiency and specificity (Figure 10B). The immunoprecipitation appeared to specifically pull out β-catenin in the assay. The lysates from cells grown in suspension versus adherent conditions were then subject to IP for β-catenin, and this reverse immunoprecipitation was performed in parallel with a β_1

Figure 9 : Immunoprecipitation of β-catenin from whole cell lysates grown under adherent or suspension conditions did not pull down β_1 integrin protein. (A) Whole cell lysates from PC3 cells were subjected to immunoprecipitation (IP) with an anti-β-catenin antibody (rabbit polyclonal, Cell Signaling) or alone with sepharose beads (negative control) to test IP efficiency. Sepharose beads used for IP were also incubated with antibody alone as a positive IgG control. Resulting precipitates were analyzed for β-catenin levels by SDS-PAGE and immunoblotting (IB) with an anti-β-catenin antibody and both mouse and rabbit secondary antibodies to assess cross-reaction with the antibody used for IP. (B) Whole cell lysates from PC3 cells grown adherently or induced to grow in suspension over a 1% solid agarose base for 24, 48 or 72 hrs were subjected to IP with an anti-β-catenin antibody (Clone 6F9, Sigma) followed by SDS-PAGE and IB with either anti- β-catenin, β_1 integrin, or E-cadherin antibodies. Total protein lysates from adherent cells were also loaded on the gels as a positive control to ensure the success of the western blot for the target protein.

A


PC3 WT w/c
IP prot. + Ab
IP prot. only
IP Ab. only
β-catenin
IgG
70ug
200ug
200ug
w/ mouse secondary
PC3 WT w/c
IP prot. + Ab.
IP prot. only
IP Ab. only
70ug
200ug
200ug
w/ rabbit secondary

B

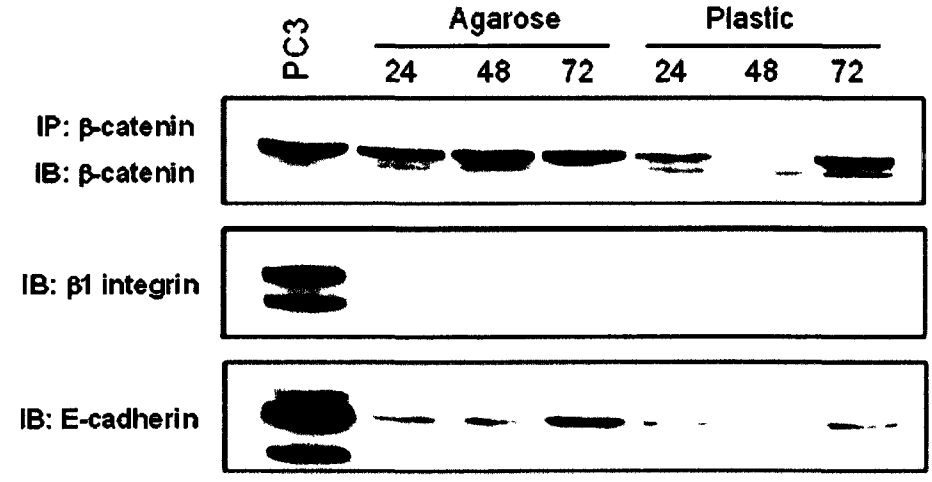
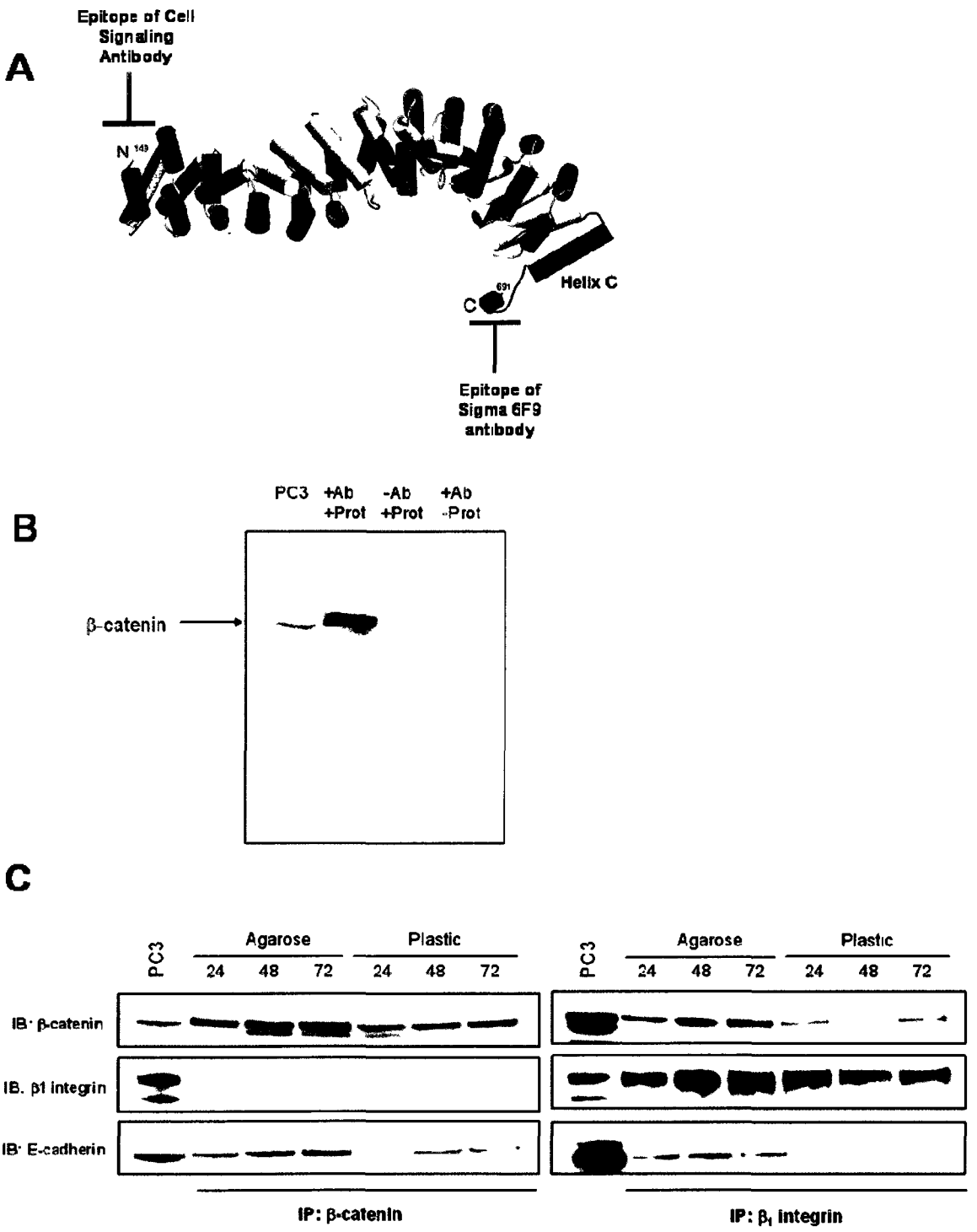

PC3
Agarose
Plastic
24
48
72
24
48
72
IP: β-catenin
IB: β-catenin
IB: β1 integrin
IB: E-cadherin

Figure 10 : Immunoprecipitation of whole cell lysates in adherent and suspension conditions with an alternative β-catenin antibody did not result in β_1 integrin coimmunoprecipitation. (A) Schematic representation of β-catenin and the approximate location of the epitopes recognized by antibodies used for immunoprecipitation (IP). Figure adapted from *Xing et al.* (174). **(B)** Whole cell lysates from PC3 cells were subjected to immunoprecipitation (IP) with an anti-β-catenin antibody (Clone 6F9, Sigma) or alone with sepharose beads (negative control) to test IP efficiency. Sepharose beads used for IP were also incubated with antibody alone as a positive IgG control. Resulting precipitates were analyzed for β-catenin levels by SDS-PAGE and immunoblotting (IB) with an anti-β-catenin antibody and both mouse and rabbit secondary antibodies to assess cross-reaction with the antibody used for IP. **(C)** Whole cell lysates from PC3 cells grown adherently or induced to grow in suspension over a 1% solid agarose base for 24, 48 or 72 hrs were subjected to IP with an anti-β-catenin (rabbit polyclonal, Cell Signaling) antibody followed by SDS-PAGE and IB with either anti- β-catenin, β_1 integrin, or E-cadherin antibodies. In parallel, the reverse IP using a β1 integrin antibody was performed followed by IB of β_1 integrin, β-catenin and E-cadherin to confirm reproducibility of initial results.

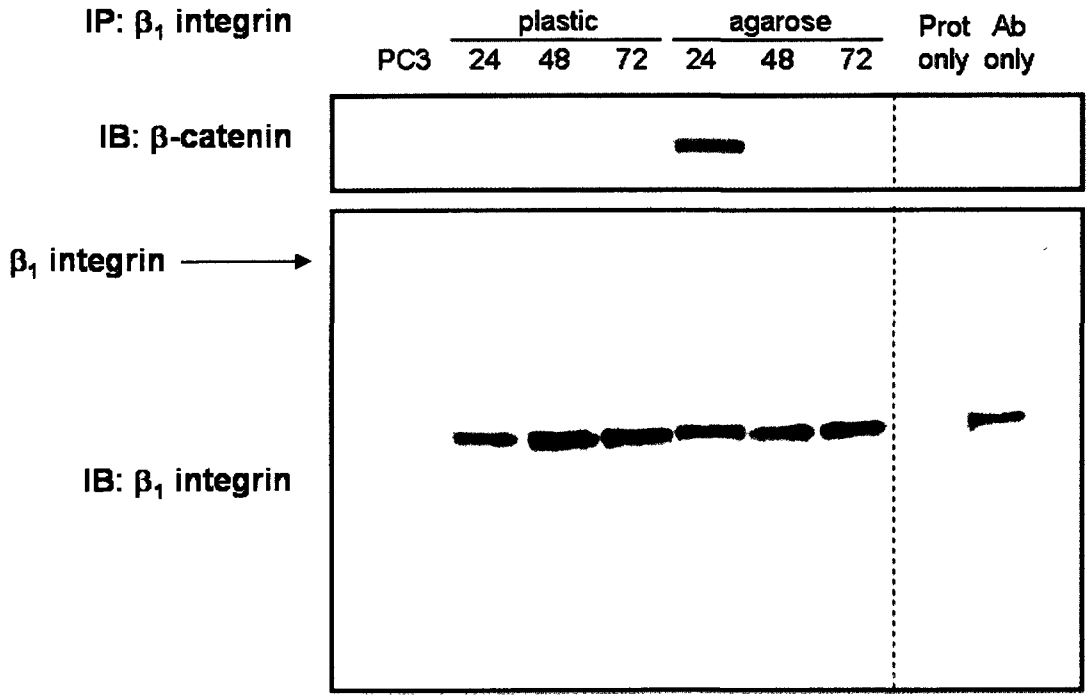

A
Epitope of Cell Signaling Antibody
N 149
Helix C
C 691
Epitope of Sigma 6F9 antibody
B
PC3 +Ab +Prot -Ab +Prot +Ab -Prot
β-catenin
C
PC3
Agarose
Plastic
24 48 72 24 48 72
IB: β-catenin
IB. β1 integrin
IB: E-cadherin
IP: β-catenin
IP: β1 integrin

integrin IP to ensure our original results could still be reproduced (Figure 10C). β_1 integrin could not be detected in either culture condition or at any time point post-plating of cells when an antibody to β-catenin was used in the IP whereas the IP using antibody to β_1 integrin still resulted in the preferential co-IP of β-catenin with $\beta_1$integrin from cells cultured in suspension. Regardless of which antibody was used for the initial IP (eg. β_1 integrin or β-catenin), E-cadherin was once again found to co-immunoprecipitate proportionally with the levels of β-catenin.

As E-cadherin is a known binding partner of β-catenin at cell-cell adherens junctions, (140) we attempted to identify β_1-integrin in a potential three way complex with β-catenin and E-cadherin by using antibody to E-cadherin in a co-IP. It was found that β-catenin can be detected when E-cadherin was immunoprecipitated. However, β_1 integrin is not detectable when E-cadherin was immunoprecipitated. (data not shown)

After three different unsuccessful approaches to confirm the co-immunoprecipitation of $\beta_1$integrin and β-catenin, it became increasingly apparent that there was likely a secondary interaction between β-catenin and the integrin blocking antibody. A second β_1 integrin adhesion-neutralizing antibody (clone P4C10) was therefore employed to repeat the initial observations. (Figure 11) This IP was not successful and although β_1 integrin was not pulled-down by the antibody, β-catenin was detected in the suspension growth conditions suggesting that there is potentially an undesirable interaction between the β_1 integrin antibodies used for the IP and β-catenin. Despite this negative result, it remains possible that adherens junction components are involved in the tumorigenic properties of prostate cancer cells. (91)

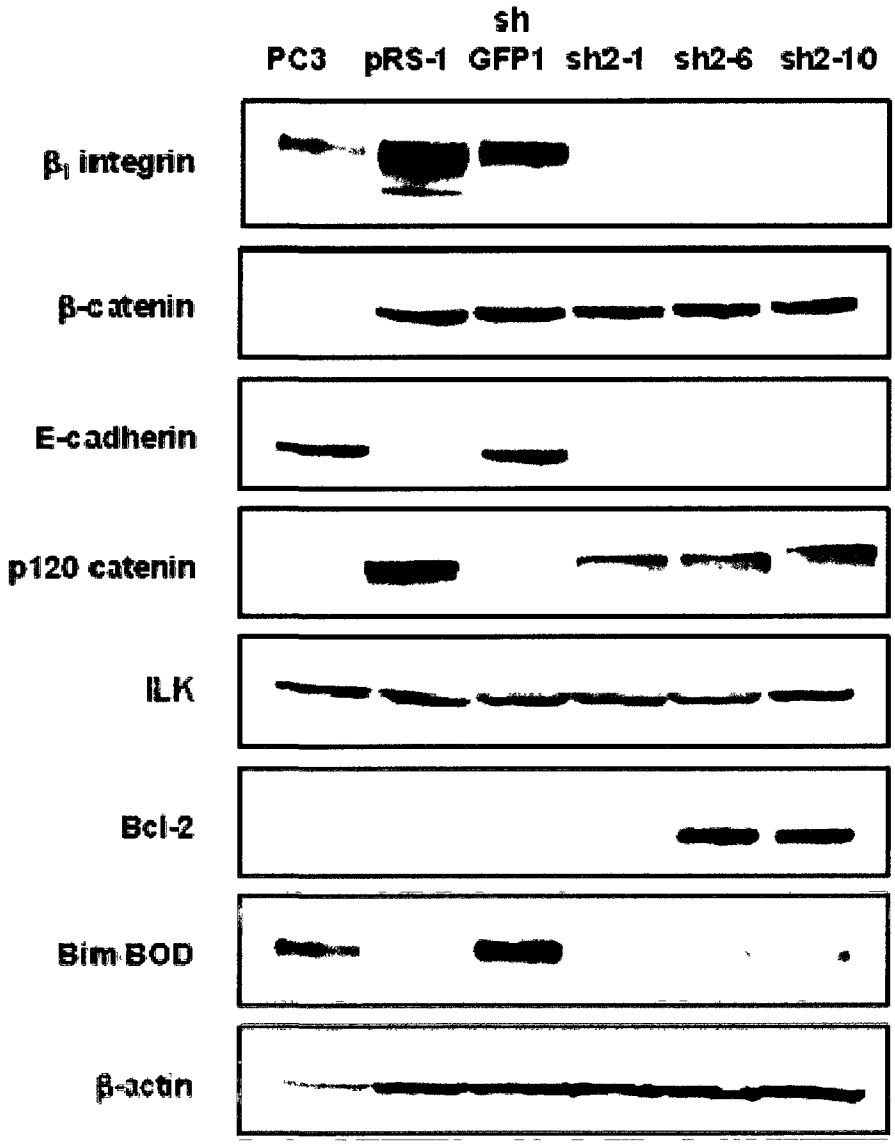
Figure 11 : β-catenin is pulled down by immunoprecipitation with β_1-integrin antibody P4C10 even in the absence of successful pull down of β_1-integrin. Whole cell lysates from PC3 cells grown adherently or induced to grow in suspension over a 1% solid agarose base for 24, 48 or 72 hrs were subjected to immunoprecipitation (IP) with a different anti-β_1 integrin antibody than previously used (Clone P4C10) followed by SDS-PAGE and immunoblotting (IB) with either anti-β_1 integrin or β-catenin antibodies. Lysates from adherent cells were incubated in the absence of β_1 integrin antibody $\pm$ mouse IgG1 and in the presence or without the sepharose binding beads to confirm the specificity of the IP.


IP: β1 integrin
plastic
agarose
Prot only
Ab only
PC3
24
48
72
24
48
72
IB: β-catenin
β1 integrin
IB: β1 integrin

3.6 - MOLECULAR CHARACTERIZATION OF β_1 INTEGRIN EXPRESSING AND STABLY DEPLETED CELL LINES

It remains important to determine the underlying mechanisms responsible for the β_1 integrin dependent growth of PC3 cells in soft agarose. A western blot analysis was performed on all stable cell lines to characterize the expression levels of various proteins of interest. Adherens junctions are important in cancer as their improper assembly, or loss of their components, results in a more motile and less epithelial cell phenotype. (91) The expression patterns of adherens junction molecules have been shown to affect the expression levels of β_1 integrin in some cancers (141) and previous studies have shown direct interactions between β_1 integrin and cell-cell junctions in cancer. (142) As E-cadherin mediates cell-cell attachment and has an established role in the malignant progression of prostate cancer, (3, 140) its expression was also evaluated in our cell lines. Endogenous levels of β-catenin were similar among the clones, however were higher than that observed in the wtPC3 cells (Figure 12). E-cadherin was strongly expressed in two of the three control cell lines (PC3 and shGFP-1) and had comparatively reduced expression in the β_1 integrin depleted cell lines as well as in the pRS cell line. Given our results with the observed differences in E-cadherin levels, we also examined p120 catenin, as it is known to interact with the cytoplasmic tail of E-cadherin and is responsible for the maintenance and turnover of E-cadherin at the cell surface.(143) Interestingly, p120 catenin was not expressed in the cell lines that did express E-cadherin, namely pRS-1, and the β_1 integrin depleted clones, but was abundantly detected in the wtPC3 and shGPF control cells. We also investigated the endogenous levels of ILK in our cell lines as it is known to modulate several β_1 integrin

Figure 12: Western Blot analysis of various protein expression levels in stable shITGB1 expressing clones suggests differential expression of E-cadherin and p120 catenin occurs in β_1 integrin depleted cells compared to controls. Whole cell lysates from PC3 cell lines with stably depleted levels of β_1 integrin were subject to an SDS-PAGE immunoblot (IB) analysis using anitbodies against β_1 integrin, its downstream effector integrin linked kinase (ILK), cell adherens junction molecules β-catenin, E-cadherin and p120 catenin (p120) and apoptotic regulators Bcl-2 and Bim/BOD. Levels of β-actin were also analyzed as a loading control.


sh
PC3
pRS-1
GFP1
sh2-1
sh2-6
sh2-10
β1 integrin
β-catenin
E-cadherin
p120 catenin
ILK
Bcl-2
Bim BOD
β-actin

downstream effector pathways, has an established role in cancer, and has been shown to be required for growth of tumor cells in an anchorage independent fashion. (65, 75) When total levels of ILK were examined, we observed no significant difference between any of the cell clones examined. However, it should be noted that the activity of ILK could be altered in our cell lines, and this remains to be determined.

Although we did not observe significant differences in cell survival between control and β_1 depleted cell lines when grown in adherent or suspension conditions, we also investigated the possible contribution of proteins regulating cell survival to the phenotype we observed in soft agar assay. As Bcl-2 is an anti-apoptotic protein that can be activated by fibronectin mediated integrin signaling, and the anchorage independent growth of PC3 cells appeared to be fibronectin signaling dependent, (144) we examined Bcl-2 expression level in our cell lines. Bcl-2 family proteins are involved in apoptosis caused by mitochondrial outer membrane permeabilization, and thus they have both pro- and anti-apoptotic functions. (145) Bcl-2 was most highly expressed in the β_1 integrin depleted cell lines sh2-6 and sh2-10 but not in the other shITGB1 cell line or in the controls. As we did not see any significant cell survival differences in the adherent and suspension growth of these cells, we were thus not surprised by a lack of Bcl-2 expression in the control cell lines when protein levels from adherent cells were analyzed. We similarly assessed the levels of Bim/BOD, a pro-apoptotic Bcl-2 antagonist. We saw that Bim/BOD was more highly expressed in wtPC3 and the shGFP-1 control cells, but was comparatively reduced in the shITGB1 cell lines and the pRS control. Again, levels of expression in adherent cell cultures may not reflect expression levels in anchorage independent growth. Furthermore, we did not assess the levels of expression of these proteins in the presence of fibronectin, and should we pursue that avenue

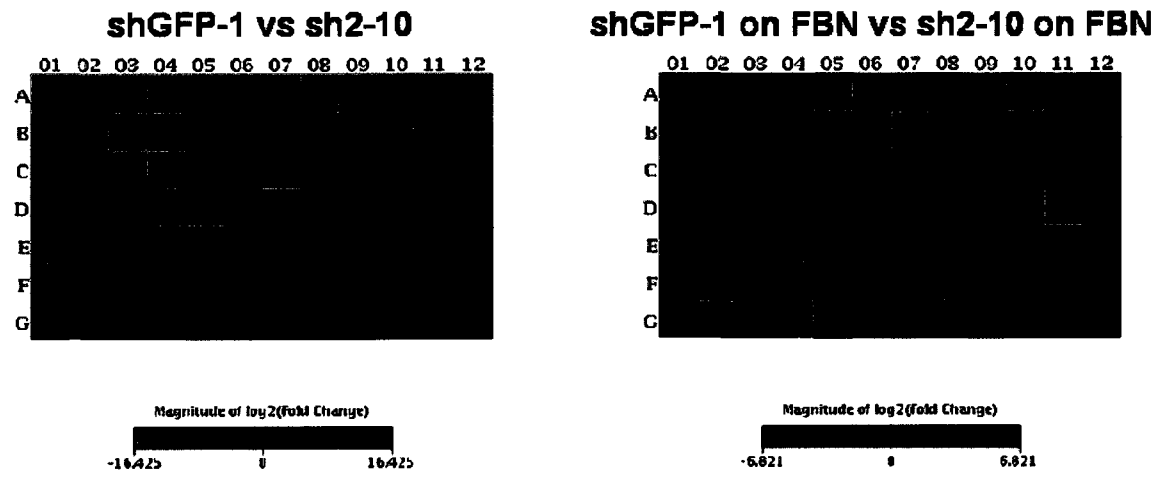
of investigation, we might find that β_1 integrin deficient clones cannot upregulate Bcl-2 in response to fibronectin which may occur in the control cell lines.

After examining these targets and finding no significant correlations to explain our anchorage independent growth phenotype, we chose a high throughput approach to potentially identify other proteins of interest that could be modulating this phenotype. To elucidate potential changes that occur in PC3 cells as a result of β_1 integrin depletion, we chose a qRT-PCR microarray to examine extracellular matrix and cell adhesion molecule gene expression patterns, primarily because many cell adhesion molecules have been shown to play essential roles in anchorage independent growth, and because of our group's previous results implicating the importance of fibronectin in mediating anchorage independent growth of PC3 cells. By comparing the RNA harvested from the shGFP-1 control and the sh2-10 cell lines grown on plastic, we were able to identify genes that had decreased expression in the shITGB1 cell line such as E-cadherin (CDH1), β_1 integrin (ITGB1), matrix metalloproteinase 9 (MMP9) and transforming growth factor βinduced (TGFBI). (Figure 13A) The aforementioned cell lines were also grown on fibronectin before RNA was harvested for the qRT-PCR array. The fibronectin dependent phenotypes included reduced expression of matrix metalloproteinase 11 (MMP11), matrix metalloproteinase 14 (MMP14) and (TIMP2). (Figure 13B) Primers for each of these targets of interest were designed and tested in real time PCR assays to confirm our pathway specific array results.

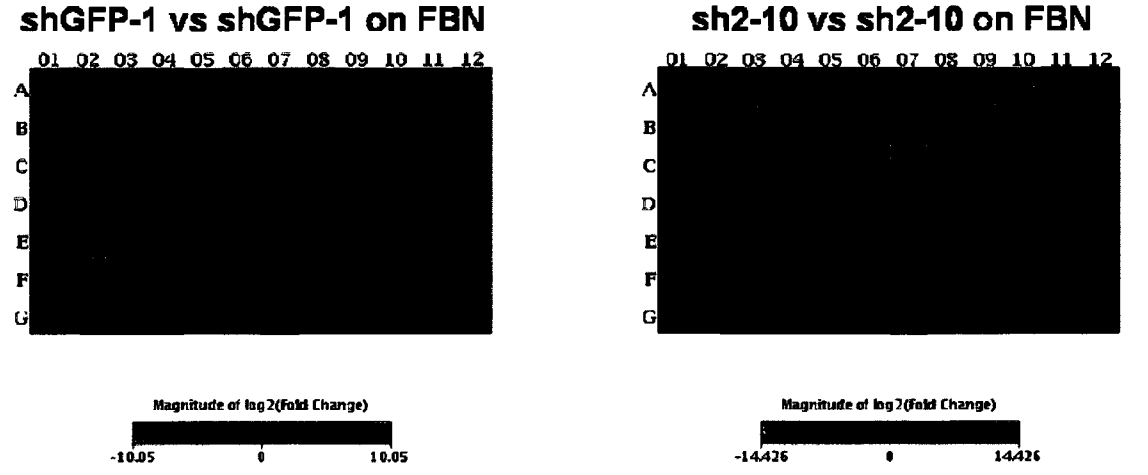
Subsequently, all cell lines (PC3, pRS-1, pRS-shGFP-1, sh2-1, sh2-6 and sh2-10) were grown in duplicate on plastic and fibronectin for 24hrs before collecting RNA and protein for analysis. The following targets were analyzed in all cell lines by comparing levels of expression to wt PC3 cells on plastic and fibronectin with endogenous levels

Figure 13 : Heat maps from qRT-PCR microarray analysis of RNA isolated from β_1 integrin depleted cell line sh2-10 compared to control cell line shGFP-1 show differential regulation of a number of gene targets. The PC3 cell clones sh2-10 and shGFP-1 were grown under adherent conditions on plastic **(A)** or fibronectin (FBN) **(B)** for 24hrs before RNA was harvested for PCR analysis using the SABiosciences Pathway specific PCR plate arrays. Relevant β_1 integrin dependent changes in gene expression to be noted in cells grown on plastic (A) are as follows: decreased expression of E-cadherin (CDH1, well A-05), β_1 integrin (ITGB1, well D-04), matrix metalloproteinase 9 (MMP9, well F-04) and transforming growth factor β_1 (TGFBI, well G-02) Relevant FBN (B) dependent changes in gene expression are as follows: increased expression of matrix metalloproteinase 11 (MMP11, well E-06), matrix metalloproteinase 14 (MMP14, well E-09) and Tissue Inhibitor of Metalloproteinases 2 (TIMP2,well G-07). In all cases, the first listed cell line is the control group while the second listed cell line is the target group in which relevant fold changes occur. The complete list of microarray targets is provided in Appendix A.

A


shGFP-1 vs sh2-10
01 02 03 04 05 06 07 08 09 10 11 12
A
B
C
D
E
F
G
Magnitude of log2(Fold Change)
-16.425
0
16.425
shGFP-1 on FBN vs sh2-10 on FBN
01 02 03 04 05 06 07 08 09 10 11 12
A
B
C
D
E
F
G
Magnitude of log2(Fold Change)
-6.821
0
6.821

B

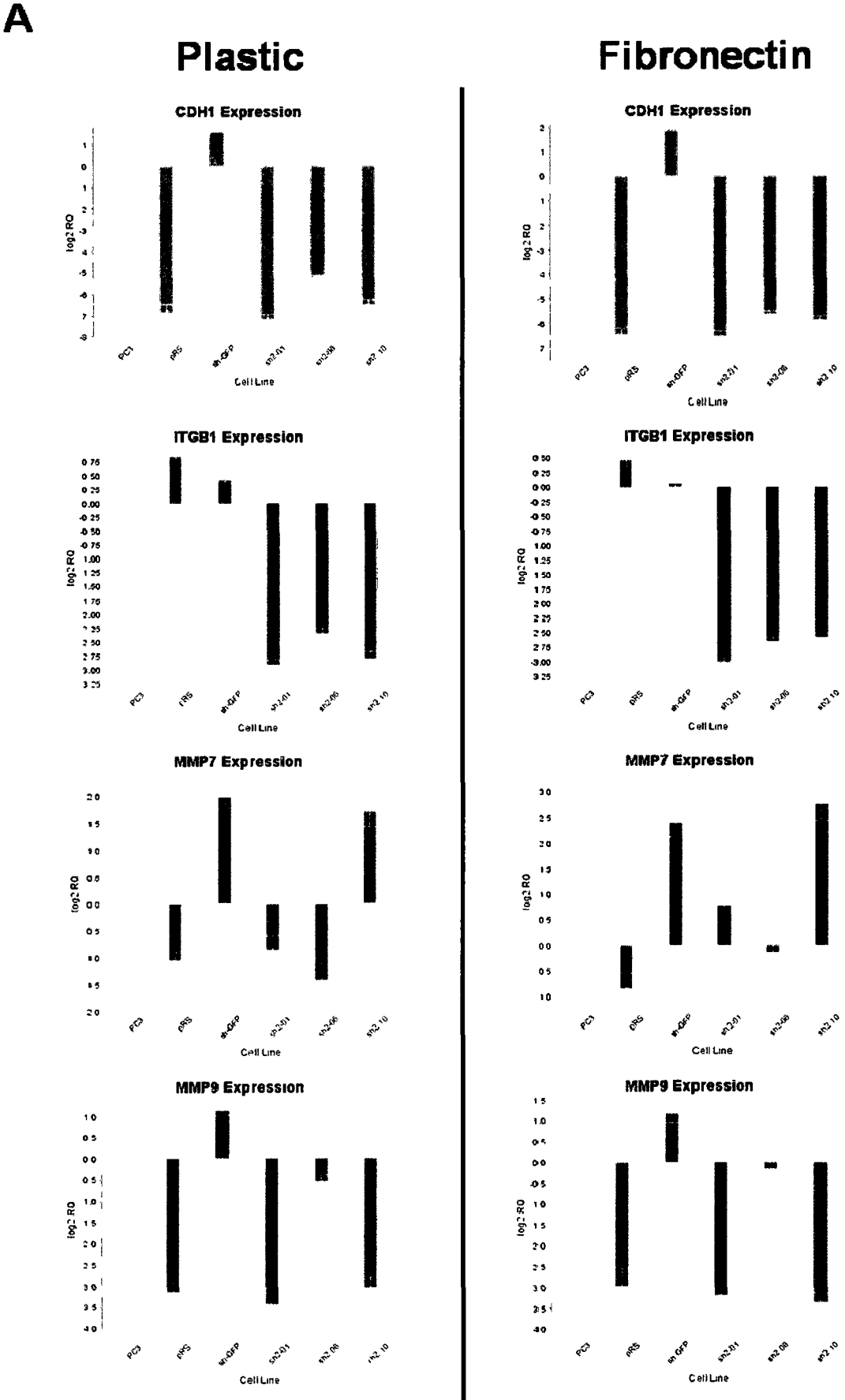

shGFP-1 vs shGFP-1 on FBN
01 02 03 04 05 06 07 08 09 10 11 12
A
B
C
D
E
F
G
Magnitude of log2(Fold Change)
-10.05
0
10.05
sh2-10 vs sh2-10 on FBN
01 02 03 04 05 06 07 08 09 10 11 12
A
B
C
D
E
F
G
Magnitude of log2(Fold Change)
-14.426
0
14.426

normalized to respective levels of β-actin for each sample: CDH1, ITGB1, MMP7, MMP9, MMP11, MMP14, TGFBI and TIMP2. (Figure 14A) It was confirmed that as well as having a decrease in β_1 integrin protein expression, shITGB1 clones showed reduced β_1 integrin RNA levels compared to all controls. The levels of E-cadherin (CDH1) and β_1 integrin (ITGB1) both reflected the observed expression patterns found at the protein level. This suggests that our qPCR method accurately reflects gene expression patterns in our cell lines. (see Figure 12)

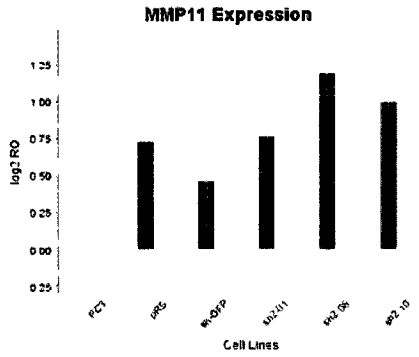
Upon examination of mRNA expression levels of our putative targets, aside from some variability between all clones, no significant trends in RNA expression between integrin β_1 depleted and control clones were found for MMP7, MMP9, MMP11, MMP14 or TIMP2. Expression of MMP9, for example, is markedly decreased in β_1 integrin depleted cell lines sh2-1 and sh2-10 compared to wtPC3 parental cells, but the levels were similar to those observed in the pRS control cell line, while MMP9 expression in the sh2-6 cell line is similar to expression levels in wtPC3 cells. As our original qPCR arrays were performed with the shGFP and sh2-10 cell lines as comparators, and the change in the expression levels of these targets between these two cell lines do validate what was observed in the initial array results, when examined in all the control and shITGB1 expressing cell lines, the changes in these markers was not consistently observed, hence emphasizing the importance of target validation. MMP7 expression appears to be slightly increased in shITGB1 cell lines upon stimulation with fibronectin compared to cells grown on plastic, however the levels of expression are still highly variable between all cell lines. MMP14 expression also appears to be altered upon fibronectin stimulation with a general decrease in expression level.

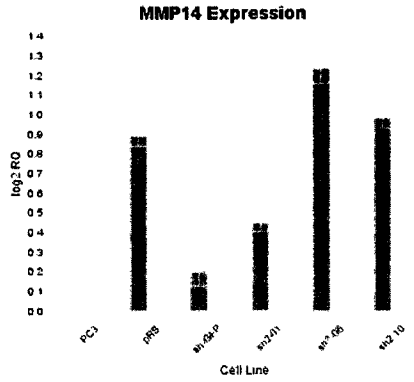
Figure 14 : RNA expression levels of putative targets validated by qPCR analysis showed consistent down-regulation of ITGB1 and TGFβ in β_1 integrin depleted compared to control cell lines. (A) All stable cell lines were grown on plastic or fibronectin coated plates for 24hrs before RNA was harvested as described in the materials and methods. Reverse transcription using random hexamers was used to generate cDNA from (1 ug) RNA of total RNA, and products were subsequently used in qPCR assays using specific primer pairs for each putative target as described in ther materials and methods. All samples were assayed in triplicate. Values shown are expressed as log2, are compared to PC3 cell values which was set at a value of 1, and were normalized to endogenous β-actin levels. Bars represent the mean relative quantity (RQ) with associated error for Pooled data from two independent biological replicates assayed in triplicate. (B) Whole cell lysates from stable cell lines grown on plastic were collected in parallel with RNA samples analyzed above, and were subject to immunoblotting for TGFβ1 and β_1 integrin to confirm differences in message level translated to differences in protein expression.

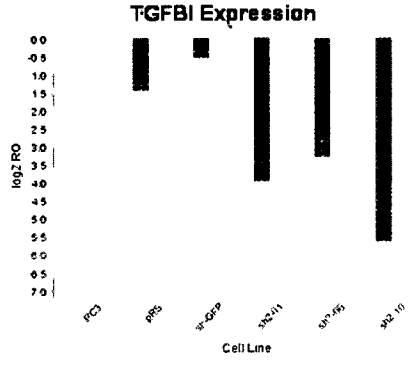
A

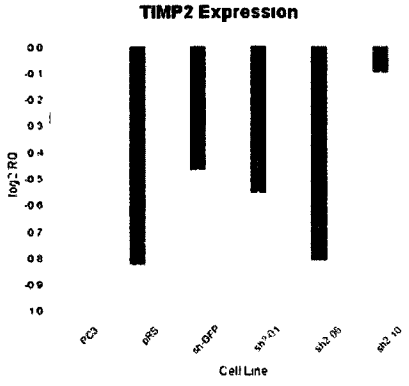

Plastic
Fibronectin
CDH1 Expression
ITGB1 Expression
MMP7 Expression
MMP9 Expression
log2 RQ
PC3
pRS
shGFP
sh2-01
sh2-08
sh2-10
Cell Line

Plastic

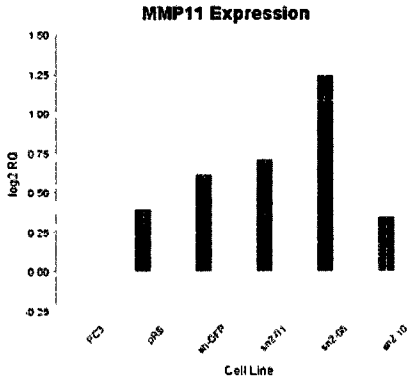


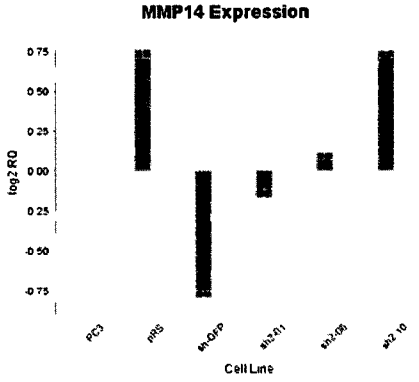


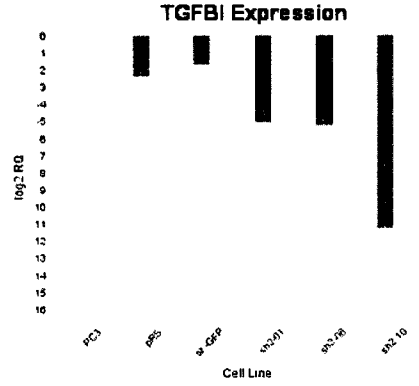


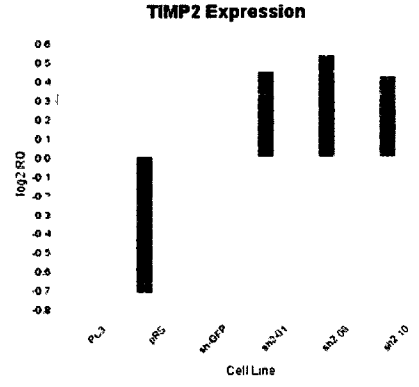


Fibronectin









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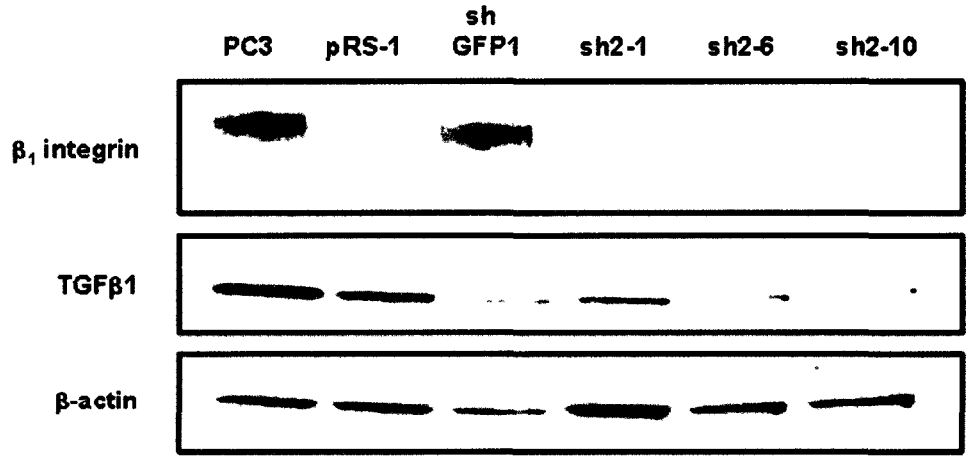


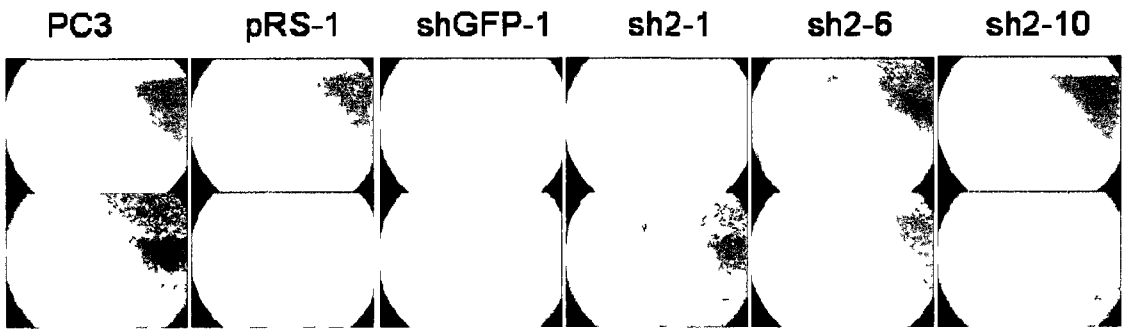
Transforming Growth Factor β-induced (TGFBI) is an extracellular matrix protein containing an integrin recognized RGD motif (178)that has been shown to interact with integrins $\alpha_v\beta_3$ and $\alpha_3\beta_1$. (179, 185)Although the specific function of TGFBI in either promoting or suppressing tumor formation is conflicting, it hasbeen shown to bedifferentially regulated in several cancer types, but not prostate cancer.(176, 177, 180, 182)TFGBI mRNA levelswere generally found to be decreased in the shITGB1 expressing cell lines compared to the controls on both plastic and fibronectin. As TGFBI expression isknown to be induced by TGFβ1 (184), protein extracts from these cell lines were analyzed by western blot to confirm concurrent decreased TGFβ1 expression in the β_1 integrin depleted cell lines. (Figure 14B)

3.7 - 2D MIGRATION MAY BE AFFECTED BY β_1 INTEGRIN EXPRESSION LEVELS OF PC3 CELLS

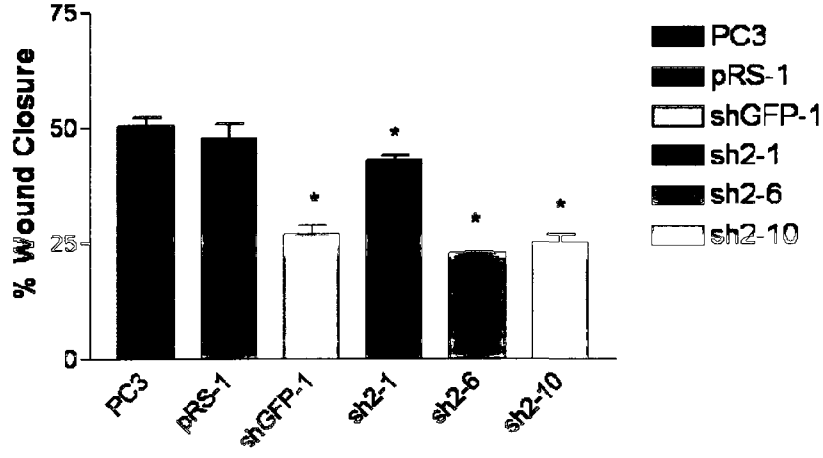
In addition to anchorage independent growth, several other phenotypes are correlated with tumorigenicity; namely increased migration and invasion *in vitro*, and increased metastasis *in vivo*. As our high throughput pathway specific expression screening indicated that there may be a possible link between fibronectin and β_1 integrin regulated expression of proteins involved in migration and invasion, we tested our various shRNA clones for these capabilities. To determine the migratory phenotypes of the stable cell lines, we performed an *in vitro* scratch wound assay. (Figure 15) Confluent monolayers of cells were scratched with

Figure 15 : Stable shITGB1 expressing cells exhibit reduced migration over plastic compared to controls in a scratch wound assay. All control and shITGB1 expressing cell lines were seeded to confluency prior to being scratched. (A) Brightfield images of wounds in cell monolayer, photographed at time 0 hrs (t_0) and 24 hrs post-wounding (t_{24}) at 40x magnification. (B) Cell migration expressed as percentage wound closure by comparing the average cell migration front between t_{24} to t_0. Bars represent mean and associated standard deviation from duplicate wells in three independent experiments. Statistical significance was measured using a non-parametric one-way ANOVA, and * represents p-value <0.05.

A


PC3
pRS-1
shGFP-1
sh2-1
sh2-6
sh2-10

B


% Wound Closure
75
50
25
0
PC3
pRS-1
shGFP-1
sh2-1
sh2-6
sh2-10
*
*
*
*
PC3
pRS-1
shGFP-1
sh2-1
sh2-6
sh2-10

a 2mm scraper and the size of the wound was measured at time 0 and again at 24 hrs post-wounding. Surprisingly, the control cell line shGFP-1 that had a similar expression levels of β_1 integrin to wtPC3 cells, had a significantly lower migration rate compared to the two other controls, however it also has the slowest proliferation rate on plastic, which could contribute to the reduced migration observed. With respect to the β_1 integrin depleted cell lines, all cell lines had significantly reduced migration compared to wtPC3 and the pRS-1 control cells. The sh2-1 cell line also had the highest proliferation rate on plastic, and thus the migration results could again be somewhat confounded by this factor. Taken together, our results suggest that cells depleted of β_1 integrin have a reduced ability to migrate, however the differences in the proliferative rates of our clones may be confounding our results to some extent, thus drawing a conclusion is difficult.

3.8 - β_1 INTEGRIN DEPLETED CELL LINES EXHIBIT REDUCED INVASION POTENITAL *IN VITRO*

The ability of a cancer cell to invade human tissues is a marker of malignancy and this process often leads to metastasis and a poor prognosis. A representative *in vitro* assay of the invasive capacity of a cell is measurement of cellular migration through matrigel towards a chemoattractant. Importantly for our work, this assay is based on a short time period of migration and invasion through matrigel, and hence results would not be confounded by differences in the proliferative rates of cells as the wounding assay can be. Cells were thus plated on a matrigel coated membrane with 8 um pores, in serum free media. The chamber

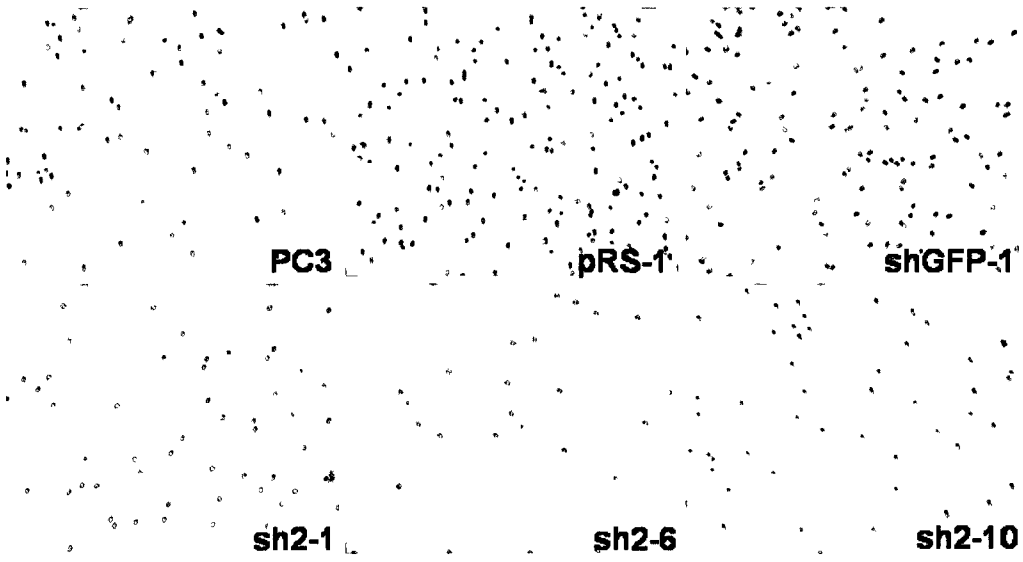
beneath the cells contained 5% fetal bovine serum (FBS) as a chemoattractant. The number of invaded cells was counted following staining with crystal violet. (Figure 16) We observed that all three shITGB1 cell lines (sh2-1, sh2-6 and sh2-10) showed significantly reduced invasion compared to all control cell lines. Although the shGFP-1 had somewhat reduced invasion in comparison to the wtPC3 cell line, this was not statistically significant, and it still remained significantly more invasive than the shITGB1 depleted cell lines. These results suggest that β_1 integrin expression plays an important role in prostate cancer cell invasion.

3.9 - β_1 INTEGRIN DEPLETION REDUCES MIGRATION OF PC3 CELLS OVER MATRIGEL

Stable shITGB1 expressing PC3 cell lines were grown over reconstituted basement membrane to assess the migratory and proliferative abilities of the stable cell lines over ECM, as this would be more representative of an *in vivo* microenvironment. Cancer cells seeded over BM have a tendency to aggregate if they are capable of migrating over the gel, forming disorganized colonies in the matrix. When total colony numbers were assessed after 4 days of growth, no significant differences were noted in colony numbers. The apparent trend, however, was that β_1 integrin depleted cells produced colonies of an apparently smaller size, which may indicate a reduced migratory ability over ECM. (Figure 17) Colonies were therefore recounted to take size differences into account. A threshold of 25 μm was imposed on all colonies counted (Figure 17B) and subsequently, only larger

Figure 16 : Invasion through matrigel is decreased in β_1 integrin depleted PC3 cells compared to controls. All stable cell lines (control and shITGB1) were seeded over matrigel coated porous membranes and allowed to migrate towards chemoattractant placed in the bottom chamber of the transwell for 4 hrs. (A) Brightfield images of invaded cells that were stained with crystal violet. Images were taken at 400x magnification. (B) Quantification of invaded cells is expressed as the mean number of invaded cells with associated standard deviation from 10 random fields of view in duplicate from three independent experiments. Statistical analysis was performed using a nonparametric one-way ANOVA, and * represents p-value <0.05.

A


PC3
pRS-1
shGFP-1
sh2-1
sh2-6
sh2-10

B

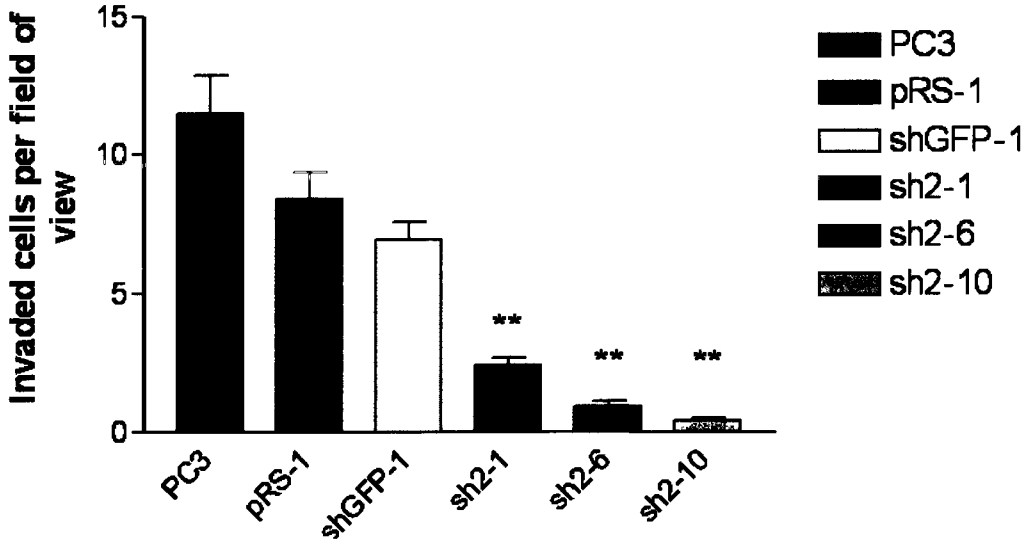
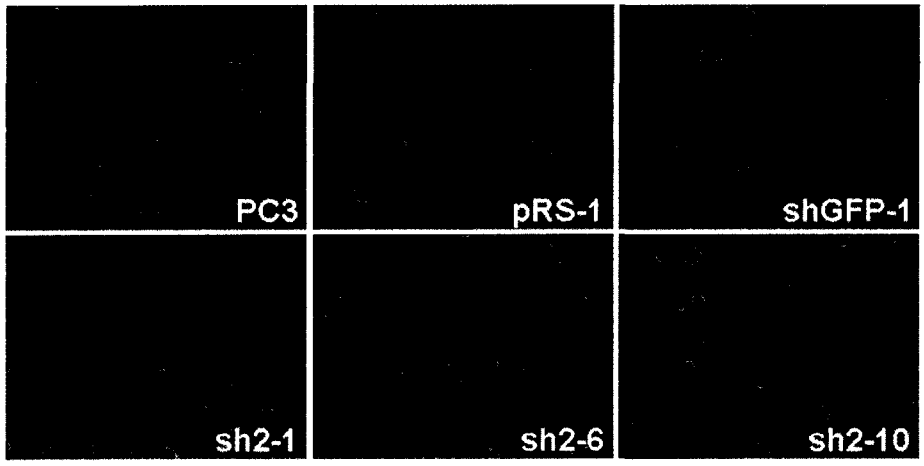
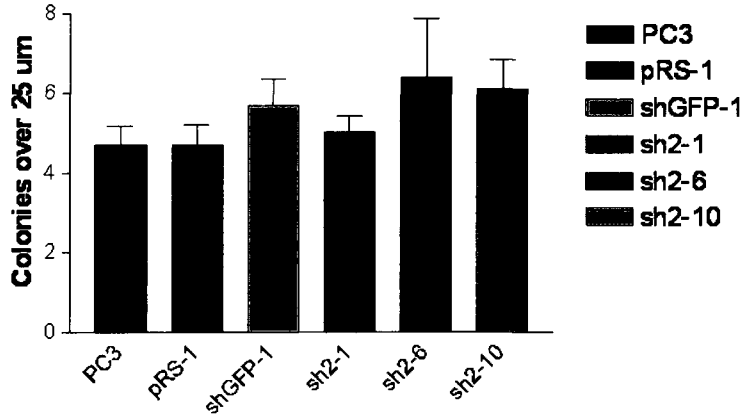

Invaded cells per field of view
15
10
5
0
**
**
**
PC3
pRS-1
shGFP-1
sh2-1
sh2-6
sh2-10
PC3
pRS-1
shGFP-1
sh2-1
sh2-6
sh2-10

Figure 17 : Migration of PC3 cells over matrigel is impeded by β_1 integrin depletion. Both control and stable shITGB1 expressing cell lines were grown over matrigel for 4 days. (A) Brightfield images at 200x magnification of colonies formed following migration and proliferation of cells. (B) Quantification of colonies measuring over 25 um in size. (C) Quantification of colonies measuring over 50um in size. For both (B) and (C), bars represent the mean and associated standard deviation from duplicate wells in two independent experiments. Statistical analysis was performed using a nonparametric one-way ANOVA, and * represents p-value <0.05.

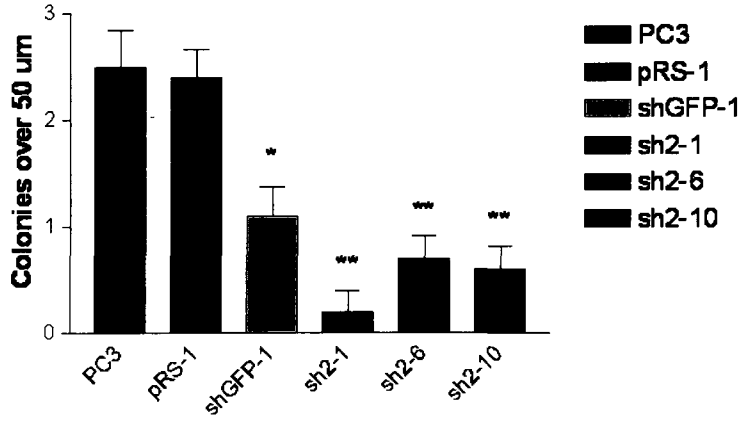
A


PC3
pRS-1
shGFP-1
sh2-1
sh2-6
sh2-10

B


Colonies over 25 um
0
2
4
6
8
PC3
pRS-1
shGFP-1
sh2-1
sh2-6
sh2-10
PC3
pRS-1
shGFP-1
sh2-1
sh2-6
sh2-10

C


Colonies over 50 um
0
1
2
3
*
**
***
**
PC3
pRS-1
shGFP-1
sh2-1
sh2-6
sh2-10
PC3
pRS-1
shGFP-1
sh2-1
sh2-6
sh2-10

colonies over 50 μm were counted. (Figure 17C) We found that control cell lines wtPC3 and pRS-1 formed more colonies over 50μm than control cell line shGFP-1 and all the $\beta_1$integrin depleted cell lines. These results would support the previous suggestion that β_1 integrin depletion may adversely affect cell migration. The decreased colony formation exhibited by the shGFP cell line could be a result of decreased growth rates, decreased migration over ECM or both. However, the relatively rapid growth rate of sh2-1 cells on plastic combined with the few threshold sized colonies are formed, suggest that proliferation is not sufficient for colony formation over matrigel and along with similar observations in the other shITGB1 expressing cell lines, these results point to a potentially decreasedexhibited by the shGFP cell line could be a result of decreased growth rates, decreased migratory ability of PC3 cells over ECM in the absence of β_1 integrin.

3.10 – A PILOT STUDY EXAMINING THE EFFECT OF β_1 INTEGRIN DEPLETION ON PC3 CELL METASTASIS TO THE BONE *IN VIVO*.

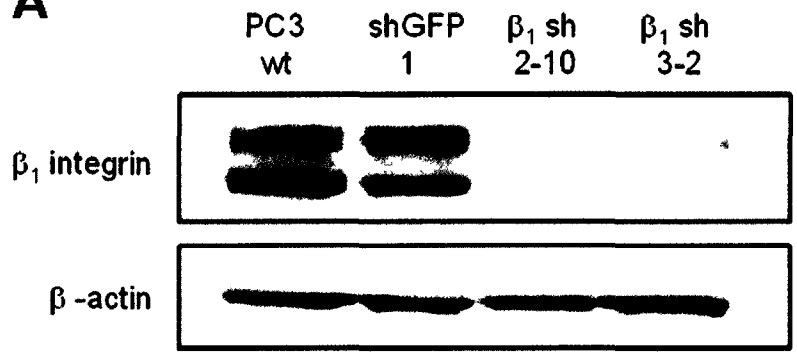
β_1 integrin is a primary receptor for collagen, a major component of the bone. It has been suggested that prostate cancer cells preferentially metastasize to the bone because of the complement of integrins they express and the favorable interactions they have with bone matrix proteins.(14, 122, 146) As the anchorage independent survival and growth and invasion ability of cells *in vitro* has been shown to correlate with tumorigenicity *in vivo*, we examined the effect of stable β_1 integrin depletion on osseous metastasis formation in nude mice. (117) In our model for prostate cancer metastasis to the bone, an intracardiac left-

ventricle injection method was chosen because of previously reported success in generating osseous lesions. (147, 148) This model is favored over direct prostate injection to ensure a systemic delivery leading to bone metastases, for ease of delivery of the cancer cells and as mouse prostates are quite morphologically different from that of humans. (149)

We chose to use the wtPC3 cell line and the shGFP-1 cell line as controls, the shGFP-1 line had similar proliferation rates to the shITGB1 cell lines used for this experiment. The β_1 integrin depleted cell lines chosen were sh2-10 and sh3-2. Clone sh2-10 formed very few colonies in soft agarose and had a similar proliferation rate to the control cell line (shGFP-1). Clone sh3-2 was chosen to show the effects of a different shRNA construct, and similarly, this cell line possessed a defect in its ability to form colonies in soft-agarose assays. Four mice were injected with each of the cell lines investigated, and after the injections, the remaining un-injected cells were collected and analyzed by western blot to ensure β_1 integrin expression levels remained depleted in shITGB1 stables, despite the manipulations required for injection (Figure 18A). Of the 16 mice injected with cancer cells, two died during surgery (one each from groups shGFP-1 and sh3-2). The remaining injected animals were left in specific pathogen free housing and were monitored for signs of ambulatory distress that would result following established tumor metastasis in bones. At 9 weeks post injection, we observed the first signs of ambulatory distress in our cohorts, and thus all animals were sacrificed and we collected tissues from limb bones and other organs which were processed as described in materials and methods. Tissues were subsequently sectioned and stained with hematoxylin and eosin (H&E) to initially characterize potential

Figure 18 : Pilot *in vivo* experiment for modeling prostate cancer bone metastasis : role of β1 integrin in the formation of osseous lesions is unclear. (A) Western blot showing the β_1 integrin levels of cell lines that were injected into the left ventricles of nude mice. (B) A representative image of osseous metastasis, 9 weeks post-intracardiac injection. Histological sections were stained with either H&E (left panel) or using a humain cytokeratin 18 antibody (right panel) to identify human PC3 cells. (C) Table summarizing the incidence of bone metastasis in this model.

A



B

H&E Human Cytokeratin 18

C

Cell Line	PC3	shGFP-1	sh2-10	sh3-2
Bone Metastasis	2/4 = 50%	0/3 = 0%	1/4 = 25%	0/3 = 0%

bone metastases. Subsequently, bone slices were stained with human cytokeratin 18 antibody to identify human PC3 cells within the mouse bone stroma. (Figure 18B) No tumors were detected in the liver or lungs of any mice. (data not shown) Of the remaining 14 mice, two mice in the PC3 group (2/4) had evidence of bone metastases in the femur of the hind-limb. No mice from the pRS-shGFP-1 group (0/3) had evidence of osseous neoplasia. One mouse from the sh2-10 injected group (1/4) had hind-limb bone metastases, and no mice from the sh3-2 group (0/3) exhibited osseous metastases. (Figure 18C) Tumor take was certainly a concern in this study and the methodology used should be reviewed before a subsequent experiment. Given previous data on the role of β_1 integrin in bone metastasis, we would have expected lesions in only the control injected mice. Hence, at this point in time, our results cannot conclusively determine whether or not stable β_1 integrin depletion of PC3 prostate cancer cells results in decreased osseous metastases *in vivo*.

4. DISCUSSION

4.1Modulation of Anchorage Independent Growth by β_1 Integrin in PC3 Prostate Cancer Cells

The altered expression of ECM in cancer and its effect on cell signaling are increasingly becoming recognized as a stumbling block for current therapeutic treatments. Prime examples are anti-angiogenic therapies that have worked in principle but not in practice. (8) As β_1 integrins are at the apex of the ECM mediated signaling cascades that regulate several pro-tumorigenic pathways, it is an attractive target for therapy. β_1 integrin has an established role in prostate cancer progression and may prove to be an effective therapeutic target, especially in light of our recent results suggesting that in its absence, prostate cancer cells have reduced invasive and anchorage independent growth capabilities. (138, 150, 151)

4.1.1. β_1 integrin Expression is Required for the Anchorage Independent Growth of PC3 Prostate Cancer Cells

Three-dimensional culture models have long been favored for their ability to predict the *in vivo* behavior of cancer cells, as anchorage independent growth is a hallmark of cancer but not of normal fibroblast or epithelial cells. (73, 152) Soft agarose culture of cancer cells has long been used as a reliable tool for prediction of *in vivo* tumorigenesis including increased invasion and metastasis in vivo in several cancer cell types. (72,

153)When β_1 integrin depleted (shITGB1) PC3 cells are cultured in soft agarose they exhibit a near complete impairment of colony formation in comparison to controls indicating that β_1 integrin is required for the anchorage independent growth of PC3 prostate cancer cells. The fate of the cells that do not produce colonies remains unclear and this technique is limited in that cells grown in agarose cannot be retrieved or extracted for protein expression analysis. As these cells cannot be retrieved, it is not possible to determine whether shITGB1 cells have undergone apoptosis as a result of being placed in an anchorage independent milieu or if they are simply senescent. Interestingly, PC3 cells with transiently depleted levels of β_1 integrin via siRNA methods that reduced expression for ~120 hrs, were not capable of re-establishing colony formation in a two-week soft agarose culture assay, during which time β_1 integrin re-expression would have occurred. (133) Although this observation does not confirm that these β_1 integrin-depleted cells are apoptotic, it does lead us to believe that if cells were viable, the resumed expression of β_1 integrin would initiate colony formation after 120hrs, which it does not. (133) This observation suggests that impeding the colony initiation through reduction of β_1 integrin expression permanently affects the growth ability of these cells in 3D culture.

Several studies involving β_1 integrin have utilized three-dimensional matrigel or laminin-rich ECM gels to facilitate post-experimental analysis of the cells. For example, β_1 integrin is necessary to support the growth of breast cancer cells in three-dimensional ECM culture. (154) These studies shed light on some of the factors that could be responsible for this type of growth and have gone further by testing *in vitro* observations *in vivo*. In three-dimensional reconstituted basement membranes (137) or laminin-rich ECM (155) culture models of breast and prostate cancer cells, inhibition of β_1 integrin signaling by blocking

antibodies decreased cell proliferation rates, lead to growth arrest (137, 138) and increased apoptosis. (155) These studies also reported a reversion in tumorigenic phenotype and re-establishment of normal E-cadherin expression patterns. (138, 150) The use of β_1 integrin blocking antibodies also corresponded with reduced malignancy *in vivo*.(138, 155) Concurrently, in a model of pancreatic beta cell carcinogenesis, ablation of β_1 integrin *in vivo* was found to impair primary tumor growth and reduce tumor cell proliferation by inducing cell cycle arrest. (156) In the three-dimensional basement membrane growth of a human breast cancer cell line, β_1 integrin and the epidermal growth factor receptor (EGFR) pathways cross modulate via the MAPK pathway and this interaction was linked to the ability of the cells to grow in an anchorage independent environment. (137) This observed reciprocal modulation does not occur in monolayer culture, suggesting that novel interactions and pathway coupling may occur in three-dimensional growth that do not occur in two-dimensional growth.

It has previously been shown that fibronectin mediated β_1 integrin signaling is required for the anchorage independent growth of PC3 cells and for soft agar growth and colony formation in a mammary cancer cell line. (119, 133) Fibronectin induced mediators of cell survival Bcl-2 and Bim/BOD have been suggested to confer resistance to anchorage independent induced apoptosis in cancer cells. (157) Despite this, expression levels of Bcl-2 and Bim/BOD were inconsistently expressed between clones suggesting that this pathway is not responsible for the observed impairment of anchorage independent growth of β_1 integrin depleted PC3 cells. However, it should also be noted that as we were not able to analyze the expression levels of these proteins in cells grown in soft agar assays due to technical limitations, we cannot exclude the possibility that the levels of these proteins may be altered

under those 3D growth conditions, a question which could perhaps be addressed using alternative 3D growth environments such as matrigel.

4.1.2. Alterations in Cell Adherent and Growth Capacities by $\beta_1$integrin does not Account for Reduced Ability to Grow in an Anchorage Independent Fashion

β_1 integrins are an important constituent of focal adhesions and it stands to reason that they are involved in the adhesion and adherent growth properties of cancer cells, including PC3 cells. (14) Functional mutagenesis studies on β_1 integrin in lymphoma cells have linked the intracellular NPXY motif to the adherent properties of the integrin receptor. (158) In our studies, a β_1 integrin targeted short-hairpin RNA treatment of PC3 cells resulted in an approximate decrease of 50% in ITGB1 protein expression. When these cells were grown either on plastic or fibronectin, there were no observed differences in adhesion. As depletion of β_1 integrin was not complete, there is potential for residual expression of β_1 integrin subunits to bind the substratum thus not producing measurable differences compared to controls. Stable shITGB1 expressing PC3 cell lines were generated to remedy this issue as stable shRNA provides a more significant and likely homogenous depletion of β_1 integrin. However, despite the confirmed decreased expression of β_1 integrin at the protein level, our stably transfected shITGB1 expressing clones were shown to have variable abilities to adhere to plastic, FBN, monomeric collagen I and vitronectin coated plates compared to controls. Although the adhesive differences noted could likely be accounted for by clonal variations in our cell lines, they did not correlate solely with the presence or

absence of β_1 integrin, nor with the anchorage independent phenotype. Hence these adhesive differences are not likely associated with the observed anchorage independent growth defect observed with shITGB1 integrin depleted cells. In contrast to our results, previous studies have shown that β_1 integrin is involved in the adherent properties of prostate cancer cells. (131, 159) Studies in breast (BT-549) and prostate cancer (Du145) cell lines have shown that β_1 integrin depletion impairs adhesion to collagen I and collagen IV but not to fibronectin or laminin. (29) The observation on fibronectin is consistent with our observations using both siRNA and shRNA approaches. It is possible that functional redundancy from other integrin pairs compensates for the adhesion to fibronectin and vitronectin. Although integrin $\alpha_5\beta_1$ has been cited as an important fibronectin receptor,(132, 152) it has been suggested that prostate cancer cell adhesion is also mediated in part by the fibronectin and vitronectin receptor $\alpha_v\beta_3$ integrin. (3) As expression of β_3 integrin is not altered by depletion of β_1 integrin by siRNA treatment (133), it is plausible that $\alpha_v\beta_3$ compensates for the decreased $\beta_1$integrin expression on the adhesion of the cells to fibronectin and vitronectin.

Given that PC3 cells are a bone metastatic derived prostate cancer cell line, it is possible that the complement of integrin receptors expressed by this cell line are already tailored to the microenvironment from which these cells originated. Although we did not examine the integrin pairings ourselves, PC3 cells have been reported to primarily express integrins $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$. (3, 160) As $\alpha_2\beta_1$ integrin is expressed in PC3 cells and is the primary collagen binding integrin, it stands to reason that a difference in adhesion on collagen I would be expected. An *in vitro* study on PC3 cells found that preferential adhesion to fibrillar collagen I over fibronectin or control substrates was attributed to the

expression of integrin $\alpha_2\beta_1$, and that adhesion to collagen I was increased in metastatic prostate cancer cell lines (PC3) compared to non-tumorigenic lines (LNCaP.) (14, 122) Arguably, in our assay, β_1 integrin ablated cells appear to show generally reduced adhesion on monomeric collagen I despite the statistical insignificance of this observation. This may be attributable to collagen structure that has been shown to affect signaling through integrins in cancer cells. (161) As monomeric collagen I is a single stranded rather than triple-helical peptide, it may be that monomeric collagen does not impede adhesion in the absence of β_1 integrin as has been reported with fibrillar collagen. The three-dimensional structure of collagens types I, III and IV, whether monomeric or fibrillar, have been previously shown to differentially affect signaling through integrins. (161, 162) As no differences were observed in the adhesive properties of shITGB1 cells compared to controls on several matrices, an observation that was supported by similar siRNA depletion of β_1 integrin, we may conclude that any effect of β_1 integrin ablation on other tumorigenic properties of PC3 cells is not attributable to adhesion.

As cancer cells are not usually dependent on adhesion to substratum for survival and their proliferation is increased by accelerated cell cycle progression, we sought to asses the proliferative variability between siRNA and stable shRNA transfected PC3 cells in adherent and suspension conditions. (87) This was also done to assess whether differences in proliferation rates in β_1 integrin depleted cells could account for the observed differences in colony formation in soft agarose. In adherent conditions, ECM signaling via β_1 integrin, and especially its downstream target ILK, has been shown to increase the proliferative rates of cancer cells. (Reviewed in (75)) ILK increases proliferation of PC3 cells grown on matrix by

driving cyclin D1 expression and promoting cell cycle progression. (79, 89) However, another study showed that LNCaP prostate cancer cells induced to express elevated levels of $\alpha_2\beta_1$ did not have increased proliferation on collagen I. (14, 122) Although a link between β_1 integrin expression and the proliferative rates of prostate cancer cells has been established, it may be that differences in cell lines and growth environments contribute to the extent of this modulation. Although we reported clonal variability, we did not observe significant differences in proliferation between β_1 integrin expressing and stable depleted cell lines in adherent or suspension conditions. Data from ITGB1 siRNA transfected PC3 cells supports this finding as no difference in proliferative rates were observed with the exception of clone ITGB1-03, which we presume was an off-target effect.

Clonal variability in tumorigenic phenotypes upon alteration of β_1 integrin expression has been previously reported in pancreatic cancer cells (163) and it should be noted that PC3 cells are reported to be a heterogeneous population, altering their phenotype based on cell culture conditions. (164) It can therefore be expected that results obtained in one study may not exactly reflect those in another and that through the highly selective process of clone generation, that certain alterations in the cell line may have occurred. This heterogeneity may explain our contradictory expression patterns, however they are likely reflective of *in vivo* tumor composition and therefore bolster our observation that despite this variability, decreased β_1 integrin expression is still sufficient to impede anchorage independent growth and invasion of PC3 cells. Potential ways to better control for clonal variability in β_1 integrin depleted cells would be to produce pooled clonal populations though these would likely become individual clones over prolonged passaging as certain clones would display higher proliferation rates than others. Another option would be to use a

tetracycline-inducible shITGB1 that could be expressed by doxycycline treatment in a naturally heterogeneous population of PC3 cells which, un-induced, would provide a comparable control population to the depleted cells. Taken together, these results suggest that despite clonal variability, the defective anchorage independent growth of β_1 integrin depleted PC3 cells is not attributable to a difference in proliferation between the control and clonal cell lines.

It must be considered that unlike adherent growth, proliferation in suspension at once prevents substratum adhesion and yet allows cells to cluster, allowing for potential differences in intracellular interactions. Although it is important to gauge the contribution of each matrix component on the cancer cell phenotype, adherent and suspension growth are not entirely representative of *in vivo* conditions. Hence, were we to grow our β_1 integrin depleted cell lines in the current types of 3-D models available for tumor cell growth, we might discover a role for β_1 integrin in this process. Although our growth over agarose coated plates argues against this possibility, growth in cell clusters as opposed to growth in cell-isolating conditions, as would be found to occur in many of the 3-D model systems currently in use, might yet reveal a dependence on β_1 integrin in controlling cell proliferation.

4.1.3 Potential Molecular Mechanisms Regulating the Anchorage Independent Growth of PC3 Prostate Cancer Cells

Soft agarose growth differs from suspension in that it requires an individual cell to initiate and sustain growth for colony formation whereas intracellular interactions may readily occur in suspension growth. Cells with significantly reduced levels of β_1 integrin are capable of surviving and even proliferating, albeit to somewhat reduced levels compared to adherent conditions, in suspension culture, but β_1 integrin depleted cells appear to be incapable of growth in isolating soft agarose culture conditions. This suggests that cell-cell interactions are important for survival of β_1 integrin depleted cells in anchorage independent conditions, and that lack of β_1 integrin might result in alterations in these cell-cell interactions. This observation led us to hypothesize that components of adherens junctions may have convergent effects on integrin function (or cross-talk) or that integrins may play a role in cell-cell junctions and not just cell-matrix junctions as initially established.

In an attempt to elucidate the mechanism responsible for β_1 integrin mediated anchorage independent growth, we examined adherens junction components β-catenin, E-cadherin and p120 catenin. Previous findings had shown that β-catenin co-immunoprecipitated with β_1 integrin in anchorage independent suspension growth but not in adherent growth (133) and other studies have reported cross modulation between integrins and components of adherens junctions. The reduced expression levels of β-catenin in wtPC3 cells that we observed are not consistent with previous findings that have shown that PC3 cells normally express β-catenin. (165)However, we could show following IP assays that our wtPC3 do in fact express β-catenin as it can be detected by IB in immunoprecipitated samples. Increasing E-cadherin expression in breast cancer cells was shown to reduce β_1 integrin expression and alter the sub-cellular localization of β-catenin from the nucleus to the cell membrane. (166) This reciprocal expression pattern was also reported in human

prostate cancer biopsies, though it was not observed in our analyses. (141) In a similar model, blocking of β_1 integrin by neutralizing antibodies restored normal E-cadherin and β-catenin expression patterns in 3D culture suggesting the existence of interactions, and potential synergy, between the integrin and adherens junctions pathways. (138, 139) In one notable study, integrin $\alpha_3\beta_1$ was shown to complex with tetraspanin CD151 as a component of a large cell-cell adhesion complex that included E-cadherin and β-catenin in epithelial cells. (142) Although the β_1 integrin subunit was not directly shown to co-immunoprecipitate with either β-catenin or E-cadherin in this complex, the α_3 stalk was. (142) This complex has been implicated in breast cancer where it is found to be highly expressed in advanced disease. (154) Although our initial observation suggesting that β-catenin did interact with β_1 integrin in suspension growth conditions could be attributable to unfavorable antibody interactions, the examination of this potential interaction was valid given the supporting literature described herein. It thus remains possible that our initial results with β_1 integrin could be attributable to its preferential association with tetraspanin CD151, which then allows detection of co-localized E-cadherin and β-catenin. When either E-cadherin or β-catenin antibodies were used in the reverse IP, it is possible that amounts of those isolated proteins are actually in association with CD151, and hence β_1 integrin, are such a small fraction of their overall cellular pools that our assay is not sensitive enough to detect the presence of β_1 integrin. Perhaps IP using antibodies against CD151 would shed light on the possible existence of a multi-protein complex containing all these members that preferentially associates in suspension versus adherent culture conditions.

E-cadherin was expressed in only two control cell lines, which is in accordance with studies that report detectable E-cadherin expression in at least a subpopulation of PC3

cells.(165, 167) Generally, E-cadherin expression is lost in prostate cancer and has been reported to be absent in other studies that report use of PC3 cells and in prostate tumors *in vivo*.(3, 141, 168) p120 catenin interacts with the cytoplasmic tail of type II cadherins, including E-cadherin, to regulate their turnover at the cell surface, thereby controlling the amount of cadherin available for cell-cell adhesion. (143) p120 is a primary regulator of cadherin abundance and activity, participating in the balance between adhesive and motile phenotypes. (143) In E-cadherin deficient cells, p120 is stranded in the cytoplasm and therefore its expression is not expected to be lost. (143) This is consistent with our results, where expression of p120 was still detected in the absence of E-cadherin. However, p120 and E-cadherin have a highly dynamic interaction and p120 is required for E-cadherin to remain at the cellular membrane, otherwise the recycling of E-cadherin is immediate. (143) Therefore in cell lines where p120 was absent, it is surprising to find strong E-cadherin expression, although this may indicate simply that E-cadherin is produced but not appropriately localized to the membrane. Similarly, it is interesting that cells expressing p120 catenin do not express E-cadherin, indicating that these cells likely lack functional adherens junctions. Cadherins and catenins are often downregulated or absent in invasive breast and prostate cancers but there are several examples of *in vivo* invasive and metastatic mammary carcinomas that express the full complement of cell-cell adhesion molecules with the exception that they are not properly assembling adherens junctions. (3, 138) Interestingly, a study in PC3 cells showed that adherens junction formation was impaired despite E-cadherin expression as a result of a lack of α-catenin – a crucial adherens junction component. (167) It is well established that cancer cells lose adherens junctions during malignant progression and gain motility in a process referred to as epithelial to

mesenchymal transition (EMT). (91) Despite the retention of E-cadherin expression, the inability of these cells to form cell-cell junctions would be in keeping with the theorized motile properties of a metastatic cancer cell line. The expression of α-catenin was not examined in our studies, and although there is evidence to suggest that catenins and cadherins are important in prostate cancer, they were not further examined in the context of our work.

4.2 Underlying Molecular Mechanisms that may Modulate β_1 integrin Mediated Tumorigenic Phenotypes

Although our *in vivo*pilot experiment cannot conclusively support a role for β_1 integrin in the metastatic establishment of bone cancer lesions in immunodeficient mice, several studies have already concluded that a relationship between the two exists as β_1 integrin function has clearly been linked to metastasis in several cancer cell types (158, 169) including prostate cancer, where β_1 integrin expression has been linked to poor prognosis. (111, 122) When technical challenges are overcome and tumor take in our model of bone cancer metastasis is optimal, the role of β_1 integrin in PC3 cancer cell metastasis to bone certainly warrants re-examination.

As invasion was found to be decreased in shITGB1 expressing cell lines, matrix metalloproteinases were a key target for validation in the qPCR experiments. MMPs have been extensively linked to the malignant progression of prostate cancer *in vivo* and to invasion *in vitro*. MMP2, MMP9, MMP11 and MMP14 as well as TIMP-2 have been found

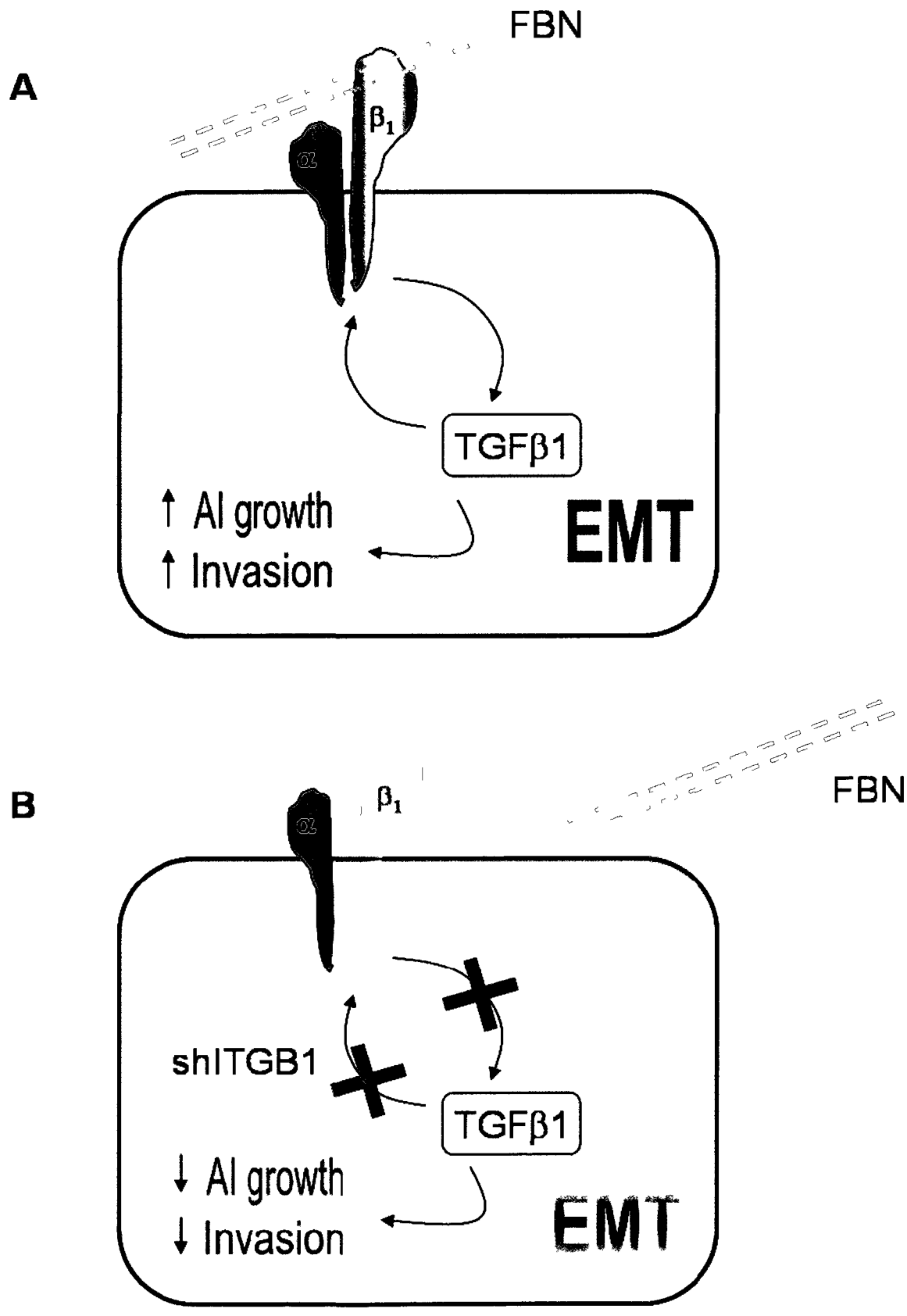
to be upregulated with the malignant progression of prostate cancer, whereas MMP7 was found to be unchanged. (3, 41, 44) In accordance with our observation that depletion of β_1 integrin expression reduces the invasive capability of PC3 cells *in vitro*, β_1 integrin has previously been shown to affect invasion by upregulating MMPs and preferentially degrading several matrices linked to healthy basement membrane composition, (29, 55, 113) while impeding β_1 integrin signaling corresponded to decreased invasion.(29, 112)Upon validation of our putative targets, we did not observe differences in MMP9 mRNA levels between control and shITGB1 cell lines. Although cell lines sh2-1 and 2-10 show reduced MMP9 expression compared to wtPC3 cells, this is likely attributable to clonal variability as the pRS-1 controls also exhibit somewhat reduced levels of MMP9. Although we did not see changes in expression at the mRNA level, we did not investigate the protein expression of these MMPs in our various cell clones, and it remains possible that changes in protein expression, or in MMP activity levels may be different amongst our clones. In support of this, it has been shown following immunohistochemical examination of prostate cancer tissues, that although gelatinases A and B (MMP2 and MMP9) are expressed, they are present in an inactive conformation. (23). Work in PC3 also showed that β_1 integrin engagement was required for the subsequent activation of MMP9. (170) Furthermore, as we validated that levels of TIMP2 mRNA were increased in the shITGB1 expressing cells compared to controls when grown in the presence of fibronectin, this may imply that MMP activity is being inhibited rather than message and protein levels being decreased in the absence of β_1 integrin. Thus further investigation with respect to the activity of the MMPs should be conducted in our cell lines using assays such as gel zymography, to conclusively determine whether $\beta_1$integrin yet modulates their activity.

Our validation of putative mRNA targets differentially expressed between β_1 integrin depleted clones and controls, did show that as seen in the original array analysis, levels of TGFβI mRNA was generally reduced in the shITGB1 cells, whereas control clones had TGFβI levels similar to wtPC3 cells.The extracellular matrix protein TGFBI has been shown to act as a tumor suppressor in studies in lung cancer (180, 182), breast cancer (176) and as a suppressor of angiogenesis and a contributor to drug resistance in cancer. (183,185) However, this protein has also been shown to have correlative expression with increasing tumor grade. In both colon cancer and renal cell carcinoma, increasing expression of TGFBI was found to increase metastasis, extravasation and lead to poor prognosis.(177)Although the role of TGFBI in cancer is not clear, our observation was accompanied by a decrease in the protein levels of the TGFBI expression inducer, TGFβ1, among β_1 integrin depleted clones. As TGFβ1 is a major regulator of EMT, and was initially characterized by its ability to induce anchorage independent growth, it is possible that reduced TGFβ1 expression in β_1 integrin depleted cells contributes to the impairment of soft agarose colony formation of these cells. (87, 123) It has been established that PC3 cells secrete TGFβ1 (128) and that this increased TGFβ1 expression leads to upregulation of the β_1 integrin subunit and to increased adhesion to collagen. (128, 131) The abundance of TGFβ1 and collagen I in the bone matrix has linked this growth factor to prostate cancer metastasis and the preferential localization of PC3 cells to the bone. (3, 131) TGFβ1 expression in prostate cancer cell lines has also been linked to invasion, (128, 171) and specifically in PC3 cells, TGFβ1 stimulation has been shown to enhance invasion. (172) Thus the concomitantly reduced expression levels of β_1 integrin together with TGFβ1 is consistent with the reduced invasion we observed in shITGB1 expressing cells. More specifically, stimulation of PC3 cells with TGFβ1 induces

MMP9 and cyclooxygenase-2 (COX-2) expression, (128, 171, 173)hence directly modulating invasive phenotypes in this manner. COX-2 is an enzyme that has been shown to degrade basement membranes in cancer and hence also contribute to invasion. (173) A separate study in squamous cell carcinoma showed that TGFβ1 stimulation did not alter MMP9 expression but instead resulted in increased MMP2 expression that modulated cell invasion. (129) This study did show, however, that with regards to cell migration, TGFβ1 enhanced the migration of cancer cells, while the inhibition of TGFβ1 impaired migration in scratch wound assays. (129) Taken together, these results suggest that lack of expression of TGFβ1 in the absence of β_1 integrin could regulate the phenotypes we observed in β_1 depleted cells, and hence may be a central mediator of β_1 integrin modulation of tumorigenesis and metastasis. Although there is strong evidence to support TGFβ1 up-regulation of β_1 integrin expression, the reverse has not been shown. Our data would suggest that a possible regulatory feedback loop exists between β_1 integrin and TGFβ1, mutually regulating EMT in PC3 prostate cancer cells. (Figure 19)

The link between anchorage independent growth, invasion and migration and β_1 integrin expression has been established, it is also clear from the literature that MMPs and TGFβ likely play an important part in modulating these tumorigenic properties. It remains that exact downstream mechanisms that are responsible for this and other malignant properties such as migration and metastasis have not been completely elucidated and although TGFβ1 could be a central player in our observed phenotypes, examination of other putative mediators, such as COX-2 levels would be warranted.

Figure 19 : Schematic representation of a potential model explaining the β_1 integrin mediated anchorage indepdnent growth and invasion of PC3 prostate cancer cells. In the presence of β_1 integrin, fibronectin mediated integrin signaling is required for anchorage independent growth. β_1 integrin expression is also required for the invasion of PC3 prostate cancer cells. As TGFβ1 is reduced in cells with depleted levels of β_1 integrin, it is conceivable that a feedback loop exists between β_1 integrin and TGFβ1 that controls tumorigenic properties such as AI growth and invasion (reported heirin) and EMT (reported in the literature). In the absence of integrins, ECM mediated downstream activation of targets is not possible.


A
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↓ Al growth
↓ Invasion
EMT

4.3 SUMMARY

In conclusion, we have shown that the anchorage independent growth of prostate cancer cell line PC3 is dependent on β_1 integrin expression. We established that this phenotype was not a result of decreased adhesion or proliferation (in suspension or adherent conditions) of clones stably depleted of β_1 integrin. In an effort to tease out the molecular mechanisms that may modulate this phenotype, we could not definitively demonstrate previously reported interactions between β_1 integrin and β-catenin in suspension growth of this cell line. (133)

We also found that invasion through matrigel and migration on plastic and reconstituted basement membrane appeared to be decreased upon loss of β_1 integrin expression. This was despite no observable trends in the RNA expression patterns of several MMP molecules between clones and controls, even upon fibronectin stimulation. However, increases in TIMP2 levels may be regulating MMP protein activity, so examination of this avenue of investigation is warranted. We suspect that a reduction in TGFβ1 expression that is concurrent with decreased β_1 integrin expression, and could likely be the cause of decreased TGFB-induced expression, may primarily be responsible for the observed phenotypes. We have certainly garnered evidence that implicates β_1 integrin in the malignant properties of PC3 prostate cancer cells, and future work will investigate putative mechanisms of regulation of these phenotypes in more detail.

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APPENDIX A

Figure S1: Representative western blot showing reduced β_1 integrin protein levels following specific mRNA depletion by stable shRNA transfection. Western blot showing β_1 integrin expression levels of all clonal populations of PC3 prostate cancer cells stably transfected with empty control vectors pRS, pRS-shGFP targeted control or shITGB1 constructs #2 or #3 compared to wild-type PC3 cells (PC3).

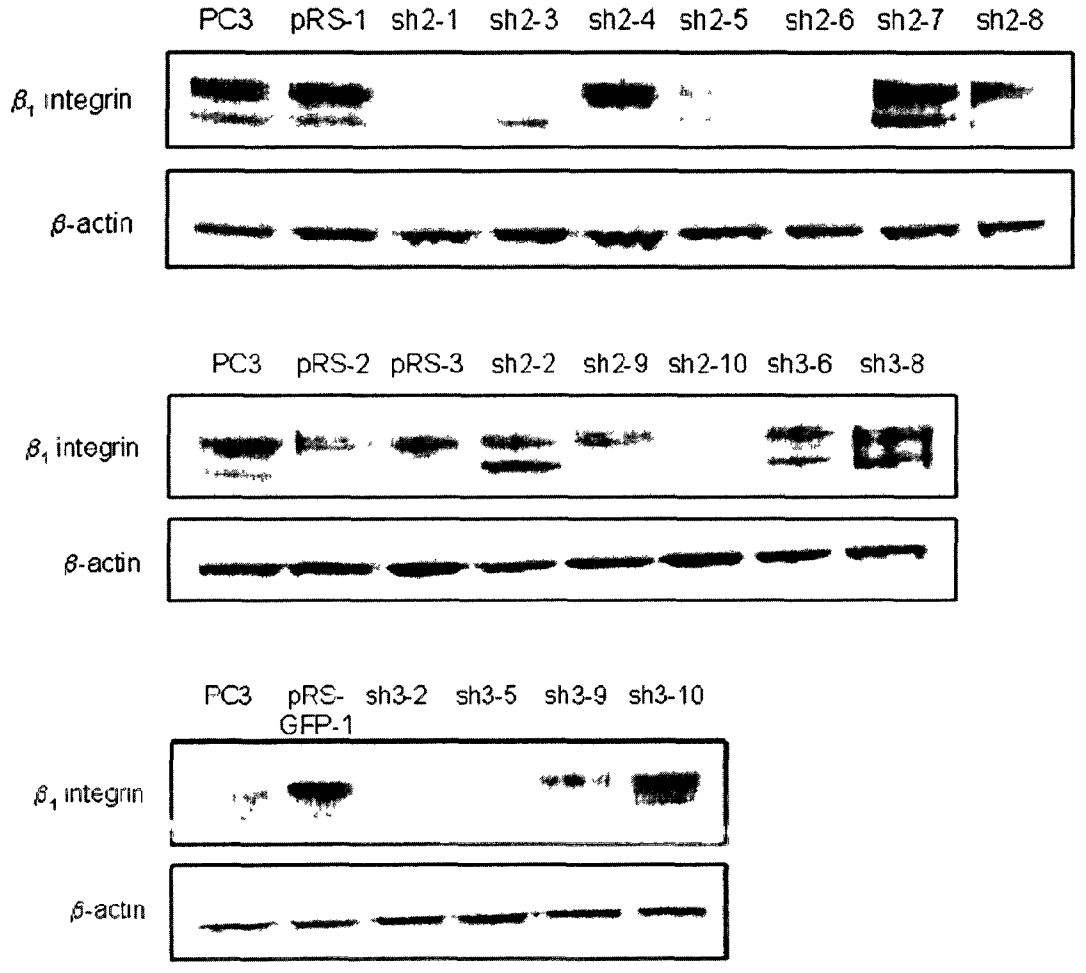
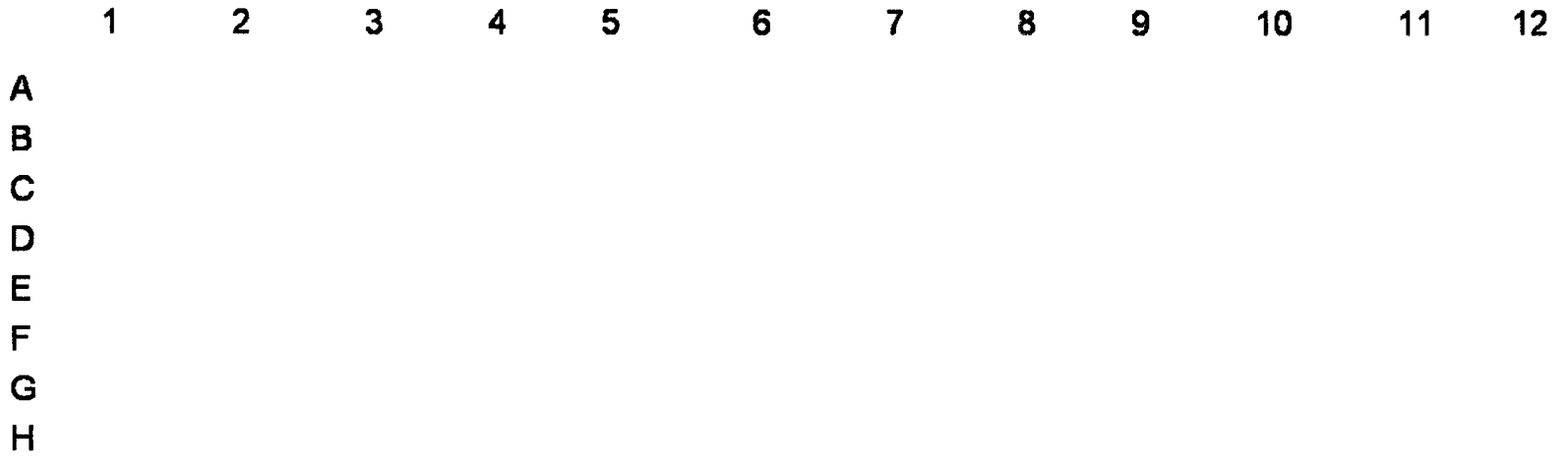

PC3
pRS-1
sh2-1
sh2-3
sh2-4
sh2-5
sh2-6
sh2-7
sh2-8
β1 integrin
β-actin
PC3
pRS-2
pRS-3
sh2-2
sh2-9
sh2-10
sh3-6
sh3-8
β1 integrin
β-actin
PC3
pRS-GFP-1
sh3-2
sh3-5
sh3-9
sh3-10
β1 integrin
β-actin

Figure S2: Table of qRT-PCR Microarray Targets used to generate Heat maps analysis of RNA isolated from β_1 integrin depleted cell line sh2-10 compared to control cell line shGFP-1 show differential regulation of a number of gene targets. All targets correspond to a coordinate within the map. Target names are abbreviated gene names.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NATALIE ANDREWS, B.Sc.

SUMMARY

• An M.Sc. Candidate in Biochemistry program at the University of Ottawa with strong initiative, sound judgement and innovative problem solving skills.
•A quick learner who can implement tasks in creative and resourceful ways.
•Well organized, observant, insightful, and friendly and maintains a positive attitude.

EDUCATION

Present — **M.Sc. Candidate in Biochemistry**
Laboratory of Dr. Christina Addison, Cancer Therapeutics
University of Ottawa, Faculty of Medicine
Ottawa, ON

May 2007 — **B.Sc. in Biochemistry with Honours**
University of Ottawa, Faculty of Science (French curriculum)
Ottawa, Ontario

June 2003 — **O.S.S.D. (new curriculum)**
École Secondaire Publique De La Salle
Ottawa, Ontario

LANGUAGES

French, English: Fluent
Spanish: Written - beginner
Spoken - beginner

SKILLS

LEADERSHIP

- Advisor for the national Let's Talk Science – Parlons Sciences program. Resolved volunteer concerns and communicated with coordinators to ensure the success of the program. Nominated for Volunteer of the Year at the National level in 2009 and 2010.
- Promoted to editor in chief of the Faculty of Science yearbook after second year on the committee. Initiated group meetings and established milestones and a timeline to meet all deadlines for the completion of the yearbook.
- Approached local businesses and secured advertising space for the 2005-

2006 University of Ottawa Faculty of Science yearbook, generating revenue for the yearbook committee.

ORGANIZATION

- Required to prioritize daily activities, assignments and commitments to meet deadlines and achieve personal goals.
- Maintained a CGPA of 8.0 throughout University undergraduate and graduate studies, requiring self-discipline and effective time management.
- Ability to maintain a balance between education, extra-curricular activities and responsibilities outside of school.

COMMUNICATION AND INTERPERSONAL

- Volunteered with a local geriatric day hospital, I was responsible for attending to patient care including encouraging communication, assisting with exercise regimes and planning social activities to promote interaction with other patients.
- Displayed a high level of sensitivity to patient needs in an effort to respect the individuals' dignity.
- Through experience, I have learned ways of ensuring that my actions are a positive reflection of the organization I represent.

AWARDS AND SCHOLARSHIPS

2010 **NationalCanadian Institutes of Health Research Synapse Award**
- Awarded to honour persons who have made exceptional contributions to the promotion of health research among Canadian secondary school students

2010 **National Let's Talk Science CIHR Synapse Award**
- Award recognizes an outstanding and innovative health research-related activity done by a Partnership Program volunteer

2009 **Sue McKee Spirit Award (Let's Talk Science – Parlons science)**
- Awarded for contribution to science outreach to one volunteer at the University of Ottawa

2009 **Best Poster - BMI Poster Day 2009**
- M.Sc. category, 2nd place

2007 - 2009 **Admission Scholarship – Graduate Studies – University of Ottawa**
- Awarded to students with a grade average of over 8.0 (A-)

2004 **Award of Excellence (Queensway Carleton Hospital)**
- Awarded to volunteers who excel at their duties: received for exceptional customer service attitude.

2003 **Renewable Admission Scholarship for Academic Achievement**
- Awarded to students with an average between 88 and 91.9%.

2003	**OSAP (RAFEO) Fellowship for Studying in French** • Awarded to students studying in French at the University level.
2003	**Bursary for studying in French** • One of five hundred bursaries awarded to students studying in French at the University level, based on a written essay.

RESEARCH

May 2009	**Biochemistry, Microbiology and Immunology (BMI) Poster Day** Poster Presentation Ottawa, ON, Canada
March 2009	**Extrinsic Control of Tumor Genesis and Progression (Keystone Symposia)** Poster Presentation Vancouver, BC, Canada
Feb 2009	**Biochemistry, Microbiology and Immunology (BMI) Seminar Symposium Day** Seminar Presentation Ottawa, ON, Canada
Nov 2008	**Ottawa Health Research Institute (OHRI) Research Day** Poster Presentation Ottawa, ON, Canada
Jan 2010 Nov 2008 Apr 2008	**Ottawa Hospital Cancer Center Work in Progress (WIP)** 3 Seminar Presentations Ottawa, ON, Canada

RELATED EXPERIENCE

April 2010 – ongoing (Part-time)	**SUPPORT Clinical Research Volunteer** (Students Undertaking a Pediatric Program of Research Training) Children's Hospital of Eastern Ontario Ottawa, ON
Sept 2008 – ongoing Sept 2007 – Sept 2008 (Part-time)	**Advisor – Let's Talk Science/ Parlons science** **Volunteer – Let's Talk Science/ Parlons science** University of Ottawa – Faculty of Science and Medicine Ottawa, ON
Sept 2009 – May 2010 Jan 2009 – May 2009 Sept 2007 – May 2008 (Part-time)	**Teaching Assistant (Chemistry, Biochemistry and Molecular Biology)** University of Ottawa Faculty of Science Ottawa, ON
Sept 2009 – Dec 2009 Sept 2006 – Jan 2007 (Part-time)	**Tutor in Math** (volunteer basis) **and Chemistry** (University level) Grade 9 math – Child with learning disability Ottawa, ON

June 2004 – May 2007 **Post Office Clerk**
(Part-time) Shoppers Drug Mart
Nepean, ON

Sept 2003 – Sept 2006 **Faculty of Science Yearbook Member, Co-editor and Editor-in-Chief**
(Part-time) University of Ottawa
Ottawa, ON

May 2005 – Sept 2005 **CR - Sales and Service Support Agent in Consumer High Speed Centre**
(Full-time) Bell Canada (BCE)
Ottawa, ON

ACTIVITIES

- cross-country skiing, painting, drawing, yoga and travelling

REFERENCES

- Available Upon Request