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FUNCTION OF THE CELL SURFACE RECEPTOR COMPONENT INTEGRIN β1 IN HUMAN TUMOUR CELLS

By

Allana M. Schooley

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ABSTRACT

The work presented herein examines the potential impact of extracellular matrix (ECM) protein-integrin receptor interactions on tumour cell chemotherapeutic sensitivity, proliferation and anchorage-independent growth. First, a screen was performed to assess the impact of ECM proteins on the survival of various tumour cell lines treated with different chemotherapeutic agents, however no significant modulation by ECM was detected. To more specifically understand potential influences of ECM on tumour cell phenotypes, a siRNA approach targeting integrin β1, an important ECM receptor component, was employed. Integrin β1 depletion studies conducted in paired cisplatin sensitive and resistant ovarian cancer cells did not support a role for ECM-integrin receptor binding in drug sensitivity. Similarly, reduction of integrin β1 protein using the siRNA approach did not impact the adhesion or adherent growth of the cancer cell lines studied. However, integrin β1 was shown to be necessary for the anchorage-independent growth of all tumour cell lines tested in soft agarose colony formation assays. The importance of integrin β1 for anchorage-independent growth of PC3 cells was further confirmed using both neutralizing antibodies to the receptor subunit and a signaling–impaired splice variant of integrin β1. Depletion of the integrin ligand, fibronectin, from the tumour cell culture medium similarly reduced soft agarose colony formation, suggesting that ligation of β1 integrins is necessary for anchorage-independence. Co-immunoprecipitation studies revealed the formation of a *de novo* interaction between β catenin and integrin β1 when tumour cells were transitioned to grow anchorage-independently. These findings suggest a model whereby ligation of the β1 integrin-containing receptors by soluble fibronectin drives the anchorage-independent growth of tumor cells through a β catenin signaling pathway.

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CONTENTS

ABBREVIATIONS

ES cells	Embryonic Stem cells
ECM	Extracellular Matrix
BM	Basement Membrane
GAP	GTPase Activating Protein
GEF	Guanine Exchange Factor
Grb2	Growth Factor Receptor Bound Protein
FAK	Focal Adhesion Kinase
αA integrin	I/A domain-containing alpha integrin
βA	I/A-like domain in beta integrins
RGD	Arginine-Glycine-Asparagine
LDV	Leucine-Asparagine-Valine
NPxY	Asparagine-Proline-(unspecified amino acid)-Tyrosine
PKC	Protein Kinase C
PNRE	Perinuclear Recycling Endosomes
GSK3β	Glycogen Synthase Kinase 3-β
PDK1	3-Phosphoinositide-Dependent protein Kinase 1
TNF-α	Tumour Necrosis Factor-α
PtdIns(4,5)P2	Phosphatidylinositol (4,5) biphosphate
RTK	Receptor Tyrosine Kinase
ILK	Integrin Linked Kinase
Bcl-2	B-Cell Lymphoma/Leukemia 2
PI3K	Phosphoinositide 3-Kinase
ERK	Extracellular Regulated Kinase
EGFR	Epidermal Growth Factor Receptor
MAPK	Mitogen Activated Protein Kinase
SH domain	Src Homology domain
MEK	Mitogen Activated Protein Kinase Kinase
TCF/LEF	T-Cell transcription Factor/Lymphoid Enhancer Binding Factor
MMP	Matrix Metalloproteinase
TGF-β	Transforming Growth Factor β
EMT	Epithelial to Mesenchymal Transition
NCI60	National Cancer Institute 60 cell panel
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal Bovine Serum
siRNA	Small Interfering Ribonucleic Acid
GFP	Green Fluorescent Protein
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate Buffered Saline
CI	Monomeric Collagen I
FN	Fibronectin
LMN	Laminin
TNC	Tenascin-C
PDL	Poly-D-Lysine
mTOR	Mammalian Target of Rapamycin
SCLC	Small Cell Lung Cancer

NSCLC	Non-Small Cell Lung Cancer
VEGF	Vascular Endothelial Growth Factor
PTEN	Phosphatase and Tensin Homolog
Wnt	Wingless
WISP-1	Wnt-Induced Secreted Protein-1

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1. INTRODUCTION

1.1 PREAMBLE

All cells contain identical genetic information but are able to give rise to very different tissues with unique functions. Equally, tissues comprised of specialized cells are able to respond to diverse stimuli during development and adult life by altering the function and structure of their component cells, presumably through changes in gene and protein expression. The programmability and plasticity of cellular phenotypes is thus central to both morphogenesis and the regulated functioning of developed organs.

The concept of context-dependent cell differentiation is evident in tissue recombination studies. In one striking example of this, mouse mesoderm which would normally promote hair follicle formation from mouse ectoderm was able to induce feather development when combined with chick corneal epithelium (1). The importance of microenvironmental cues during development can also be inferred from embryonic stem (ES) cell transplantation studies. When injected into adult mice, ES cells isolated from normal pre-implantation mice form teratomas (2). However, cells isolated from teratomas retain the ability to contribute to normal development when re-implanted into an embryo (3), indicating that the phenotype of these pluripotent cells is entirely dependent on the extracellular context.

The tissue microenvironment generally consists of stromal cells and their secreted factors, the vasculature, and the extracellular matrix (ECM). The ECM provides not only the physical framework for cells but also regulates cellular functioning through storage of secreted factors such as growth factors and cytokines, and through direct interactions with

cell surface receptors. The impact of ECM proteins on cell phenotypes has been extensively studied in culture. Losses in differentiated functions of cultured cells can often be re-acquired when cells are grown on naturally occurring ECM or surfaces coated with ECM components (4). The importance of the structural impact of the ECM is reflected in studies of mammary epithelial cells plated on thick collagen gels. When the gels are suspended in culture medium, cells derived from pregnant or lactating mice regain their polarity, functional morphology, and secrete factors characteristic of the differentiated epithelial cells, however cells grown on immobilized collagen gels were found to exhibit a phenotype intermediate between the floating cells and cell adhered to plastic (5, 6). In addition, ECM production can be regulated by positive feedback (7). Cellular functions are thus dependent on the structure and composition of the ECM which is in turn subject to cellular regulation. Accounting for these phenomena, Bissell's dynamic reciprocity model describes the bidirectional communication from the ECM with the cell membrane anchored adhesion receptors to the actin cytoskeleton, nuclear matrix and chromatin (4) and has been extended to include many more extracellular components including cell-cell communication as well as the role of intracellular signaling molecules (8).

Molecular aspects of dynamic reciprocity between cells and the extracellular matrix will be discussed in the following section beginning with the components of the ECM and the focal contacts mediating adhesion, as well as the identification of integrins as the main family of transmembrane receptors enabling bidirectional signaling events. A comprehensive review of known functions of the important integrin receptor subunit under investigation in this study, integrin β1, will also be included in these sections. Finally, implications for ECM proteins and integrin receptors in human cancers and the importance

of adhesion in malignant transformation will be discussed with an emphasis on integrin β1 mediated events.

1.2 THE EXTRACELLULAR MATRIX

Cells of all tissues are supported and coordinated by the surrounding ECM which can be divided into two broad categories; the interstitial matrix most prevalent in connective tissues and the basement membrane which supports cells of the epithelium and endothelium as well as muscle fibres and nerves. The mechanical or tensile requirements of extracellular matrices is variable between tissues ranging from the highly vascularized fluid matrix surrounding blood cells to the avascular calcified matrices of bone and cartilage. A large degree of interconnectivity between ECM components with each other, with soluble factors such as growth factors, and with cell receptors accounts for the complexity of the ECM as an entity. Although the matrix surrounding and supporting cells is best understood in the context of a complex and dynamic network of effectors, analyses of individual microenvironmental components serves to identify basic motifs of bidirectional cell-ECM modulation. Fibrils or networks composed predominately of collagen entrap proteoglycans, elastic fibrils, and glycoproteins such as laminin and fibronectin. These collagen structures are the molecular scaffolds which provide the architecture characteristic of the various connective tissues and basement membranes. Accordingly, the collagen super-family can be subdivided into at least 9 subfamilies, including fibril forming and network forming collagens, which reflect their different structural capacities (9). While some collagens such as I, III, and V have a broad distribution, others are more specific in their expression, such as the basement membrane collagens IV and VII (10) or cartilage collagens II, IX, X, and XI (11). Mouse strains with engineered mutations in a wide variety of collagen genes display

abnormalities in nearly every tissue type, many of which halt development in the mouse as early as E9.5 (12). Mutational analyses in mice have revealed a range of functions for collagen molecules including collagen fibril stability and organization (13, 14, 15), and interactions with other ECM proteins which are particularly important for maintaining the integrity of the basement membrane (16). Both fibrillar and basement membrane collagens are also known to interact with cell transmembrane receptors such as integrins (17) and discoidin domain receptors (18) to activate intracellular pathways affecting cell proliferation and ECM remodeling. Unsurprisingly, association with other ECM components and cellular signaling events are particularly dependent on the 3-D conformation of collagen structures (19).

The basement membrane (BM) is composed of a collagen fibril-rich reticular lamina proximal to connective tissues, and a cell-adjacent layer called the basal lamina which consists primarily of collagen IV, laminin, proteoglycans, and fibronectin. Collagen IV provides the basement membrane structural integrity necessary for mouse development beyond E9.5 (20) however, heterotrimeric laminin glycoproteins are necessary much earlier and are therefore thought to comprise the functional framework initiating basement membrane assembly during development (21, 22). While only two are absolutely required for early embyogenesis, 16 laminins identified to date are expressed in developmental and tissue specific ways for proper organogenesis and organ function (23). The first laminin protein was purified from the basement membrane of Engelbreth-Holm-Swarm sarcoma mouse tumours and subsequently identified as a basement membrane component in normal mouse tissues using immunofluorescence (24). Although laminin, collagen IV, heparin sulphate proteoglycan (perlecan), and entactin components are able to self assemble into basement membrane-like structure in vitro, a phenomenon presumably based on homophylic

and heterophilic interactions (25), there is some evidence that interactions with cell membrane lipids (26) increase the efficiency of this process. Laminins interact with a variety of ECM components including collagen IV and perlecan, and with cell surface receptors. Laminins therefore impact intracellular signaling through indirect interactions with ECM cell ligands as well as through direct binding to integrin and α-dystroglycan (27) transmembrane cell receptors to regulate adhesion, differentiation (28, 29), proliferation (30), and migration (31) in normal and transformed cells.

Much like other major components of the ECM, fibronectin is an absolute requirement for early mouse development and mice lacking all isoforms die at E8.5 with severe defects in mesodermal cell migration and mesoderm formation (32). Ubiquitously expressed by fibroblasts, epithelial cells, and most other cell types, fibronectin dimers are secreted as soluble molecules which assemble into stable multimeric fibrils at sites called fibrillar adhesions (33). Fibrillogenesis is a cell dependent process mediated primarily by activated α5β1 integrin (34), although other integrin receptors may also possess this capacity (35, 36). Fibrillogenesis couples the contractility of the actin cytoskeleton to the conformational changes neccessary to promote coordination of fibronectin molecules (37). The inward movement of α5β1 integrin-fibronectin complexes is mediated by actin stress fibers (38, 39) and is thought to sufficiently stretch individual fibronectin dimers to enable fibronectin-fibronectin interactions (40). The discovery and subsequent purification of fibronectin (41, 42) and the ability of exogenous fibronectin to strongly induce focal adhesion formation (43) and in turn adhesion to ECM proteins (44) has greatly advanced the study of cell adhesion and ECM-cell interactions in vitro. Fibronectin molecules include binding domains for collagen, heparin sulphate proteoglycan, and fibrin which synergistically promote fibronectin mediated cell adhesion (45). An *Arg-Gly-Asp* (RGD)

domain mediates fibronectin binding to α5β1, and other integrin binding domains have more recently been identified (46). Fibronectin therefore also modulates a variety of cellular phenotypes by impacting integrin mediated signaling cascades involved in migration, proliferation, and survival (47, 48) as well as cell deposition of ECM proteins such as laminin and collagen (49).

The fibronectin gene gives rise to a single pre-mRNA species which through alternative splicing is known to generate 20 known human isoforms. Cellular fibronectin can be alternatively spliced in several domains to give rise to tissue specific isoforms with unique adhesive and binding properties but which generally, with the exception of the monomeric fibronectins, interact with cells and the ECM as fibrils (46). Alternatively, plasma fibronectin is secreted from hepatocytes in a structurally closed form and therefore circulates as a soluble dimer at a plasma concentration of 300ug/ml (50). Plasma fibronectin contributes to repair processes by mediating, in conjunction with fibrinogen and Von Willbrand factor, the adhesion of activated platelets and its deficiency has been found to reduce thrombus growth and stability in a mouse model of arterial injury (51). Plasma fibronectin also interacts with integrin receptors of other cell types (52), regulates the secretion of cellular fibronectin (53), and has been found to incorporate into fibronectin fibrils assembled by cultured cells (54).

1.3 CONNECTING THE ECM TO CELLULAR FUNCTIONS

1.3.1 Focal Contacts

The existence of structural cell adhesions in live cells was first described for contacts between cells observed in contact dependent growth inhibition studies. The inhibition was thought to be related to the formation of focal contacts observed between chick heart or

mouse muscle fibroblasts but not between cultured mouse sarcoma cells and either each other or in co-culture with fibroblasts (55). Adhesion structures between cultured cells and the substratum were identified using electron microscopy several years later in 1971 (56) and confirmed by interference-reflection microscopy (57, 58) in fibroblasts spreading along 2-D planar surfaces. The regions of adhesion are surprisingly few in number, approximately 10-100 per cell, and consist of 5-10 micron segments of plasma membrane in close (10-15nm) connection to the underlying substratum. These focal contacts are characterized intracellularly by the presence of bundled actin microfilaments associated with electron dense submembrane plaques (58, 59). Immunofluorescence studies have confirmed the alignment of the actin-containing microfilament bundles inside the cell with fibronectin containing ECM fibrils (60, 61, 62). The bidirectional nature of the association between cell surface fibronectin and intracellular actin bundles has been demonstrated in several early studies. Disruption of actin filaments with cytochalasin B treatment led to a loss of fibronectin associated with the cell surface in hamster fibroblasts (62). In the opposite direction, oncogenically transformed fibroblasts which do not express fibronectin are described as having disorganized actin cytoskeletons. Interestingly, these fibroblasts could be induced to assume a more strongly adherent and flattened phenotype with reorganized actin bundles 24-48h following treatment with exogenous purified fibronectin (63, 64, 65). Bundling of actin microfilaments 1h following the addition of fibronectin protein and before formation of focal adhesions, indicates that the actin reorganization was a direct consequence of exogenous ECM protein and not a consequence of heightened cell adhesion (66). The submembrane plaques or focal adhesions observed at regions of contact between ECM proteins and the actin cytoskeleton comprise a complex assembly of signaling and scaffold proteins connecting ECM cues to intracellular events which regulate ECM composition, and

adhesion dependent cell polarity, spreading, motility, and growth. Cytoskeleton associated proteins such as α-actinin (67), vinculin (68, 69, 70), and talin (71) were the first adhesion plaque proteins to be identified mainly because they presented as contaminants of actin and subsequently α-actinin purification procedures. It was however, the identification of the transmembrane link between actin and the substratum, members of the integrin superfamily, which facilitated subsequent discoveries of a large variety of molecular components localizing to areas of focal contact. The current model depicting focal adhesions consists of a dynamic network of 156 components, "including 25 adaptor proteins, 24 cytoskeletal proteins, nine actin-binding proteins, 10 serine/threonine kinases, three serine/threonine phosphatases, eight tyrosine kinases, nine tyrosine phosphatases, eight GAPs, eight GEFs, seven transmembrane receptors, six adhesion proteins, five GTPases, and 32 other types of components" (72). The adhesome map described by Zaidel-Bar *et al* (72) is based on pooled published data and is characterized by a high number of links per node, perhaps owing to the high degree of interconnectivity between focal adhesion components. It is also suggested that the density of this network, consisting of 379 binding, 213 activating, and 98 inhibitory interactions between the 156 components, may contribute to the adhesome's stability due to many redundant interactions. Generally, it was found that proteins described as "hubs", having greater than 20 interactions, in the adhesome were more essential for its function. Such hubs include integrins, paxillin, growth factor receptor-bound protein 2 (Grb2), and focal adhesion kinase (FAK). The full interaction database described by Zaidel-bar *et al* can be accessed at http://www.adhesome.org.

Although they are difficult to detect in tissues compared to other adhesion structures (73), focal adhesions have more recently been observed by immunoelectron microscopy in mouse (74) and human (75) tissue samples and are therefore not thought to be simply an

artifact of 2D culture. Focal adhesions are, however, highly dynamic structures displaying more heterogeneity in molecular composition than may have been previously expected. Two major types of focal adhesions have been classified in rat embryo fibroblasts according to the quantitative presence of various components using fluorescence ratio imaging (76). The "classical focal adhesion" is described as approximately arrowhead shaped with the tip connecting the ECM outside the membrane to a submembrane protein dense plaque and tightly packed actin bundles at the terminal ends of stress fibres (77, 78). Zamir *et al* found these to contain high levels of integrin $\alpha v\beta 3$, phosphotyrosine, paxillin, and vinculin, but relatively low levels of tensin, another actin binding protein. Elongated fibrillar adhesions containing high levels of integrin $\alpha 5\beta 1$ and tensin, and relatively low phosphotyrosine, were also identified. Fibronectin fibrillogenesis was subsequently found to occur at these latter sites in a tensin-dependant way (79). Many subtle variations of focal or fibrillar adhesion molecular compositions were also observed by fluorescence intensity labeling, underlining the vast heterogeneity which likely exists for adhesion structures even within cells. Other cell-ECM adhesion structures identified to date include focal complexes at edges of filopodia and lamellopodia (80) which can develop into actin stress fibre-containing focal contacts upon Rho activation (81), and podosomes, identified in Rous-sarcoma-virus-transformed cells (82, 83) as well as in a variety of normal cells (83).

The exact nature of *in vitro* focal adhesion formation and composition varies among cell types (83) but is also dependent upon culture conditions. The importance of understanding focal adhesion biology in 3-dimensional matrix systems is underlined by the changes in polarity, an adhesion-dependent process, observed for both epithelial cells (84) and fibroblasts (84, 85) when they are studied in 2-D culture but which can be normalized in some 3-D culturing models. Focal adhesions are tension dependent structures, evidenced by

treatment of cells with the kinase inhibitor H-7 which can reversibly decrease local phosphotyrosine (76), inhibit actomyosin contractility and cause the disassembly of focal adhesion complexes (76, 86). Because the alterations in actin structure and cell shape were more analogous to those observed for a myosin light chain kinase inhibitor than to other PKC inhibitors, the loss of focal adhesion complexes was attributed to the change in actomyosin contractility and therefore cell tension. Thus it seems likely that mechanical changes in tension applied by rigid 2-D surfaces govern the altered phenotypes of cultured cells and that a 3-D model, given it is of a relevant elasticity, may be a useful alternative for *in vitro* studies of focal adhesion complexes. In agreement with this idea, Cukierman *et al* describes a α5 integrin and paxillin rich adhesion complex present *in vivo* but not in 2-D culture conditions (75). The complex formed *in vivo* includes features of both classical focal and fibrillar adhesions combined and can be recapitulated in 3-D tissue derived matrices but not in 2-D flattened matrices. The "3-D adhesion" complex is contingent on three dimensionality, pliability, fibronectin, and other unidentified components of cell derived matrix. Interestingly, in contrast to 2-D adhesion complexes (87), the 3-D adhesion was dependent on a single adhesion receptor, integrin α5β1.

1.3.2 Identification of ECM-Adhesion Receptors

Several important glycoproteins mediate the connection between extracellular cues and intracellular signaling by acting as adhesion receptors. These include glycoproteins such as CD36 and dystroglycan, as well as surface proteoglycans such as CD44, and members of the syndecan family (88). However, the best characterized ECM interactions governing cytoskeletal structure and downstream intracellular signaling events are mediated by a family of integrin receptors.

The first approach used to identify adhesion receptors was based on slime mold aggregation studies (89) in which antibodies capable of disrupting adhesion were used in immunoprecipitaion, biochemical purification, or affinity chromatography to identify molecules with which they interacted. In this way, cell glycoproteins which could interact with the ECM were identified using broad-spectrum cell surface directed anti-serum (90, 91). A complex from avian cells was the first to be identified and extensively characterized using monoclonal antibodies. Two adhesion disrupting antibodies, CSAT (92), and JG22 (93) were used to identify avian integrin in a variety of cell types including muscle, fibroblasts, and neurons. These antibodies inhibit adhesion in a cell type and ECM specific way (94, 95) foreshadowing the complexity of integrin-ECM interactions studied today. Additional evidence for the role of avian integrins as transmembrane cell-ECM links includes their localization to focal adhesion structures, where fibronectin fibrils align with the actin cytoskeleton (96, 97), and their ability to bind known ECM proteins such as laminin and fibronectin (98).

Identification of the mammalian integrin receptor was achieved by ligand-affinity chromatography. An RGDS tetrapeptide known to mimic fibronectin binding activity was able to isolate a glycoprotein complex which, when incorporated into lipid vesicles, was found to mediate attachment to fibronectin but not laminin (99). This fibronectin receptor is now known as integrin $\alpha 5\beta 1$. Receptors for vitronectin (100), and collagen I (101) were also identified in this way, using matrix specific peptides. At the same time, specialized receptors were being identified in platelets (102), and lymphocytes (103). In all cases, mammalian cell-ECM receptors were found to consist of two distinct non-covalently linked glycoproteins designated α and β, one of which is generally rich in intramolecular disulfide bonds. Sequence information for various avian and mammalian receptors points out many

homologies between receptor subunits and also led to the realization that integrin receptor α and β subunits comprise a large family of transmembrane glycoproteins which dimerize to mediate a variety of important cell adhesion events (104).

1.4 INTEGRIN RECEPTORS

1.4.1 The Integrin Receptor Family

Integrin receptors are heterodimers generated by the non-covalent association of alpha and beta integrin glycoproteins. They belong to the type I transmembrane family of cell surface receptors, containing a large N-terminal extracellular domain, one hydrophobic membrane spanning section and a relatively short but important C-terminal cytoplasmic domain (105). All metazoa have integrins to facilitate cell matrix adhesion, and the expression of at least two distinct heterodimeric receptors in higher metazoa may reflect the need for asymmetric interactions with the basal lamina (106, 107). The mammalian complement of integrin subunits is expanded compared to basic bilateral metazoa to include 18α and 8β subunits, comprising 24 unique integrin receptor heterodimers of which 12 contain a β1 integrin subunit (108). Each individual integrin contributes in a unique way to mammalian development and life as evidenced by phenotypes of knockout mice ranging from embryonic lethality as early as E6.5 for integrin β1, to impaired functions in a large variety of adult tissues (108, 109). Despite the diversity of roles for integrins and their implications in nearly every biological process, there is some redundancy in receptor interactions. Accordingly, integrin receptors can be categorized based on ligand specificities (Figure 1B) for ECM, cell surface, or soluble proteins which crucially depend on the distinct association between α and β subunits (110, 111, 112).

Electron micrographs of integrin extracellular domains reveal a globular "head" comprising both α and β integrins which is anchored to the lipid bilayer by the stalk-like domains of each integrin subunit (113, 114, see Figure 1A). The first domain of integrins to be crystallized was the I/A domain of an alpha integrin. This domain contains a Metal Ion Dependent Adhesion Site (MIDAS) responsible for ligand binding (115). While I/A domain containing alpha (αA) integrins are a more recent evolutionary addition to the integrin family, all beta integrins contain the analogous I/A-like domain (βA) (116) which, in coordination with the alpha subunit beta-propeller domain, directly mediates ligand binding in non-αA containing receptors such as in β3-containing RGD-binding integrins (117, 118). The RGD family of integrin receptors, including two β1 integrins (α5β1 and α8β1), recognize the short peptide sequence, Arginine-Glycine-Asparagine, within larger proteins such as fibronectin and vitronectin, a specificity which is conserved throughout metazoan evolution. In addition to RGD binding integrins, ancient non-αA containing laminin receptors are conserved in the model invertebrates *Drosophila Melanogaster* and *Caenorhabditis Elegans* (106). The mammalian receptors of this class include three β1 integrins and α6β4 which are highly specific for the laminin ligand but for which a defined site or motif mediating this interaction has not been determined (109). Dual specificity laminin/collagen binding receptors appear in vertebrate species with the appearance of fibrillar collagens (107) and to date include four unique receptors each comprising one αA-domain containing alpha and one β1 integrin subunit (109, 119). Vertebrates also express LDV-binding integrins which recognize the acidic motif (Leucine-Asparagine-Valine) functionally related to RGD (120) but occupying a unique site in ligands such as fibronectin, VCAM-1, and MAdCAM-1 (121). Three β2 integrin receptors specific to white blood cells are also included in the LDV-binding family due to the structurally similiar ligand site

recognized (122). However, the mode of interaction necessary to mediate heterotypic cell-cell adhesion employs alpha I/A domains for integrin binding to Ig-superfamily counter-receptors (123). A complete summary of known integrin-ligand interactions is provided in Figure 1B. Due to variation in the exact nature of unique integrin active site pockets modulated in cell and receptor specific ways by contextual factors such as lipids (124) and divalent cations (125, 126), not all receptors bear equal affinity for a given ligand (127). Most importantly ligand affinity is tightly regulated; at the cell level by modulation of integrin expression and localization, and at the level of individual receptors by conformational modulation from inside the cell as depicted in figure 2A.

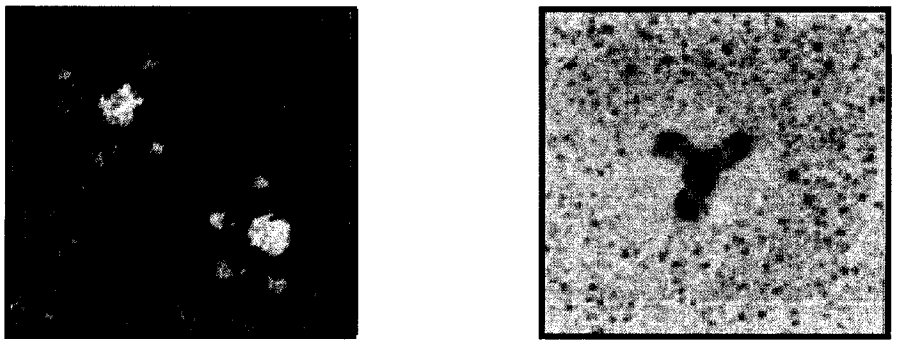
1.4.2 Integrin Regulation and "Inside-Out" Signaling

Expression at the Cell Surface

Adhesion mediated processes and signaling events are regulated at the level of integrin receptor display and function in several ways. The promoter regions for many α and β integrins have been characterized and transcriptional control of protein expression has been shown to be modulated by extracellular stimuli such as growth factors and pharmacologic agents (128). While expression of integrin β1 can be modulated in this way, the expression of functional β1 containing receptors, also depends on synthesis of its α subunit partner (129, 130), and on integrin β1 maturation involving glycosylation (131), as it has been suggested that integrin β subunits may be generally expressed in excess. Mechanical factors may also influence integrin expression, evidenced by altered mRNA levels and protein distribution of β1 integrin in cells under mechanical strain compared to unstrained counterparts (132, 133). Expression of active integrin heterodimers at the cell surface depends on a complex series of spatially regulated endo- and exo-cytosis events which promote adhesion, migration, and the maintenance of cell polarity (134). Exocytosis of integrin receptor stores in intracellular

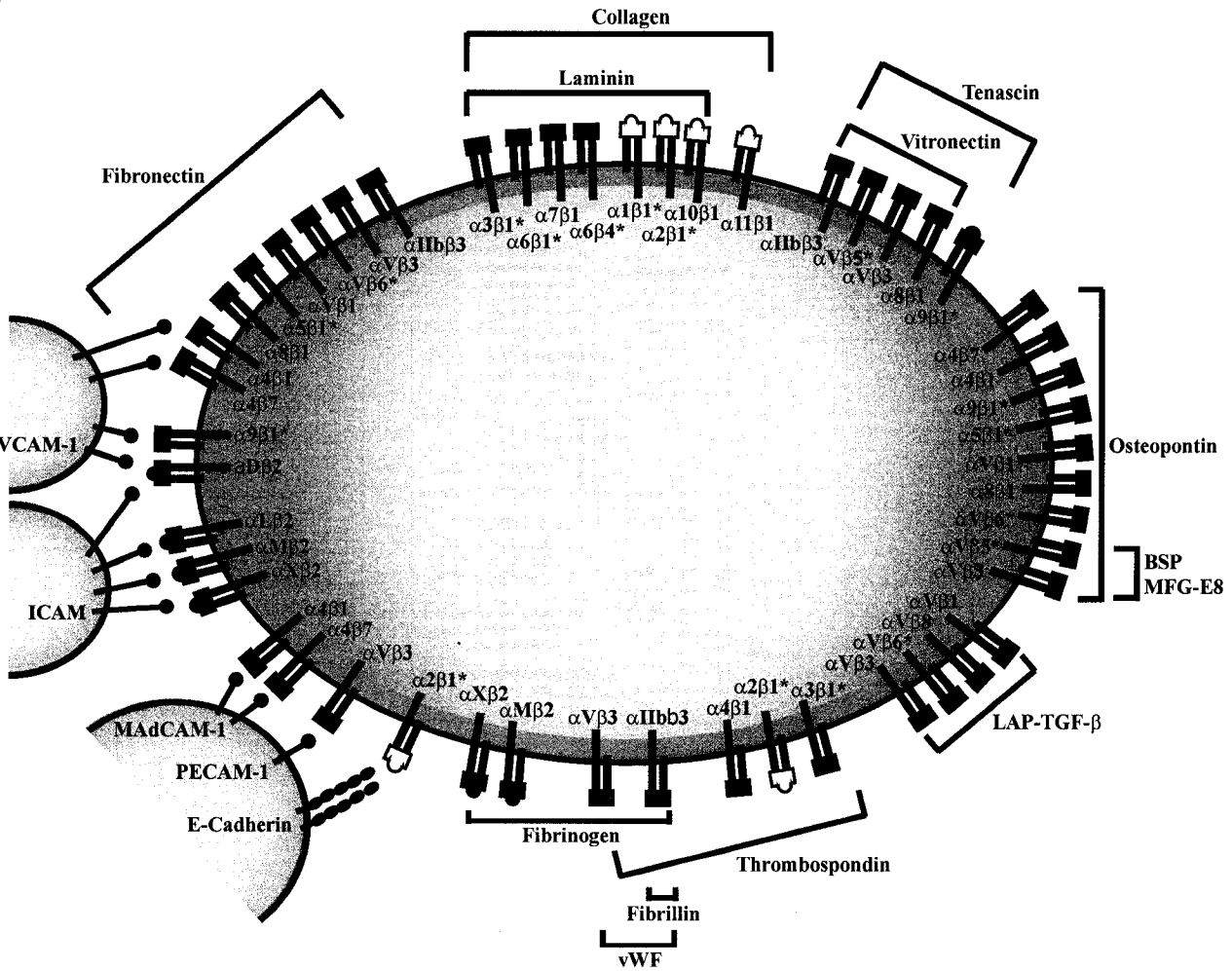
Figure 1. The Integrin Receptor Family. (**A**) Electron micrographs of integrin αIIbβ3 (Carell *et al* 1985 (113)) and of the fibronectin receptor, integrin α5β1 (Nermut *et al* 1988 (114)) heterodimeric receptors which form clusters in the absence of detergent such that two tails per receptor, or two cytoplasmic domains, interact and the globular head portion, or ligand binding domain, of each dimer is directed away from the interacting tails. (**B**) As adapted from Humphries *et al* 2006 (121), integrin receptors are classified in the schematic based on interaction motifs and organized according to their capacity to bind the ligands shown. Integrin receptors known to be expressed by untransformed epithelial cells are shown with an asterisk.

A



αIIbβ3 α5β1

B



granules has been reported for granulocytes and monocytes (135), enabling a rapid mobilization of cell surface integrin expression following stimulation. In most cell types, a complete endo-exocytic cycle involves focal contact dissassembly in which integrin-actin connections are lost (136), integrins are internalized to endosomal compartments, and Rab GTPases mediate recycling back to the plasma membrane (134). Internalization and recycling of β1 integrins partially depends on Cyto 1, 2, and 3 cytoplasmic motifs in the β integrin tails which are also responsible for focal adhesion localization (137). Specifically, the two NPxY domains (cyto 2, and cyto 3) are implicated in activated Protein Kinase C α (PKCα) (138), Rab21 GTPase (139), and microtubule (140) mediated internalization of β1 integrins. Alpha integrin subunits interact with Rab21 to mediate endocytosis (140). While some β3 integrins are recycled via Rab4 and the short loop recycling pathway (141), recycling of many integrins, including the β1 integrins occurs via perinuclear recycling endosomes (PNRE) of the long loop and is mediated by Rab11 (142). In tumour cells, Rab11c (or Rab25) trafficking of α5β1 has a particular role in promoting the assembly of fibronectin containing fibrils at cell junctions (143) and at the leading edge of migrating cells (144). There is evidence to suggest that these recycling loops interact such that integrin α5β1 recycling by the Rab11 loop is dependent on integrin αVβ3 recycling by the Rab4 loop (145). Several intracellular kinases have also been shown to interact with integrin recycling pahways, including PKCα (138), AKT via inactivation of glycogen synthase kinase 3β (GSK3β) (146) and PKCε via activation of 3-phosphoinositide-dependent protein kinase 1 (PDK1) (147), thus regulating from inside the cell integrin surface expression and adhesion dependent processes.

Alternative Splicing

Alternative splicing of integrin mRNA contributes to the diversity of functional receptors. Much like expression and sub-cellular localization, alternative splicing of extracellular and cytoplasmic domains of α and β integrin subunits is specific to both tissue type and developmental stage (148). Extracellular splice variants of the alpha integrin subunit, αPS2, in drosophila dimerize with the same integrin β subunit, βPS, but their heterodimeric receptors possess different affinities for fibronectin and vitronectin, as well as altered cell spreading characteristics (149). Similarly, mammalian extracellular splice variants of alpha subunits possess different ligand affinities when complexed with the same beta subunit, such as for α7β1 (150), or in the case of α6, alternative splicing can alter preferential association with β subunits, thus altering ligand specifity and affinity (151). Alternative splicing of cytoplasmic domains has been observed for both α and β subunits and due to conservation of the membrane proximal domains known to regulate ligand affinity, these splice variants generally impact intracellular events following receptor ligation (148). Human integrin β1 exists as at least five distinct cytoplasmic splice variants. Splice variant β1A, generally referred to as the wild-type β1 integrin, is highly conserved (152), and is abundantly expressed in all tissues with the exception of mature erythrocytes. It is the main β1 integrin expressed and studied. The isoform displaying the most significant homology to integrin β1A is the β1D variant, differing by only a few amino acids (153). Although it lacks two sequential threonine residues which are thought to regulate wildtype ligand binding (154), β1D localizes to focal adhesions and mediates strong cytoskeleton-to-ECM connections via increased fibronectin fibril formation, affinity for extracellular ligands, as well as comparatively stronger intracellular binding to talin, filamin, and actin associations (155, 156) in later embryonic and adult skeletal and cardiac muscle tissues where it is

expressed (153, 157). Neither β1C nor the β1B human-specific splice variants contain Cyto-2 or 3 motifs which mediate focal adhesion localization of the A and B variants (158), thus these isoforms are diffusely localized without connections to talin or filamin and the cytoskeleton (156). When overexpressed to high levels, integrin β1B, which is mostly found in adult keratinocytes and hepatocytes (159), acts as a dominant negative to the wild type A isoform and causes decreased attachment and cell spreading on fibronectin and laminin (160, 161), however the physiological relevance of this function is unknown. Differing from each other by six amino acids, two integrin β1C variants, arising from a splicing mediated frameshift compared to the wildtype sequence, have been identified (162, 163), although most studies have focused on variant C-1 which will henceforth be described as integrin β1C. At the message level, no obvious specificities exist for β1C expression and accordingly integrin β1C protein has been detected in the erythroleukemia cell line HEL, tumour necrosis factor-α (TNF-α) treated endothelial cells (HUVEC), prostate carcinoma, and benign prostate tissue (164). Integrin β1C is endogenously expressed in differentiated epithelial tissue, such as fetal differentiated prostate glands and the luminal prostate tissue of adults, but not in budding glands or basal epithelia of the prostate (165). Ectopic expression of integrin β1C is associated with decreased proliferation of several cell lines in vitro (166, 167, 168) including prostate cancer cells (165, 168), and in agreement with its role as a growth inhibitor, β1C expression is reduced in neoplastic prostate tissue compared to the neighbouring benign prostate epithelium (169). Levels of integrin β1C but not β1A are also reduced in transformed prostate cell lines compared to normal immortilized prostate epithelial cells, a down-regulation which is thought to occur by transcriptional and post-transcriptional mechanisms (170). Because expression of β1C does not affect integrin dimerization, adhesion to fibronectin and to an extracellular antibody for integrin β1, or phosphorylation of

focal adhesion proteins such as Focal Adhesion Kinase (FAK) and AKT, it has been suggested that a distinct mechanism is employed by β1C in order to inhibit growth (171). In support of this, a specific domain of integrin β1C has been found to be sufficient to inhibit proliferation independently of membrane or focal adhesion localization (167, 168).

Activation

For integrin receptors expressed at the cell surface, modulation of integrin activity by inside-out signaling refers to the ability of signaling that occurs inside the cell to impact the extracellular conformation and therefore ligand affinity. The first evidence of the allosteric properties of integrins came from studies of the human platelet receptor, integrin αIIbβ3, based on the fact that a resting circulating platelet does not bind its soluble ligands but must be able to do so when activated, to initiate the adhesive events necessary for clot formation (172, 173). Integrin αIIbβ3 demonstrates the ability to differentially recognize antibody epitopes dependent on activation by known platelet activators such as thrombin (174, 175). Subsequently, crystal structures of CD11 (leukocyte) αA domains (176, 177), and the αA domain from integrin α2β1 (178) revealed the existence of "open" and "closed" conformations which differentially coordinate metal at the MIDAS site (179, 180). When αA domains were locked in either conformation they were shown to exhibit very different ligand affinity (180, 181, 182). The crystal structure of the integrin αVβ3 unligated (116) and RGD bound (117) extracellular domain reveals a dramatically bent conformation for the unbound receptor, which has been confirmed by electron microscopy (182, 183). Crystal structures for integrin αIIbβ3 complexed with fibrinogen agree with the observation of a "closed" or bent structure for unligated receptor which is extended from the membrane in the presence of ligand (118). Because the change in integrin affinity for ligand in the active conformation is characterized by a 30-100 fold decrease in dissociation and only a modest

increase in association, it has been proposed that the conformation and not the accessibility of the binding pocket is responsible for the activation state of integrin receptors (184). This suggests that the extended or open conformation is the result of ligand binding, however mutational and structural analyses have also implicated intracellular integrin activity in allosteric activation (185). The current model describes a salt bridge between the membrane proximal segments of α and β integrin cytoplasmic tails which stabilizes the low affinity bent conformation of the integrin receptor (183, 186, 187, 188). Interactions between an intracellular protein, talin, and the unphosphorylated NPxY domain of a β integrin cytoplasmic tail (189, 190) results in the loss of this salt bridge (191, 192) and is thought to be the final event enabling the conformational switch of the integrin receptor to the high affinity state (190, 193). In addition to the well studied activation of integrin αIIbβ3 in platelets and β2 integrins in monocytes, neutrophils, and lymphocytes, integrin activation has also been reported for several β1 integrins (126). Beta-1 integrins are activated in lymphocytes in response to immune activation (194), but integrin α5β1 is deactivated during the terminal differentiation of cultured teratocarcinoma cells (195), keratinocytes (196), and retinal neurons (197). Additionally, inactivation of β1 integrins by phosphorylation has been associated with cell rounding and reduced adhesion to fibronectin in Rous sarcoma virus transformed (198) and mitotic (199) cells. While it is clear that intracellular signaling pathways, such as the Ras GTPase signaling cascades, can regulate the spatial and temporal activation of integrins (200) and talin function (190), a definitive link between signaling, talin, and the final conformational switch has not been made.

Integrin activation may not always be initiated by an intracellular cue but instead depends on the overall avidity or functional affinity of the receptors present which includes both activation and valency or clustering of receptors (201, 202, 203) as well as the specific

requirements of the ligand itself (202). It has been proposed that integrin receptors exist in dynamic equilibrium between high and low affinity conformations, and that activation and cytoskeletal clustering serve to shift the balance toward a higher affinity state (200). This may help to explain why crystallized integrin αVβ3 could bind ligand when in a bent conformation which differed only slightly from the unbound receptor (182) and why intermediate conformations of leukocyte integrins mediate distinct adhesive events (204). Most importantly, because integrins signal bidirectionally, initial activation and sustained signaling is likely to fundamentally require the coordination of both extracellular and intracellular input. While the exact nature of affinity modulation remains to be determined, it is clear that the stabilization of separated α and β tails, which can result from multiple bidirectional events, is sufficient to facilitate subsequent phosphorylation events and intracellular signal transduction to modulate adhesion-dependent processes (188, 205, 206).

1.4.3 "Outside-In" Signaling

Integrin receptors represent a major convergence point in context-dependent cell signaling. They contribute to adhesion-dependent growth and survival, differentiation, and migration by modulating the expression and activity of intracellular proteins in conjunction with regulating the cytoskeleton in response to extracellular cues. Ligation specifies conformational alterations (188), redistribution, and phosphorylation of integrin receptors and the concomitant redistribution and phosphorylation of a large variety of cell signaling molecules, or effector proteins. The formation of focal or fibrillar contacts, collectively termed focal complexes, is initiated at the level of integrin self-associations (207) and ligand dependent microcluster formation (208). Subsequently, members of the Rho GTPase family can promote firm adhesion, mitosis, or motility through specific actin polymerization events and regulated focal complex assembly and dissassembly (209). The nucleation of actin

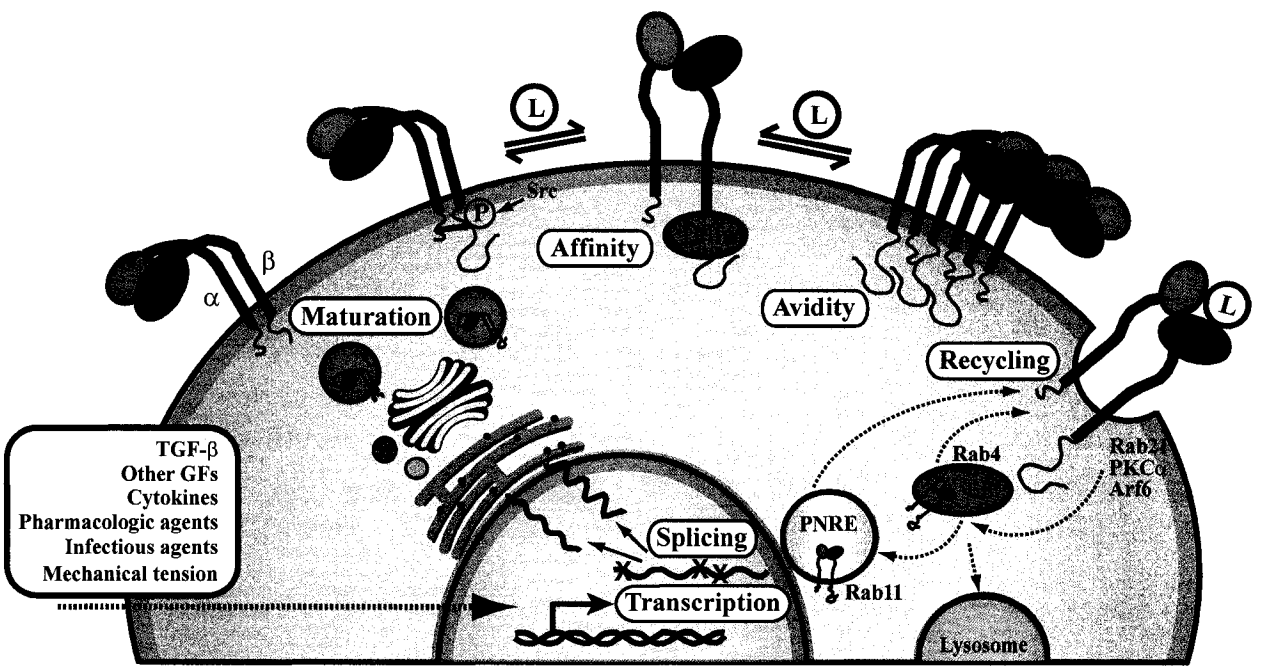
fibres at sites of focal contact, as well as the formation of distinct complexes formed at the tips of filopodia and lamellipodia (82) is mediated by the conformational regulation of vinculin by phosphatidylinositol (4,5) biphosphate (PtdIns(4,5)P2) (210) and talin, and the assembly of other structural adaptors and actin regulators of which 75 have been identified (71). These include α-actinin and filamin (211), as well as paxillin at focal adhesions (212) and tensin at integrin α5β1-rich fibrillar adhesions (75). Integrin localization as well as binding and recruitment by integrin receptors of a wide variety of functional proteins can be regulated by a dynamic code established by tyrosine, threonine, and serine phosphorylation of integrin beta cytoplasmic tails (213), and downstream protein-protein interactions. As integrins do not possess intrinsic kinase activity, it is the assembly of protein complexes by direct and indirect integrin interactions which promotes the activation of various signaling pathways. Indeed, the colocalization of clustered integrin receptors with FAK is sufficient to initiate kinase activation and downstream signaling events (214). Many known focal complex proteins and the crucial linkages between them are included in Figure 2B and described in further detail below with respect to the integrin-mediated phenotypes they contribute to. A significant degree of overlap between integrin and growth factor receptor mediated signaling exists and there is much evidence to support the hypothesis that anchorage and integrin dependent signaling necessarily supports growth factor dependent expansion and survival of untransformed cells (215).

Survival and Growth

The ECM coordinates growth factor dependent signaling via integrin phosphorylation and activation of receptor tyrosine kinases (RTKs) which recognize soluble growth factors and cytokines, and via the integrin dependent localization of RTKs to multi-protein

Figure 2. Integrin Signaling. (**A**) Schematic demonstrating major modes of "inside-out" integrin signaling which may be initiated by a variety of extracellular stimuli. Intracellular pathways governing transcription, splicing, maturation, and recycling of receptor components act to regulate the nature and quantity of integrin heterodimers present at the cell surface. Quantity is, in turn, one factor which contributes to integrin avidity for extracellular ligands, mediated by integrin clustering at the cell membrane. Individual receptors are also regulated individually by cytoplasmic binding proteins, such as talin, which cause a shift in the conformational equilibrium of the receptor structure towards the "open" or high affinity state presumably by inhibiting the presence of a salt bridge between membrane proximal segments of the α and β cytoplasmic tails. Phosphorylation of the β integrin cytoplasmic tail such as by the kinase Src, regulates integrin activity by preventing talin binding. (**B**) Schematic highlighting major intracellular signaling cascades mediated by integrin receptors upon extracellular ligand engagement. Adhesion links the ECM to the actin cytoskeleton via integrin receptors and a dynamic assembly of adaptor proteins and actin regulators. Specific adhesion structures can be identified based on the presence of these components, such as focal adhesions with increased paxillin and vinculin vs fibrillar adhesions, characterized by high levels of the protein tensin. Integrin activity can stimulate receptor tyrosine kinase (RTK) signaling both directly and indirectly, and both receptor types converge at key signaling nodes to promote growth and survival. Proliferation is mediated by the indicated integrins in epithelial cells and requires the sustained activation of cyclin D1 transcription dependent on the convergence of integrin and RTK signaling. The most directly involved signaling molecules at the level of integrin signaling, focal adhesion kinase (FAK) and integrin linked kinase (ILK), can interact with the integrin cytoplasmic tails themselves. Migration relies on firm integrin adhesion at the leading edge of the cell regulated mainly by FAK and the small Rho GTPases Rac and Cdc42, as well as myosin contractility mediated by integrin-dependent Rho kinase signaling. Detachment at the lagging edge of the cell involves focal complex disassembly, and in some cases calpain activity mediated by FAK. Differential integrin localization and/or activity is therefore essential to the different integrin-dependent signaling events mediating cell adhesion.

A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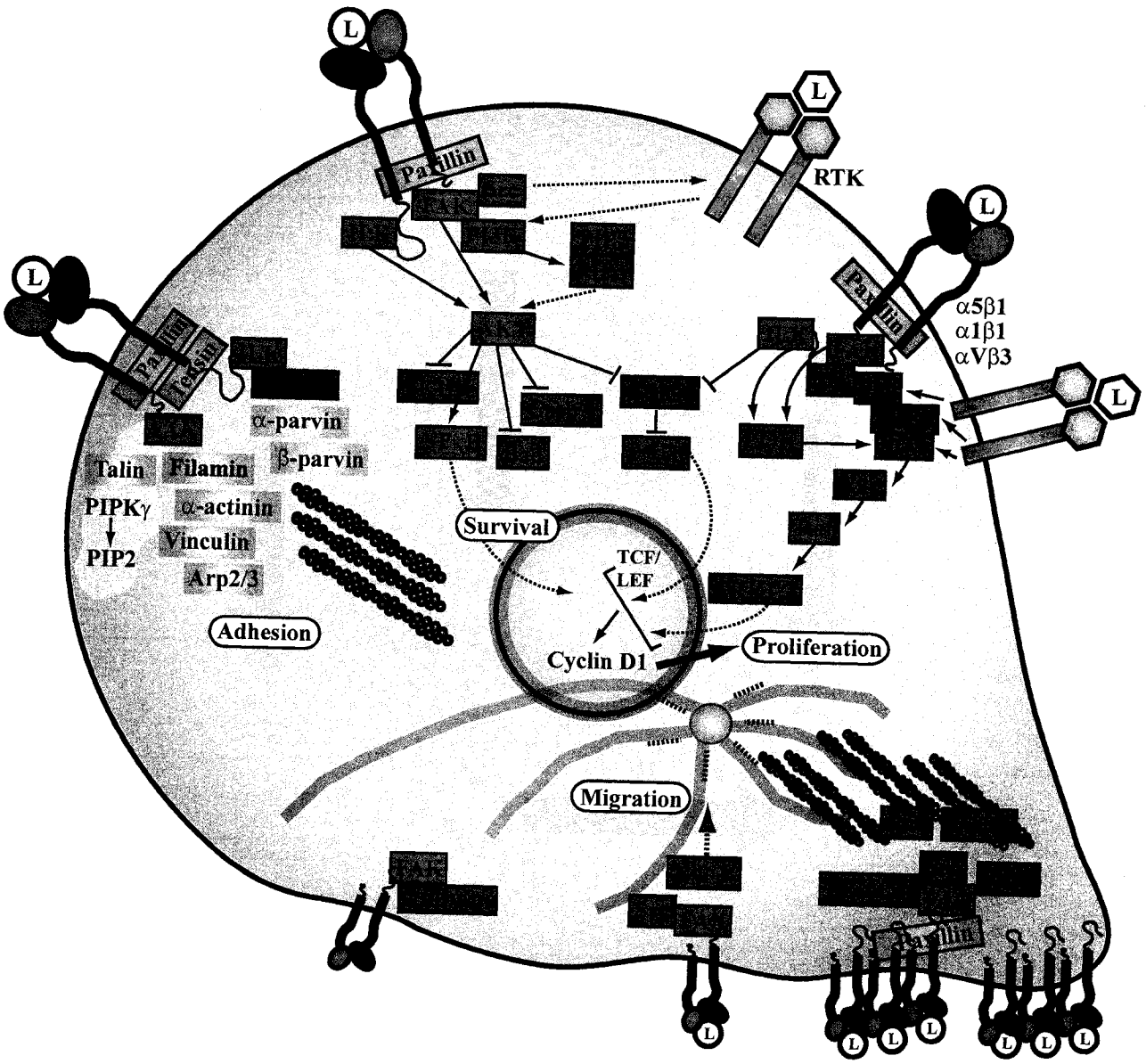
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PIP2
Vinculin
Arp2/3
Adhesion
Survival
TCF/
LEF
Cyclin D1
Proliferation
Migration

complexes (216, 217, 218), most likely in lipid rafts (219, 220, 221). Indirectly, stimulation of shared downstream signaling pathways by both integrins and growth factor receptors is necessary for full activation of anti-apoptotic and mitogenic programs (222). This is evident in the anchorage dependent survival of untransformed cells (223) which is thought to be mediated by both pro-survival and anti-apoptotic signaling. In the absence of soluble growth factors, RTKs can be sufficiently activated by integrins in a Src dependent manner to promote survival but not proliferation (224). "Anoikis", or homelessness in Greek, refers to the programmed cell death which occurs in epithelial cells that lose contact with an appropriate ECM (225). Importantly, the survival impact of ECM engagement is ECM, integrin, and cell specific (226). In the presence of the appropriate ECM, cell adhesion results in the recruitment and activation by integrins, including β1 and αV containing integrins, of two important kinases: FAK (227, 228) and Integrin Linked Kinase (ILK) (229) which in turn help to recruit PI3 Kinase to focal adhesions and subsequently contribute to the activation of PKB/AKT (230, 231). Downstream targeting of AKT includes activation of NFκB and transcription of its pro-survival effectors, as well as inhibition of forkhead transcription factors which promote transcription of pro-apoptotic genes, a pro-apoptotic member of the Bcl-2 family called Bad, and Caspase-9 which acts as a late effector in apoptotic cascades (232). Unsurprisingly, the constitutive activation of AKT signaling is sufficient to abrogate anchorage dependence as evidenced by the ability of cells overexpressing ILK (229), activated FAK (233), Ras, or constitutively active phosphoinositide 3-kinase (PI3K) (234) to grow independently of anchorage. Similarly, activation of Extracellular Regulated Kinase (ERK) promotes cell survival in epithelial cells by downregulating another pro-apoptotic factor, Bim, and detachment from the ECM is accompanied by decreased Epidermal Growth Factor Receptor (EGFR) expression and

resulting increased Bim expression and apoptosis (235) Detachment from ECM not only relieves anti-death signals but additionally unattached cells have been found to undergo programmed cell death through Fas ligand and receptor expression (236), and Caspase-8 recruitment by unligated β1 integrins (237) although the general applicability of the latter phenomenon has yet to be determined. Similarly, cytoskeleton dependent alterations in some detached cells may directly impact the localization and function of death receptors and signaling effectors (238, 239).

Cell shape, which is initially conveyed to the cell via adhesion receptors bound to the substratum, has been shown to regulate the growth of untransformed cells (240). Accordingly, G1 progression and cell proliferation crucially depends on both integrins and growth factor receptors for sustained activation of the Mitogen Activated Protein Kinase (MAPK) signaling cascade (215). Not all integrin receptors contribute to proliferation but those which do, such as integrins α5β1, α1β1, and αvβ3, act in a cytoskeleton dependent way (241) through Shc activation of Ras and Raf signaling (242). Almost all β integrin tails interact with FAK to recruit Shc via the Src SH2 domain (243, 244), while the alpha integrin-Shc interaction mediated by caveolins (244, 245) has only been reported for the receptors mentioned above. Phosphorylation of Shc by both mechanisms opens an SH3 domain which interacts with a binding complex containing adaptor protein Grb2 and Ras-GTP-exchange factor mSOS (243). Subsequent activation of Ras GTPase (a MAPK), Raf-1, and MEK converge with growth factor receptor signaling and with a FAK/p130Cas initiated cascade. MEK phosphorylates and activates ERK which translocates to the nucleus in an integrin and cytoskeleton dependent way (246) to activate nuclear transcription factors and genes encoding cyclin D1 and other cell cycle proteins (247). Sustained activation of ERK1/2 by integrin dependent signaling is essential for cyclin D1 biosynthesis (248) and thus adhesion

regulated G1 progression. Other regulators of cyclin D1 include FAK or ILK-activated PI3 Kinase/AKT which impact cyclin D1 expression at several steps along the MAP kinase pathway (249, 222) and via stabilization of β-catenin which mediates cyclin D1 transcription in conjunction with the T-cell transcription factor/lymphoid enhancer binding factor (TCF/LEF) transcriptional complex (250), as well as Rho GTPases which together regulate the timing and sustainment of ERK signaling (251, 252). In contrast to the integrin receptors mentioned above, ECM ligation of other integrin receptors fails to activate Shc and downsream Erk signaling resulting in cell cycle arrest (242, 253). As differentiation depends on cell cycle exit, this may provide one explanation for the importance of integrin-ECM interactions in the control of cell fate.

Migration

In stationary fibroblasts, GFP-labelling of integrin β1 has revealed continual centripetal movement of dispersed focal adhesions. Conversely, motile or migrating cells were found to contain stationary focal contacts relative to the substratum (254). In agreement with this idea, migration events during development, wound repair, immune surveillance, and cancer, occurs by means of dynamic assembly and dissassembly of focal complexes and contact points (255). Migration is directional and involves the establishment of polarity with respect to cell adhesion and an accompanying gradient of signaling intensity (256). Integrin containing focal complexes accumulate at the leading edge of lamellipodia and filopodia which extend due to the localized decrease in tension (257) and the onset of actin polymerization events (258). These nascent complexes represent regions of high integrin-ECM affinity which are primed through the activity of the Rac, and Cdc42 GTPases and renewed via FAK dependent dissassembly (259) to enable firm adhesion (260, 261). In response to ECM attachment, integrins initiate positive feedback loops regulating, Cdc42,

and Rac (262) and may be responsible for the amplification of PI3 Kinase signaling at the leading edge (263). Finally, contractility by Rho GTPase and Rho Kinase activation of of Myosin II light chain (264) moves the cell forward in conjunction with FAK dependent focal complex dissassembly and the loss of integrin attachment at the rear end of the cell (265, 266). While overexpression of β1 integrins increases adhesion dependent Rac activation and lamellipodia formation in CHO cells, β3 integrin promotes adhesion dependent Rho activity and stress fibre formation (267) implicating specific integrin localization in the polarized cellular events during migration. Migration encompasses many aspects of integrin signaling, including localized focal complex formation, dissassembly, signal amplification, and mechanoreceptor function. The latter is particularly important as successful migration depends on the coordination of traction forces, directly dependent on integrin density and affinity, and contractility which is at least in part modulated by integrin signaling (268).

1.5 ADHESION AND CANCER

1.5.1 The Extracellular Matrix

Invasion and migration is facilitated by degradation of the extracellular matrix by integrin regulated matrix metalloproteinases (MMPs) (269). At the same time, as has been mentioned previously, integrins, such as α5β1 can signal to modulate secretion and organization of the ECM (33, 34). Thus, in normal cells, integrins participate in a bidirectional homeostasis with the extracellular environment which is disturbed in various ways in cancerous tissues. Several studies have reported increased levels of MMPs in tumour cell lines (270) and in tumour tissues (271), and it has recently been established that the expression of MMPs in stromal cells, stimulated by but not produced by the tumour cells themselves, contributes to the latter phenomenon (272, 273). Although, alternative roles for

MMPs in cell signaling have been identified (274), their role in ECM degradation is considered an important step for angiogenesis (275) and tumour cell invasion (276). Despite a decreased dependence of tumour cells for ECM survival cues (277), the site of colonization following dissemination from the primary tumour will also depend on compatibility with the ECM present in the new organ. This idea of compatibility between "seed", or favoured tumour cells in the heterogeneous population which disseminate, and "soil", or the environment provided by certain organs, was first reported by Stephen Paget, who noted in 1889 that sites of metastasis observed in patients were not in agreement with the first organ reached by blood flow (278). The importance of the ECM in cancer is thus reflected in the need of tumour cells to migrate and invade through it, and its ability to provide a distinct niche of preference for specific metastatic cells. This is of course unsurprising, as ECM composition is known to regulate cell shape and behaviour, integrin affinity and distribution, and signaling mediated by soluble factors (279).

Unregulated thinning of the basement membrane and the resultant disorganization of ECM and tissue architecture are hallmarks of tumour stroma (280, 281). Specific ECM proteins, such as tenascin-C, fibronectin, collagen, and isoforms of laminin are aberrently expressed in several tumour types (282, 283, 284, 285), however clear indication for the tumourigenic or anti-tumourigenic nature of any particular protein has not been observed, most likely due to the heterogeneity of tumour cell requirements. Many studies have proposed expression of ECM components, such as tenascin-C which is only expressed during would healing, invasion, and metastasis (286), fibronectin and an oncofeatal isoform of fibronectin, isoforms of laminin such as laminin-5, and some collagens, as prognostic markers in a wide variety of cancers. Because of the heterogeneity between and within cancer types, consistent and specific ECM expression patterns may be difficult to obtain and

this area certainly requires further investigation. However in some cases extracellular matrix signatures have been found to correlate with a prognosis in human patients when detected both as circulating moieties (287, 288), sometimes reflective of angiogenesis (281) and in immunohistochemical sections of tissue (289, 290) or ascites fluid (291). Additionally, the mammographic density of breast tissue correlates with collagen and fibronectin expression (292) and is predictive of risk for breast cancer development (293), reinforcing the importance of ECM composition and the subsequent impact of tension on cell functions.

Several experiments have implicated normal ECM composition and specific ECM proteins in the reversion from a malignant to normal phenotype both in culture (3, 294, 295, 296) and in vivo (297). Clearly, a capacity for tumour cell interaction with the ECM exists and impacts tumour cell phenotype. Such an impact can also be inferred from analyses of data from drug-screening trials in which regression of tumour metastases in response to chemotherapy differed according to the organ of metastasis (298). Mouse studies have similarly revealed that implanted tumour metastasis derived from the same primary tumour are not equally responsive to chemotherapy (299, 300) and that tumour cells are differentially sensitive to chemotherapeutic drugs, such as alkylating agents, in vivo compared to explants from drug-resistant mouse metastases (301) suggesting that, in vivo, the tumour microenvironment may be an important determinant of chemotherapeutic response. In recent years, much effort has been directed at context specific drug screening, reflected in the volume of in vitro evidence for ECM modulation of drug sensitivity. In vitro chemotherapeutic cytotoxicity is reportedly modulated by at least one ECM protein in at least one cell line derived from transformed glial (302), uveal (303), breast (304), lung (305, 306), pancreatic (307), and ovarian (308) tissues, as well as for cells from heamatological malignancies (309) Whether a central mechanism exists to explain the specific protective

impact of individual ECM proteins in the drug response of distinct tumour types is currently unclear, however the importance of integrin receptors, particularly β1 integrins, and many of their downstream signaling targets in ECM-mediated protection from apoptosis is becoming well established (310).

1.5.2 Integrins

Integrin expression is not unlike ECM expression in tumourigenic compared to normal cells in that aberrent expression of specific integrins and their ability to contribute to tumour phenotypes is well documented, however a consistent expression profile and function for integrins in cancer cannot be clearly defined (311). Tissue specific alterations of integrin receptors in tumourigenic cells include increased integrin αvβ3 expression in melanomas (312) and increased expression of integrin α6β4 in invasive thyroid carcinomas (313) and papillomas (314). Conversely, surface expression of integrin α5β1 was found to decrease in SV40-transformed human lung fibroblasts (315) and in rodent cell lines transformed with Rous Sarcoma virus or murine sarcoma viruses encoding Ras-oncogenes while levels of α3β1 were unchanged (316). Overexpression of the integrin α5β1 fibronectin receptor was also found to normalize the transformed phenotype of CHO cells (317). Recently, integrin α5β1 expression was found to increase in HA-Ras-transformed mammary epithelial cells with a more pronounced elevation following transforming growth factor β (TGF-β) induced epithelial to mesenchymal transition (EMT) (318) suggesting that the role of this receptor may be species or tissue specific with respect to transformation. Interestingly and similarly to human fibrosarcoma cells and SV40 transformed lung fibroblasts (319), the post-EMT expression of integrin α5β1 in transformed mammary epithelial cells is diffuse throughout the plasma membrane, implicating abberrent localization of integrin receptors in transformed cells. The localization of integrin αvβ3 at the invasive front of malignant skin cells (312),

may help to explain why induction of either αv (320) or β3 (321) integrin subunits increase the metastatic potential of melanoma cell lines. These changes in localization may reflect a cause or effect of alterations in integrin phosphorylation and affinity which have also been reported in tumourigenic cells (311).

Mechanistically, tumour growth and survival pathways are generally not dependent on integrin expression (322), perhaps due to the constitutive activity of integrin downstream signaling molecules which include ILK (323) and FAK (324), both attractive targets for clinical therapeutics. Nevertheless, expression of integrin β1 was found to increase in response to ionizing radiation (325) and has been found to essentially contribute to the survival of normal fibroblastic and tumour cell lines exposed to radiation-induced genotoxic injury (326). Beta-1 integrins, as well as integrins αvβ3 and αvβ5 in gliomas, have also been implicated in ECM-dependent survival strategies of various cancer cell lines treated with different classes of chemotherapeutic drugs, usually in a PI3 kinase dependent manner (310, 327, 328). Why integrin ligation and activation of pathways which are often already activated in human cancers contribute to drug resistance is unclear but suggests that tumour-derived cells rely on a type of adhesion-independent homeostasis which in the presence of extracellular stress can be shifted towards increased survival by ECM-integrin engagement.

As integrin expression profiles have been found to correlate clinically with invasive and metastatic disease (329), much effort has been directed towards understanding the mechanisms by which integrin receptors contribute to these phenotypes. Metastasis for many cancer types represents the main obstacle to successful therapy and recovery (277). The loss of cell-cell adhesions in EMT is a prerequisite for epithelial tumour cell invasion and is characterized by a loss of E-cadherin at these junctions as well as changes in integrin

receptor expression (330). Although expression patterns are likely to be cell specific, β1 integrins can regulate the formation (331) and disruption (332) of these junctions in malignant and untransformed cells, respectively. The impact of integrin attachment and signaling during cell migration is more complex. While integrins αvβ3, αvβ6, and α6β4 generally promote tumour progression (333), the basement membrane integrins, α2β1 and α3β1, suppress migration and progression when overexpressed in mouse tumour cell implantation studies (331, 334). Interestingly, integrins α3β1 and α2β1 have a positive impact on adhesive events involved in cell arrest and attachment to endothelial cells (335), and subsequent colonization of a new organ (336), respectively, underlining the importance of different receptors at various stages during cancer progression. The activation of migration specific pathways, and not merely mechanical adhesion, also depends on integrin ligation. For example, integrin α5β1 antagonists suppress fibroblast, endothelial, and tumour cell migration on vitronectin without affecting adhesion to this matrix protein (337).

Tumour growth much beyond a volume of $1mm^3$ and subsequent metastasis depends on the integrin-dependent recruitment of new blood vessels from pre-existing ones, a process called angiogenesis (338). The survival, growth, and migration of endothelial cells relies on adhesion and integrins such as α1β5 and αvβ3, which are known to be important for endothelial cell migration in vitro and angiogenesis in vivo (339). Chemotherapeutics targeting integrins such as αvβ3, αvβ5, and α5β1, have been developed as anti-angiogenic therapies for a variety of cancer types, although given evidence for the role of integrins in tumourigenesis, efficacy of these drugs alone or in combination therapy is likely to depend on not only the impact of inhibiting endothelial cells in the angiogenic process but also inhibition of tumour cells which might also express the target receptors at various stages of tumour progression (340).

1.5.3 Anchorage Independence

The ability of cells to grow into spherical colonies in semisolid medium is strongly correlated with their in vivo tumorigenicity (341). Cells subjected to viral, carcinogenic or oncogene induced transformation, as well as tumour derived cell lines, are often able to grow in the absence of anchorage, where their untransformed counterparts are not (342). Taken together, anchorage independence is perhaps the most obvious defining property of tumour cells in vitro. It has already been mentioned that untransformed cells depend on integrin-mediated adhesion to a mechanically stable ECM, and not only growth factor induced, signaling for survival and growth. The fibronectin-integrin α5β1 adhesion system has been implicated in much of the signaling which regulates anti-apoptotic and cell cycle progression pathways for nonadherent cells. The idea of integrins as dependence receptors may build on their contribution to anoikis in normal cells, while suggesting that tumour cells are also subject to cell death in the absence of specific adhesive events. By this mechanism, some integrin receptors have been found to initiate programmed cell death in both normal and transformed cells in the absence of an appropriate ligand (343). For example, antagonists to integrin β1 were found to activate caspase-8 and induce cell death in human keratinocytes (344). In tumour cells, a similar mechanism may regulate proliferation and explain the growth arrest and loss of tumourigenicity observed for colon carcinoma cells overexpressing α5β1, an integrin receptor they do not endogenously express (345). The phenotype observed was reversed by the addition of exogenous fibronectin, the known ligand for integrin α5β1. Conversely, untransformed cells, such as fibroblasts, display adhesion dependent expression of cell surface integrin α5β1 and total extracellular fibronectin, both of which are reduced in

the absence of adhesion (346). Mutant rat kidney fibroblasts which express cell surface integrin α5β1 in suspension at levels comparable to their adherent counterparts do not undergo anoikis and proliferate in the absence of adhesion (347). Additionally, TGF-β stimulation of these cells was sufficient to abrogate adhesion dependence for growth and survival by causing an upregulation of cell surface integrin α5β1 expression. Importantly, TGF-β successfully initiates transformation and acquisition of an anchorage independent phenotype only in cells which no longer depend on adhesion for cyclin D1 expression and G1/S progression (348, 349). These observations emphasize the importance of constitutively activated signaling molecules downstream of integrins, such as FAK, ILK, and cdc42, which are thought to relieve the requirement for integrin attachment to the ECM in combination with constitutive growth factor signaling cascades (350) but does not establish integrin independence as a feature of transformed and anchorage independent cells.

1.6 SUMMARY AND HYPOTHESIS

Extracellular matrix proteins and integrin adhesion receptors comprise a powerful bidirectional system which dually affects intracellular signaling and extracellular composition in order to direct cellular phenotypes and tissue function. A family of heterodimeric integrin receptors is expressed in a cell and context dependent way and bind ECM proteins to initiate the assembly of multi-protein signaling complexes inside the cell which are connected to the cytoskeleton. This allows for integrin-mediated signaling to regulate cellular behaviour such as adhesion, differentiation, migration, and invasion, as well as the expression and secretion of ECM proteins and remodelling enzymes. Integrin signaling is also crucial for the growth and survival of adherent and anchorage independent cells. Malignant transformation is characterized by the loss of regulated cell behaviour and

many important signaling events downstream of integrin receptors are activated in transformed cells such that adhesion is no longer required for growth and survival. Because of this, although cancer cells also express functional integrin receptors which have been found to mediate adhesion and contribute to many aspects of tumourigenic phenotypes, the overall necessity of these receptors in cancer cells is controversial. Beta-1 integrins account for half of the integrin receptor family and the integrin β1 knockout mouse is the most lethal of the integrin knockout mouse phenotypes. Beta-1 integrins are important and well understood components of cell-matrix adhesions involved in signaling cascades leading to a variety of cellular behaviours. As a receptor subunit widely expressed by both normal and transformed cells, integrin β1 represents an attractive target in determining the impact of integrin ligation in tumour cells. This thesis describes experiments conducted in order to test the following hypothesis: ***Interactions between cellular β1 integrin receptors and ECM proteins contribute to the growth and survival of tumour-derived cell lines.***

1.7 APPROACH

1) *Investigation of the impact of extracellular matrix proteins and integrin β1 ligation on the adherent growth and survival of human cancer cell lines.* Cancer cells from the NCI-60 cell panel were screened for their response to standard chemotherapeutics of different classes in the presence or absence of different ECM proteins known to interact with various β1 integrins using an MTT reduction assay to assess cell viability. Targeted siRNA was also employed to deplete cancer cell lines of cell surface β1 integrins in order to assess their role in adhesion and in the growth and survival of adherent cell populations.

2) *Investigation of the impact of β1 integrin expression in the anchorage independent growth of cancer cell lines.* Targeted siRNA was employed to deplete cancer cell lines of cell surface β1 integrins. Soft agarose colony formation assays were employed to assess anchorage independent growth of different cancer cell lines in the presence or absence of β1 integrins. Soft agarose colony formation was also assessed in PC3 prostate carcinoma cells overexpressing either integrin β1A (wildtype) or the splice variant β1C, which is expressed in normal prostate epithelial cells and lacks important cytoplasmic signaling motifs. Neutralizing antibodies to the integrin β1 subunit and to a major β1 integrin ligand, fibronectin, were also used in soft agarose cloning assays of PC3 cells. Finally, PC3 cells were induced to grow in suspension on agarose-coated plates to allow for isolation and assessment of differences in protein expression and protein-protein interactions in the adherent and non-adherent cells.

2. MATERIALS AND METHODS

2.1 CELL LINES AND PLASMIDS

The following human cell lines from the US National Cancer Institute 60 cell panel (NCI 60) were obtained from the American Type Culture Collection (ATCC): A549 (CCL-185) lung adenocarcinoma cells, PC3 (CRL-1435) grade IV prostate adenocarcinoma cells isolated from bone metastasis, and SKNAS (CRL-2137) neuroblastoma cells isolated from bone metastasis. Ovarian cancer A2780S cisplatin sensitive cells and A2780CP cisplatin resistant cells (351) were a gift from B. C. Vanderhyden (Ottawa Health Research Institute, Ottawa, ON). The SF295 glioblastoma cell line, also from the NCI 60 cell panel, was a gift from J. C. Bell (Ottawa Health Research Institute, Ottawa, ON). Cells were routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone, Logan UT, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan UT, USA) at 37°C in the presence of 5% CO_2. PC3 cells were also cultured in suspension on 1% agarose (UltraPure, Invitrogen, Carlsbad CA, USA) coated plates.

Plasmids; pBJ-β1A and pBJ-β1C were a gift from L. R. Languino (University of Massachusetts School of Medicine; Worcester MA, USA). A 2.9kb fragment of wildtype integrin β1 cDNA consisting of 2.334 kb of coding sequence flanked by 3' and 5' noncoding regions was isolated from a pBLUESCRIPT vector containing the full-length human integrin β1 cDNA (351) and cloned into the pBJ vector using XbaI to produce pBJ-β1A (6.1kb). To construct a pBJ vector encoding the variant cytoplasmic domain of integrin β1C, the 5' end of wild-type integrin β1 cDNA was isolated from the pBLUESCRIPT vector and the 3' end of integrin β1C was isolated from the PCR-1000 vector into which PCR products had been

previously cloned (352) and digested to obtain a HindIII/EagI fragment beginning at nucleotide 2358. The 5' wild-type Xba/HindIII fragment (2.3kb) ending at nucleotide 2357 was then directionally ligated in frame with the 3' HindIII/EagI fragment encoding the variant cytoplasmic domain cDNA from integrin β1C (250bp) into the XbaI/NotI-linearized pBJ-1 vector to produce pBJ-β1C (5.8kb) (353, 354, Appendix A: S1). An empty pBJ-1 vector control was generated by excision of the β1C insert from pBJ-β1C at EcoR1 (Invitrogen) sites, gel purification using the QIAquick Gel Extraction kit (Qiagen, Mississauga ON), and religation of the linear 3.2kb vector sequence using T4 DNA Ligase (Fermentas Canada, Burlington ON). All plasmids were amplified in *E. Coli* DH5α and subsequently purified using a CompactPrep Plasmid Midi kit (Qiagen) and spin protocol.

For plasmid transfections of PC3 cells, 1e6 cells were plated into 10cm plates 24 hours prior to transfection in order to reach a confluency of approximately 80% at the time of transfection. Lipofectamine 2000 (Invitrogen) was diluted in Optimem (Gibco, Invitrogen) and added to 12ug of plasmid DNA at a ratio of 2:1 according to the manufacturer's instructions. The lipid/plasmid mixture was then added dropwise to cells in a final volume of 5ml. Approximately four hours following transfection, cells were maintained in DMEM containing 10% FBS at 37°C.

2.2 REAGENTS AND ANTIBODIES

Chemotherapy agents cisplatin (Mayne Pharma Canada Inc) and etoposide (Bristol-Meyers Squibb Company Inc) were obtained from the Ottawa Hospital Cancer Centre Pharmacy and staurosporine was from Sigma (Saint Louis MO, USA). The staurosporine analogue UCN-01 (Kyowa Hakko Kogyo Co. Ltd., Tokyo Japan) was obtained with permission from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute.

Activating (P4G11) and adhesion neutralizing (P4C10, and 6S6) antibodies to Integrin β1 used in soft agarose assays, as well as the antibody to integrin β3 (B3A), and purified mouse IgG1 were from Chemicon International Inc (Temecula CA, USA). The adhesion blocking integrin β1 antibody used in immunoprecipitation and flow cytometry experiments was clone 6S6 and the secondary antibody used in flow cytometry experiments was goat anti-mouse Alexa-488-conjugated IgG from Molecular Probes (Invitrogen). The integrin β1 antibody (clone 18) used in immunoblotting, and the fibronectin antibody used in soft agarose experiments were both from BD transduction laboratories (BD Biosciences, San Jose CA, USA). Antibodies to β-catenin (6F9), and β-actin were from Sigma and rabbit polyclonal antibodies to AKT and pAKT (ser473) were from Cell Signaling Technology (Danvers MA, USA). Goat anti-mouse and anti-rabbit IgG peroxidase conjugated secondary antibodies were from Calbiochem (San Diego CA, USA).

2.3 SIRNA TRANSFECTIONS

Oligonucleotides were purchased from Dharmacon (Chicago IL, USA). Sequences of the siGENOME duplexes used to target human integrin β1 (ITGB1*)* were as follows: ITGB1-1 (D-004506-01); sense, GAACAGAUCUGAUGAAUGAUU; antisense, UCAUUCAUCAGAUCUGUUCUU; ITGB1-2 (D-004506-02); sense, CAAGAGAGCUGAAGACUAUUU; antisense, AUAGUCUUCAGCUCUCUUGUU; ITGB1-3 (D-004506-03); sense, GAAGGGAGUUUGCUAAAUUUU; antisense, AAUUUAGCAAACUCCCUUCUU; ITGB1-4 (D-004506-04); sense, CCACAGACAUUUACAUUAAUU; antisense, UUAAUGUAAAUGUCUGUGGUU. Duplexes were also commonly used as a pool of the mentioned oligonucleotides (siGENOME SMARTpool) to target integrin β1. Two scrambled oligonucleotide controls from Dharmacon were used: siCONTROL Non-Targeting siRNA #1 and GFP control-2;

sense, AGAACGGAAUCAAGGUUAAUU; antisense, UUAACCUUGAUUCCGGUUCUUU. A non-targeting Cy3 labelled oligonucleotide was also used to assess transfection efficiency in PC3 cells by flow cytometry.

For siRNA transfections of tumour cell lines, 6e5 cells were seeded in 10 cm plates 24 hours before transfection, reaching 60-70% confluency at the time of transfection. Oligofectamine (Invitrogen) reagent was diluted in Optimem according to the manufacturer's instructions and incubated for 8 minutes. The lipid mixture was then added to oligonucleotides diluted in Optimem and subsequently incubated for 25 minutes before being added dropwise to cells in Optimem. Working concentrations of siRNA duplexes varied from 2.5nM to 5nM. Cells were maintained in DMEM containing 10% FBS starting approximately 12-16 hours following transfection.

2.4 EXTRACELLULAR MATRIX-COATED PLATES

Standard 24-well and 96-well tissue culture plates were coated with ECM proteins in 500ul or 50ul of liquid no more than 24 hours prior to plating cells. Laminin, from human placenta (Sigma) was diluted in phosphate-buffered saline (PBS, Hyclone) at a concentration of 1ug/cm^2 and dried overnight at room temperature. Fibronectin (Sigma), diluted at a concentration of 1ug/cm^2 in PBS, and monomeric collagen I (PureCol, Inamed, Freemont CA, USA), diluted in 0.01N HCl at a concentration of 5ug/cm^2, were coated at room temperature for 2-3 hours, after which remaining liquid was removed. All matrices were washed twice with PBS prior to cell seeding.

2.5 MTT CELL VIABILITY ASSAY

Based on standard curves constructed for tumour cell lines (A549: Appendix B: S2), 7e3 trypsinized cells were seeded into each well of 96-well ECM coated or uncoated plates and allowed to adhere overnight at 37°C. Chemotherapeutic drugs were added 24 hours

following seeding. At endpoint, cells were treated with approximately 0.75 mg/ml MTT reagent, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in PBS (Sigma), for 4 hours at 37°C and lysed in acidic SDS buffer (0.05mM HCl, 10% SDS) at 37°C overnight. Cell viability was determined by measuring the absorbance at 570nM using a Multiskan Ascent Microplate Photometer (Thermo Fisher Scientific, Waltham MA, USA).

2.6 GROWTH EXPERIMENTS

Trypsinized cells were stained with trypan blue and counted using a heamocytometer, and 3e4 viable cells were seeded in DMEM containing 10% FBS into each well of uncoated 24 well plates or plates coated with monomeric collagen I, fibronectin, or laminin and incubated at 37°C. Cells were trypsinized at regular intervals and viable cells were counted using trypan blue exclusion with a Beckman Coulter Vi-cell-XR analyzer (Beckman Coulter, Fullerton CA, USA) and the following parameters; images:50, cell diameter:10-50 microns, cell brightness:85%, cell sharpness:100%, viable spot area: 5%, viable spot brightness: 75%, minimum circularity: 0, aspirate:1 cycle, trypan blue mixing: 3 cycles. For growth assays measuring response to chemotherapeutics, drugs were added in the amounts indicated 24 hours following seeding of cells in 24 well plates. Growth experiments were plated 24 hours following transfection with siRNA.

2.7 ADHESION ASSAYS

To retain integrity of cell surface molecules, all cells were harvested with ice cold PBS containing 1mM EDTA and subsequently counted using a heamocytometer and trypan blue exclusion. Aliquots containing 3e4 viable cells were seeded in each well of uncoated 24 well plates or plates coated with monomeric collagen I, fibronectin, or laminin and incubated at 37°C. Adherent cells were trypsinized 1-2 hours after seeding and viable cells were counted using either a heamocytometer, or the Beckman Coulter Vi-cell-XR cell viability

analyzer and trypan blue exclusion. Incubation with 2.5ug/ml activating or neutralizing antibodies to integrin β1 for 0.5h at room temperature was performed prior to seeding. Adhesion experiments were performed 48 hours following transfection with siRNA.

2.8 INTEGRIN β1 IMMUNOPRECIPITATION

Cells were washed once with PBS (Hyclone) and lysed with ice cold NP40 lysis buffer (150mM NaCl, 1% NP40, 0.5% Deoxycholic acid, 50mM Hepes, pH 7.5) containing 2mM sodium fluoride, 2mM sodium pyrophosphate, 0.2mM phenylmethanesulphonylfluoride, 500uM ammonium vanadate, 2ug/ml aprotinin, and 5ug/ml leupeptin. Lysates were sheared by passage 5 times through a 20-gauge needle, kept on ice for 30 minutes, and centrifuged at 4°C for 30 minutes. Protein concentrations were determined using the Bio-Rad Protein Assay (Biorad, Hercules CA, USA) based on the Bradford assay (355), and absorbances were determined using a Beckman DU 640 spectrophotometer at 595nm. Final protein concentrations were calculated following interpolation of a standard curve generated from known quantities of bovine serum albumin (BSA). A total of 200ug of protein was incubated with 2ug of Integrin β1 antibody (clone 6S6, Chemicon International) in 100ul of NP40 lysis buffer overnight at 4°C on a rotator platform. Following overnight incubation, 50ul of a mixture containing 60% pre-washed GammaBind G Sepharose beads (GE Healthcare Biosciences AB, Uppsala, Sweden) in NP40 lysis buffer was incubated with each sample for 6 hours, rotating at 4°C. Beads were collected by centrifugation, washed 3 times with NP40 buffer, and resuspended in 25ul of 2X sample buffer (125 mM Tris-HCl pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 0.004% (w/v) bromophenol blue) in NP40 lysis buffer. Bound protein was released from beads by boiling samples for 10 minutes and supernatants were isolated for SDS-PAGE and subsequent immunoblotting.

2.9 IMMUNOBLOTTING

Cells were washed once with PBS and lysed with ice cold Radioimmunoprecipitation (RIPA) buffer (30mM Tris-HCl pH 7.2, 150mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100 (Sigma) containing 2mM sodium fluoride, 2mM sodium pyrophosphate, 0.2mM phenylmethanesulphonylfluoride, 500uM ammonium vanadate, 2ug/ml aprotinin, and 5ug/ml leupeptin. Lysates were sheared by passage 5 times through a 20-gauge needle, kept on ice for 30 minutes, and centrifuged at 4°C for 30 minutes. Protein concentrations were determined using the Bio-Rad Protein Assay and measurement of absorbances in a Beckman DU 640 spectrophotometer at 595nm, followed by interpolation of protein concentration from a generated standard curve of known protein concentrations. For most antibodies, 60-75 ug of total protein was diluted in a 4X sample buffer (250mM Tris-HCl pH 6.8, 40% (v/v) glycerol, 8% (w/v) SDS, 20%(v/v) β-mercaptoethanol, 0.008% (w/v) bromophenol blue), and separated on a 10% polyacrylamide gel with Tris-glycine-SDS running buffer (24mM Tris base, 190mM Glycine, 0.1% SDS, pH 8.3). For the β-catenin blots 25-30ug of protein from PC3 cells was used. Protein was transferred to a Hybond-C Extra nitrocellulose membrane (Amersham Biosciences UK Limited, Bucks, UK) in Tris-glycine transfer buffer (12mM Tris base, 95mM glycine, 20% methanol) for 1.5 hours at 100V. Membranes were blocked for 1 hour at room temperature in a solution containing 5%(w/v) skimmed milk powder or 5%(w/v) BSA, for pAKT and AKT blots, and 0.05%(v/v) Tween 20 in PBS, pH 7.5. Incubation with primary antibodies was generally performed overnight at 4°C, with the exception of β-actin, for which a 1 hour incubation at room temperature was used. Membranes were washed by shaking on an orbital shaker in 0.05% Tween 20 in PBS before addition of the appropriate horseradish peroxidase (HRP)-labelled secondary antibody and subsequent incubation for 1 hour at room temperature. Following

washing of secondary antibody, detection of bound HRP-conjugated antibody was made with either ECL (1:1 mixture of 5mM hydrogen peroxide, and 100mM Tris, pH8.5: 2.5mM luminol, 0.4mM p-coumaric acid, and 100mM Tris, pH 8.5) or Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica MA, USA), using the GeneGnome Syngene bio imaging system and Genesnap software (Product version 6.05, Synoptics Ltd, Cambridge UK) to capture images. For some loading controls, membranes were incubated with 1% Amido Black protein stain (50% methanol, 10% acetic acid, 1% amido black) at room temperature for 10 minutes, washed once with water and photographed using the GeneGnome Syngene bio imaging system and Genesnap software.

2.10 FLOW CYTOMETRY

To stain for cell surface integrin β1, adherent and nonadherent PC3 cells were treated with ice cold PBS containing 5mM EDTA until cells could be collected, washed, and diluted in PBS containing 0.03% BSA. Aliquots containing 5e5 cells/ml were incubated with 2.5 ug/ml integrin β1 antibody (clone 6S6, Chemicon International) in 0.03% BSA-PBS for 2h at 4°C. Cells were collected and resuspended with 2ug/ml Alexa-488 conjugated anti-mouse IgG (Molecular Probes, Invitrogen) in 0.03% BSA-PBS for 0.5h at 4°C. Following one wash, 5e5 cells/ml in 0.03% BSA-PBS were analyzed using a BD LSR II benchtop flow cytometer (BD Biosciences) at 488nm (FL1). Background fluorescence was determined by analysis of cells stained with Alexa-488-conjugated secondary antibody only.

To determine transfection efficiency, PC3 cells were transfected with Cy3 labelled siRNA duplexes. Forty-eight hours following transfection, 5e5 trypsinized PC3 cells were diluted in PBS containing 0.3% BSA and immediately analysed using the BD LSR II at 633nm (FL3). Untransfected PC3 cells were used as a control.

2.11 SOFT AGAROSE CLONING

Twenty-four hours following siRNA or plasmid transfection, viable trypsinized cells were counted by haemocytometer using trypan blue exclusion and resuspended in 10ml of alpha-Minimal Essential Media (α-MEM, containing 10% FBS and 0.5% Low Melting Point (LMP) agarose (UltaPure, GIBCO BRL, Carlsbad CA, USA)) at 37°C. For integrin β1-specific antibody experiments, viable untransfected PC3 cells were incubated with 2.5ug/ml integrin β1 activating or neutralizing antibody or mouse IgG-1 for 30 minutes at room temperature before resuspension in 0.5% LMP agarose. For fibronectin depletion experiments, PC3 cells were suspended directly in 0.5% LMP agarose containing 2ug/ml fibronectin antibody in 0.09% azide, 2 ug/ml mouse IgG-1, or 0.09% (w/v) azide. Cell suspensions were then serially diluted two-fold and 700ul of each cell suspension was then seeded into 24-well plates onto an underlay of 1% LMP agarose in α-MEM containing 10% FBS. Dilutions ranged from 62 to 2000 cells plated per well. Progression of colony formation was noted at various time points and colonies greater than 25 and 50 cells in size were scored after 10 (untransfected cells and transfected SKNAS cells) or 15 days (transfected cells). Colony numbers were obtained using an Olympus CK2 inverted phase-contrast microscope at a total magnification of 100X and photographed using a Nikon digital camera. Minimally, six counts from at least two dilutions in each condition were used in statistical analyses.

2.12 STATISTICAL ANALYSIS

Variances within groups were assessed by F-test and where equal, significant differences between groups were ascertained by unpaired two tailed *t*-test with a confidence interval of 95% using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego California USA). Means were considered significantly different where $P<0.05$*.

3. RESULTS

To test whether interactions between cellular integrin β1 receptors and ECM proteins contribute to the malignant phenotypes of human tumour cell lines we employed several investigative approaches. The first approach was indirect. It questioned the ability of ECM-mediated adhesion and presupposed ligation of cell surface integrin receptors to modulate the response of various tumour cell lines to several classes of chemotherapeutic agents. To more specifically examine the impact of β1 integrins on adherent cell growth and survival, a second approach was employed using targeted siRNA to deplete cells of surface integrin β1 receptors. Thirdly, this siRNA approach, as well as the use neutralizing antibodies to integrin β1 and transfection of a plasmid construct to overexpress a growth inhibitory splice variant, integrin β1C, was used to study the importance of β1 integrins in the anchorage independent growth of human tumour cell lines using a soft agarose colony formation assay. An antibody against fibronectin was also used to examine the role of cellular and exogenous fibronectin on the soft agarose colony forming ability of one cell line, PC3. Finally, PC3 cells were induced to grow in suspension over a 1% agarose base in order to easily isolate protein for biochemical analyses which might provide insight into important signaling events mediating the anchorage independence of human cancer cell lines.

3.1 MODULATION OF CHEMOSENSITIVITY BY ECM PROTEINS DOES NOT OCCUR IN SEVERAL HUMAN CANCER CELL LINES.

In order to address the idea that extracellular matrix protein in the tumour microenvironment impacts the efficacy of chemotherapy, human tumour cell lines were

tested for their response to chemotherapeutic drugs in the presence of ECM protein. To this end, an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay was employed to test cell viability and to enable quick screening of many cell lines from different origin tissues, several classes of drugs, and different ECM proteins. Cells, mostly from the NCI 60 cell line panel, were seeded overnight into 96 well plates pre-coated with collagen I (CI), fibronectin (FN), laminin (LMN), or tenascin-C (TNC). The influence of ECM proteins was examined by comparison to either uncoated plastic plates, or plates coated with poly-d-lysine (PDL) to promote non-integrin mediated cell adhesion. Following seeding, cells were treated for 48 hours with cisplatin; a DNA crosslinking agent which promotes the accumulation of DNA damage leading to apoptosis (356), etoposide; a topoisomerase II inhibitor which similarly causes apoptosis (357), rapamycin; an inhibitor of the mammalian target of rapamycin (mTOR) which halts proliferation by inhibiting protein translation (358), staurosporine; a nonspecific PKC kinase inhibitor which inhibits proliferation, or UCN-01 (7-hydroxy-staurosporine), a soluble staurosporine analogue (359). Cells were then incubated for 4 hours with MTT reagent, and lysed overnight. The colorimetric change associated with mitochondrial reduction of MTT was quantified by measuring the absorption in each well at 570nm and the percentage of viable cells was determined by normalizing the absorption observed at various drug concentrations to the absorption of untreated cells. Table 1 lists the types of drugs and ECM proteins compared in this screening and summarizes results indicating whether ECM modulates the response in the cell lines tested. Surprisingly, there was no modulation of drug sensitivity by 1-5 ug/cm^2 of immobilized ECM protein compared to plastic or poly-d-lysine (PDL) coated controls observed in PC3 prostate carcinoma cells (Table 1, Figure 3), or any of the other cell lines tested (Table 1, Appendix B: S3-8), regardless of class of drug or cell type. In contrast to

observations reported in small cell lung cancer cells (305), the non-small cell lung cancer cell line, A549, did not demonstrate a survival advantage following treatment with etoposide in the presence of ECM proteins (Table 1, Appendix B: S3, iii-iv). Given the redundancy between growth factor and integrin signaling pathways (249), the ability of the ECM to modulate the response of A549 cells to etoposide was also examined in reduced serum conditions in order to increase the dependence of these cells on adhesion-mediated signals. However, fibronectin failed to modulate the cytoxicity of etoposide compared to the PDL control even in reduced serum conditions (Figure 4C, D). It was also confirmed that A549 non-small cell lung adenocarcinoma cells grew with similar kinetics regardless of whether adherent to fibronectin or PDL coated plates (Figure 4A, B), suggesting that basal growth kinetics did not impact cell number following drug treatment. In addition to staurosporine, A549 cells were also screened for ECM modulation of response to the water soluble analogue, UCN-01, but similarly to other cell lines and drugs tested, growth on various ECM proteins did not impact cell survival in response to drug (Table 1, Appendix B: S3, ii).

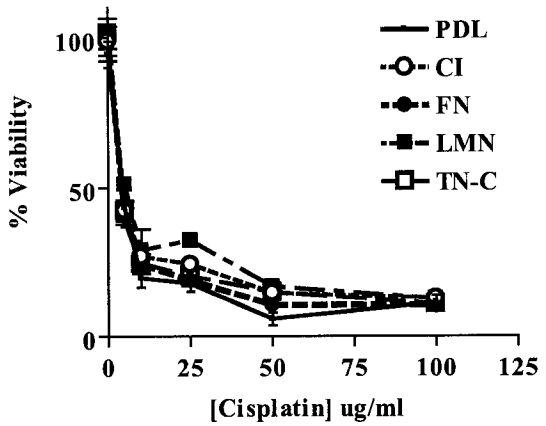
Table 1. ECM protein does not modulate the cytotoxicity of many chemotherapeutic drugs in a variety of cancer cell lines. Different cancer cell lines were screened using an MTT reduction assay for their response to chemotherapeutic drugs in the presence of ECM proteins. Cancer cells were seeded overnight onto plastic, fibronectin (FN), collagen I (CI), laminin (LMN) or tenascin-C (TNC) coated 96 well plates and treated with various concentrations of cisplatin, etoposide, rapamycin, staurosporine or UCN-01 for 48 hours. Cytotoxicity was determined by measuring the colorimetric change in absorbance at 570nM associated with MTT reduction in cells treated for four hours with MTT reagent and lysed overnight. Drug names, mechanisms of action, cell lines, and ECM protein combinations tested are indicated. Response to ECM was determined by comparing the mean absorbance and standard deviation from eight replicate wells for each drug concentration on the different matrices pair wise to the PDL control using an unpaired two-tailed t-test with a confidence interval of 95%. Means were considered significantly different where $P<0.05$.

Chemotherapeutic Drug	**Drug Action**	**Malignancy**	**ECM protein**	**ECM Modulation? (Y/N)**
Cis-Platinum	DNA damage:	**Neuroblastoma**: SKNAS	FN, CI, LMN	N
		Glioblastoma: SF295	FN, CI, LMN	N
	crosslinks DNA	**NSCLC**: A549, H460,	FN, CI, LMN, TNC	N
		Prostate: PC3, Du145	FN, CI, LMN, TNC	N
		Ovarian: A2780S, A2780CP	FN, CI, LMN, TNC	N
Etoposide	DNA damage:	**Neuroblastoma**: SKNAS	FN, CI, LMN	N
		Glioblastoma: SF295	FN, CI, LMN	N
	topoisomerase II	**NSCLC**: A549, H460,	FN, CI, LMN, TNC	N
	inhibitor	**Prostate**: PC3, Du145	FN, CI, LMN, TNC	N
		Ovarian: A2780CP	FN, CI, LMN	N
Rapamycin	Anti-proliferative:	**NSCLC**: A549, H460,	FN, CI, LMN, TNC	N
		Prostate: PC3, Du145	FN, CI, LMN, TNC	N
	mTOR inhibitor			
Staurosporine	Induction of apoptosis:	**Neuroblastoma**: SKNAS	FN, CI	N
		Glioblastoma: SF295	FN, CI	N
		NSCLC: A549	FN, CI	N
	kinase inhibitor	**Prostate**: PC3	FN, CI	N
7-hydroxystaurosporine (UCN-01)	Several modes: kinase inhibitor	**NSCLC**: A549	FN, CI	N

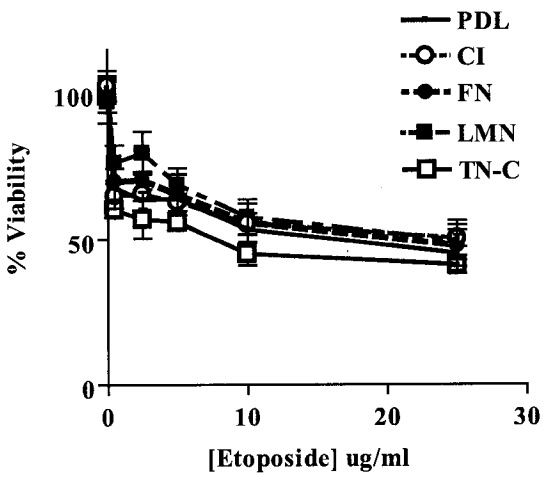
FN: Fibronectin, CI: Collagen I, LMN: Laminin, TNC: Tenascin-C

Figure 3. ECM protein does not modulate the response of PC3 cells to chemotherapeutic drugs. PC3 cells were seeded overnight onto plastic, fibronectin (FN), collagen I (CI), laminin (LMN) or tenascin-C (TNC) coated 96 well plates and treated with various concentrations of cisplatin (**A**), etoposide (**B**), rapamycin (**C**), or staurosporine (**D**), in 10% FBS-containing media for 48 hours. Cytotoxicity was determined by measuring the colorimetric change in absorbance at 570nM associated with MTT reduction in eight replicate wells of cells treated for four hours with MTT reagent and lysed overnight. Cell viability is expressed as the mean percentage of MTT activity in untreated wells $\pm$ SD.

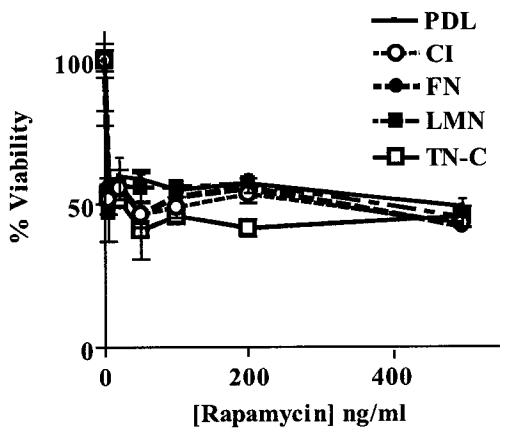
A


PDL
CI
FN
LMN
TN-C
% Viability
100
50
0
0
25
50
75
100
125
[Cisplatin] ug/ml

B


PDL
CI
FN
LMN
TN-C
% Viability
100
50
0
0
10
20
30
[Etoposide] ug/ml

C


PDL
CI
FN
LMN
TN-C
% Viability
100
50
0
0
200
400
[Rapamycin] ng/ml

D

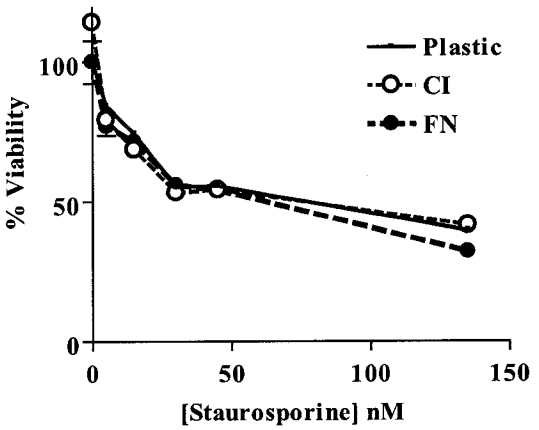
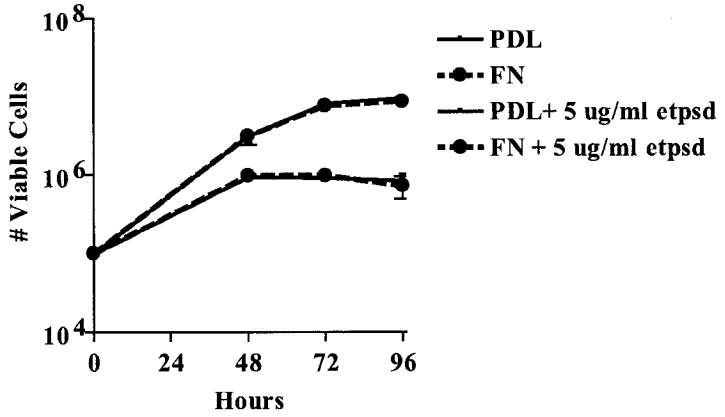
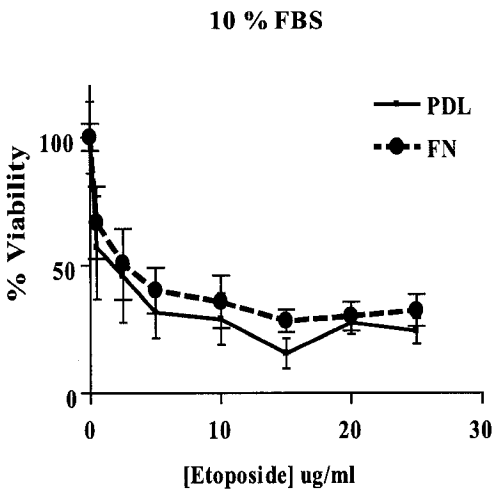

Plastic
CI
FN
% Viability
100
50
0
0
50
100
150
[Staurosporine] nM

Figure 4. Fibronectin does not modulate the growth of A549 cells in the presence or absence of Etoposide at reduced serum protein concentration. For growth curves, A549 cells were seeded overnight into poly-d-lysine (PDL), or fibronectin (FN) coated 24 well plates in 10% FBS-containing media. Media was exchanged the following day to contain either 10% FBS (**A**) or 5% FBS (**B**) $\pm$ 5 ug/ml etoposide. Beginning at 48 hours, cells from three replicates were trypsinized and counted every 24 hours. Viable cells, determined by trypan blue exclusion, are expressed as means $\pm$ SD. For MTT viability assays, A549 cells were seeded overnight into poly-d-lysine (PDL), or fibronectin (FN) coated 96 well plates in 10% FBS-containing media. Media was exchanged the following day to contain either 10% FBS (**C**) or 5% FBS (**D**) and varying concentrations of etoposide. After 48 hours of drug treatment, cytotoxicity was determined by measuring the colorimetric change in absorbance at 570nM associated with MTT reduction in eight replicate wells of cells treated for four hours with MTT reagent and lysed overnight. Cell viability is expressed as the mean percent of MTT activity in untreated wells averaged over three independent experiments $\pm$ SD.

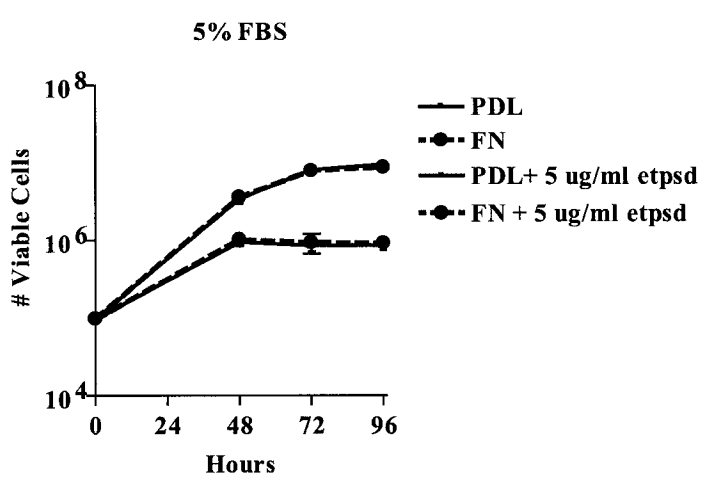
A


10 % FBS
Viable Cells
10^8
10^6
10^4
0
24
48
72
96
Hours
PDL
FN
PDL+ 5 ug/ml etpsd
FN + 5 ug/ml etpsd

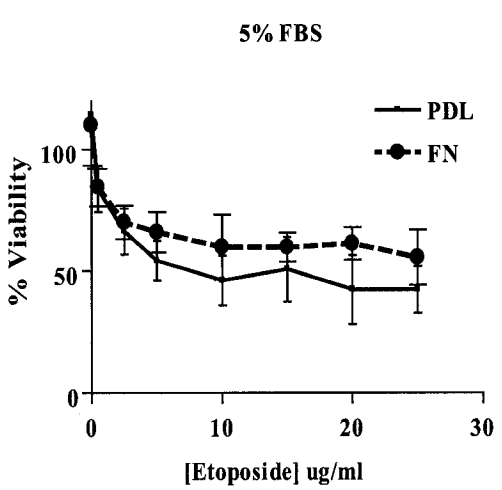
C


10 % FBS
% Viability
100
50
0
0
10
20
30
[Etoposide] ug/ml
PDL
FN

B


5% FBS
Viable Cells
10^8
10^6
10^4
0
24
48
72
96
Hours
PDL
FN
PDL+ 5 ug/ml etpsd
FN + 5 ug/ml etpsd

D

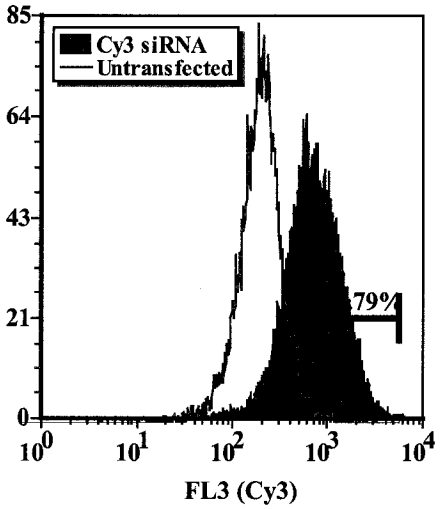

5% FBS
% Viability
100
50
0
0
10
20
30
[Etoposide] ug/ml
PDL
FN

3.2 INTEGRIN β1 DEPLETION DOES NOT IMPACT THE ADHESION OR ADHERENT GROWTH OF PC3 CELLS.

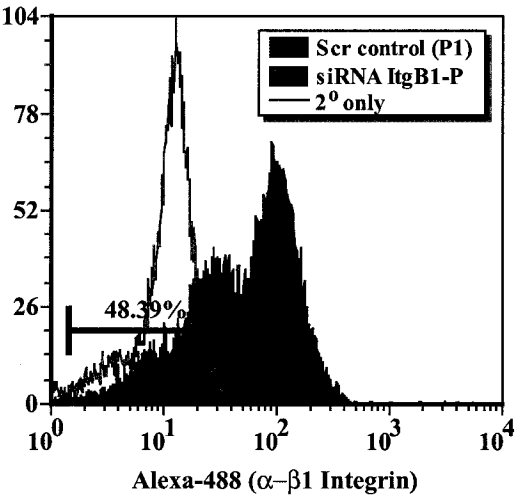
Despite the fact that we did not observe ECM-dependent modulation of tumour cell survival and given the central position of integrin receptors in ECM mediated signaling, a direct study of one major adhesion receptor subunit, integrin β1, and its impact on tumour cell characteristics, was undertaken. Although multiple integrin receptors redundantly bind the same ECM proteins to promote downstream signaling, β1 integrins comprise a large proportion of the integrin receptor family and have been found to essentially contribute to a wide variety of cellular phenotypes (108). A siRNA approach was employed in order to specifically deplete PC3 cells of integrin β1 protein. To optimize siRNA transfections, PC3 cells were first transfected with Cy3-labelled scrambled duplexes. Fluorescence was measured and compared to untransfected cells by flow cytometry and it was determined that under the most efficient conditions approximately 50 percent of cells were transfected with siRNA duplexes (Figure 5A). We also examined the efficiency of integrin β1 surface expression depletion by siRNA in PC3 cells transfected with either a pooled scramble control or a pool of four integrin β1 targeted siRNA duplexes (Dharmacon Smartpool) by staining for surface protein expression. Flow cytometry analysis revealed that, corresponding to the observed 50% transfection efficiency using Cy3-conjugated siRNA, integrin β1 targeted siRNA could reduce the proportion of cells expressing detectable surface levels of the integrin heterodimer subunit by nearly 50% (Figure 5B). Western blot analysis also confirmed reduced integrin β1 protein expression for up to 120h post-transfection in PC3 cells treated with three different individual targeted siRNA duplexes compared to either the pooled scramble control or a GFP-targeting control (Figure 5C).

Figure 5. Targeted siRNA reduces the surface expression of integrin β1 protein in PC3 cells. (**A**) PC3 cells were transfected with 2.5nM Cy3-labelled scrambled siRNA duplexes for 48 hours before being analyzed by flow cytometry. Cells were gated based on forward and side-scatter data to eliminate debris and the percentage of transfected cells was determined by comparing Cy3-siRNA transfectants to the background fluorescence associated with untransfected PC3 cells, as shown in the histogram. (**B**) PC3 cells were transfected with 2.5nM pooled integrin β1 targeted siRNA (siRNA ItgB1-P) or a pool of scrambled siRNA (Scr control (P1)) duplexes and collected 48 hours later with EDTA. Cell surface protein was stained with an integrin β1 specific antibody and alexa-488 conjugated secondary antibody and analyzed by flow cytometry. Cells treated with conjugated secondary only were used to determine background fluorescence. Cell populations gated by forward and side scatter to eliminate debris, were used to construct the histograms shown. (**C**) Whole cell lysates from PC3 cells transfected with 5nM individual integrin β1 siRNA targeted (ItgB1-01, ItgB1-02, or ItgB1-03) or control (scrambled siRNA: Scr control (P1) or GFP-targeting control (GFP)) duplexes were collected at various time-points, separated by SDS-PAGE, and immunoblotted using an integrin β1 antibody. Amido Black protein stain was used as a loading control.

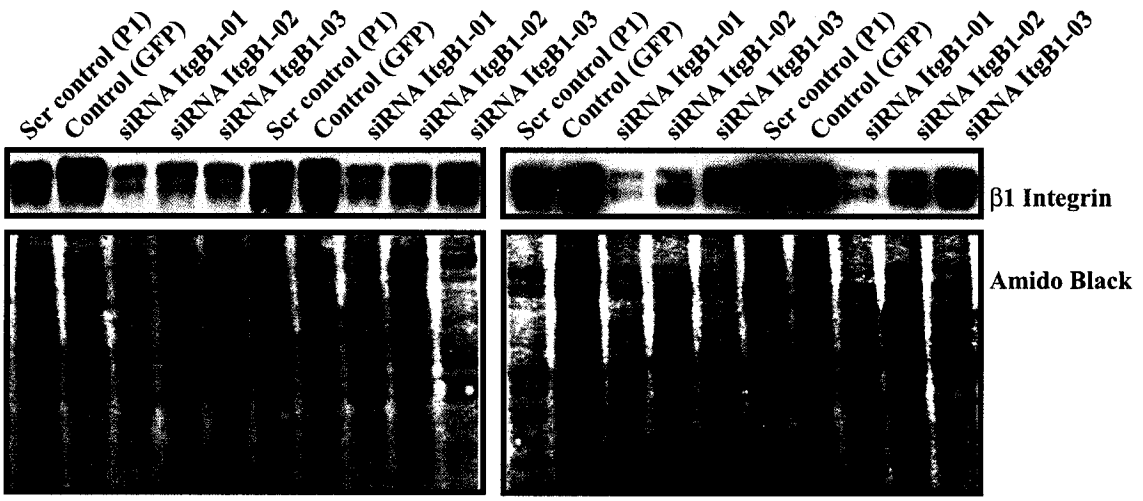
A


Cy3 siRNA
Untransfected
85
64
43
21
0
79%
10^0 10^1 10^2 10^3 10^4
FL3 (Cy3)

B


Scr control (P1)
siRNA ItgB1-P
2^0 only
104
78
52
26
0
48.39%
10^0 10^1 10^2 10^3 10^4
Alexa-488 (α–β1 Integrin)

C

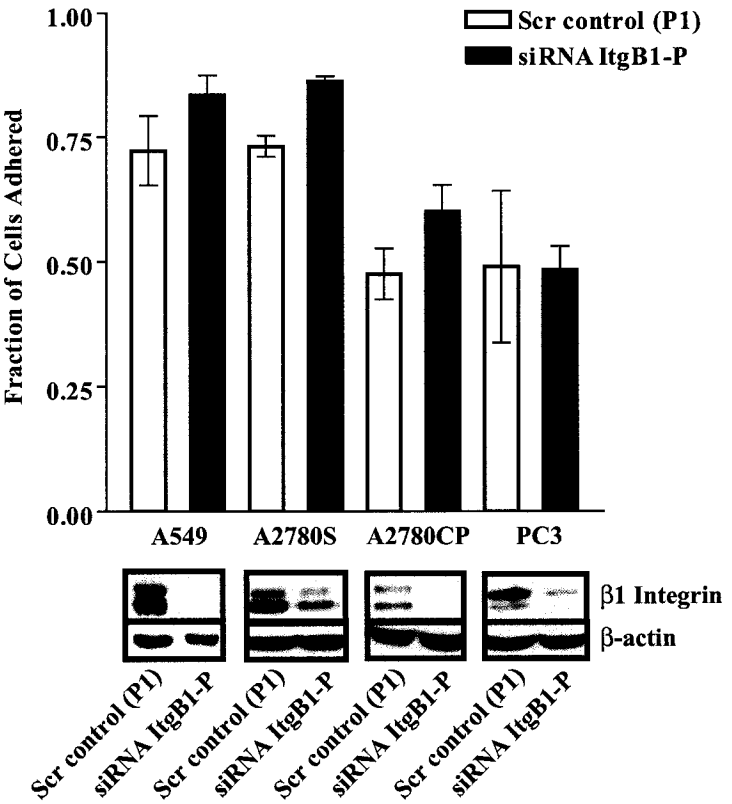

Scr control (P1)
Control (GFP)
siRNA ItgB1-01
siRNA ItgB1-02
siRNA ItgB1-03
Scr control (P1)
Control (GFP)
siRNA ItgB1-01
siRNA ItgB1-02
siRNA ItgB1-03
Scr control (P1)
Control (GFP)
siRNA ItgB1-01
siRNA ItgB1-02
siRNA ItgB1-03
Scr control (P1)
Control (GFP)
siRNA ItgB1-01
siRNA ItgB1-02
siRNA ItgB1-03
β1 Integrin
Amido Black

We first examined tumour cell adhesion in the absence of integrin β1 ligation. A549, A2780S (cisplatin sensitive ovarian carcinoma cells), A2780CP (cisplatin resistant ovarian carcinoma cells), and PC3 cells were depleted of integrin β1 using siRNA and collected 48 hours post-transfection, using EDTA in order to maintain the integrity of adhesion receptors, before being seeded in 24 well plates and allowed to adhere for 1 hour. The number of cells adhered to plastic was quantified and found to be not significantly different in any cell line tested when integrin β1 was depleted compared to scrambled controls (Figure 6A). It was also found that there was no difference in PC3 cell adhesion to fibronectin when transfected with two individual integrin β1 targeted siRNA duplexes compared to two different scrambled control transfectants (Figure 6B).

To investigate the importance of integrin β1 in tumour cell growth, PC3 cells transfected with pooled targeted siRNA, individual targeted duplexes, or scrambled controls were seeded and counted every 24 hours. Whether pooled (Figure 7C) or individual (Figure 7A) duplexes were employed, integrin β1 depletion using siRNA did not affect the growth over time of adherent PC3 cells compared to control non-targeting duplexes. Similarly, growth of PC3 cells on plates coated with fibronectin protein, to which cells attach via integrin receptors, including α5β1, αVβ1, and α8β1 (see Figure 1B), was not impacted by integrin β1 depletion (Figure 7B). Notably, western blot analysis confirmed that effective integrin β1 depletion using this approach was not accompanied by a decrease in protein expression of β3 integrin (Figure 7C), another major β subunit expressed in tumour cells which pairs with alpha integrins to bind fibronectin (Figure 1B). To further explore the role of integrin β1 in tumour cell growth, clonogenic expansion was examined in PC3 cells depleted of integrin β1 using pooled siRNA duplexes. In agreement with monolayer

Figure 6. Adhesion of various cancer cell lines is not dependent on integrin β1 expression. **(A)** A549, A2780S, A2780CP, and PC3 cells transfected with either 2.5 nM pooled integrin β1 targeted siRNA (siRNA ItgB1-P) (filled bars) or a pool of scrambled siRNA (Scr control (P1)) (clear bars) duplexes for 48 hours were collected with EDTA and allowed to adhere to plastic plates. Cells which adhered after 1 hour were counted and are expressed as the mean percentages of total cells plated ± SD for one representative experiment. Two independent experiments confirmed similar results. Depletion of integrin β1 protein was ascertained by western blot analysis 48 hours following transfection. **(B)** Individual integrin β1 siRNA targeted (5nM: ItgB1-01, ItgB1-02, or ItgB1-03) and control (5nM scrambled siRNA: Scr control (P1) or GFP-targeting control (GFP)) PC3 transfectants were collected with EDTA and allowed to adhere to plastic, or fibronectin (FN) coated plates for 1 hour. Adherent cells were counted and are expressed as the mean percentages of total cells plated ± SD for one representative experiment. Two independent experiments confirmed similar results. Depletion of β1 integrin protein was ascertained by western blot analysis 48 hours following transfection.

A


Scr control (P1)
siRNA ItgB1-P
Fraction of Cells Adhered
1.00
0.75
0.50
0.25
0.00
A549
A2780S
A2780CP
PC3
β1 Integrin
β-actin
Scr control (P1)
siRNA ItgB1-P
Scr control (P1)
siRNA ItgB1-P
Scr control (P1)
siRNA ItgB1-P
Scr control (P1)
siRNA ItgB1-P

B

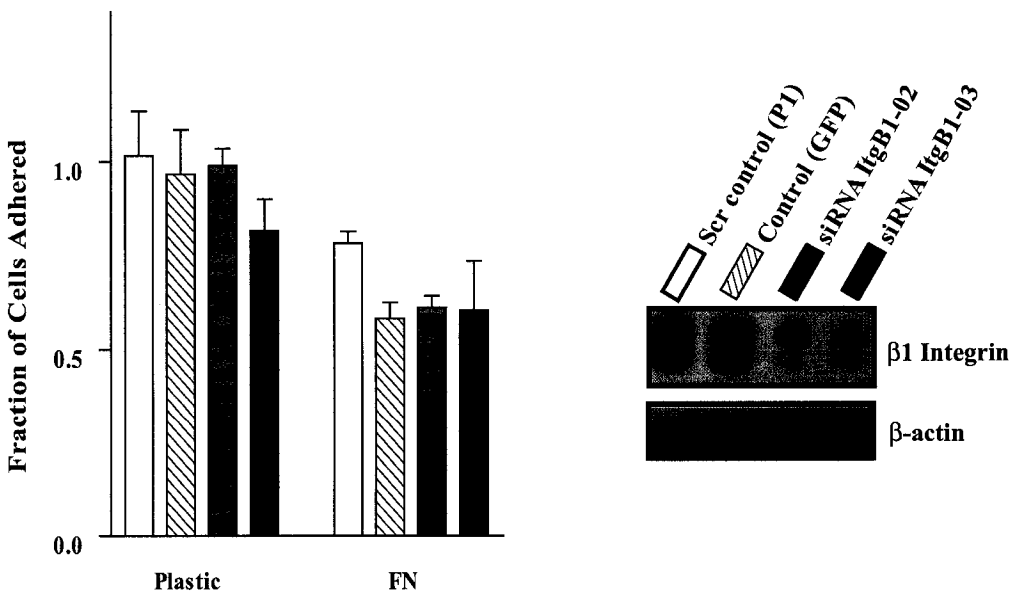
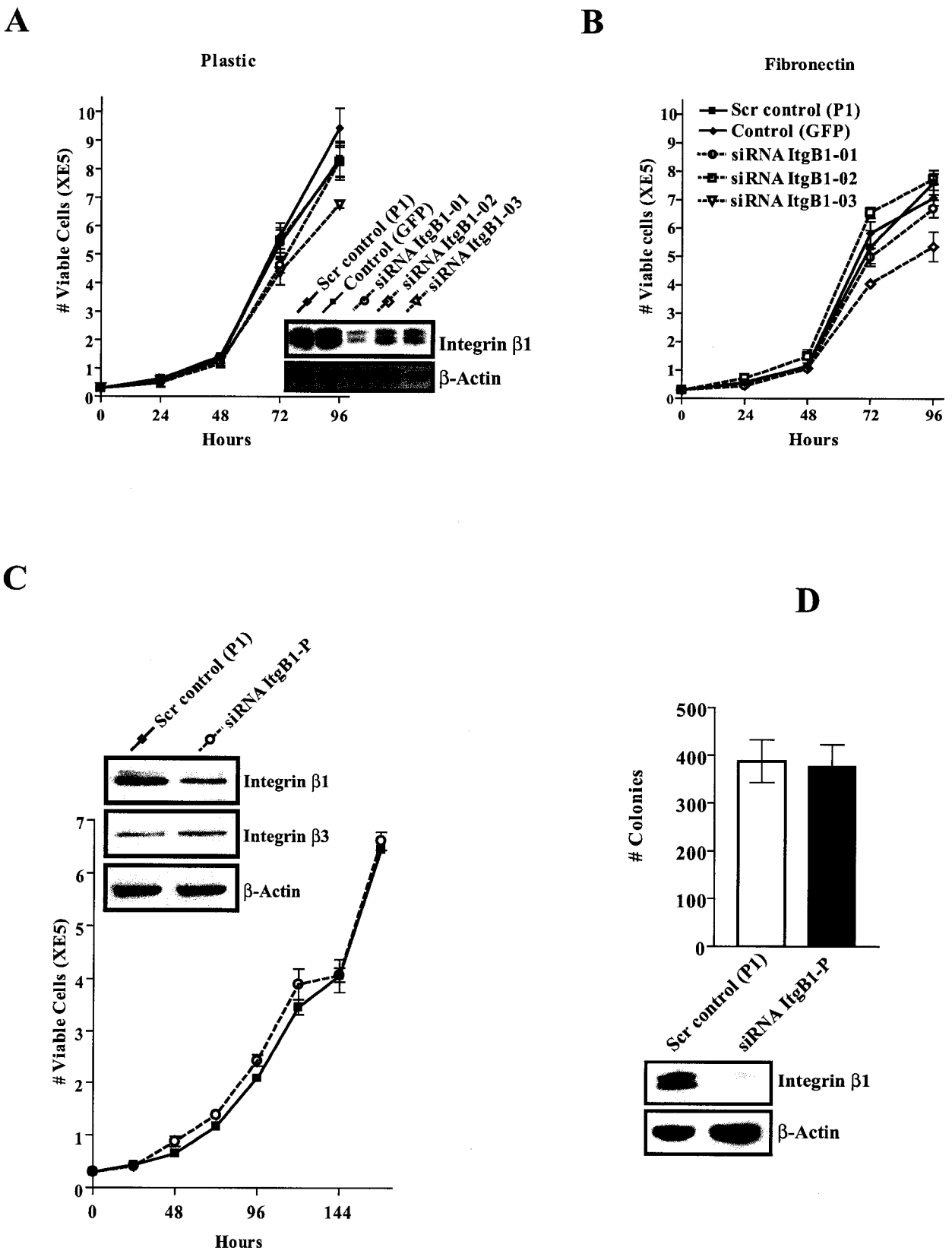

Fraction of Cells Adhered
1.0
0.5
0.0
Plastic
FN
Scr control (P1)
Control (GFP)
siRNA ItgB1-02
siRNA ItgB1-03
β1 Integrin
β-actin

Figure 7. Integrin β1 depletion does not modulate the growth of adherent PC3 cells. (**A**) Individual integrin β1 siRNA targeted (5nM: ItgB1-01, ItgB1-02, or ItgB1-03) and control (5nM scrambled siRNA: Scr control (P1) or GFP-targeting control (GFP)) PC3 transfectants were grown adherently on uncoated plastic (**A**) or fibronetin (**B**) coated plates. Cells from three replicates for each condition were trypsinized and counted every 24 hours. Viable cells, determined by trypan blue exclusion, are expressed as means ± SD for one representative experiment. Two independent experiments confirmed similar results. Depletion of integrin β1 protein was ascertained by western blot analysis 48 hours following transfection. (**C**) 2.5 nM pooled integrin β1 siRNA targeted (siRNA ItgB1-P) and pooled scramble control (Scr control (P1)) PC3 transfectants were grown adherently on plastic and counted in triplicate every 24 hours. Viable cells are expressed as means ± SD for one representative experiment. Three independent experiments confirmed similar results. Depletion of β1 integrin protein expression and accompanying β3 integrin levels were ascertained by western blot analysis 48 hours following transfection. (**D**) 2.5nM pooled integrin β1 targeted siRNA (siRNA ItgB1-P) and pooled scramble control (Scr control (P1)) PC3 transfectants were cultured at low density and allowed to form colonies over 10 days. Cells were stained with methylene blue and colonies >50 cells in size were counted in 6 replicates from 2 dilutions, normalized to 1000 cells for comparison, and expressed as means ± SD. Depletion of integrin β1 protein was ascertained by western blot analysis 48 hours following transfection.


A
Plastic
Viable Cells (XE5)
Hours
Scr control (P1)
Control (GFP)
siRNA ItgB1-01
siRNA ItgB1-02
siRNA ItgB1-03
Integrin β1
β-Actin
B
Fibronectin
Viable cells (XE5)
Hours
Scr control (P1)
Control (GFP)
siRNA ItgB1-01
siRNA ItgB1-02
siRNA ItgB1-03
C
Viable Cells (XE5)
Hours
Scr control (P1)
siRNA ItgB1-P
Integrin β1
Integrin β3
β-Actin
D
Colonies
Scr control (P1)
siRNA ItgB1-P
Integrin β1
β-Actin

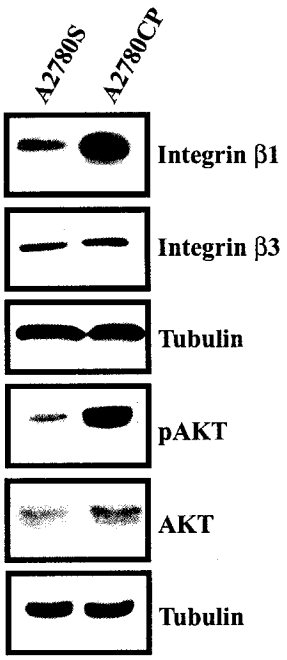
expansion studies, PC3 cells transfected with integrin β1 targeted siRNA duplexes were equally able to form adherent colonies as scrambled control transfectants (Figure 7D) indicating that the proliferation of PC3 cells is not dependent on integrin β1 mediated attachment in a 2D culture system.

3.3 INCREASED EXPRESSION OF INTEGRIN β1 IN CISPLATIN RESISTANT OVARIAN CANCER CELLS IS NOT RESPONSIBLE FOR THEIR DECREASED CHEMOSENSITIVITY.

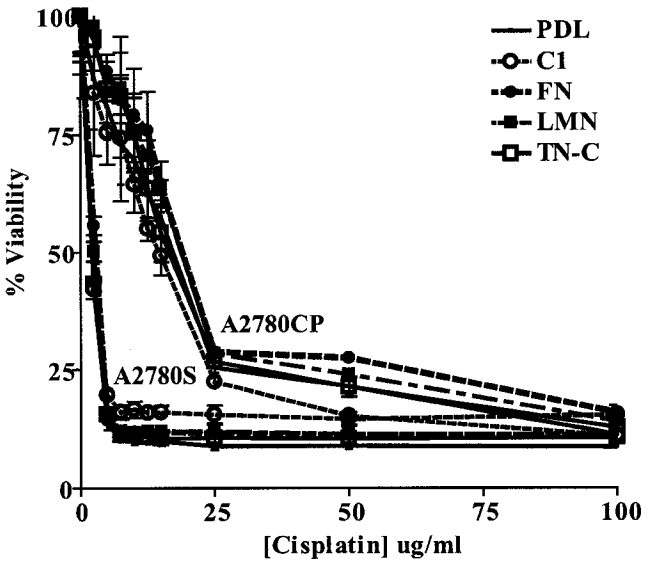
The ovarian cancer cell line A2780CP was developed for its ability to survive cisplatin treatment compared to its paired counterpart cell line A2780S. This model was chosen to study the impact of integrin β1 depletion on tumour cell survival because A2780CP cells were found to overexpress integrin β1 compared to A2780S parental cells (Figure 8A). This increase in expression of the integrin β1 subunit was accompanied by increased phosphorylation of AKT, a major integrin and growth factor downstream signaling node, in A2780CP cells (Figure 8A). As reported in Table 1, the survival of paired ovarian cancer cell lines treated with cisplatin was not affected by the presence of ECM protein (Figure 8B). Similarly, and despite the observed overexpression of integrin β1 in A2780CP cells, adherent A2780S and cisplatin resistant A2780CP cells depleted of integrin β1 using pooled targeted siRNA duplexes did not respond differently to cisplatin treatment compared to each of their scrambled control transfectants (Figure 8C). Western blot analysis confirmed that integrin β1 was reduced to a similar degree in both cell lines.

Figure 8. Integrin β1 expression is upregulated in A2780CP cells but does not mediate cisplatin resistance. (**A**) Whole cell lysates from A2780S and A2780CP cells were separated by SDS-PAGE and immunoblotted using antibodies to integrins β1 and β3, pAKT, and AKT to detect endogenous protein levels. Tubulin was used as a loading control. (**B**) Cells were seeded overnight onto poly-d-lysine (PDL), fibronectin (FN), collagen I (CI), laminin (LMN) or tenascin-C (TNC) coated 96 well plates and treated with various concentrations of cisplatin for 48 hours. Cytotoxicity was determined by measuring the colorimetric change in absorbance associated with MTT reduction at 570nm in cells from eight replicate wells treated for four hours with MTT reagent and lysed overnight. Cell viability is expressed as the mean percent of MTT activity in untreated wells ± SD. (**C**) A2780S and A2780CP cells transfected with 2.5 nM pooled integrin β1 targeted siRNA (siRNA ItgB1-P) or a pool of scrambled control (Scr control (P1)) duplexes for 24 hours were subsequently treated with various concentrations of cisplatin for 48 hours. Viable cells were counted in triplicate using trypan blue exclusion and are expressed as the mean percentage of untreated cells ± SD for one representative experiment. Two independent experiments confirmed similar results.

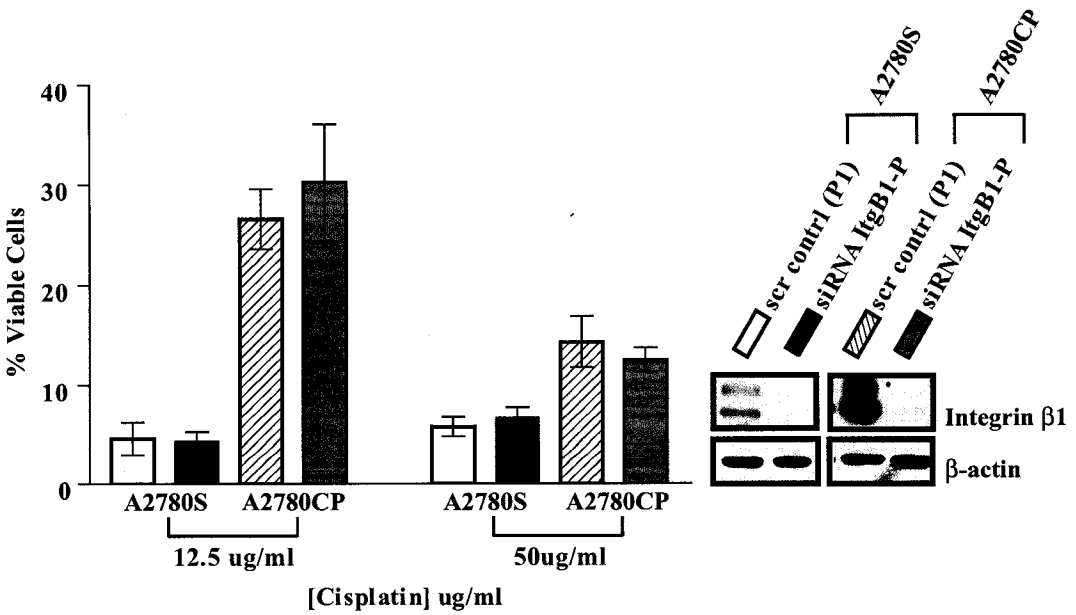
A


A2780S
A2780CP
Integrin β1
Integrin β3
Tubulin
pAKT
AKT
Tubulin

B


PDL
C1
FN
LMN
TN-C
% Viability
100
75
50
25
0
A2780CP
A2780S
0
25
50
75
100
[Cisplatin] ug/ml

C

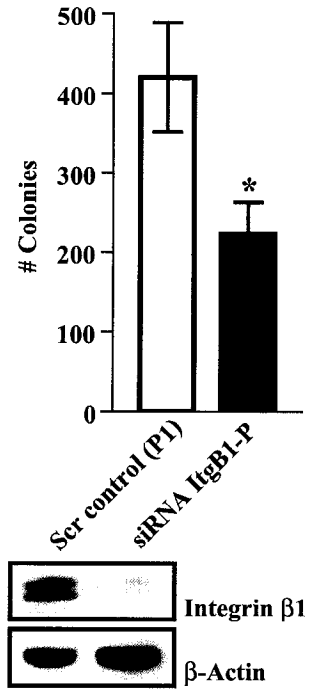

% Viable Cells
40
30
20
10
0
A2780S
A2780CP
A2780S
A2780CP
12.5 ug/ml
50ug/ml
[Cisplatin] ug/ml
A2780S
A2780CP
scr contrl (P1)
siRNA ItgB1-P
scr contrl (P1)
siRNA ItgB1-P
Integrin β1
β-actin

3.4 INTEGRIN β1 DEPLETION REDUCES THE ANCHORAGE INDEPENDENT GROWTH OF HUMAN CANCER CELLS.

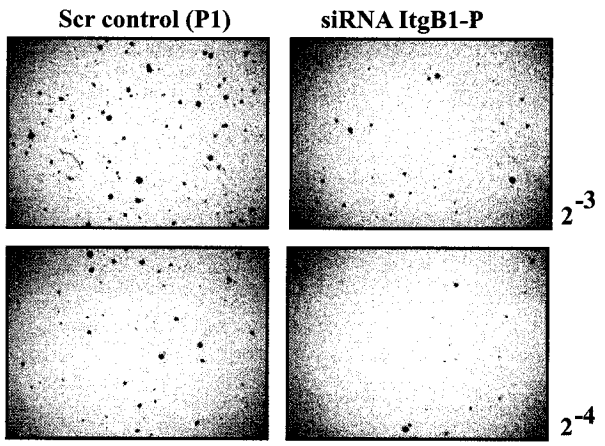
While several integrins mediate focal complex formation and signaling in adherent cells, the main focal complex observed in 3-dimensional cell culture of untransformed fibroblasts is dependent on one integrin receptor, integrin α5β1 (74). The hypothesis that integrin β1 is important for the anchorage independent growth aspect of tumour cell characteristics was therefore tested using a soft agarose colony formation assay. Cells were transfected with integrin β1 targeted or control siRNA duplexes. Twenty-four hours later, cells were plated in 0.5% agarose onto a 0.75% agarose underlay and incubated for 15 days to allow colonies to form. Reduction of integrin β1 protein levels using pooled targeted siRNA duplexes which was verified by western blot, resulted in a significant decrease in the number of PC3 cell colonies formed compared to cell populations transfected with pooled non-targeting siRNA (Figure 9A and 9B). Although integrin β1-targeted duplexes caused a reduction in anchorage independent colony formation by approximately two-fold, those PC3 cells which were able to form colonies in soft agarose from the integrin β1 depleted population, did so with identical kinetics to cells transfected with control siRNA duplexes. The number of cells in each colony greater than five cells was recorded every second day and the average number of cells in each colony was plotted as a function of time. Depletion of integrin β1 in PC3 cells using the pool of four siRNA duplexes did not affect the average colony size over time compared to cells transfected with control siRNA (Figure 9C) but rather reduced the total number of colonies formed. Given the observed 50% depletion efficiency of integrin β1 surface expression accomplished by pooled targeted siRNA (Figure 5B), the kinetic information suggests that integrin β1 expressing cells from the untransfected

Figure 9. Integrin β1 depleted PC3 cell populations demonstrate decreased anchorage independent growth but with identical kinetics to β1 integrin expressing populations. (**A**) 2.5nM pooled integrin β1 targeted siRNA (siRNA ItgB1-P) and pooled scramble control (Scr control (P1)) PC3 transfectants were serially diluted in 0.5% soft agarose in the presence of 10% FBS and cultured for 15 days. The number of colonies >50 cells in size were counted in 6 replicates from 2 dilutions, normalized to 1000 cells for comparison, and are expressed as means ± SD for one representative experiment. P values of <0.05 are shown with an asterisk. Four independent experiments confirmed similar results. Depletion of β1 integrin protein was ascertained by western blot analysis 48 hours following transfection (**B**) Bright field photographs of PC3 cell colonies from two dilutions were taken at 40X magnification after 15 days in soft agarose culture. (**C**) PC3 cell colonies >5 cells were counted in at least 6 replicates from 2 dilutions starting at day 5 and are expressed as mean colony size ± SD.

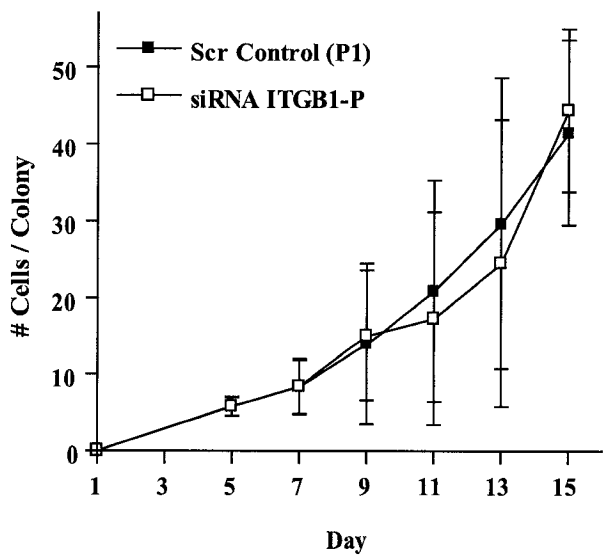
A


500
400
300
200
100
0
Colonies
*
Scr control (P1)
siRNA ItgB1-P
Integrin β1
β-Actin

B


Scr control (P1)
siRNA ItgB1-P
2-3
2-4

C


Scr Control (P1)
siRNA ITGB1-P
Cells / Colony
50
40
30
20
10
0
1
3
5
7
9
11
13
15
Day

population formed colonies in soft agarose normally while cells not expressing integrin β1 were unable to do so.

To determine whether integrin β1-contingent anchorage independent growth was a cell line-specific phenomenon, other tumour cell lines were tested. A2780S ovarian carcinoma and SF295 glioblastoma cells were transfected with pooled integrin β1-targeted or scrambled control duplexes and plated, 24 hours later, in 0.5% agarose unto a 0.75% agarose underlay and incubated for fifteen days. Reduction in integrin β1 protein for each experiment was ascertained by western blot and, as for PC3 cells, all cell lines tested exhibited markedly reduced colony formation in soft agarose assays when integrin β1 was depleted compared to control siRNA transfected cells (Figure 10).

Given recent concern over non-specific effects attributed to the use of pooled siRNA duplexes, integrin β1-dependent colony formation was tested in PC3 cells using individual targeted duplexes from the previously described pool. Depletion of integrin β1 in PC3 cells using either siRNA-ItgB1-02, or siRNA-ItgB1-03 significantly reduced colony formation compared to either the pooled scramble or individual GFP-scrambled control duplexes (Figure 11A). Both targeted duplexes decreased the number of PC3 colonies by approximately two-fold compared to control siRNA. Additionally, and in agreement with observations made for the pooled siRNA duplexes, targeted siRNA duplexes did not appear to influence the size of colonies formed over time (Figure 11B) but only the number of colonies which were formed.

Figure 10. Integrin β1 depletion reduces the anchorage independent growth of cancer cell populations derived from different tissue types. 5nM pooled integrin β1 targeted siRNA (siRNA ItgB1-P) and pooled scramble control (Scr control (P1)) transfected A2780S (**A**) or SF295 (**B**) cells were cultured in 0.5% soft agarose in the presence of 10% FBS for 15 days. The number of colonies >50 cells in size were counted in 6 replicates from 2 dilutions, normalized to 1000 cells for comparison, and are expressed as means $\pm$ SD. P values of <0.05 are shown with an asterisk. Minimally, two independent experiments confirmed similar results. Depletion of integrin β1 protein was ascertained by western blot analysis 48 hours following transfection.

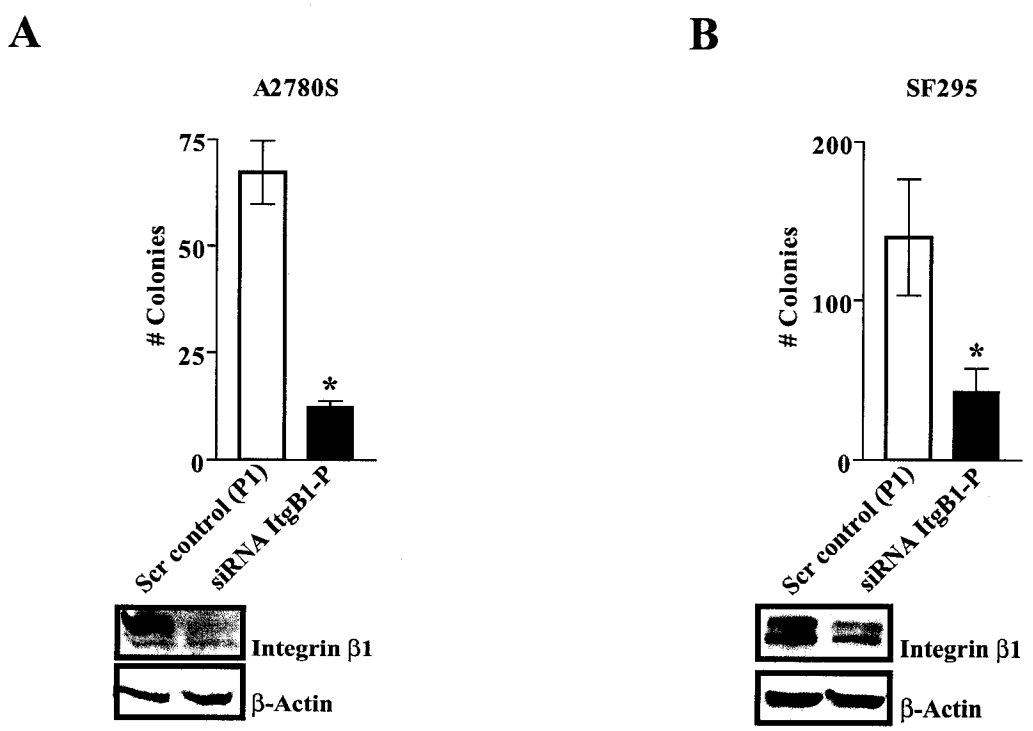
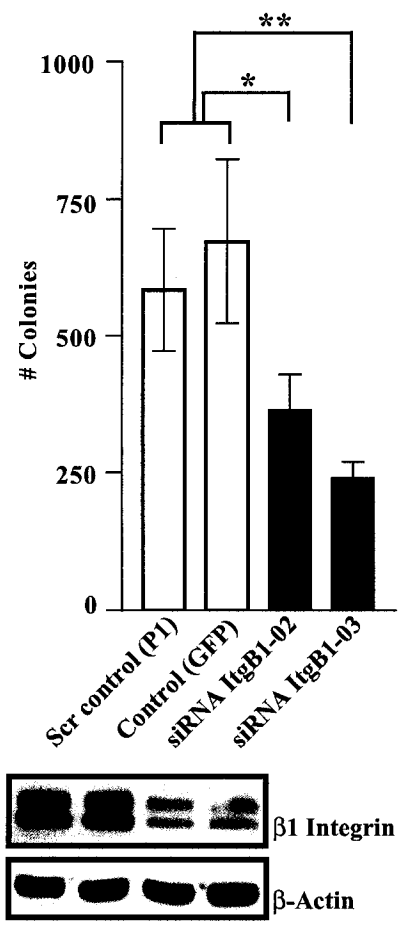
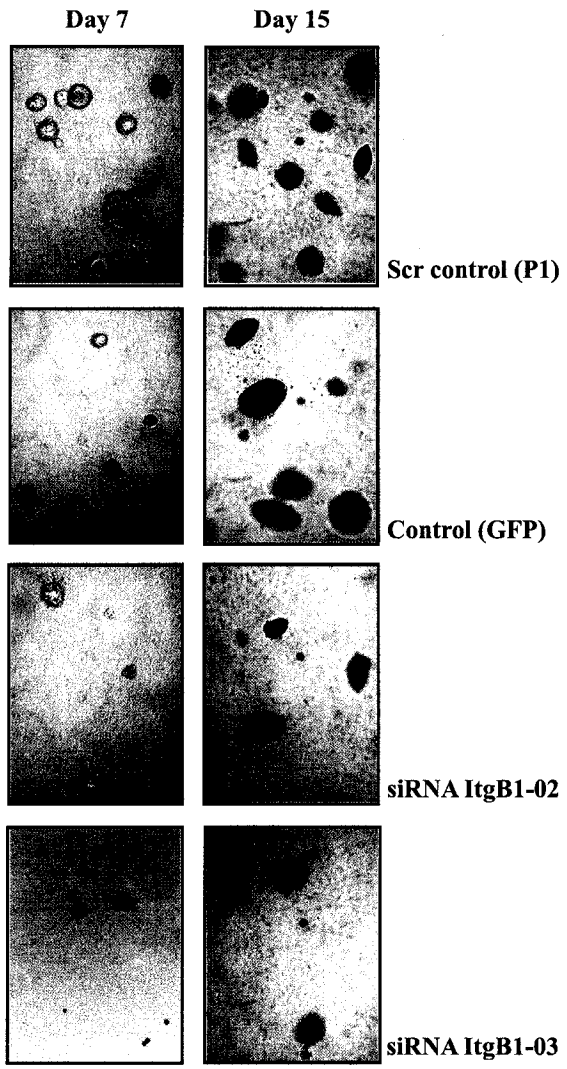

A
A2780S
75
50
25
0
Colonies
*
Scr control (P1)
siRNA ItgB1-P
Integrin β1
β-Actin
B
SF295
200
100
0
Colonies
*
Scr control (P1)
siRNA ItgB1-P
Integrin β1
β-Actin

Figure 11. PC3 cell populations depleted of integrin β1 using different targeted siRNA duplexes demonstrate reduced anchorage independent growth. (**A**) Individual integrin β1 siRNA targeted (5nM: ItgB1-01, ItgB1-02, or ItgB1-03) and control (5nM scrambled siRNA: Scr control (P1) or GFP-targeting control (GFP)) PC3 transfectants were cultured in 0.5% soft agarose in the presence of 10% FBS for 15 days. The number of colonies >50 cells in size were counted in 6 replicates from 2 dilutions, normalized to 1000 cells for comparison, and are expressed as means ± SD for one representative experiment. Significant differences between scramble controls and targeted integrin β1 duplexes were determined by unpaired t-tests ($P<0.05$) and are denoted by * and **. Three independent experiments confirmed similar results. Depletion of integrin β1 protein was ascertained by western blot analysis 48 hours following transfection. (**B**) Bright field photographs of PC3 cell colonies were taken at various times during the 15 day incubation at 400X magnification.

A


**
*
1000
750
500
250
0
Colonies
Scr control (P1)
Control (GFP)
siRNA ItgB1-02
siRNA ItgB1-03
β1 Integrin
β-Actin

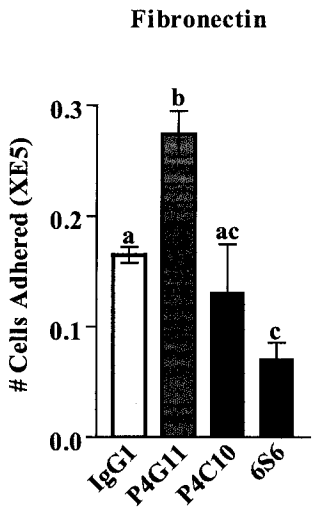
B


Day 7
Day 15
Scr control (P1)
Control (GFP)
siRNA ItgB1-02
siRNA ItgB1-03

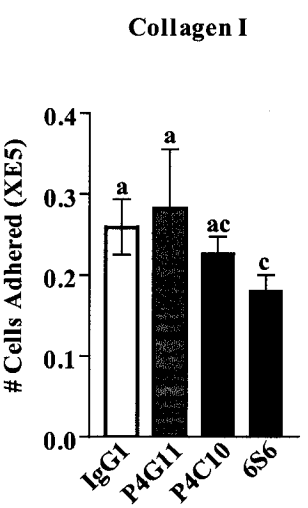
In order to further confirm the importance of β1 integrins in anchorage independent growth, an alternative strategy was employed. Several integrin β1 binding antibodies, which have been shown to either activate (P4G11), or neutralize (P4C10, and 6S6) β1 integrin-mediated adhesion in various cell types (360, 361, 362) were tested for their ability to modulate the anchorage independent growth of PC3 cells. To verify the function of these antibodies, PC3 cells were collected using EDTA, in order to preserve surface integrin β1 function, and were pre-incubated with antibody or isotype matched IgG (IgG1) control for 30 minutes before being seeded onto either fibronectin or collagen-I coated plates. Activating antibody significantly increased the number of cells adhered to fibronectin after 1 hour but did not increase adhesion to collagen I compared to IgG1 controls (Figure 12A and 12B). Pre-incubation with either neutralizing antibody resulted in a decrease in the average number of cells adhered to fibronectin and collagen I after one hour compared to IgG1 treated cells, however, only 6S6 and not P4C10 significantly reduced adhesion to both matrices based on unpaired two-tailed t-tests with a P value of 0.05 (Figure 12A and 12B). To test the impact of integrin β1-targeting antibodies on soft agarose colony formation, PC3 cells were pre-incubated with antibody or IgG1 for 30 minutes and plated in 0.5% agarose onto 0.75% overlays and incubated for 10 days. Although the adhesion activating antibody, P4G11, did not influence PC3 colony formation, both neutralizing antibodies to integrin β1, P4C10 and 6S6, significantly reduced the ability of PC3 cells to form colonies in soft agarose compared to the IgG1 control (Figure 12C and 12D). This reduction in anchorage independent growth is consistent with the decrease in anchorage independence observed in PC3 cells depleted of integrin β1 using siRNA (Figure 9A and 11A).

Figure 12. Integrin β1 adhesion neutralizing antibodies reduce the ability of PC3 cells to demonstrate anchorage independent growth. PC3 cells were collected with EDTA and incubated with mouse IgG1, integrin β1 adhesion activating antibody: P4G11, or integrin β1 adhesion neutralizing antibodies: P4C10 or 6S6 at room temperature for 30 minutes. Cells were then seeded into plates coated with either fibronectin (**A**) or Collagen I (**B**). Adherent cells were counted after 1 hour and are expressed as means ± SD. Unpaired t-tests ($P<0.05$) were conducted between conditions and means which are significantly different are assigned unique letter values while congruent means are denoted by the same letter. (**C**) PC3 cells pre-incubated with mouse IgG1, integrin β1 adhesion activating antibody: P4G11, or integrin β1 adhesion neutralizing antibodies: P4C10 or 6S6, were cultured in 0.5% soft agarose in the presence of 10% FBS for 15 days. The number of colonies >50 cells in size were counted in 6 replicates from 2 dilutions, normalized to 1000 cells for comparison and are expressed as means ± SD for one representative experiment. Differences in mean colony formation ($P<0.05$) are indicated by * and **. Three independent experiments confirmed similar results. (**D**) Bright field photographs of PC3 cell colonies from two dilutions were taken at 400X magnification after 15 days in soft agarose culture.

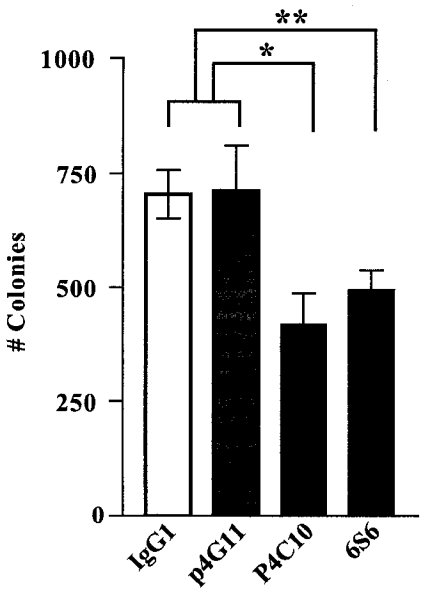
A


Fibronectin
Cells Adhered (XE5)
0.3
0.2
0.1
0.0
a
b
ac
c
IgG1
P4G11
P4C10
6S6

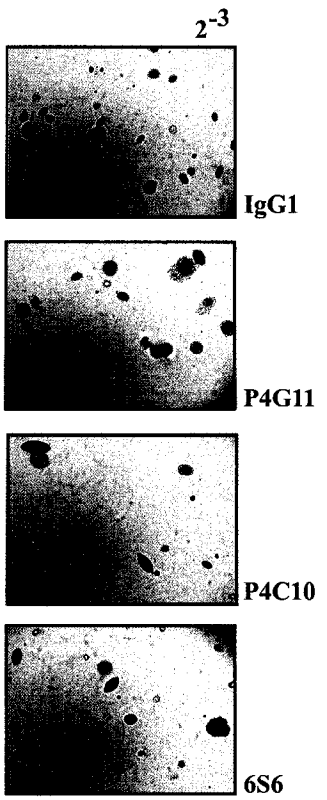
B


Collagen I
Cells Adhered (XE5)
0.4
0.3
0.2
0.1
0.0
a
a
ac
c
IgG1
P4G11
P4C10
6S6

C


**
*
1000
750
500
250
0
Colonies
IgG1
p4G11
P4C10
6S6

D


2-3
IgG1
P4G11
P4C10
6S6

3.5 EXPRESSION OF THE INTEGRIN β1C CYTOPLASMIC SPLICE VARIANT DECREASES THE ANCHORAGE INDEPENDENT GROWTH OF PC3 CELL POPULATIONS.

The impact of integrin β1 on the anchorage independent phenotype of PC3 cells was further characterized by transiently overexpressing pBJ plasmids expressing either the wild-type β1A receptor subunit or the cytoplasmic splice variant β1C integrin. The unique 48 amino acid C-termini of β1A and B1C notably differ in several key functional domains (Figure 13A). Integrin β1A but not β1C contains two NPxY interaction domains, Cyto-2 and Cyto-3, which are best understood in their capacity to mediate localization to focal adhesions (136). The recruitment of cytoskeletal interacting proteins such as talin, α-actinin, and filamin, as well as the kinase ILK to integrin β1 cytoplasmic tails also depends on residues which are absent from the β1C splice variant (363, 364). However, recruitment of several signaling proteins, including paxillin, are not clearly specific to integrin β1A and FAK phosphorylation is not impaired in integrin β1C expressing cells despite the fact that integrin β1C does not localize to focal adhesions (165, 170). Wild-type integrin β1 also contains two threonine residues involved in regulating conformation dependent extracellular ligand binding which are absent from the integrin β1C variant (365).

In agreement with its expression pattern in normal but not malignant prostate epithelial cells (168), the integrin β1C protein contains a unique 8 amino acid peptide sequence which is essential and sufficient to enable its function in inhibiting cell cycle progression (166), although the exact mechanism by which this occurs is currently unknown. It is of interest that while integrin β1A activates the MAPK and AKT signaling cascades in CHO cells, integrin β1C will stimulate AKT signaling but not ERK activity. Accordingly overexpression of activated Ras or MEK is sufficient to rescue the anti-proliferative

phenotype of integrin β1C transfectants (170). Perhaps more importantly, integrin β1C is able to associate with alpha integrin subunits and does not inhibit adhesion to fibronectin or to a β1 integrin antibody (170) although there is no evidence that the integrin β1C receptor binds extracellular ligands. This suggests that although it likely contributes directly to inhibiting cell proliferation, integrin β1C could potentially also compete with endogenous integrin β1A for dimerization partners and inhibit the subsequent formation and localization of functional receptors if overexpressed in cell lines.

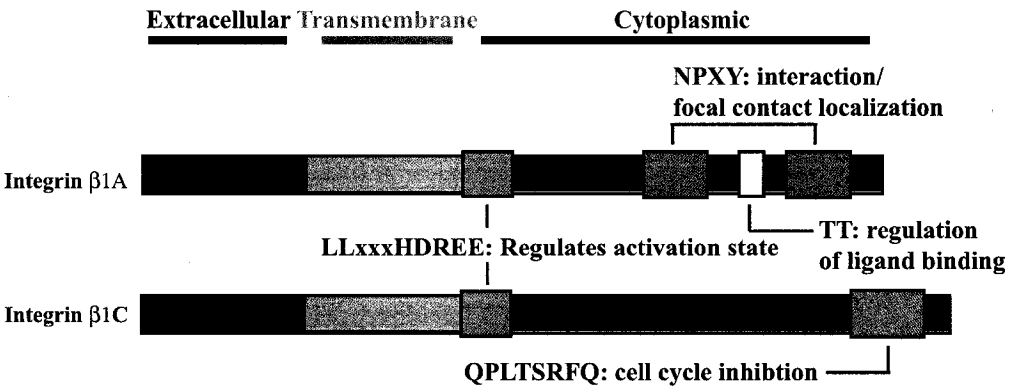
To determine the impact of overexpressing either wildtype integrin β1 or the integrin β1C variant isoform, PC3 cells were transiently transfected with empty vector (pBJ) plasmid, pBJ-β_{1A}, or pBJ-β_{1C} for 24 hour prior to culture in soft agarose for 15 days. Although overexpression of the wild-type β1A integrin did not affect the ability of PC3 cells to form colonies in soft agarose, expression of pBJ-β_{1C} significantly reduced anchorage independent colony formation in PC3 cells compared to the empty vector control transfected population even though overexpression of integrin β1 was not highly detected by western blot analysis for either splice variant (Figure 13B). Noticeable differences in colony size at end-point were not observed, however this was not quantified.

3.6 A FIBRONECTIN-SPECIFIC ANTIBODY REDUCES THE ABILITY OF PC3 CELLS TO FORM COLONIES IN SOFT AGAROSE.

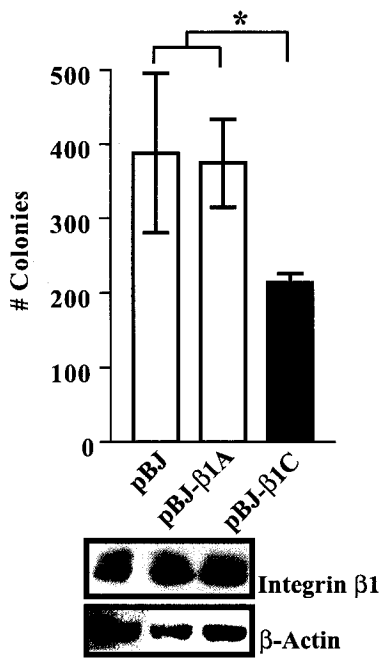
Although cells grown in soft agarose do not adhere to a solid surface, ECM components are synthesized by cells and are therefore available for ligation of integrin heterodimers. Additionally, fetal calf serum, which is indispensable for soft agarose growth of PC3 cells (data not shown), contains a variety of protein components, including fibronectin. To investigate the importance of integrin ligation by ECM in anchorage

Figure 13. Expression of integrin β1 isoform C reduces anchorage independent growth of PC3 cell populations. (**A**) Schematic representation of the integrin β1A and β1C variant proteins resulting from alternative splicing events. Known functional components of the cytoplasmic domain are indicated. (**B**) PC3 cells transfected with pBJ-β1A, pBJ-β1C, or pBJ empty vector were cultured in 0.5% soft agarose in the presence of 10% FBS for 15 days. The number of colonies >50 cells in size were counted in 6 replicates from 2 dilutions, normalized to 1000 cells for comparison, and are expressed as means ± SD. Differences between pBJ and pBJ-β1 transfectants were determined by unpaired t-tests ($P<0.05$) and are denoted by an asterisk. Two independent experiments confirmed similar results. Overexpression of integrin β1 protein was ascertained by western blot analysis 48 hours following transfection.

A


Extracellular
Transmembrane
Cytoplasmic
NPXY: interaction/
focal contact localization
Integrin β1A
TT: regulation
of ligand binding
LLxxxHDREE: Regulates activation state
Integrin β1C
QPLTSRFQ: cell cycle inhibtion

B


*
500
400
300
200
100
0
Colonies
pBJ
pBJ-β1A
pBJ-β1C
Integrin β1
β-Actin

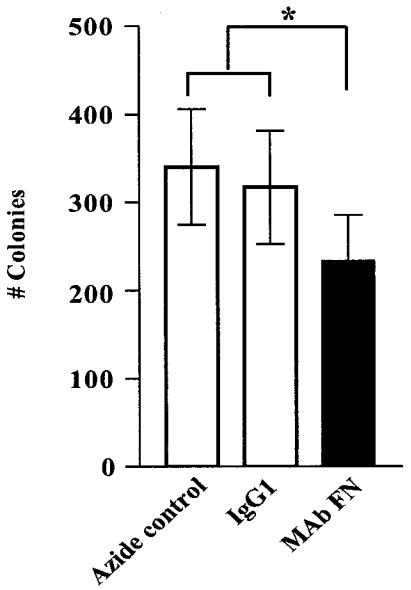
independent growth, fibronectin present in the assay system was competitively bound using a monoclonal anti-fibronectin antibody. The presence of fibronectin antibody (2ug/ml) diluted directly into 0.5% agarose containing 10% FBS was sufficient to significantly inhibit the number of anchorage independent PC3 colonies formed by approximately one third compared to azide and IgG1 controls (Figure 14A). As in β1 integrin depletion studies, the size of colonies was not obviously different between groups and only the total number of colonies formed was reduced in the presence of the fibronectin antibody (Figure 14B).

3.7 SURFACE EXPRESSION OF INTEGRIN β1 IS NOT SIGNIFICANTLY ALTERED IN PC3 CELLS INDUCED TO GROW IN SUSPENSION.

PC3 cells can be induced to grow in suspension by plating them onto a 1% solid agarose base to which they will not attach (Figure 15A). This system was used to examine molecular and biochemical characteristics of PC3 cells which might clarify the necessity of integrin β1 for anchorage independent growth. PC3 cells grown adherently or in suspension for 48 hours were collected with EDTA and stained for surface expression of integrin β1. Flow cytometry analysis revealed that surface expression of integrin β1 was equivalent in PC3 cells grown either adherently or in suspension (Figure 15B). Densitometry from two independent western blot experiments demonstrated that total integrin β1 protein levels normalized to β-actin were slightly reduced in PC3 cells grown in suspension. Densitometry also revealed that PC3 cells transfected with individual integrin β1-targeted siRNA duplexes were able to maintain a similar degree of integrin β1 depletion compared to scrambled controls after 48 hours of adherent or induced suspension growth (Figure 15C and 16A).

Figure 14. Anchorage independent growth of PC3 cells is diminished in the presence of an anti-fibronectin antibody. **(A)** PC3 cells were cultured in 0.5% soft agarose in the presence of 10% FBS and 0.01% azide (control), 2 ug/ml mouse IgG1 (control), or 2 ug/ml monoclonal antibody to fibronectin. After 15 days, the number of colonies >50 cells in size were counted in 6 replicates from 2 dilutions and are expressed as means $\pm$ SD for one representative experiment. P values of <0.05 are shown with an asterisk. Three independent experiments confirmed similar results. **(B)** Bright field photographs of PC3 cell colonies at two dilutions were taken at 400X magnification after 15 days in soft agarose culture.

A


*
500
400
300
200
100
0
Colonies
Azide control
IgG1
MAb FN

B

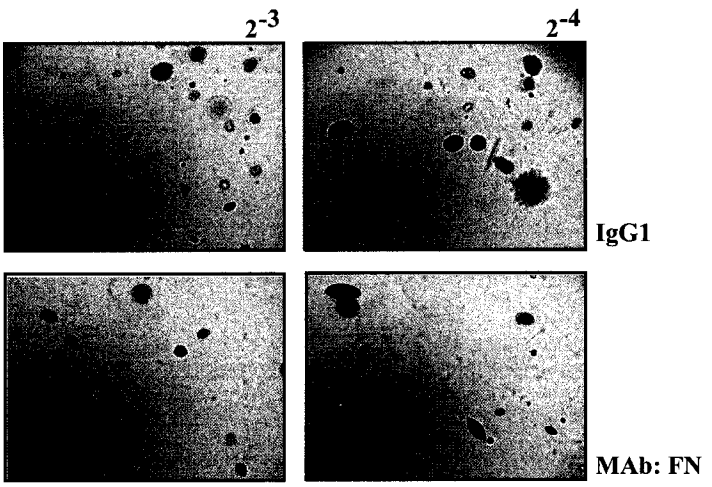
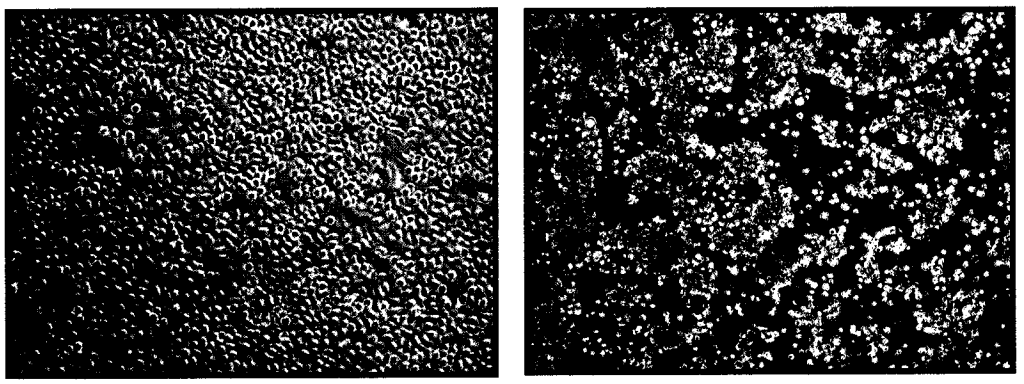

2-3
2-4
IgG1
MAb: FN

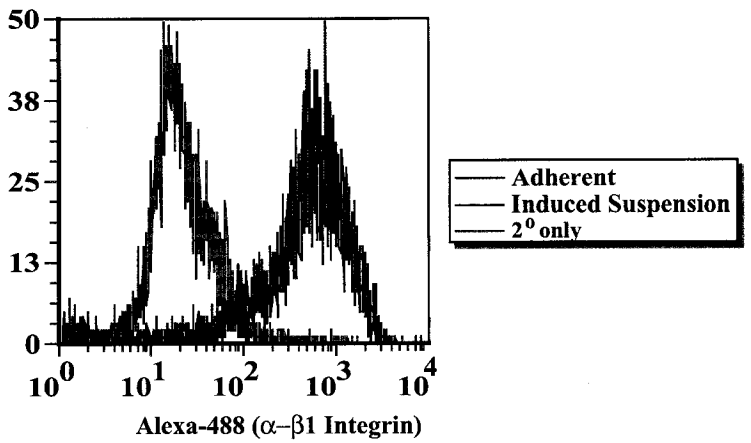
Figure 15. Induced suspension of PC3 cells does not impact surface expression of integrin β1 protein. (**A**) Bright field photographs of PC3 cells grown adherently and in suspension over a solid 1% agarose base were taken at 400X magnification after 48 hours in culture. (**B**) PC3 cells grown adherently or in suspension for 48 hours were collected with EDTA. Cell surface protein was stained with an integrin β1 specific antibody and alexa-488 conjugated secondary antibody and used for flow cytometry analysis. Cells treated with conjugated secondary only were used to determine background fluorescence. Cells gated using forward and side scatter plots to eliminate debris, were used to construct the histograms shown. (**C**) Whole cell lysates were collected from individual integrin β1 (5nM: ItgB1-01, ItgB1-02, or ItgB1-03) or control (5nM scrambled siRNA: Scr control (P1) or GFP-targeting control (GFP)) siRNA treated PC3 cells which were replated 24 hours following transfection to be grown adherently or in suspension for 48 hours. Protein was separated by SDS-PAGE, and immmunoblotted using an antibody to integrin β1. Detection of β-actin was used as a loading control and for the normalization of densitometry values for integrin β1 expression averaged over two independent experiments. The percentage decrease in intensity of the integrin β1 band for siRNA treated cells is compared to the mean intensity of the two scrambled control bands for each growth condition.

A

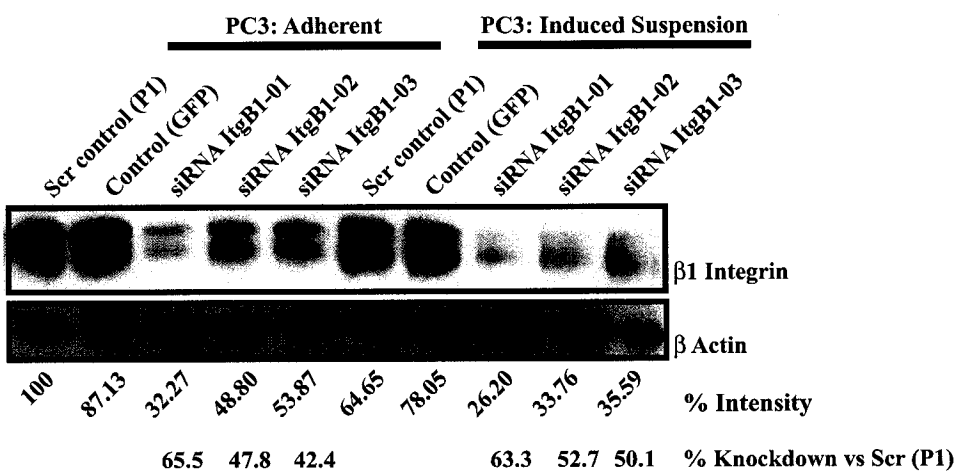


PC3: Adherent PC3: Induced Suspension

B



C



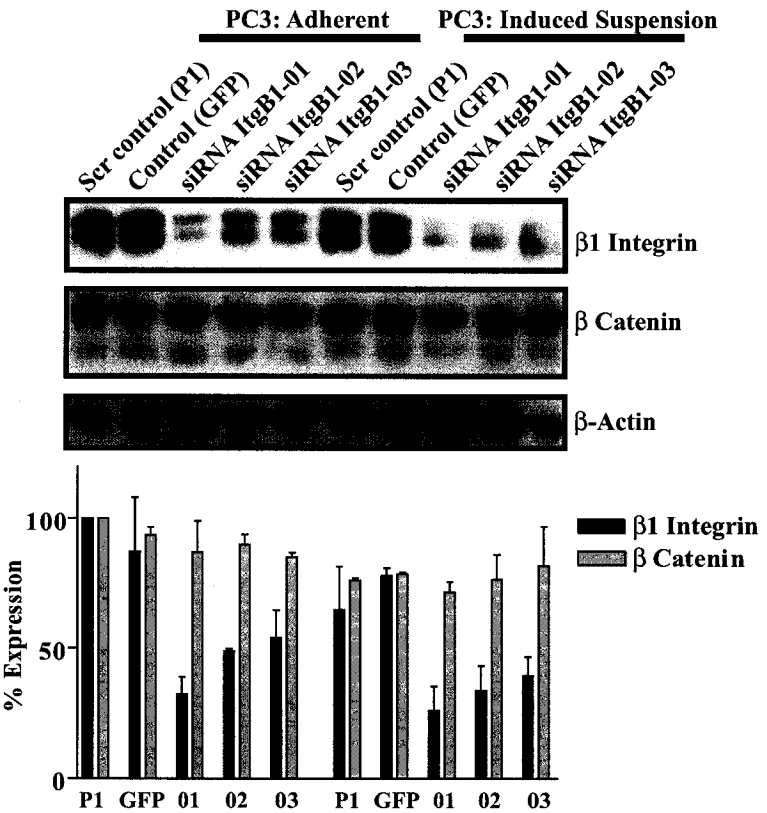
3.8 β CATENIN CO-IMMUNOPRECIPITATES WITH INTEGRIN β1 IN PC3 CELLS GROWN IN SUSPENSION BUT NOT IN ADHERENT PC3 CELLS.

B-catenin was examined as a potential mediator of integrin β1 dependent anchorage independent colony formation because of its position as a downstream effector of integrin β1 signaling (322) with known aberrant activity in many cancer models and more invasive tumour cells (366). Overexpression of β-catenin has also been found to induce an anchorage independent phenotype in untransformed epithelial cells (367). To this end, whole cell lysates from adherent and non-adherent PC3 cells which had first been depleted of integrin β1 using individual targeted siRNA duplexes or treated with control duplexes were subjected to immunoblotting. Densitometry from two independent experiments comparing β-catenin levels to a β-actin control revealed that β-catenin protein expression did not rely on integrin β1 expression in either adherent and suspension PC3 cells (Figure 16A).

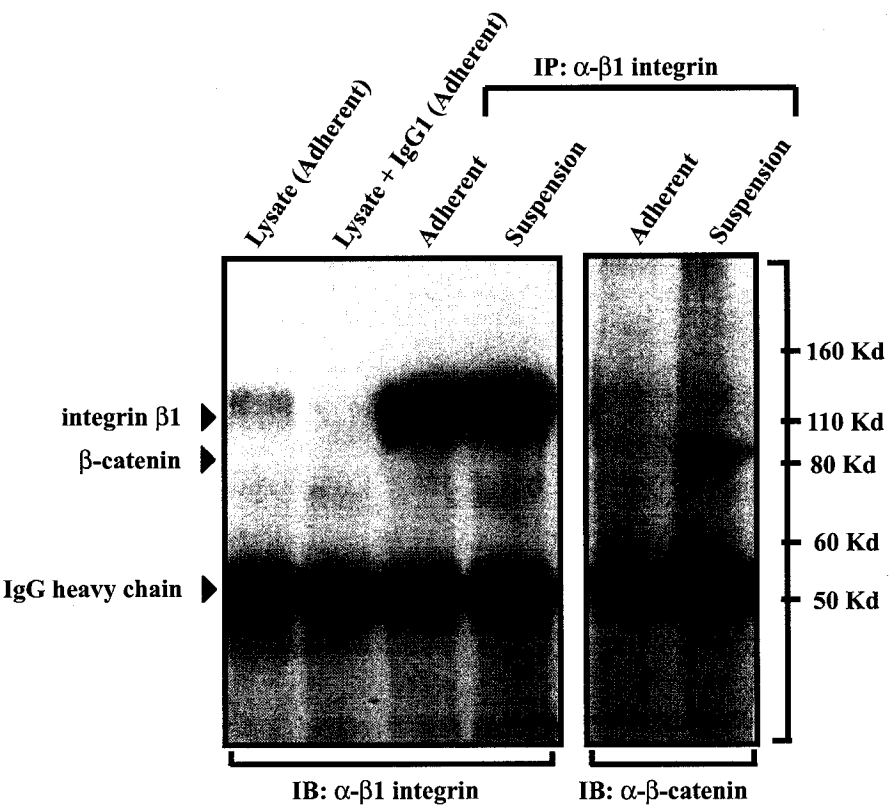
To further examine whether integrin β1 occupies an inherently different role in non-adherent PC3 cells compared to 2-D adherent cells, lysates from cells grown either adherently or in suspension were subjected to immunoprecipitation using an anti-integrin β1 antibody. Antibody-free and IgG1 controls were used to assure the specificity of the pull-down. Subsequent immunoblotting confirmed the specificity of the IP and demonstrated that β-catenin associated with integrin β1 in PC3 cells induced to grow in suspension for 48 hours but not in adherent PC3 cells (Figure 16B). The lack of a significant reduction in β-catenin expression when β1 integrin is depleted in PC3 cells grown either adherently or in suspension (Figure 16A), suggests that co-localization with integrin β1 does not impact on the total amount of β-catenin protein present in the cell. As this interaction only occurs in suspension cells, it is plausible that an interaction between integrin β1 and β-catenin may be functionally important for anchorage independent colony formation.

Figure 16. β-catenin and β1 integrin co-immunoprecipitate in PC3 cells induced to grow in suspension. (**A**) Individual integrin β1 (5nM: ItgB1-01, ItgB1-02, or ItgB1-03) or control (5nM scrambled siRNA: Scr control (P1) or GFP-targeting control (GFP)) PC3 transfectants were grown adherently or in suspension for 48 hours beginning 24 hours following transfection. Whole cell lysates were separated by SDS-PAGE and immunoblotted with anti-β1 integrin, anti-β-catenin, or anti-β actin antibodies. Band intensities for integrin β1 and β-catenin blots were analyzed and compared to β-actin as a loading control, and are expressed as the mean percentage expression ± SD of the first scrambled control band for the adherent growth condition and are averaged over two independent experiments. **(B),** Whole cell lysates from PC3 cells grown adherently or induced to grow in suspension over a 1% solid agarose base for 48 hours were subjected to immunoprecipitation (IP) with an anti-β1 integrin antibody followed by SDS-PAGE and immunoblotting (IB) with either anti-β1 integrin or β-catenin antibodies. Lysates from adherent cells were incubated in the absence of β1 integrin antibody ± mouse IgG1 to confirm the specificity of the IP.

A


PC3: Adherent
PC3: Induced Suspension
Scr control (P1)
Control (GFP)
siRNA ItgB1-01
siRNA ItgB1-02
siRNA ItgB1-03
Scr control (P1)
Control (GFP)
siRNA ItgB1-01
siRNA ItgB1-02
siRNA ItgB1-03
β1 Integrin
β Catenin
β-Actin
% Expression
100
50
0
P1 GFP 01 02 03 P1 GFP 01 02 03
β1 Integrin
β Catenin

B


IP: α-β1 integrin
Lysate (Adherent)
Lysate + IgG1 (Adherent)
Adherent
Suspension
Adherent
Suspension
160 Kd
110 Kd
80 Kd
60 Kd
50 Kd
integrin β1
β-catenin
IgG heavy chain
IB: α-β1 integrin
IB: α-β-catenin

3.9 SUMMARY.

In contrast to previous studies, we did not observe a role for ECM-mediated adhesion in the survival of human tumour cells treated with various chemotherapeutic agents. In agreement with this, integrin β1, a component of several integrin adhesion receptors capable of binding collagen, fibronectin, tenascin, and laminin, was not found to significantly contribute to the adhesion, growth, or survival of the tumour cell lines tested under 2-D adherent conditions. This was determined by comparing the phenotype of cells depleted of integrin β1 using siRNA to integrin β1-expressing controls. We also used the siRNA strategy to study anchorage independent growth using a soft agarose assay, and in contrast to adherent cell phenotypes, integrin β1 depletion inhibited anchorage independent colony formation in three different cancer cell lines. Similarly, neutralizing antibodies to integrin β1, or overexpression of a growth inhibitory isoform of integrin β1 which is not normally expressed in transformed prostate tissue, integrin β1C, reduced anchorage independent colony formation in PC3 cells. Use of an antibody to fibronectin, a major β1 integrin ligand, also reduced soft agarose colony formation by PC3 cells. Finally, we grew PC3 cells in suspension by culturing them over a solid 1% agarose base and found that surface expression was equivalent in both conditions and that integrin β1 depletion did not impact protein levels of β-catenin in either adherent or suspension PC3 cells. However, integrin β1 preferentially associated with β-catenin in suspension cells but not in PC3 cells grown adherently suggesting that an association between these two molecules may contribute to the importance of integrin β1 in the anchorage independent colony formation of tumour cells.

4. DISCUSSION

4.1 ADHERENT CANCER CELL PHENOTYPES

4.1.1 ECM and Chemotherapeutic Response of Human Cancer Cell Lines

In vitro drug screening is a useful tool in the identification of novel chemotherapeutics and combinations thereof which might, upon further study, be efficacious in human cancer patients. Perhaps the most ambitious example of this is the NCI60 anticancer drug screen which began to assemble lung cancer cell lines in 1986 and has since assembled a panel of cells from a plethora of tissue and cancer origins and tested their response to an unprecedented variety of cytoxic and cytostatic agents (368). Today the NCI60 screening database serves mainly to support the coordination of smaller scale screens and the identification of cellular mechanisms involved in drug sensitivity. A need for more clinically relevant screening tools, however, has been identified and culture models aimed at better recapitulating *in vivo* phenotypes are a subject of much investigation. The ability of protein from the ECM, an important component of the tumour microenvironment, to modulate cancer cell drug sensitivity has been reported for several tumour types in response to different chemotherapeutic drugs and to radiation, as mentioned previously (302-309, 369). In our study, laminin, monomeric collagen-I, fibronectin, and tenascin-C, all known components of the ECM, were used to coat tissue culture plates on which human cancer cell lines were grown adherently and subsequently challenged with chemotherapeutics. An MTT assay in which viable cells mediate the reduction of tetrazolium salt to provide a quantifiable colourimetric readout was employed with the potential for more sensitive measurements of

viability and proliferation to be used in the verification and further study of promising results. Surprisingly, and despite the precedent for ECM modulation of drug response described in the literature, our culture and screening system did not identify any reproducible modulation of drug response by ECM protein for the various drug classes and tissue types tested. Although tumour cells secrete ECM proteins and coordinate the organization of soluble ECM proteins which are also found in serum, it has been shown that many cell types respond to supplementation with additional ECM proteins using coating procedures analogous to ours resulting in increased survival upon drug treatment (303-309, 369). For example, small cell lung cancer (SCLC) cell lines treated with etoposide in the presence of fibronectin or laminin ECM proteins demonstrate increased survival and decreased apoptosis compared to cells treated in the absence of ECM (305). However, we did not observe such modulation in the non-small cell lung cancer (NSCLC) cell lines, A549 and H460, nor in any of the other human cancer cell lines tested, regardless of the chemotherapeutic employed. The ability of ECM proteins to impact the basal phenotype of cancer cells such as growth and clonogenic survival, compared to plastic culturing conditions, has also been demonstrated for SCLC (305) as well as hepatocarcinoma and Ewing's sarcoma human cancer cell lines (370), however no modulation of basal growth kinetics was observed in our system in A549 cells by the ECM protein fibronectin.

It may be of note that the ECM protein used in our experiments was coated at a reliably decreased concentration compared to published experiments demonstrating ECM-mediated drug resistance in tumour cells despite similar concentrations being sufficient modulate the sensitivity of vascular endothelial growth factor (VEGF) stimulated endothelial cells treated with the angiogenesis inhibitor, endostatin (371). This suggests that tumour cells may be less sensitive to ECM signals than primary endothelial cells and underlines the

need for a positive control in order to assure that coating procedures are in fact depositing functional protein capable of contributing to cellular phenotypes. To this end, FAK phosphorylation, which depends on integrin ligation (372) and has been shown to correspond to the presence of ECM proteins (373) could be employed. Alternatively, because signalling pathways regulating growth and survival are characteristically abberrent at several nodes in tumour cell lines, a more sensitive and reliable control for these experiments might be the phosphorylation of total cellular protein. Accordingly, total tyrosine phosphorylation has been shown to increase in the presence of fibronectin in these cells (374), as well as in fibroblasts (375), and SCLC cells (305) even in the presence of 10% FBS.

Importantly, the modulation of tumour cell chemosensitivity by individual ECM proteins reported to date has been fairly modest, rarely exceeding two-fold and not encompassing a rescue from cytotoxity of a chemotherapeutic in most cases but rather increasing the threshold beyond which growth arrest or apoptosis is inevitable. One reason for the diminutive nature of the survival benefit may be that much of the pro-survival and growth signaling machinery in a cell is redundantly impacted by ECM and growth factor dependent signaling cascades. Serum added to the media of cultured cells is abundant in growth factors, as well as providing a potential source of exogenous ECM proteins, such as fibronectin and vitronectin (376), therefore repeating these experiments in decreased serum conditions may help to clarify the potential for ECM modulation of cancer cell survival. In our experimental system A549 cells were assayed for their ability to grow and survive following treatment with etoposide in the presence of 5% FBS (reduced serum) but an ECM-mediated survival advantage remained elusive. Although further optimization of a cell culture system to study drug response of tumour cell lines in the context of ECM proteins is needed, such as further decreasing serum concentrations in the event that even in 5% FBS

conditions saturate growth factor dependent signaling of the cell lines studied, the idea that many cell lines are not responsive to cues from individual matrix components agrees with the ability of most tumourigenic cell lines to grow in the absence of anchorage (350).

The prevalent mechanism proposed for ECM mediated drug resistance depends on integrin β1 and AKT signaling but not activation of the MAPK/ERK cascade (304, 305, 306, 377, 378). Tumour cell lines, including PC3 cells which are null for expression of the PI3 Kinase inhibitor, phosphatase and tensin homolog (PTEN), are often characterized by increased or constitutive AKT signaling (379) which may help to explain why many cell lines will not demonstrate ECM-mediated drug resistance.

Given the promiscuous nature of individual integrin receptors for different ECM ligands and the fact that specific ligands differentially mediate drug response reported in the literature, it seems that such modulation is not only the result of ligation of a particular receptor but is also tension dependent. In agreement with the idea that mechanical contributions of shape contribute to cancer cell survival it has been suggested that drug screens in vitro might be more representative of in vivo efficacy were they to be done using 3-D culture models (380, 381, 382). Such models may also more accurately recapitulate in vivo tissue architecture which takes advantage of cell-cell interactions. In fact, several studies have demonstrated altered chemosensitivity for cell lines grown as 3-dimensional spheroids compared to monolayer cultures (383, 384, 385) and ECM synthesis in spheroid cultures is more reflective of in vivo ECM composition, at least for some cell lines (386, 387, 388), suggesting that studies focused on the impact of ECM proteins on drug resistance in 3-D models might also be useful.

Taken together, the ability of the ECM to impact tumour cell response to chemotherapeutics likely depends on specific interactions with integrin receptors and the

impact of integrin engagement on the specific molecular phenotype of the cell in question. As such, a more powerful approach to investigate the impact of ECM-cell interactions may involve determining the impact of integrin receptors on important tumourigenic properties of cancer cells. Integrin β1 can pair with twelve different alpha subunits and β1 integrins mediate adhesion to every matrix protein investigated in this study. The integrin β1 subunit thus represents an attractive target for the *in vitro* study of integrin-mediated signaling in cancer cell lines.

4.1.2 The Impact of Integrin β1 Depletion on the Growth and Survival of Adherent Human Cancer Cell Lines.

Integrin β1 depletion was accomplished in several tumour cell lines using small interfering double stranded RNA oligonucleotides (siRNA). Original experiments employed a pool of four different oligonucleotides targeting integrin β1 (Dharmacon Smartpool) or a pool of four non-targeting scrambled control oligonucleotides. The transfection efficiency of PC3 cells determined by flow cytometry corresponds to an approximately 50% decrease in protein expression determined by western blot analysis. Similarly, the individual integrin β1-targeted siRNA oligonucleotides achieved a substantial decrease in protein expression by western blot up to 120 hours following transfection. Thus siRNA uptake would seem to be the limiting step to depletion in this particular system. In support of this, the histogram constructed from the flow cytometry analysis of surface integrin β1 expression demonstrates that approximately 50% of cells are depleted of integrin β1 using pooled siRNA duplexes and not that each cell is reduced of integrin β1 protein by 50%.

In order to address concerns about the specificity of siRNA (389, 390), duplexes from the pool of integrin β1-targeting oligonucleotides were used individually in PC3 cells and compared to two scrambled controls. Furthermore, to diminish the probability of off-target

gene silencing by mismatched duplexes interfering with translation, the concentration of siRNA used did not exceed 5nM. Generally, Dharmacon siRNA-02 and siRNA-03 were most reliably efficient at reducing integrin β1 protein levels and thus were employed in the study of adherent and anchorage independent growth and survival of PC3 cells.

Integrin β1 depletion did not compromise the ability of tumour cells from several tissue origins to adhere to plastic. Similarly, although a trend towards decreased adhesion after 1 hour was observed for integrin β1-siRNA transfectants compared to at least one control in two independent experiments, no significant difference in adhesion to either plastic or fibronectin was detected for PC3 cells depleted of integrin β1 protein. These observations are most easily explained by the functional redundancy at the level of adhesion for non-integrin β1 containing receptors which mediate binding to a variety of ECM protein ligands including fibronectin (see Figure 1B). This may seem surprising as ES cells (391) and F9 embryonal carcinoma cells (392) which are null for integrin β1 demonstrate a decreased ability to attach to the ECM proteins laminin and fibronectin compared to integrin β1-expressing counterparts. Several tumour cell lines have also been found to be dependent on β1 integrins for adhesion to collagens I and IV, laminin, and fibronectin (393, 394, 395). In these studies, integrin β1 depletion did not alter the ability of the various cell lines to adhere to the ECM protein vitronectin, suggesting that adhesion to plastic in which several ECM components are potentially available for integrin mediated attachment could occur equally well in integrin β1-depleted cell populations.

When plates are coated with fibronectin, attachment is presumably promoted by cells adhering to this protein, and therefore it might be expected that, as integrin α5β1 is the main fibronectin adhesion receptor in most epithelial cells (98, 396), depletion of integrin β1 would compromise the ability of PC3 cells to adhere to this substrate. Interestingly,

androgen independent prostate carcinoma metastasis-derived cell lines, such as Du145 and PC3 were found to express elevated levels of integrin α5β1 as well as αVβ3, a receptor which is generally absent from normal epithelial cells (397). Because it binds to fibronectin, and integrin β3 protein expression was not affected by integrin β1 depletion, it is plausible that integrin αVβ3 mediates adhesion of PC3 cells to fibronectin in the absence of integrin α5β1 expression. Additionally, β3 integrins are capable of mediating many of the same cell spreading events ascribed to β1 integrins which are necessary for cell adhesion following ECM engagement (396). However, the fact that monoclonal antibodies designed to neutralize integrin β1 function were effective at significantly inhibiting PC3 cell adhesion to fibronectin in contrast to the siRNA strategy employed indicates that the less than complete knockdown efficiency achieved by integrin β1 siRNA may also play a role in dampening the observed impact of integrin β1-mediated adhesion. Although shorter adhesion times, increased concentrations of ECM protein, such as fibronectin, or a more efficient knockdown of integrin β1 protein expression may reveal a more substantial role for β1 integrins in early adhesive events, such as ligand binding, as well as cell spreading which depends on focal adhesion formation (396), adhesion will likely not play a role in the context of subsequent studies on integrin β1-mediated tumour cell growth in our system.

Malignant cells are generally less dependent on adhesion to the extracellular matrix for expansion due to the constitutive activity of kinases regulating cyclin D1 expression and, in turn, cell cycle progression (350). This may evolve from a need to evade growth inhibitory signals from specific ECM components in primary sites of tumour growth or during the process of colonization of distant organs characterized by different ECM compositions (396, 398). However, it has also been suggested that amplified or activated RTKs for growth factors can cooperate with integrin receptors to enhance mitogenic

signaling, particularly for cells secreting growth factors in an autocrine manner, and even when downstream signaling molecules are constitutively active (333). By this mechanism, tumour cells, which according to the hallmarks of cancer are anchorage and growth factor independent, could exhibit growth modulation dependent on integrin expression.

In the case of integrin β1-containing receptors in PC3 cells, however, integrin-mediated growth modulation was not observed in 2-D culture systems. Depletion of integrin β1 protein by either individual or pooled targeted siRNA did not significantly impact the adherent growth of PC3 cells on plastic. Similarly, adherent colony formation was not impeded in PC3 cells depleted of integrin β1 confirming that β1 integrins do not collectively modulate proliferation in this setting. Growth of integrin β1-depleted PC3 cells grown on fibronectin was also not significantly different compared to scrambled control duplexes. Although the siRNA duplex, siRNA-ItgB1-01, apparently impeded growth of PC3 cells on fibronectin, this was not considered significant in either of two experiments. It is tempting to infer that the trend observed for ItgB1-01 in PC3 cells grown on fibronectin is attributable to the fact that it more effectively depleted integrin β1 protein levels in this experiment, based on western blot analysis. This would suggest that a greater efficiency of protein depletion could reveal a role for β1 integrins in modulating the proliferation of PC3 cells and potentially other cancer cell lines in the presence of fibronectin. However, our flow cytometry data indicates that the 50% decrease in integrin β1 protein observed for PC3 cells transfected with siRNA is attributable to 50% of cells in the population losing expression of surface integrin β1 protein and it therefore seems unlikely, if integrin β1 surface expression and ligation of fibronectin is important for tumour cell proliferation, that we would not be able to observe a similar decrease in proliferation for integrin β1 siRNA-treated cells in our studies.

As described previously, integrin β1 depletion does not affect the expression of integrin β3, and therefore β3 integrins may either compensate or sufficiently modulate growth of PC3 cells such that in either case β1 integrins do not increase growth potential. Alternatively, as specific alpha-beta1 integrin dimers have been reported to both promote or antagonize aspects of the tumourigenic phenotype in cancer cell lines (317, 318, 331, 334-336), it is possible that depletion of all β1 integrin-containing receptors maintains the established balance of growth signaling in PC3 cells. The fact that PC3 cells grow in the absence of anchorage, however, suggests that integrin and specifically integrin β1-mediated adhesion to an immobilized ECM may simply not impact the ability of the prostate carcinoma cell line to proliferate.

The cisplatin-resistant ovarian carcinoma cell line, A2780CP, was developed by serial passage and exposure to cisplatin-containing medium of the parental cell line, A2780S (351). These paired cell lines represent an in vitro model developed primarily to address mechanisms of acquired drug resistance to cisplatin regimens, a common barrier to the successful treatment of ovarian cancer in human patients. Although the presence of various ECM proteins did not modulate the response of either A2780S or A2780CP ovarian cancer cells to cisplatin, integrin β1 protein expression was found to be elevated by western blot analysis in the cisplatin-resistant cells compared to their parental counterpart. This was accompanied by an increase in the phosphorylation of AKT in A2780CP cells compared to A2780S. Different ovarian cancer cell lines expressing high levels of pAKT have been found to become more sensitive to cisplatin treatment when either AKT or a downstream target mTOR is inhibited (399) and there is some indication that active AKT promotes the chemoresistance of A2780CP cells (400, 401). The chemosensitivity of A2780S cells is mediated by AKT dependent p53 function, however inhibition of PTEN, which in turn

inhibits AKT, was not sufficient to induce apoptosis in A2780CP cells treated with cisplatin (402), suggesting that several mechanisms are involved in the chemoresistance of these cells. As AKT is an important downstream target of integrin β1, and as integrin β1 potentially regulates multiple anti-apoptotic pathways, this seemed to be an attractive model for testing the impact of integrin β1 depletion on tumour cell survival. However, in agreement with other adherent phenotypes examined herein, integrin β1 depletion had no impact on the survival of either sensitive or resistant ovarian cancer cells treated with cisplatin. The possibility that integrin β1-mediated adhesion or signaling is not an important factor in tumour cell chemotherapeutic response was not further examined although it must be acknowledged that such modulation by integrins could be tumour cell and drug-specific.

4.2 NON-ADHERENT OR 3-DIMENSIONAL CANCER CELL PHENOTYPES

4.2.1 The Impact of Integrin β1 Depletion by siRNA on the Growth and/or Survival of Human Cancer Cell Lines Grown in Soft Agarose.

Our study of integrin β1-dependent phenotypes using an *in vitro* adherent cell culture system, as described above, suggested that tumour cells are able to grow and survive independently of ECM-integrin interactions. However, integrin receptors, and specifically β1 integrins, have been found to contribute to or mediate tumour growth and invasion *in vivo* (403). Metastasis of ras-myc-transformed fibroblasts, for example, was found to be decreased on a integrin β1-null background in a murine model (404). Interestingly, sites of metastasis were different for tumours lacking integrin β1 expression. Other studies have also underlined the requirement for integrin β1 expression in both tumour growth and spread *in*

vivo (403). Taken together, integrin β1 contributes to *in vivo* malignancy in a context-dependent way, as evidenced by various animal studies, but does not contribute to the growth or survival of tumour cells we examined grown adherently in culture. Therefore, as anchorage independent growth *in vitro* has been established to correlate with tumourigenicity *in vivo* (341), we sought to determine whether integrin β1 was contributing to the anchorage independent phenotype of cultured tumour cells using several approaches to manipulate the functional activity of this protein.

The ability of PC3 cells to form colonies in soft agarose was ascertained by counting spheroid colonies and clusters greater than 50 cells in size after fifteen days in culture. Untransfected and scrambled control transfected PC3 cells formed colonies from approximately 50% of the cells plated. In contrast to adherent colony formation assays, PC3 cells depleted of integrin β1 using pooled siRNA duplexes reliably formed approximately 50% fewer colonies in soft agarose compared to the scrambled control. Similarly, PC3 cells depleted of integrin β1 using two individual targeted oligonucleotides were significantly impaired in colony formation compared to two separate scrambled controls. As adherent colony formation was not affected by integrin β1 depletion, this phenomenon is specific to anchorage independent growth in a 3-D culture system.

The fact that transfection and knockdown efficiencies parallel the 50% decrease in colony formation associated with integrin β1 depletion suggests that cells not expressing integrin β1 cannot form colonies. Further support of this idea was achieved by counting the size of each colony formed over time. The kinetics with which colonies formed in control and integrin β1 depleted PC3 cell populations were identical, indicating that the cells able to form colonies in both populations did so in the same way. The difference in total colony number at day 15 is therefore attributable to a decrease in the number of cells capable of

forming colonies in the integrin β1 siRNA transfected population and, given the knockdown efficiency determined by flow cytometry analysis; this suggests that integrin β1 is essential for soft agarose colony formation in PC3 cells. Furthermore, the fact that the transient depletion of integrin β1 achieved by siRNA transfection was sufficient to inhibit colony formation suggests that initial events of anchorage independent growth are dependent on this receptor subunit. Future investigations focused on the impact of integrin β1 on the anchorage independent survival versus growth of tumour cell lines will be necessary in order to determine the mechanism by which integrin β1 mediates anchorage independent colony formation.

The importance of integrin β1 expression for the anchorage independent growth and survival of human cancer cells was also examined in other tumour cell lines. SF295 glioma and A2780S ovarian carcinoma cells were tested for their ability to form colonies in soft agarose, and it was found that for both cell lines, depletion of integrin β1 using siRNA significantly impaired anchorage independent growth. Oncogenic mutations in p53 and PTEN are common to the PC3 and SF295 cells lines (405), however, A2780S cells express functional p53 (399, 402) and do not express constitutively active AKT (401), a usual consequence of a loss of PTEN inhibition. Thus the necessity of integrin β1 for anchorage independent growth is not specifically associated with activating or inactivating mutations of AKT signaling or p53 respectively, but rather appears to be a general characteristic of diverse tumour cell types.

Efforts directed at obtaining a more homogeneous tumour cell population in which integrin β1 is depleted could serve to further emphasize the importance of this receptor subunit in anchorage independent growth. A co-transfection strategy using fluorescent-labelled duplexes, for example, could be used to sort by flow cytometry for cells which were

successfully transfected with integrin β1 siRNA, thus eliminating untransfected cells from the cell population. Approaches aimed at stably suppressing integrin β1 protein expression, such as the use of short-hairpin RNA (shRNA) expression constructs (406), could also effectively demonstrate the importance of integrin β1 in anchorage independent colony formation. Although transient suppression of integrin β1 seems to be sufficient to inhibit colony formation, clonal populations of cells could be derived from stable transfectants thus eliminating the presence of integrin β1-expressing cells. Cell lines stably depleted of integrin β1 will be particularly useful in ascertaining the role of integrin β1 in tumour growth and metastasis *in vivo*, predicted based on the anchorage independent phenotypes of tumour cells studied here.

As an alternative approach for inhibiting integrin β1 function, we employed monoclonal antibodies which have been established to specifically activate or neutralize integrin β1-mediated adhesion. Although the activating antibody employed significantly increased PC3 cell adhesion to fibronectin, it did not impact anchorage independent colony formation. However, both adhesion neutralizing antibodies employed significantly reduced PC3 cell colony formation in soft agarose despite the fact that P4C10 did not significantly reduce adhesion to either fibronectin or collagen I in our adherent assay. Thus, the importance of integrin β1 in the anchorage independent colony formation of PC3 cells is not a siRNA-specific phenomenon, as confirmed by the ability of integrin β1 function-blocking antibodies to repress colony formation.

4.2.2 Modulation of Anchorage Independent Colony Formation in PC3 Cells by the Forced Expression of Integrin β1A and β1C.

Integrin β1C is found in non-neoplastic tissue and normal prostate epithelial cells but is absent at the protein level in neoplastic prostate tissue and in prostate cancer cell lines,

including PC3 (169, 170). The integrin β1C splice variant lacks important signaling motifs, such as the cyto-2 and cyto-3 NPxY domains, which are particularly important for localization of integrin β1 to focal adhesions (137). Accordingly, integrin β1C expression is growth inhibitory in several cancer cell lines, including PC3 cells (165) and it has been proposed that either the full length polypeptide or cytoplasmic domain may prove therapeutically useful for proliferative disorders, such as cancer (354). Importantly, the growth inhibitory activity of integrin β1C may depend on the presence of a short cytoplasmic sequence and not a dominant negative effect directed at integrin β1A function (167).

We sought to determine how the over-expression of wildtype integrin β1A or the growth inhibitory variant, β1C, would impact anchorage independent colony formation in PC3 cells. To this end, pBJ expression constructs for the integrin β1A and β1C isoforms driven by an SR alpha promoter (407) were obtained from Dr. Languino for use in soft agarose cloning experiments. It is clear, from western blot analyses using an antibody which recognizes both isoforms, that over-expression of either integrin β1 isoform was only modest. This may explain why anchorage independent colony formation was not different for pBJ-β1A-transfected PC3 cells compared to the empty vector control transfectants. Alternatively, this result agrees with the inability of an integrin β1 activating antibody to modulate colony formation in PC3 cells and may indicate that basal levels of expression and activity are sufficient to maximally promote integrin β1-dependent soft agarose colony formation.

Despite an apparently modest degree of over-expression, integrin β1C transfected PC3 cell populations consistently demonstrated a significantly reduced ability to form colonies in soft agarose compared to the empty vector transfectants. Whether this is the result of a dominant negative effect or a gain of function is not apparent and further

experimentation aimed at characterizing the impact of integrin β1C expression on adherent growth in our system is required to determine such an impact. However, because it has been shown to reduce thymidine incorporation of PC3 cells grown adherently to fibronectin (165) and we have shown that endogenous integrin β1 does not significantly modulate the proliferation of adherent PC3 cells, integrin β1C most likely inhibits proliferation independently of any impact on integrin β1A function. As such, integrin β1 variants or mutants which lack specific functional domains such as the cyto 2 and cyto 3 motifs or residues implicated in specific protein-protein interactions (137), but which do not contain the putative growth inhibitory sequence present in the integrin β1C cytoplasmic domain, would be useful to further elucidate the role of integrin β1 in anchorage independent colony formation. It is nonetheless of interest that expression in PC3 cells of an integrin β1 splice variant endogenous to untransformed prostate epithelial cells is sufficient to reverse the anchorage independent phenotype characteristic of malignant cells and certainly supports the future exploitation of this phenomenon for therapeutic purposes.

4.2.3 The Importance of Fibronectin in the Anchorage Independent Growth of PC3 cells.

Although spheroids that form in semisolid medium are characterized by intimate cell-cell contacts, they also contain ECM proteins, including fibronectin (408, 409). This is functionally important as several experiments have demonstrated a requirement for fibronectin in the anchorage independent growth of human breast cancer cell lines (54, 410). Additionally, it has been hypothesized that one reason for the dependence of some cancer cell lines on TGF-β in combination with other growth factors for anchorage independent colony formation (411) may be its role in stimulating the assembly of exogenous fibronectin, as well as cellular fibronectin production (54, 412, 413). Like their untransformed

counterparts, malignant cells synthesize fibronectin which they subsequently incorporate into fibrils (10). However, tumour cells and fibroblasts grown in suspension have been found to produce decreased levels of fibronectin compared to their adherent counterparts (54, 345). While fibroblasts also downregulate expression of integrin α5β1 in suspension (345) this has not been reported for tumour cell lines which may therefore continue to grow in the absence of anchorage by becoming more dependent on exogenous sources of fibronectin, such as from a feeder layer of cells or in the FBS commonly added to culture medium (54). In fact, bovine plasma fibronectin is readily incorporated into the fibrils associated with mammary tumour cell spheroids in soft agar and is indispensable for the anchorage independent survival and growth of these cells (54).

Because we had found that integrin β1 was important for the anchorage independent colony formation of tumour cells lines grown in 10% FBS-enriched medium but that PC3 cells were unable to form colonies in the absence of serum (data not shown) we asked whether fibronectin, as a major ligand of several β1 integrin heterodimers, was required for the soft agarose colony formation of PC3 cells. In agreement with previous studies demonstrating a role for fibronectin in anchorage independence, we found that the presence of a monoclonal antibody to fibronectin in the soft agarose medium significantly reduced the number of PC3 cell colonies formed compared to azide and IgG controls. Further experimentation in which fibronectin is directly removed from the agarose culture medium and re-introduced in a controlled manner will be necessary to more directly confirm the importance of fibronectin in this system and to establish whether it is primarily exogenous fibronectin mediating anchorage independent growth and survival. The relationship between β1 integrins and fibronectin in the context of anchorage independence is also a subject for

future investigation which could be explored using an integrin β1 mutant in the RGD or fibronectin-binding motif (352, 414).

Considering the impact of siRNA depletion and functional blocking of integrin β1 on soft agarose colony formation, together with a requirement for fibronectin in this process, a model in which ligation of β1 integrins by fibronectin is required for the anchorage independence of tumour cells is plausible. In this sense, anchorage independence is indicative of the ability of tumour cells to grow and survive in the absence of cell spreading but not in the absence of integrin ligation *per se*. Whether fibronectin fibrils provide necessary mechanical support to tumour spheroids is also unknown. Because soft agar assays for anchorage independence do not typically address the presence of fibronectin in serum-supplemented media, there is surprisingly little precedence for the idea of integrin-ligation dependent anchorage independence, however the general applicability of this idea is suggested by our findings indicating the dependence of several tumour cell lines of different tissue origins on integrin β1 expression for soft agarose colony formation.

Because anchorage independence in several tumour cell lines depends on it, integrin β1 may represent an important factor facilitating this characteristically malignant phenotype. It is unclear why tumour cells depend on integrin β1 specifically in the absence of anchorage. One possibility is that compensation for the loss of integrin β1 in adherent cells is mediated by the activity of other adhesion receptors, such as β3 integrins, which do not maintain this function in suspension. However, it is equally possible that integrin β1 performs a novel function, which is likely regulated by mechanical tension albeit still dependent on fibronectin ligation, and is specific to suspension survival and growth. Further study aimed at determining whether integrin β1 is specifically necessary for anchorage independent growth versus survival would facilitate our understanding of the mechanism by which integrin β1

modulates soft agarose colony formation. In this sense, others have observed the ability of an integrin β1-blocking antibody to reverse the malignant characteristics of spontaneously transformed breast epithelial cells in 3-dimensional culture (330). Importantly, although blocking integrin β1 strongly reduced the survival of untransformed mammary epithelial cells in 3-D, only growth and not survival of transformed cells was inhibited. Therefore we might infer that fibronectin ligation by β1 integrins necessarily promotes anchorage independent growth and not survival in tumour cells, although future experimentation will be required to address this issue.

4.2.4 Induced Suspension of PC3 cells and Modulation of Integrin β1-Mediated Events.

The ability of PC3 cells to grow in suspension was exploited in order to more easily understand the integrin-dependent molecular events which occur in the absence of anchorage. PC3 cells grown in suspension in the absence of serum have been found to depend on cell-cell contacts in order to escape apoptosis (415). In our system, PC3 cells readily formed aggregates in media containing 10% FBS when induced to grow in suspension over a solid 1% agarose base. Analysis by flow cytometry revealed that integrin β1 protein was equally expressed at the surface in PC3 cells grown adherently or induced to grow in suspension. While PC3 cells survive and proliferate in the absence of anchorage, untransformed fibroblasts, which are not able to undergo mitosis in the absence of anchorage, were found to down-regulate integrin α5β1 in suspension under otherwise usual culturing conditions (346). Because fibroblasts treated with TGF-β which elevated integrin α5β1 expression or clonal populations selected for increased integrin α5β1 protein levels were able to form anchorage independent colonies (346), it is sensible that PC3 prostate carcinoma cells which characteristically grow anchorage independently would not

downregulate integrin β1 in suspension. We did observe a modest but insignificant decrease in total integrin β1 expression in suspension compared to adherent PC3 cells based on average densitometry values from two independent western blot experiments, however given the surface expression data it is likely that this is not of functional importance.

Activation or overexpression of numerous integrin β1 downstream targets, including FAK, ILK, and β-catenin (367), has been shown to mediate anchorage independence in untransformed cells. The contribution of β-catenin to cell behaviour is twofold. First, it supports cell-cell-contacts by physically linking E-cadherin and α-actinin to the cytoskeleton. This is generally abrogated in malignant epithelial cells, and the loss of tight cell-cell connections is indicative of EMT and a subsequent invasive phenotype (366). As a result of release from E-cadherin, cytosolic pools of β-catenin are free to mediate pro-survival and growth signaling events. This second function of β-catenin is best understood in its capacity as a downstream target of the canonical Wnt (Wnt-1 being the human wingless homolog) signaling pathway, where upon cytosolic stabilization it translocates to the nucleus in association with the TCF/LEF transcriptional complex, and facilitates transcription of several known genes including cyclin D1, c-myc and many anti-apoptotic effectors thus contributing to both growth and survival mechanisms (416, 417). Degradation of β-catenin represents a major mode of its regulation and is initiated by adenomatosis polyposis coli (APC) and GSK 3 dependent serine phosphorylation. Accordingly, in addition to activating mutations in β-catenin, tumourigenesis is also associated with loss of function mutations in the tumour suppressor APC and in GSK-3 (366, 418). Previous work in breast epithelial cells linked decreased integrin β1 function and reversion of malignant characteristics in 3-dimensional culture with restored localization of E-cadherin-bound β-catenin at cell-cell junctions (330). In agreement with this, several important signaling molecules downstream of integrin β1,

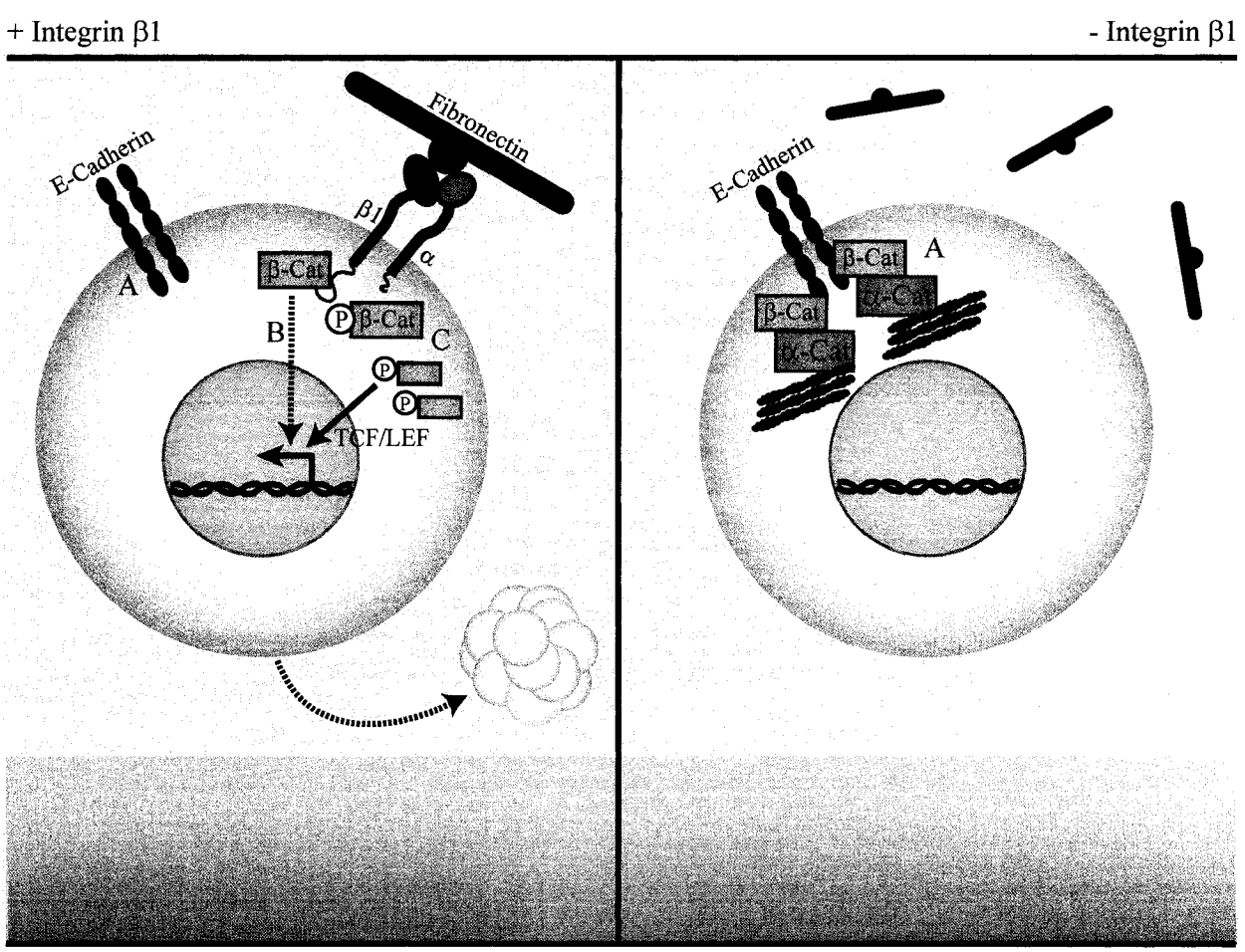
such as AKT and ILK, positively modulate the activity of β-catenin, primarily via their inhibition of GSK 3 or E-cadherin (232, 323). Thus as β-catenin is a known downstream effector of integrin signaling and is sufficient to enable anchorage independent growth in untransformed epithelial cells when overexpressed, we began our investigation by studying the impact of integrin β1 depletion and suspension growth on the expression and localization of β-catenin. Surprisingly, we did not observe a reduction in the total protein expression of β-catenin to accompany integrin β1 depletion in either adherent or suspension cultured PC3 cells. This indicates that for PC3 cells integrin β1 signaling likely does not modulate the stability of β-catenin. Because β-catenin classically activates transcription following nuclear translocation, it would be interesting to determine by further experimentation whether β-catenin is differentially distributed in the nuclear and cytoplasmic fractions when integrin β1 is depleted. Furthermore, as E-cadherin adhesion complexes sequester β-catenin rather than degrade it, a better understanding with regard to the membrane-bound, cytosolic, and nuclear localization of β-catenin in PC3 cells depleted of integrin β1 and cultured under different growth conditions may aid in understanding whether β-catenin activity is functionally linked to integrin β1 in the anchorage independent phenotype of cancer cell lines.

Despite the inability of integrin β1 protein expression to modulate cellular levels of β-catenin we made one important observation regarding its potential localization. When PC3 cells were grown in suspension β-catenin was found to co-immunoprecipitate with integrin β1, but this association was not observed in adherent cultures. This represents a novel interaction between integrin β1 and β-catenin which may function to facilitate survival or growth in a specifically anchorage independent way.

While inhibition of molecules mediating degradation results in its cytoplasmic accumulation, β-catenin must also avoid complexing with E-cadherin at cell-cell junctions in

order to mediate transcriptional events. One study has suggested that E-cadherin expression is induced in PC3 cells grown in suspension in the absence of serum (415), emphasizing the importance of actively maintaining a free pool of cytoplasmic β-catenin. Tyrosine phosphorylation of β-catenin has been shown to abrogate its interaction with E-cadherin and thus assembly of these junctions (419). Importantly, two signaling molecules with which integrin β1 interacts functionally, epidermal growth factor receptor (EGFR) and Src kinase, have been shown to phosphorylate β-catenin. As E-cadherin can suppress the anchorage independent growth of tumour cells (420) and both β-catenin and integrin β1 have been found to promote such a phenotype, it is possible that an integrin β1-dependent complex is responsible for anchorage independent growth in its capacity to render β-catenin unavailable for cell-junctional complexes and therefore able to mediate signaling events. In this way, accumulation of free cytoplasmic β-catenin via integrin β1-dependent phosphorylation may promote anchorage independent growth through the classical wnt pathway, by activating TCF-LEF-dependent gene transcription. Alternatively, β-catenin has been found to mediate transcriptional activation of Wnt-Induced Secreted Protein-1 (WISP-1), a gene overexpressed in many tumour cells including Wnt-1 transformed lines (421), in the absence of nuclear translocation (422). Although how this occurs is unclear, intermediate kinases are likely involved, and its participation in a novel integrin β1 containing complex may represent a general mechanism of cytoplasmic signaling for β-catenin. Therefore ECM-integrin β1 dependent events such as tyrosine phosphorylation at multiprotein complexes may modulate: A) the absence of β-catenin in cadherin-containing adhesion complexes, B) cytoplasmic signaling, and C) its translocation to the nucleus, potentially in a non-mutually exclusive way, in order to promote anchorage independent colony formation (see Figure 18). Further experimentation will be required to confirm the functional importance of an association

Figure 17. Model for Integrin β1-Dependent Anchorage Independence. Based on the findings described herein, this schematic highlights a proposed mechanism for integrin β1-mediated growth and survival of tumour cells in the absence of anchorage. Fibronectin ligation by integrin β1-containing receptors initiates specific intracellular signaling events which are necessary for anchorage independent colony formation but not colony formation in adherent cells. Recruitment of β-catenin to an integrin β1-containing complex (left panel) may regulate its function by A) impeding its interaction with E-cadherin and α-catenin at cell-cell junctions, B) promoting a novel signaling function for β-catenin at these focal complexes leading to downstream transcriptional events, and C) initiating tyrosine phosphorylation of β-catenin and promoting its translocation to the nucleus and transcription of downstream targets in conjunction with TCF/LEF DNA binding proteins. In the absence of integrin β1 protein at the cell surface (right panel), β-catenin is not recruited to the cell-suspension specific complex and can associate with E-caderin/α catenin cell-cell junctional complexes (A). Potentially novel signaling functions for β-catenin are abrogated (B) and free cytoplasmic β-catenin fails to accumulate (C), thus inhibiting the ability of cancer cells to form anchorage independent colonies.


+ Integrin β1
- Integrin β1
E-Cadherin
Fibronectin
β1
α
β-Cat
A
B
C
P
TCF/LEF
α-Cat

between integrin β1 and β-catenin in the anchorage independence of human tumour cells, and to identify other components of this novel complex. This will facilitate future endeavours aimed at elucidating a mechanism by which integrin β1 promotes anchorage independence in tumour cell lines.

4.3 SUMMARY

Our investigation into the role of the ECM-dependent activity of integrin β1 has led us to conclude that although ECM proteins and integrin β1-dependent signaling do not generally impact the adherent growth and survival of human tumour cell lines, anchorage independent colony formation seems to occur in a fibronectin and integrin β1-dependent way. As an *in vitro* assay intimately linked to tumourigenicity, the dependence of soft agarose colony formation on integrin β1 suggests that tumour cells maintain some dependency on extracellular matrix mediated signaling *in vivo*. Our work has also identified a novel association between integrin β1 and β-catenin which occurs only in non-adherent PC3 cells. As a specifically anchorage independent phenomenon, this association between β-catenin and integrin β1 may be relevant to the requirement for integrin β1 in anchorage independent growth. The preferential importance of integrin β1 in suspension and the existence of a suspension-specific complex containg integrin β1 and β-catenin suggest that tumour cells may activate novel signaling pathways in order to grow and survive in the absence of firm attachment to 2-D substrates. This in turn underlines the importance of understanding tumour cell phenotypes in 3-dimensional systems which have demonstrated a heightened potential to recapitulate *in vivo* phenotypes, in order to better address means of therapeutic intervention.

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Introduction

Preamble

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APPENDIX A

Figure S1. Plasmid Map for pBJ-β1A and pBJ-β1C. The plasmids, pBJ-β1A and pBJ-β1C were a gift from L. R. Languino. A 2.9kb fragment of wildtype integrin β1 cDNA was isolated from a pBLUESCRIPT vector and cloned into the pBJ vector using XbaI to produce pBJ-β1A (6.1kb). To construct pBJ-β1C, the 5' end of wild-type integrin β1 cDNA ending at bp 2357 was isolated from the same pBLUESCRIPT vector and the 3' end of integrin β1C (250bp) beginning at nucleotide 2358 was isolated from a PCR-1000 vector into which PCR products had been previously cloned. The 5' wild-type Xba/HindIII fragment (2.3kb) was then directionally ligated in frame with the 3' HindIII/EagI fragment encoding the variant cytoplasmic domain cDNA from integrin β1C (250bp) into the XbaI/NotI-linearized pBJ-1 vector to produce pBJ-β1C (5.8kb).

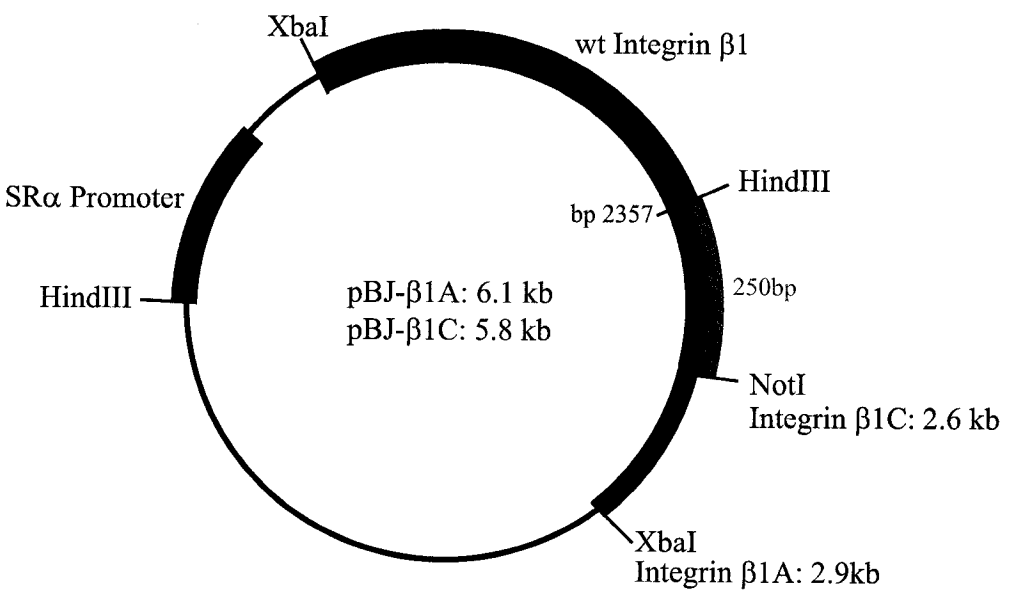
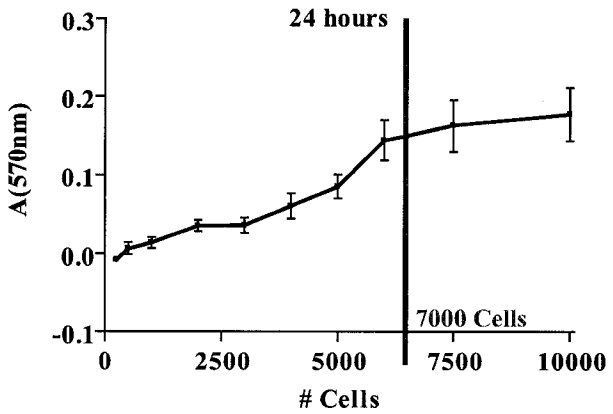
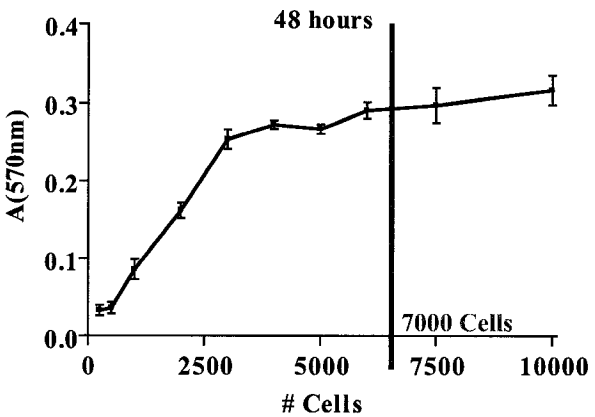

XbaI
wt Integrin β1
HindIII
bp 2357
250bp
SRα Promoter
HindIII
pBJ-β1A: 6.1 kb
pBJ-β1C: 5.8 kb
NotI
Integrin β1C: 2.6 kb
XbaI
Integrin β1A: 2.9kb

Figure S2. Optimal Cell Density for MTT Reduction Assay, A549 cells. A549 cells were counted and seeded at various densities, as indicated, onto plastic 96 well plates in 10% FBS-containing media. 24 (**i**), 48 (**ii**), or 72 (**iii**) hours following plating, the colorimetric change in absorbance at 570nM associated with MTT reduction in eight replicate wells of cells treated for four hours with MTT reagent and lysed overnight was measured. The mean absorbance value $\pm$ SD is depicted for each starting density at each time-point. An optimal density of 7000 cells was selected for cytotoxic assays because it provided a reading at the top of the linear range (the range in which two different values can be accurately differentiated) of absorbances when measured 72 hours post-plating.

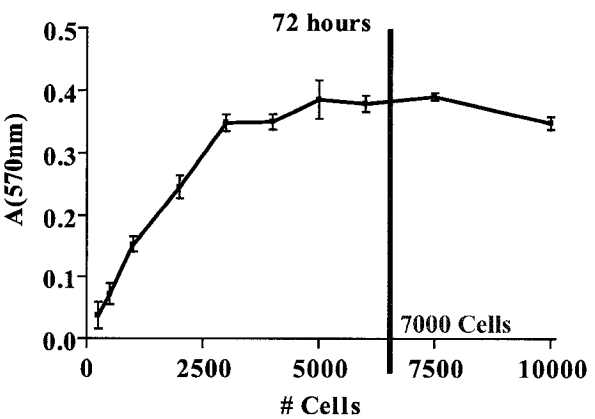
i)


24 hours
0.3
0.2
0.1
0.0
-0.1
A(570nm)
7000 Cells
0
2500
5000
7500
10000
Cells

ii)


48 hours
0.4
0.3
0.2
0.1
0.0
A(570nm)
7000 Cells
0
2500
5000
7500
10000
Cells

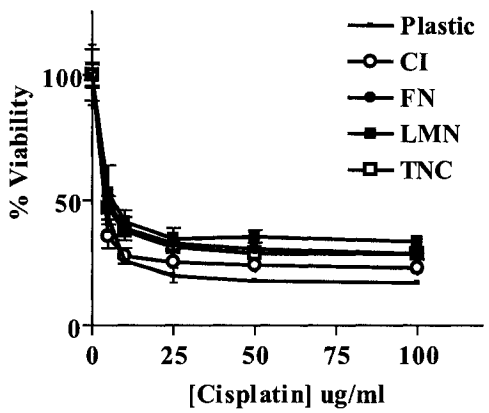
iii)


72 hours
0.5
0.4
0.3
0.2
0.1
0.0
A(570nm)
7000 Cells
0
2500
5000
7500
10000
Cells

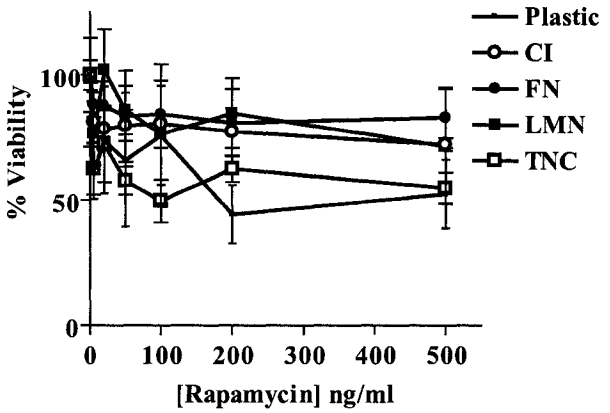
APPENDIX B

Figure S3. ECM protein does not modulate the response of A549 cells to chemotherapeutic drugs. A549 cells were seeded overnight onto plastic, fibronectin (FN), collagen I (CI), laminin (LMN) or tenascin-C (TNC) coated 96 well plates and treated with various concentrations of cisplatin **(i)**, rapamycin **(ii)**, or staurosporine **(iii)** in 10% FBS-containing media for 48 hours. Cytotoxicity was determined by measuring the colorimetric change in absorbance at 570nM associated with MTT reduction in eight replicate wells of cells treated for four hours with MTT reagent and lysed overnight. Cell viability is expressed as the mean percentage of MTT activity in untreated wells $\pm$ SD. **(iv)**, A549 cells were seeded overnight onto plastic, fibronectin (FN), collagen I (CI), laminin (LMN) or tenascin-C (TNC) coated plates and treated with various concentrations of etoposide in 10% FBS-containing media for 2 hours. Cells were subsequently cultured at low density and allowed to form colonies over 10 days. Cells were stained with methylene blue and colonies >50 cells in size were counted in 2 replicates from 4 dilutions and expressed as the mean percentage of colony forming cells in untreated samples $\pm$ SD.

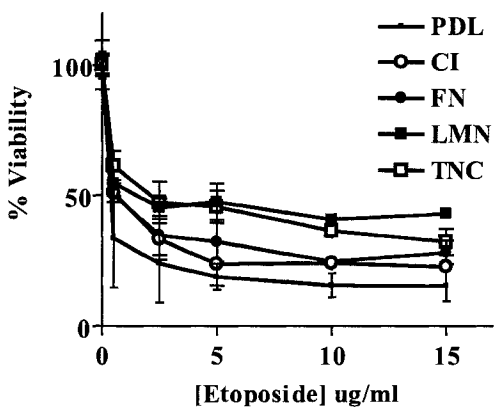
i)


Plastic
CI
FN
LMN
TNC
% Viability
100
50
0
0
25
50
75
100
[Cisplatin] ug/ml

ii)


Plastic
CI
FN
LMN
TNC
% Viability
100
50
0
0
100
200
300
400
500
[Rapamycin] ng/ml

iii)


PDL
CI
FN
LMN
TNC
% Viability
100
50
0
0
5
10
15
[Etoposide] ug/ml

iv)

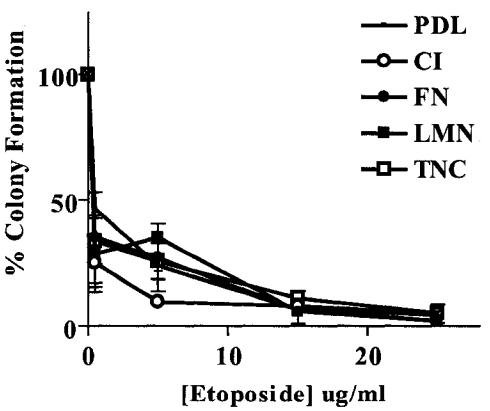
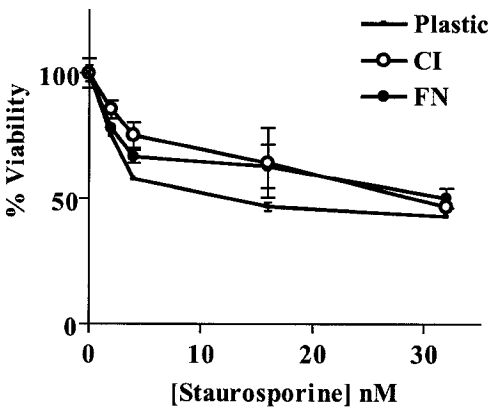

PDL
CI
FN
LMN
TNC
% Colony Formation
100
50
0
0
10
20
[Etoposide] ug/ml

Figure S4. ECM protein does not modulate the response of A549 cells to staurosporine or the water soluble analogue UCN-01. A549 cells were seeded overnight onto plastic, fibronectin (FN), or collagen I (CI) coated 96 well plates as indicated and treated with various concentrations of staurosporine **(i)**, or UCN-01 **(ii)** in 10% FBS-containing media for 48 hours. Cytotoxicity was determined by measuring the colorimetric change in absorbance at 570nM associated with MTT reduction in eight replicate wells of cells treated for four hours with MTT reagent and lysed overnight. Cell viability is expressed as the mean percentage of MTT activity in untreated wells $\pm$ SD.

i)


Plastic
CI
FN
100
50
0
% Viability
0
10
20
30
[Staurosporine] nM

ii)

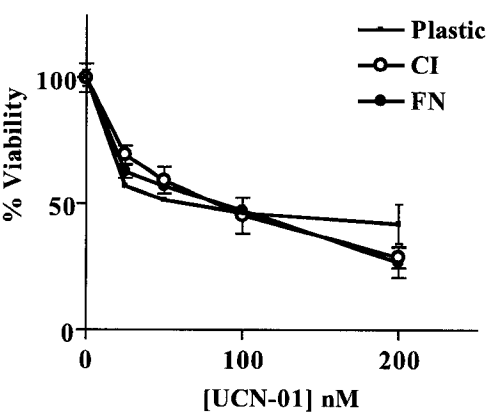
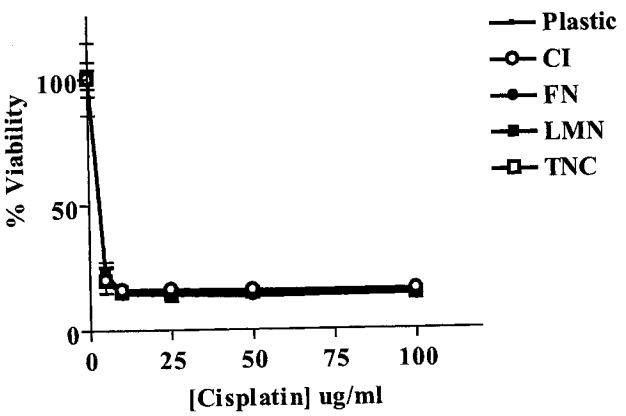
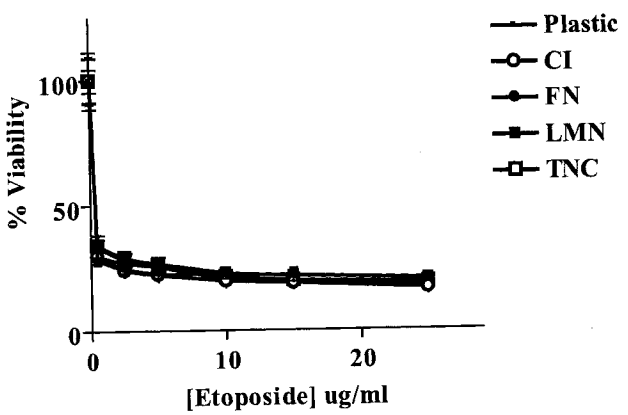

Plastic
CI
FN
100
50
0
% Viability
0
100
200
[UCN-01] nM

Figure S5. ECM protein does not modulate the response of H460 cells to chemotherapeutic drugs. H460 cells were seeded overnight onto plastic, fibronectin (FN), collagen I (CI), laminin (LMN) or tenascin-C (TNC) coated 96 well plates and treated with various concentrations of cisplatin **(i)**, etoposide **(ii)**, or rapamycin **(iii)** in 10% FBS-containing media for 48 hours. Cytotoxicity was determined by measuring the colorimetric change in absorbance at 570nM associated with MTT reduction in eight replicate wells of cells treated for four hours with MTT reagent and lysed overnight. Cell viability is expressed as the mean percentage of MTT activity in untreated wells $\pm$ SD.

i)


Plastic
CI
FN
LMN
TNC
% Viability
100
50
0
0
25
50
75
100
[Cisplatin] ug/ml

ii)


Plastic
CI
FN
LMN
TNC
% Viability
100
50
0
0
10
20
[Etoposide] ug/ml

iii)

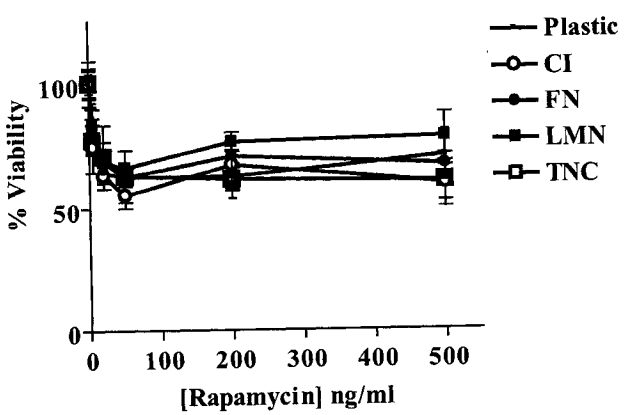
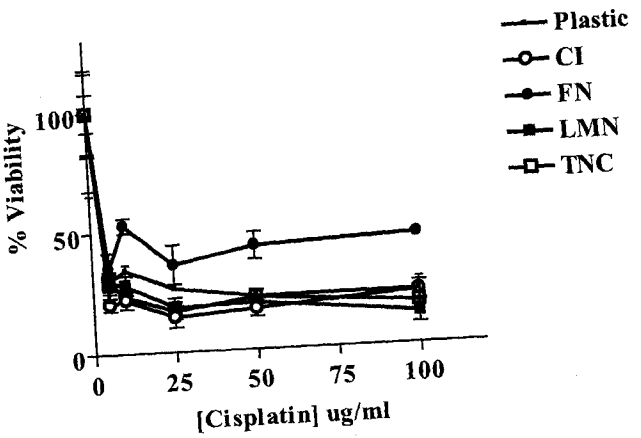
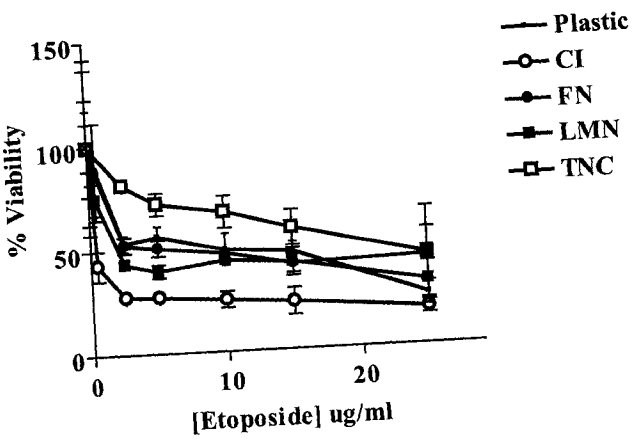

Plastic
CI
FN
LMN
TNC
% Viability
100
50
0
0
100
200
300
400
500
[Rapamycin] ng/ml

Figure S6. ECM protein does not modulate the response of Du145 cells to chemotherapeutic drugs. Du145 cells were seeded overnight onto plastic, fibronectin (FN), collagen I (CI), laminin (LMN) or tenascin-C (TNC) coated 96 well plates and treated with various concentrations of cisplatin (**i**), etoposide (**ii**), or rapamycin (**iii**) in 10% FBS-containing media for 48 hours. Cytotoxicity was determined by measuring the colorimetric change in absorbance at 570nM associated with MTT reduction in eight replicate wells of cells treated for four hours with MTT reagent and lysed overnight. Cell viability is expressed as the mean percentage of MTT activity in untreated wells $\pm$ SD.

i)


Plastic
CI
FN
LMN
TNC
% Viability
100
50
0
0
25
50
75
100
[Cisplatin] ug/ml

ii)


Plastic
CI
FN
LMN
TNC
% Viability
150
100
50
0
0
10
20
[Etoposide] ug/ml

iii)

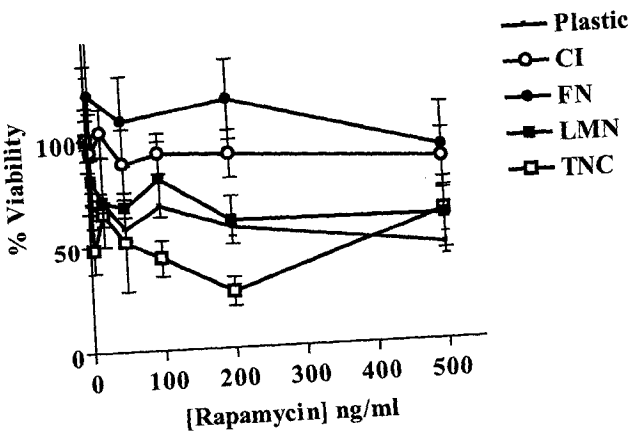
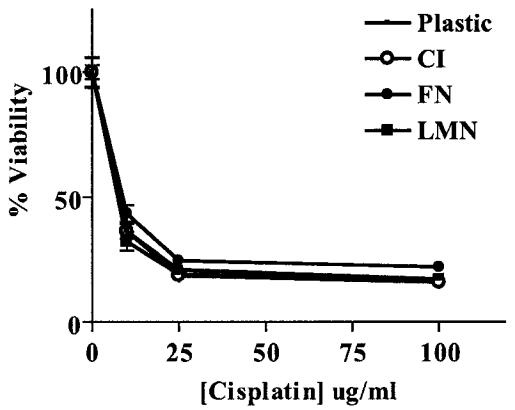
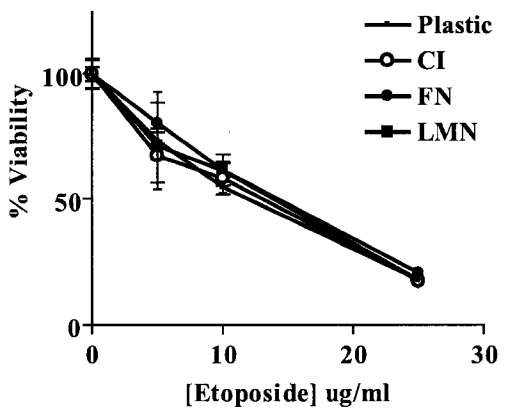

Plastic
CI
FN
LMN
TNC
% Viability
100
50
0
0
100
200
300
400
500
[Rapamycin] ng/ml

Figure S7. ECM protein does not modulate the response of SKNAS cells to chemotherapeutic drugs. SKNAS cells were seeded overnight onto plastic, fibronectin (FN), collagen I (CI), or laminin (LMN) coated 96 well plates as indicated and treated with various concentrations of cisplatin **(i)**, etoposide **(ii)**, or staurosporine **(iii)** in 10% FBS-containing media for 48 hours. Cytotoxicity was determined by measuring the colorimetric change in absorbance at 570nM associated with MTT reduction in eight replicate wells of cells treated for four hours with MTT reagent and lysed overnight. Cell viability is expressed as the mean percentage of MTT activity in untreated wells $\pm$ SD.

i)


Plastic
CI
FN
LMN
% Viability
100
50
0
0
25
50
75
100
[Cisplatin] ug/ml

ii)


Plastic
CI
FN
LMN
% Viability
100
50
0
0
10
20
30
[Etoposide] ug/ml

iii)

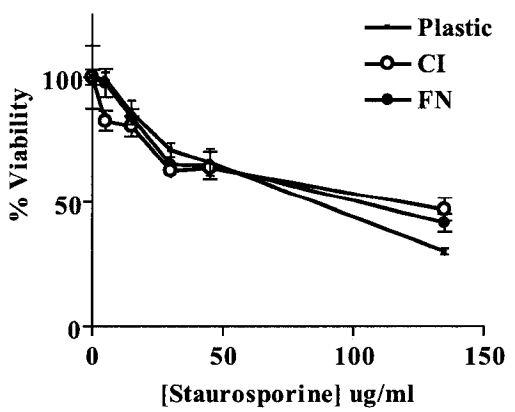
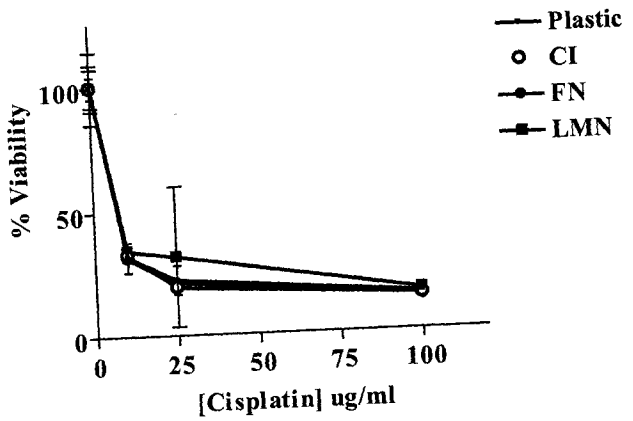
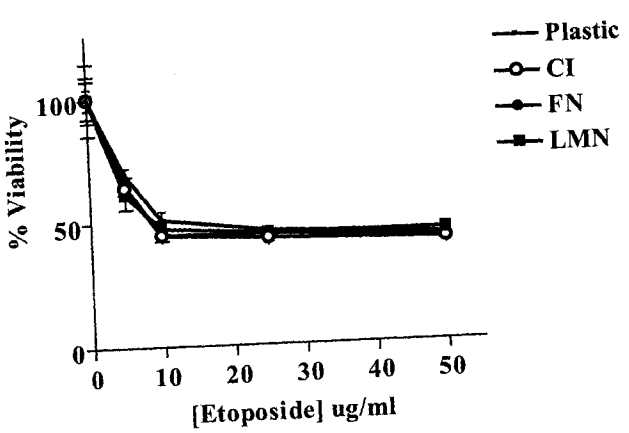

Plastic
CI
FN
% Viability
100
50
0
0
50
100
150
[Staurosporine] ug/ml

Figure S8. ECM protein does not modulate the response of SF295 cells to chemotherapeutic drugs. SF295 cells were seeded overnight onto plastic, fibronectin (FN), collagen I (CI), or laminin (LMN) coated 96 well plates as indicated and treated with various concentrations of cisplatin (**i**), etoposide (**ii**), or staurosporine (**iii**) in 10% FBS-containing media for 48 hours. Cytotoxicity was determined by measuring the colorimetric change in absorbance at 570nM associated with MTT reduction in eight replicate wells of cells treated for four hours with MTT reagent and lysed overnight. Cell viability is expressed as the mean percentage of MTT activity in untreated wells $\pm$ SD.

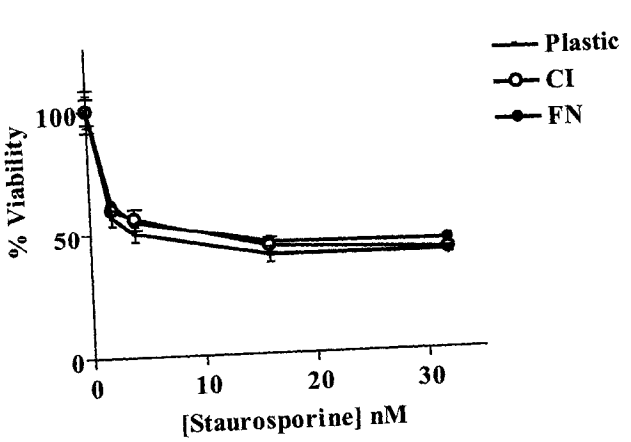
i)


Plastic
CI
FN
LMN
% Viability
100
50
0
0
25
50
75
100
[Cisplatin] ug/ml

ii)


Plastic
CI
FN
LMN
% Viability
100
50
0
0
10
20
30
40
50
[Etoposide] ug/ml

iii)


Plastic
CI
FN
% Viability
100
50
0
0
10
20
30
[Staurosporine] nM

ALLANA M. SCHOOLEY

LANGUAGES

English, French

ACADEMIC TRAINING

Master of Science, Biochemistry **2004-2008**
University of Ottawa, Canada
Function of the Cell Surface Receptor Component Integrin β1 in Human Tumour Cells.
Supervised by Dr. Christina Addison, Ottawa Health Research Institute

Bachelor of Medical Science, Honours Biochemistry **2000-2004**
University of Western Ontario (UWO), Canada

Honours Project, UWO **2003-2004**
Exploring the Importance of Methylation of the Myogenin Promoter in Early Mouse Myogenesis.
Supervised by Dr. Siu Pok Yee, London Regional Cancer Program

West Carleton Secondary School, Ottawa, Canada **1998-2000**
Received Ontario Secondary School Diploma

Canterbury High School for the Performing Arts **1995-1998**

ACADEMIC AWARDS

- Ontario Graduate Studies in Science and Technology Graduate Studies Award $30,000 CAD over two years. **2005-2007**
- Named to dean's honours list University of Western Ontario. **2002, 2004**
- Named Ontario Scholar, OAC **2000**

DEGREES GRANTED

Bachelor of Medical Science, Honours Biochemistry **2004**
University of Western Ontario, Canada
Graduated with Honours (>80%)

OTHER TRAINING AND INTERESTS

Les Petits Ballets **1996-2000**
Studied ballet at the pre-professional ballet school and company

Carp Quality Cleaners **1999-2000**
Part time Cashier

Richmond Nursery garden centre and strawberry farm **2001**
Full-time summer employment

Amnesty International **1998-2008**

University of Western Ontario Dance Company **2002-2004**

Oxfam Canada (University of Western Ontario) **2001-2004**

Dental Assistant
Part-time summer employment, Dr. C. Davidson **2004**

Dental Assistant **2002-2004**
Full-time summer employment, Dr. D.F. Schooley

Let's Talk Science Volunteer **2005-2007**