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INTEGRIN α6β4 CONTRIBUTIONS IN PANCREATIC ADENOCARCINOMA

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INTEGRIN α6β4 CONTRIBUTIONS IN PANCREATIC ADENOCARCINOMA

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Dedication

I will like to dedicate my dissertation work to my loving husband and soul mate who has always believed in me more than I believe in myself.
Te amo con todo mi corazón, gracias por tu apoyo incondicional. Sin ti no lo hubiera podido lograr. Gracias MI AMOR.

I also dedicate this work to all those pancreatic cancer patients who have lost the battle with this deadly disease, hopefully my small contribution to the field will help in understanding this cancer a bit more, so that in the future we can provide new tools for those who will join this battle!
May your souls rest in peace!

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INTEGRIN α6β4 CONTRIBUTIONS IN PANCREATIC ADENOCARCINOMA

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Pancreatic adenocarcinomas are among the most lethal human cancers due to an elevated incidence of tumor cell invasion and metastasis for reasons that are currently unclear. In this dissertation, I determined how the pro-invasive integrin α6β4 expression was related to pancreatic adenocarcinoma tumor progression in tumor samples and assessed *in-vitro* if the expression of this integrin was required for the migration and invasion of pancreatic cancer cells. In addition, I explored the mechanisms of how the α6β4 integrin could contribute to an invasive and migratory phenotype. To examine if integrin α6β4 expression was related to cancer progression, immunohistochemical analysis was performed in normal pancreas, pancreatic intraepithelial neoplasia (PanIN) lesions, pancreatic adenocarcinomas, and chronic pancreatitis. In normal pancreatic ducts, integrin α6β4 was noted only at the cell's basal interface with the basement membrane. In pancreatic adenocarcinomas, 92% (104/113) demonstrated overexpression

of integrin α6β4 and altered localization to the cytoplasm and membranous regions. This pattern of expression was observed in all PanIN lesions as early as PanIN-1A. In contrast, 93% (13/14) of chronic pancreatitis samples resembled the staining pattern of normal pancreas. When cancer was present in areas of chronic pancreatitis, this altered expression of α6β4 integrin identified the cancer. These results indicate that the early overexpression of the α6β4 integrin in pancreatic adenocarcinoma progression may contribute to the malignancy of this disease.

To understand the contribution of integrin α6β4 expression in cell migration and invasion, cell lines were screened for the presence of integrin α6β4 by immunoblotting and fluorescence activated cell sorting and correlated with their ability to migrate towards hepatocyte growth factor (HGF), a known mitogenic and motility factor of pancreatic carcinomas. I found that cell surface expression positively correlated with the cell line ability to migrate and invade towards HGF. When cells expressing high levels of integrin α6β4 were treated with siRNAs targeting the α6 or β4 integrin subunits, I observed a reduction in HGF-induced migration and invasion. Furthermore, the activity of the small GTPase Rac-1 decreased when α6β4 integrin expression was reduced. In addition, expression of the Rac-specific nucleotide exchange factor, Tiam-1 was upregulated by the integrin α6β4 and required for Rac-1 activity. These results suggest that the integrin α6β4 plays an important role in the migratory and invasive phenotype of pancreatic carcinoma cells and that the Tiam-1-Rac1 pathway is an important mediator of integrin α6β4-mediated HGF-induced migration and invasion. Overall this study provided evidence that integrin α6β4 is an important contributor to the migratory and invasive phenotype that characterize pancreatic adenocarcinomas.

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CHAPTER ONE: INTRODUCTION

Pancreatic Cancer Biology

The pancreas is the organ responsible for regulating protein and carbohydrate digestion as well as glucose homeostasis. It is regionally divided into the head, neck, body and tail and is composed of two systems: the exocrine and endocrine pancreas. The exocrine pancreas is arranged in a branching network of acinar and ductal cells that produce digestive enzymes which are secreted into the duodenum. The endocrine pancreas is composed of islet cells that produce hormones that are transported via the bloodstream and regulate metabolism and glucose homeostasis.

Most pancreatic cancers (around 75%) develop specifically in the exocrine pancreas and are confirmed by histology as adenocarcinomas (Lillemoe et al., 2000). Pancreatic adenocarcinomas from the exocrine pancreas are the most common type of pancreatic malignancy and 65% of them occur in the head of the pancreas (Solcia et al., 1997). They represent 5% of all cancer related deaths in virtually all industrialized countries and have the worst prognosis of all human cancers causing more than 31,000 deaths per year in the United States alone (Jemal et al., 2006). The overall 5-year survival rate for patients diagnosed with pancreatic adenocarcinoma has not changed in decades and remains less than 5% (Wray et al., 2005). The location of the pancreas in the retroperitoneum makes it difficult for physicians to detect pancreatic cancers during routine physical examinations which contribute to the late diagnosis of most cases. The most common reason for such poor prognosis comes in part from the lack of effective and sensitive methods to detect this cancer at an early stage. Patients often present symptoms only when the cancer is already in an advanced state and curative resection is no longer possible.

Pancreatic adenocarcinomas are often unresectable at the time of diagnosis because of invasion to nearby organs or large vessels and metastasis to distant sites such as the liver,

lungs, skin, peritoneum and adrenals (Solcia et al., 1997). Pancreatic adenocarcinomas can metastasize to other organs via lymphatic channels, blood vessels, and nerves. Nerve invasion, commonly referred to as perineural invasion, is a widely accepted route for the spread of pancreatic adenocarcinomas, it is present in more than 90% of the cases and has been correlated with poor prognosis of the disease (Hirai et al., 2002; Pour et al., 1991; Takahashi et al., 1997). Resection in patients with perineural invasion is difficult because their tumor margins are not well defined. One of the reasons for the often dissemination of pancreatic adenocarcinomas is that the posterior pancreatic surface lacks a restraining covering and is in close contact with the retroperitoneal structures, including the inferior vena cava, abdominal aorta, and a profuse network of lymphatic channels and neural plexus (Ohta et al., 1993). Interestingly, rarely is pancreatic cancer due to metastasis of a cancer that originated in another organ; although a case was reported in which a patient diagnosed with lung cancer had a tumor form in the head of the pancreas that was around 3 cm and was identified as a metastasis from the lung (Kubota et al., 2003).

The causes for pancreatic adenocarcinoma are still under investigation but some risk factors have been identified, such as age (80% of pancreatic adenocarcinoma patients are between the ages of 60 – 80), race (there is a higher incidence in African Americans than in whites), and sex (it more frequently occurs in men) (Tominaga and Kuroishi, 1998). Other factors such as chronic pancreatitis, smoking, obesity, nutrition and hereditary genetic alterations, like the carriers of the BRCA2 and p16 germline mutations have a 10-20 fold increased risks, (Lillemoe et al., 2000).

Pancreatic adenocarcinomas are classified by histology as *well-differentiated, moderately differentiated,* and *poorly differentiated*. However, the different state of differentiation is often not relevant to the patient if the cancer has already metastasized to other sites. Another histological feature that characterizes pancreatic adenocarcinomas is the presence of desmoplasia. Desmoplasia is described as an increase in dense fibrous

connective tissue (predominantly collagen type I and other glycoproteins) caused by the proliferation of fibroblastic cells (Omary et al., 2007). This desmoplastic reaction can infiltrate and surround the carcinoma were it sometimes can outnumber the local tumor cells. Interestingly, desmoplasia is a feature that characterizes the pancreas of chronic pancreatitis patients. Chronic pancreatitis is an inflammatory disease of the pancreas that results in irreversible deterioration of pancreatic structure and function. Therefore, desmoplasia in pancreatic adenocarcinoma and chronic pancreatitis often results in increase confusion in diagnosing these diseases (Iacobuzio-Donahue et al., 2002).

Pancreatic adenocarcinomas are thought to arise from noninvasive epithelial precursor lesions. There are three types of lesions, the noninvasive lesions, which include the intraductal papillary mucinous neoplasms that are usually more than 1 cm and the mucinous cystic neoplasms, and the proliferative pre-malignant lesions termed pancreatic intraepithelial neoplasia (PanINs) which are less than 5 mm in size (Hruban et al., 2000). PanIN lesions are grouped into three histologic stages (PanIN 1-3) based on increasing degrees of architectural and nuclear atypia (Kern et al., 2001). The knowledge of PanIN lesions dates back to the 1900s (Hulst, 1905) and are more commonly found in pancreatic adenocarcinomas than in normal pancreas (Cubilla and Fitzgerald, 1976). There have been patients with PanIN lesions who years later develop pancreatic adenocarcinoma and the genetic alterations in such lesions resemble the associated pancreatic adenocarcinoma. Although early PanINs (PanIN-1A and PanIN-1B) are considered carcinoma in situ, molecular studies have revealed that PanIN-2 and PanIN-3 lesions represent a distinct step towards invasive carcinomas (Kloppel and Luttges, 2004). PanIN lesions have been associated with genetic mutations or gene deletions that aid in the development of pancreatic adenocarcinomas. Telomere shortening is one of the initial changes associated with early PanIN lesions and has been correlated with the genomic instability that characterizes pancreatic adenocarcinomas that can help them develop towards an invasive carcinoma

stage (van Heek et al., 2002). Activated K-ras mutations have been documented to be present in 80-100% of infiltrating carcinomas and PanIN lesions and is the first known genetic alteration that occurs sporadically in normal pancreas tissue (Caldas et al., 1994). Point mutations in the K-ras gene that lead to its constitutive activation are the substitution of a glycine with aspartate, valine or arginine (codon 12, from GGT to GAT or GTT, and more rarely CGT). Some of the consequences of K-ras constitutive activation are the activation of multiple signaling pathways such as the RAF-mitogen-activated kinase, phosphoinositide-3-kinase (PI3-K) and Ral GDS pathways which can promote changes in cell growth, differentiation, apoptosis, migration and invasion of cancer cells (Campbell et al., 1998). Furthermore, loss of tumor suppressor genes such as p16Ink4a, p53, and DPC4/SMAD (Caldas et al., 1994; Kloppel and Luttges, 2004) and overexpression of growth factors like hepatocyte growth factor, transforming growth factor β, fibroblast growth factor, epidermal growth factor and vascular endothelial growth factor (Lillemoe et al., 2000) are among some of the changes seen during pancreatic adenocarcinoma progression. Interestingly, pathways that are known to regulate development and are subsequently turned off are now becoming important during cancer development. In the case of pancreatic adenocarcinomas, signaling in the Hedgehog and Notch pathways seems to be necessary for tumor maintenance and malignant progression (Murtaugh et al., 2003; Thayer et al., 2003).

However, the molecular mechanisms linking these genetic changes to the aggressive nature of pancreatic cancers remains poorly understood. In Chapter 2 of this dissertation I focus on the discovery of another molecular maker (integrin α6β4) at an early stage in pancreatic adenocarcinoma development that could help provide an understating of why this cancer has such a poor prognosis.

Pancreatic cancer treatment

Most pancreatic adenocarcinoma cases are discovered when the cancer is at an advance metastatic stage and are not eligible for surgical resection. Pancreatic resection is by far the only possible cure for this disease but only 15-20% of patients are eligible. However, even if a patient undergoes surgical resection the 5-year survival rate remains at approximately 20%, suggesting that even patients with localized tumors have the possibility of dying from a metastatic disease (Yeo et al., 1997). The only option for patients with a metastatic disease is chemotherapeutic treatment. Currently there are only two therapies approved to treat unresectable and metastatic pancreatic adenocarcinomas: gemcitabine (a drug that replaces cytosine during DNA replication and promotes arrest of tumor growth as new nucleotides cannot be attached, resulting in cell apoptosis) and more recently gemcitabine in combination with Tarceva (an epidermal growth factor receptor tyrosine kinase inhibitor). However, these therapies do not cure the patients and can only provide at best an additional 3 to 6 months of survival (Burris et al., 1997; Moore et al., 2007). In addition, most pancreatic adenocarcinomas have the ability to acquire resistance to these chemotherapeutic treatments. This resistance can be caused by many processes such as, changes in membrane receptors, alteration in drug transport at the cell membrane, ineffective metabolic conversion to active compounds, increased DNA repair mechanism and/or deregulation of the apoptosis pathway (Zalatnai and Molnar, 2007).

Metastasis

It is clear that what kills cancer patients is not the localized primary tumor, but the subsequent metastasis, as it occurs with pancreatic adenocarcinoma patients. Metastasis is the process by which tumor cells spread to other parts of the body through the blood stream or lymphatic vasculature, and grow relentlessly at secondary sites. The process of metastasis has been studied for more than 100 years and only recently have scientists

begun to understand some of the mechanisms involved. It is the result of genetic and epigenetic changes that occur in cancer cells of a primary tumor. Metastasis is not a random process. Mechanical arrest of tumor cells in the capillary bed of distant organs occurs as well as proliferation and growth in the secondary lesions which are influenced by organ specific cells (Hart and Fidler, 1980). Success of the metastatic process depends on the properties of tumor cells, the responses from target sites and the completion of a series of sequential steps (Fig. 1.1). Each step is rate limiting as failure to accomplish one can stop the process. For cells to become metastatic they must exchange signals that can modify the local environment to promote: 1) neoplastic transformation, proliferation and angiogenesis, 2) detachment of tumor cells from the primary tumor lesion and invasion of the local stroma, where they must first penetrate the basement membrane and then invade by active proteolysis, 3) embolization of the tumor cells to invade the subendothelial basement membrane or cell aggregates into the circulation, 4) adherence of tumor cells to the subendothelial basement membrane (extravasation), 5) evasion of the host immune defenses, and 6) invasion into the organ parenchyma with subsequent progressive growth (Fidler, 1990). Clearly the metastatic cascade includes a series of steps, all of which require molecular controls that are extremely diverse from tumor to tumor and due to their complexity are poorly understood. A better understanding of how all these steps are regulated, in combination with the known genetic changes that occur which regulate progression and metastasis during pancreatic adenocarcinoma, is necessary.

Cell Migration and Invasion

It is fundamental for metastatic tumor cells to acquire migratory and invasive properties in order to reach a secondary site away from the primary tumor. Understanding the cellular and molecular basis involved has been a challenge, as these processes seem to be cell and tumor type specific. Recent studies have provide unexpected evidence of

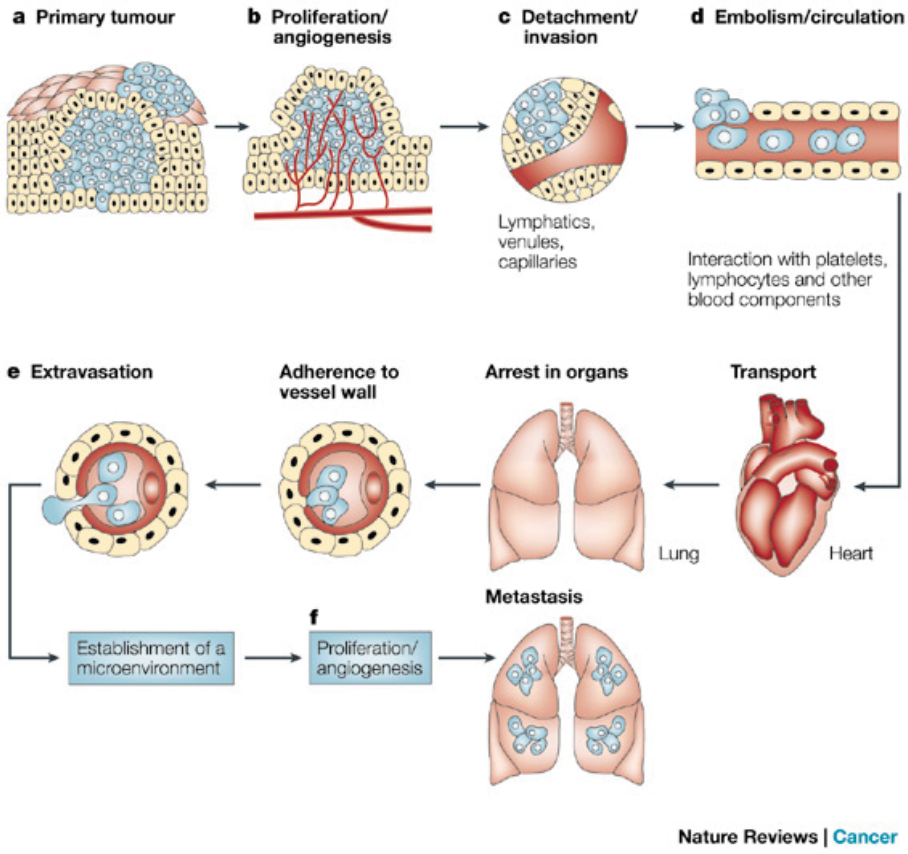


Figure 1.1. The main steps in the formation of metastasis. (A) Cellular transformation and tumor growth. (B) Formation of vascularization if the tumor mass excees 1–2 mm. (C) Local invasion of the host stroma by some tumor cells. Thin-walled venules, such as lymphatic channels, provide the most common route for the entry of tumor cells to the circulation. (D) Detachment and embolization of single tumor cells or aggregates occurs, were most circulating tumor cells are rapidly destroyed. After the tumor cells survived the circulation, they become trapped in the capillary beds of distant organs by adhering either to capillary endothelial cells or to subendothelial basement membrane that might be exposed. (E) Extravasation occurs probably by similar mechanisms from those used during invasion. (F) Proliferation at the new site completes the process. Micrometastasis can then follow the same steps and produce additional metastases. Reprinted and modified with permission from Macmillan Publishers Ltd: Nature Reviews Cancer, The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Fidler, I.J. Vol. 3 Pages 453-458. Copyright (2003).

adaptation responses resulting in a degree of plasticity where cells can switch to alternative mechanisms of migration (e.g. collective, mesenchymal, or amoeboid migration) in response to different conditions in their microenvironment (Wolf and Friedl, 2006). Nevertheless, the more we learn about all these processes, the more we will understand how cancer cells disseminate. In this section I will describe some of the known mechanisms of tumor cell migration and invasion that allow them to metastasize to distant sites.

Mechanisms of cell migration

Cell migration is a critical process in all living organism. In multicellular organisms, cell migration is essential for physiological and pathological processes, including embryological morphogenesis, wound healing, immune responses, inflammation, vascular diseases, chronic inflammatory diseases, tumor formation, tumor cell dissemination and metastasis. Understanding the mechanisms of cell migration has been a challenge because of the multiple integrated processes that are been regulated simultaneously. Most of what is known about the mechanisms of cell migration come from studies in two-dimensional (2-D) surfaces using non-neoplastic fibroblasts, keratinocytes and myoblasts, although it is believed that most of the same basic mechanisms are retained by tumor cells to spread within tissues (Friedl and Brocker, 2000; Lauffenburger and Horwitz, 1996).

The current model of cell migration as a dynamic process was described by Lauffenburger and Horwitz in 1996 and more recently by Friedl and Wolf in 2003, and summarizes an immense amount of research performed in multiple systems, into a single model that encompasses five interdependent steps of cell migration. The five-step model of cell migration describes a cyclical process that usually starts as a response to an external stimulus and leads to the polarization and extension of cell protrusions towards the stimulus. Cellular protrusions are actin-rich membrane processes that lack organelles and differ in their morphology and dynamics. They include sheet-like lamellipodia, thin spike-like

filopodia, or cylindrical finger-like pseudopods (thicker than filopodia) (Adams, 2001) and are responsible for initiating the cells extracellular matrix recognition. In step one of the five-step model, moving cells become polarized and elongated. Cellular protrusions are formed in the leading edge by the connection of actin filaments to adaptor proteins that help push the membrane outward in an adhesion independent manner. Actin polymerization at the leading edge to promote membrane protrusions requires the interactions of molecules such as the Arp2/3 complex, Wiscott-Aldrich syndrome protein (WASP), phosphoinosides and small GTPases such as Rac, cdc42, and Rho, as well as Ras (Kaibuchi et al., 1999; Rohatgi et al., 1999). In step 2, integrin molecules (which are discussed in detail in the next section) such as α5β1, α6β1, αvβ3 and α2β1, mediate adhesion to extracellular matrices that promote the formation of focal contacts which act like "clutches" to provide traction for cells to move. Focal contacts are clusters of integrins that can recruit adaptor and signaling molecules via their intracellular domains to control the regulation of downstream molecules and promote the recruitment of actin binding proteins such as, vinculin, paxillin and α-actinin, and regulatory molecules such as, PI3-K and Rho small GTPases (Lauffenburger and Horwitz, 1996). In step 3 (this step applies when migration occurs in three-dimensional (3-D) matrixes), proteases, if expressed at the cell surface, are recruited to attachment sites where focalized proteolysis occurs to help degrade extracellular molecules (ECM), such as collagen, fibronectin and laminins, which are close to the cell surface. Proteases can also promote cleavage of pro-matrix metalloproteinases (MMPs), to form active MMPs, which are enzymes such as MMP2, involved in the degradation of ECM (Ohuchi et al., 1997). In step 4, the binding of actin filaments with active myosin II (actomyosin) creates contraction and tension along the cell body and promotes cell length shortening creating a gradual gliding of the rear end of the cell. This process is dependant on calcium and the calmodulin-dependent myosin light-chain kinase (MLCK). Rho is known to regulate MLCK predominantly through its effector, ROCK. Phosphorylation of ROCK inhibits

the phosphatase that inactivates MLCK, MLCPtase. This results in increased contractility (Riento and Ridley, 2003). Lastly in step 5, detachment of the cells trailing edge occurs by the disassembly of the focal contacts that were initially formed in step 2. One mechanism for detachment of the trailing edge is by actin binding and severing proteins such as, gelsolin and cofilin which promote actin filament breakage and therefore filament turnover.

The speed of movement in a given cell type is dependent on the turnover rates of adhesion and de-adhesion events. Stabilization of focal contacts increases attachment of a cell to the substrate and impairs cell movement. Weakening of these contacts can promote migration. However, the balance between these events and molecules is required for cells to move in a particular direction (Lauffenburger and Horwitz, 1996). Tumor cells can use different types of migration mechanisms where they can migrate either as individuals moving by an amoeboid or mesenchymal-type movement, or as collective cell sheets, strands and clusters (Friedl and Wolf, 2003). Cells follow a specific strategy depending on the expression of integrins, proteases, and cadherins, as well as their state of differentiation. In many tumors, both types of movement (individual and collective) have been seen. Epithelial tumors commonly migrate in a collective manner, but the lower the differentiation stage, the more likely the tumor cells are to move individually (Thiery, 2002). Epithelial tumors that migrate as individuals tend to lose their cell contacts, detach, and migrate through connective tissue, a process called epithelial-mesenchymal transition (Thiery, 2002). Collective migration (cluster or cohorts) is seen in epithelial cancers that keep a high or intermediate state of differentiation, such as breast and colon carcinomas, although *in vivo* studies show that mammary tumor cells migrate as solitary amoeboids as well (Condeelis and Segall, 2003). This method of migration provides an added advantage because cells can work together to move towards a particular direction and survive the process. Cells cluster could secrete factors to increase autocrine signals, stimulate pro-migratory factors and matrix proteases that could help the migratory process.

In addition, these factors could help protect cells in the inner regions of the cluster from an immunological response that could kill them. The five-step model of migration previously described applies for both individual and collective migration. Many of the molecules involved in the processes of cell migration have been found to be altered in pancreatic cancers.

Mechanisms of tumor invasion

Tumor cell invasion is a process that requires the coordination of adhesion, proteolysis of ECM components and migration. It commonly occurs during normal cell morphogenesis and wound healing. Tumor cells rely on invasion to complete multiple steps of the metastatic cascade (e.g. angiogenesis, intravasation, and extravasation) to penetrate tissue barriers, such as the basement membrane and interstitial stromal connective tissue of the surrounding tissues and vasculature (Condeelis and Segall, 2003). The basement membrane, the first barrier of invading cells, is an insoluble continuous but flexible structure that underlies epithelia and endothelia and surrounds, muscles, testis and eye lens (Kalluri, 2003). The stromal connective tissue is a gelatinous extracellular matrix rich in proteoglycans that surrounds glands and blood vessels. For tumor cells to invade, cells must first attach to their surrounding matrix via cell surface receptors (e.g. integrins) to provide a cell-matrix anchor. Then tumor cells must have the capability of secreting proteolytic enzymes to help degrade the encountered matrix and use their migratory properties to move along the degraded matrix (Liotta, 1986). Tumor cells must continue this cyclical process until cells reach secondary sites, where they are then classified as micrometastases.

For epithelial tumor cell invasion to occur, cells must lose their junctional contacts between adjacent cells, unless they are using a method of collective migration previously described. This could be mediated by the alteration in expression and function of several adhesion molecules whose disruptions can increase the motility of tumor cells. Some

specific cell surface-associated molecules involved include the family of integrin receptors (discussed below), the cadherins, and the immunoglobulin (IgG) superfamily. For example, E-cadherin is an important suppressor of invasion and metastasis and in the majority of pancreatic cancer tumors there is a correlation between loss of E-cadherin expression and poor prognosis (Karayiannakis et al., 2001; Yonemasu et al., 2001). In addition, loss of β catenin (a subunit of the cadherin protein complex) expression may help predict poor prognosis in pancreatic cancers (Julkunen et al., 2003).

During the invasion and metastasis of many cancers the MMPs, enzymes responsible for ECM degradation, play a critical role (Deryugina and Quigley, 2006). These molecules are important in migration, invasion and metastasis because they can degrade the extracellular matrices allowing cancer cells to invade and migrate through the basement membrane, stromal components, and endothelial layer for access to the circulation. In addition, the tissue inhibitors of metalloproteinases (TIMPs), which control the activity of MMPs, are important as they provide balance between these two to maintain tissue homeostasis. Pancreatic cancer cells are known to produce high amounts of MMPs, especially MMP2 (Bloomston et al., 2002) and the MMP inhibitor prinomastat (AG3340) can inhibit pancreatic tumor growth and progression in an orthotopic model (Alves et al., 2001).

Overall, combinations of tightly regulated factors that control migration and invasion events are necessary for tumor cells to complete the steps of the metastasic cascade that eventually led to patient death.

Integrins

The integrin family of heterodimeric cell surface receptors binds to ECMs (such as laminin, collagen and fibronectin). Integrins are one of the major families of cell adhesion receptors for extracellular matrix proteins in multicellular animals (Humphries et al., 2006;

Hynes, 2002). They are composed of two type I transmembrane glycoproteins subunits, α and β, that transmit both mechanical and chemical signals to cells. Each of these subunits has a large extracellular domain that contains the ligand binding site, a single transmembrane region, and a short cytoplasmic tail (except for the β4 subunit, described below) (Arnaout et al., 2005). The mammalian genome consists of 18 α and 8 β subunit genes that are associated in various combinations to form at least 24 integrins that can bind to different extracellular matrix components or ligands on the surfaces of other cells (Hynes, 2002). The cytoplasmic region organizes the assembly of cytoskeletal polymers and signaling complexes.

Integrins play a key role in the transmission of bidirectional signals between the extracellular and intracellular environments that can control processes such as cell migration, invasion, adhesion, survival, proliferation, apoptosis, phagocytosis and transcriptional regulation. The name comes from their ability to integrate signals from both environments (Hynes, 1987). Most integrins, except the α6β4 integrin (discussed below), regulate cytoskeletal dynamics, adhesion, and migration events through associated proteins such as focal adhesion kinase (FAK), paxillin and p130Cas. Integrins mediate traction and signal transduction cascades that are required for invasion to the tissue stroma and migration of cells to new tissues. Moreover, signaling from and adhesion to extracellular matrices by the alteration of integrin expression is associated with tumor development and metastasis (Guo and Giancotti, 2004). One example, is the upregulation of the α6β4 integrin in many types of metastatic cancers (Mercurio and Rabinovitz, 2001).

Integrin α6β4

Of relevance to this dissertation is the α6β4 integrin. This integrin is expressed primarily on the basal surface of most epithelia. Its primary function is to maintain the integrity of epithelia through its ability to mediate the formation of stable and rigid multi-

protein structures called hemidesmosomes. Hemidesmosomes link intermediate filaments, cytokeratins, with laminins in the basement membrane (Borradori and Sonnenberg, 1999; van der Neut et al., 1996). The integrin α6β4 is a receptor for the laminin family of basement membrane components (Lee et al., 1992; Niessen et al., 1994); however it can signal independently of ligand binding possibly by the self-association of the β4 cytoplasmic domain and/or cooperation with other signaling molecules (Chao et al., 1996; Lipscomb and Mercurio, 2005; Rezniczek et al., 1998). In contrast to other integrin subunits that contain small cytoplasmic domains of around 50 amino acids, the β4 integrin subunit contains a large cytoplasmic domain of approximately 1000 amino acids (in most of its splice variants). It contains two pairs of type III fibronectin repeats that are separated by a connecting segment. (Fig. 1.2) The extracellular domain of the β4 integrin

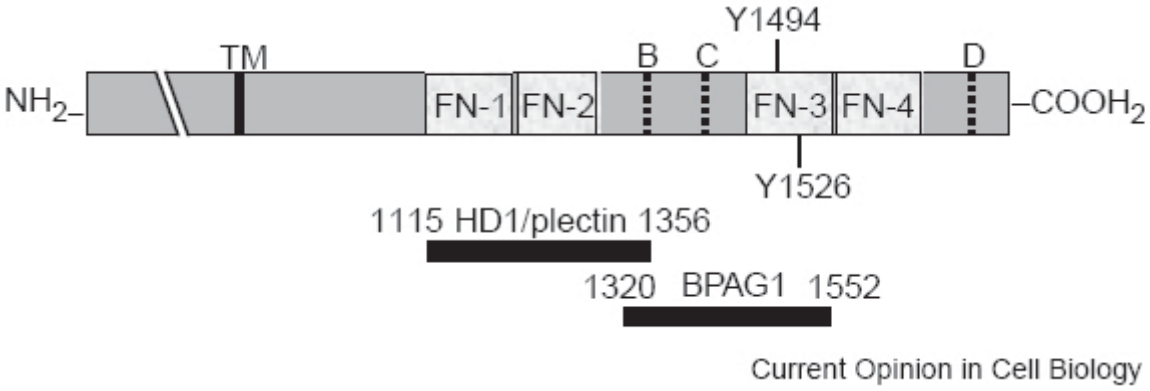


Figure 1.2. Diagram of the β4A integrin subunit. The β4 cytoplasmic domain contains two sets of fibronectin Type III repeats (FN) separated by a connecting segment. Shown are the tyrosine residues that have been implicated in α6β4-dependent activation of PI3-K (Y1494) and MAPK (Y1526). The black bars denote regions of the β4 cytoplasmic domain that are essential for hemidesmosome formation. The segment encompassing amino acids 1115–1356 is required for HD1/plectin interactions and the segment spanning amino acids 1320–1552 is required for BPAG1 binding. Dotted lines indicate the location of insertions or a deletion (carboxy-terminal dotted line) that result from differential splicing and that give rise to the three alternative isoforms of the βA subunit; β4B, β4C, and β4D. TM, transmembrane domain. Reprinted with permission from Current Opinion in Cell Biology, The α6β4 integrin and epithelial cell migration, Vol. 13, Arthur M. Mercurio, Issac Rabinovitz and Leslie Shaw, Pages 541-545, Copyright Elsevier 2001

subunit associates exclusively with the α6 integrin subunit (Hemler et al., 1989) and has signaling functions distinct from those of other integrins (Clarke et al., 1994; Hogervorst et al., 1990). Both α6 and β4 integrin subunits have multiple known splice variants. The α6 subunit has two splice variants, the α6A and α6B (Hogervorst et al., 1991). Some functional differences and expression localization have been reported for the α6 variants but mostly from its association with the β1 integrin subunit (Gimond et al., 1998). There are five known cytoplasmic splice variants for the β4 subunit, β4 A-E, but little is known about there functional significance. The most common variant is the β4A. The β4B and β4C integrin subunits contain inserts of 53 and 70 amino acids, respectively compared to the β4A, the β4D lacks twenty one base pairs and the last variant β4E has a truncated cytoplasmic domain (Tamura et al., 1990).

The β4 integrin subunit was identified as a tumor-related antigen associated with metastasis, (TSP-180) (Falcioni et al., 1986; Kennel et al., 1989) and provided the first evidence of this integrin with tumor spread. Since then, it has been shown to be increased in several types of invasive and metastatic carcinomas with levels of expression correlating to disease progression and reduced patient survival of some tumors (Grossman et al., 2000; Mercurio and Rabinovitz, 2001; Van Waes and Carey, 1992). Furthermore, there is now evidence that this integrin is involved in the formation of some carcinomas (Dajee et al., 2003) and in breast cancer for example, tumor formation is regulate by tumor cell survival in a VEGF-dependent manner (Lipscomb et al., 2005). Translation of VEGF can be regulated by the integrin α6β4 to promote cell survival by directly regulating the eukaryotic translation initiation factor 4E by phosphorylating and inactivating the repressor 4E-binding protein 1 (Chung et al., 2002).

In vitro studies confirmed the role of α6β4 integrin in migration and invasion by exogenous expression of this integrin in colon and breast cancer cells that lacked expression of the β4 integrin subunit (Chao et al., 1996; Shaw et al., 1997). Low levels of the β4

integrin subunit were enough to elicit major changes in the invasive phenotype of the colon cancer cell line RKO (Chao et al., 1996). Furthermore, mechanistic studies on how α6β4 integrins promote invasion of cancer cells have revealed that activation of the PI3-K pathway, the small GTP-binding protein Rac-1, as well as cAMP-specific phosphodiesterase are required (Keely et al., 1997; O'Connor et al., 1998; Shaw et al., 1997). Rac-1 is a member of the Rho family of GTPases, which are known to be signaling intermediates for cell migration by the regulation of actin polymerization and actin-myosin contraction (Horwitz and Parsons, 1999). Furthermore, in other cell models, PI3-K is known to be crucial for initiation of growth factor-induced cell motility and transformation responses; and the small GTPase Rac1 is a prerequisite that acts downstream of the PI3-K pathway (Hooshmand-Rad et al., 1997; Rosenmuller et al., 2001).

Integrins can cooperate with growth factors to regulate signaling of multiple cellular events. This cooperation can occur by the sharing of downstream signaling molecules where both receptors are needed for a specific kinase or adaptor protein to trigger a downstream signal or through direct interaction with each other, causing physical alterations within the complexes (Yamada and Even-Ram, 2002). These interactions are important during development and tumor progression. In the case of α6β4 integrin, the cooperation with growth factors is becoming extremely important in understanding its role in many tumors. This integrin can associate with a member of the family of epidermal growth factor receptors, ErbB-2 (Falcioni et al., 1997) which enhances cell invasion, and this association promotes the activation of the transcription factors STAT3 and c-Jun in mammary tumors (Guo et al., 2006). In a recent report, the integrin α6β4 was shown to regulate the expression of ErbB-3 via translation and the heterodimer formation of ErbB-2/ErbB-3 promoted activation of the PI3-K/Akt pathway which prevented the apoptosis of carcinoma cells that was dependent of α6β4 integrin (Folgiero et al., 2007). In addition, the HGF receptor c-Met tyrosine kinase can cooperate with α6β4 integrin to enhance migration and invasion, as well as

tumorigenesis (Bertotti et al., 2005; Chung et al., 2004; Trusolino et al., 2001). Among other growth factors that interact with α6β4 integrin are the Ron receptor (Santoro et al., 2003), EGFR and Fyn (Mariotti et al., 2001). Integrin α6β4-dependent activation of PI3-K can be induced by both growth factor-dependent and independent signaling mechanisms (Mercurio and Rabinovitz, 2001). The cooperation of α6β4 integrin with growth factor receptors is important for understanding its ability to activate PI3-K to promote migration and invasion, because this integrin lacks a binding motif to the regulatory subunit of PI3-K via SH2 domains. Nevertheless, a PI3-K binding motif is present in several growth factor receptors that can activate PI3-K (Hu et al., 1992). Synergistic activation by the α6β4 integrin and growth factor receptors is suggested to activate signaling intermediates that could then promote PI3-K activation and consequently invasion and migration of cancer cells. Other mechanisms that could account for PI3-K activation, are the interactions with insulin receptor substrate adaptor proteins (IRS-1 and IRS-2) (Shaw, 2001), and the compartmentalization of integrin α6β4 into membrane microdomains, such as lipid rafts and tetraspanin-enriched microdomains (Gagnoux-Palacios et al., 2003; Yang et al., 2004).

The involvement of α6β4 integrin in the formation of stable adhesion complexes (hemidesmosomes) in normal epithelia becomes a paradox as to its involvement in migration and invasion and consequent tumor spread. For this reason it was assumed that the disruption of these complexes was necessary. Evidence of this disruption came by the demonstration that integrin α6β4 colocalized and promoted the formation of lamellipodia and membrane ruffles after EGF stimulation where it interacted with filamentous actin (F-actin) (Rabinovitz and Mercurio, 1997; Rabinovitz et al., 1999) (Fig. 1.3). These is dependent on PKCα-mediated phosphorylation of the integrin β4 subunit in serine residues (Rabinovitz et al., 1999). In addition, to aid in tumor invasion, the α6β4 integrin mediates compression forces that can promote the remodeling of the basement membrane. This process required the packaging of basement membrane material under the cells which

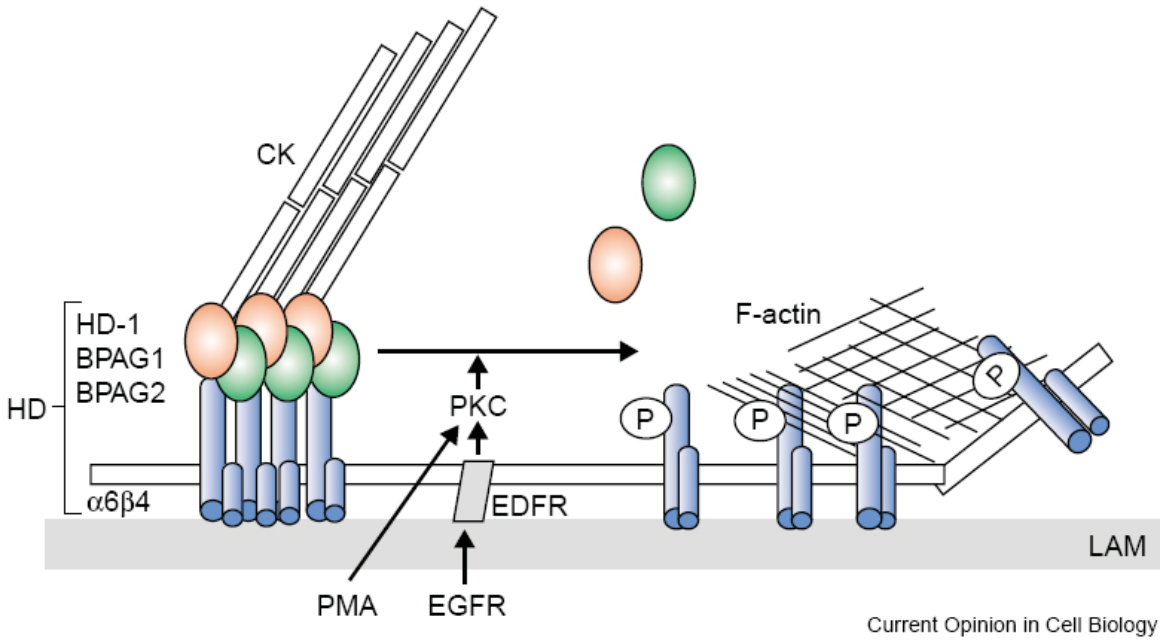


Figure 1.3. Mobilization of the α6β4 integrin from its association with cytokeratins in hemidesmosomes to F-actin in lamellae and lamellipodia. A chemotactic stimulus such as EGF or PMA can disassemble hemidesmosomes (HD) and promote the formation of α6β4-containing lamellipodia and lamellae. This process is dependent on PKC-α and may involve phosphorylation of the β4 integrin. These findings might explain the dichotomy observed in α6β4 function in stably adherent and migrating epithelial derived cells. This model suggests that chemotactic factors can drive migration by mobilizing α6β4 and disassembling hemidesmosomes. Cellular components of the hemidesmosome: Bullous Pemphigoid Antigens 1 and 2 (BPAG1, BPAG2); HD1/Plectin; α6β4 integrin; cytokeratins (CK). Reprinted with permission from Seminars in Cancer Biology, Towards a mechanistic understanding of tumor invasion--lessons from the α6β4 integrin, Vol. 11, Arthur M. Mercurio and Issac Rabinovitz, Pages 129-141, Copyright Elsevier 2001.

promotes the removal of the basement membrane from the cells nearby (Rabinovitz et al., 2001). These traction forces and basement membrane remodeling can promote gaps between cells were tumor cells can escape via invasion mechanisms.

Experimental Rationale and Hypothesis

The acquisition of a motile and invasive phenotype is a crucial event in the progression of localized malignancies to widespread metastatic disease, a common phenomenon of

pancreatic adenocarcinomas. A greater understanding of the basic mechanisms that drive pancreatic adenocarcinomas to a deadly metastatic phenotype will help us develop better molecular therapies targeted to help cure this disease, prolong the life of the patients and/or even prevent the disease. In order to achieve this goal, studies are needed that will focus on the mechanisms by which tumor cells become motile and invasive so that we can identify effective ways of blocking these steps.

Given these needs, the overall goal of this dissertation was to study the significance of integrin α6β4 expression and signaling in pancreatic adenocarcinoma that contribute to increased cell migration and invasion. Interestingly, although this integrin is increased in several types of invasive and metastatic carcinomas and has been shown to be associated with an invasive phenotype (Mercurio and Rabinovitz, 2001), few studies have looked at its role in pancreatic adenocarcinoma. Recent genetic profiling and immunohistochemistry suggested that the integrin α6β4 is selectively over expressed in pancreatic adenocarcinomas (Crnogorac-Jurcevic et al., 2003; Gleason et al., 2005; Logsdon et al., 2003; Nakamura et al., 2004). These studies provided evidence of the potential role of this integrin in pancreatic adenocarcinoma progression, cell migration and invasion.

Therefore, the major hypothesis of this dissertation was that expression of and signaling from integrin α6β4, in cooperation with hepatocyte growth factor (HGF) stimulation, a known mitogenic and motility factor of pancreatic carcinomas, facilitates cell migration and invasion of pancreatic adenocarcinomas. The aim of this work was to delineate the importance of α6β4 integrin expression in pancreatic adenocarcinomas and to understand some of the signaling mechanisms by which it contributes to increased migration and invasion of pancreatic cancer cells.

In Chapter Two my objectives were 1) to compare the localization and expression levels of integrin α6β4 in normal pancreas, chronic pancreatitis and pancreatic

adenocarcinomas; 2) to establish if integrin α6β4 expression and localization is associated with pancreatic cancer tumor progression; and 3) to determine if integrin α6β4 can serve as a diagnostic marker to distinguish pancreatic cancer from chronic pancreatitis, a pancreatic disease that is histologically similar to pancreatic adenocarcinoma and therefore hard to distinguish (from each other).

In Chapter Three my objectives were 1) to investigate if the integrin α6β4 contributes to the migration and invasion phenotype in pancreatic cancer cell lines and 2) to assess some of the mechanism of how integrin α6β4 contributes to the migratory and invasive phenotype in these cells.

CHAPTER TWO: UPREGULATION AND REDISTRIBUTION OF INTEGRIN α6β4 EXPRESSION OCCURS AT AN EARLY STAGE IN PANCREATIC ADENOCARCINOMA PROGRESSION[1]

Introduction

Pancreatic carcinoma is the fourth leading cause of cancer death in the US and has the highest death to incidence ratio of all cancers (Jemal et al., 2006). Poor prognosis of pancreatic cancer patients relates to a high incidence of tumor cell invasion and metastasis. Treatment options are limited and patients succumb to the disease shortly after diagnosis unless eligible for tumor resection (Li et al., 2004). Of those patients that undergo resection, only 15-20% will survive to 5 years (Jemal et al., 2006).

Over 90% of pancreatic cancers are adenocarcinomas that are thought to arise from proliferative premalignant pancreatic intraepithelial neoplasia (PanIN) of the ductal epithelium. PanIN lesions start as low cuboidal epithelial cells that become columnar due to increased mucin production. Cells then present with nuclear atypia and enhanced proliferation, that lead to luminal shedding and/or invasion into the stroma (Hruban et al., 2001). These morphological alterations correlate with increased genetic abnormalities such as the activation of K-ras, loss of tumor suppressors (e.g., p16, p53, and DPC4) and upregulation of telomerases (Bardeesy Hruban et al., 2000). Although PanIN-1A and PanIN-1B are considered early cancer precursor lesions, molecular studies have shown that PanIN-2 and PanIN-3 lesions represent a distinct step toward invasive carcinoma (Kloppel and Luttges, 2004). Moreover, PanIN lesions can be found in patients with chronic pancreatitis (Volkholz et al., 1982) and these patients have an increased risk of developing pancreatic cancer (Lowenfels et al., 1993; Malka et al., 2002).

1 Reprinted from Modern Pathology Cruz-Monserrate, Z., Qiu, S., Evers, B. M., and O'Connor, K. L. 2007. Upregulation and redistribution of integrin α6β4 expression occurs at an early stage in pancreatic adenocarcinoma progression. 20: 656-667, Copyright 2007 with permission from Nature Publishing Group.

Infiltrating duct-like and tubular structures embedded in a highly desmoplastic stroma are characteristic of pancreatic cancers but also are features of chronic pancreatitis. The overlap in the clinical presentation and histopathological features between chronic pancreatitis and pancreatic cancer can lead to confusion in the diagnosis and management of both diseases (Taylor, 2003). While histological parameters are useful, more specific markers are needed to detect pancreatic cancer at an early stage and distinguish pancreatic cancer from chronic pancreatitis. The quest for such markers has led to the use of high throughput technology such as genetic profiling and proteomics. Recent studies that included the used of genetic profiling and immunohistochemistry suggested that the integrin α6β4 is selectively over expressed in pancreatic adenocarcinomas (Crnogorac-Jurcevic et al., 2003; Gleason et al., 2005; Logsdon et al., 2003; Nakamura et al., 2004) thus providing evidence of the potential role of this integrin in pancreatic cancer progression.

Integrins are receptors for extracellular matrices that transmit mechanical and biochemical signals to regulate cellular functions including survival, proliferation, motility, transcription and protein translation. They consist of two type I transmembrane α and β subunits that associate in various combinations to form at least 25 receptors (Hynes, 2002). Integrin adhesion to extracellular matrices and signaling have been associated with tumor development, invasion and metastasis (Guo and Giancotti, 2004). In particular, the α6β4 integrin is increased in several types of invasive and metastatic carcinomas with increased levels of expression correlating with a highly invasive and motile phenotype (Mercurio and Rabinovitz, 2001) as well as reduced patient survival (Grossman et al., 2000). The β4 integrin subunit is unique in that its cytoplasmic domain is 1000 amino acids longer than that of other integrins and its extracellular domain associates exclusively with the α6 integrin subunit (Hemler et al., 1989). One function of the α6β4 integrin is to maintain the structure and integrity of epithelia through the formation of hemidesmosomes (Borradori and Sonnenberg, 1999). In cancers, integrin α6β4 is released from the hemidesmosomes in

epithelial cells and associates with the actin cytoskeleton (Rabinovitz et al., 1999). When released from hemidesmosomes, the α6β4 integrin contributes to an invasive phenotype by signaling to molecules such as PI3-K and cooperating with growth factors (Lipscomb and Mercurio, 2005).

The strong link between the α6β4 integrin and an invasive phenotype coupled with gene array data that shows upregulation of this integrin in pancreatic adenocarcinomas prompted me to investigate if the α6β4 integrin is associated with the aggressiveness and progression of pancreatic cancers. Therefore, the goals of this study were: 1) to compare the localization and expression levels of integrin α6β4 in normal pancreas, chronic pancreatitis and pancreatic adenocarcinomas 2) to establish if integrin α6β4 expression and localization is associated with pancreatic cancer tumor progression and 3) to determine if integrin α6β4 can serve as a diagnostic marker to distinguish pancreatic cancer from chronic pancreatitis.

Materials and Methods

Tissue Specimens

Paraffin embedded archival tissues from patients with histological normal pancreas (n=4; 2 cases underwent tumor resections for other gastrointestinal cancers in which the pancreas was free of tumor, 1 suspected pancreatic cancer case was found to be normal after resection was performed and 1 case was a benign pancreatic tumor which contained ample normal pancreatic tissue for analysis), pancreatic adenocarcinomas (n=20), and chronic pancreatitis (n=14) were obtained from the UTMB Surgical Pathology Department using an IRB approved protocol. Of the 20 pancreatic adenocarcinoma cases, 12 cases included regions of normal uninvolved tissue and 15 cases presented with both pancreatic adenocarcinoma and chronic pancreatitis. In most of these cases, multiple tissue specimens were used in the analysis with tissue size varying from a few mm to 2 inches. In addition,

three pancreatic cancer tissue microarrays embedded in paraffin were used: AccuMax Array A207 (II) (1.5mm spots, 2 spots per case), AccuMax Array A207 (III) (1mm spots, 2 spots per case) (ISU ABXIS Co., Seoul, South Korea) and the PA801 array (1.5mm spots, 1 spot per case) (US Biomax, Inc., Rockville, MD). The cases included in my study analyzes that came from these tissue arrays were 93 pancreatic adenocarcinomas for which 23 cases included normal uninvolved tissue). Pathology of all specimens was determined based on the histology of serial sections stained with hematoxylin and eosin (H&E) using standard criteria.

Overall, my study utilized 131 cases, which included 4 cases with histological normal pancreas, 14 chronic pancreatitis and 113 pancreatic adenocarcinomas (which included 35 cases with regions of normal uninvolved tissue and 15 cases for which the specimens presented with pancreatic adenocarcinoma and chronic pancreatitis). The average age of all cases was 57 (range 22-78) including 43 females and 88 males.

Immunohistochemical staining

Tissue sections (4 μm) and tissue microarrays (5 μm) were deparaffinized with xylene and rehydrated in decreasing concentrations of alcohol. Endogenous peroxidase activity was blocked by incubating slides with 3% hydrogen peroxide in 1X Dulbecco's Phosphate Buffer Saline, (DPBS; pH 7.4) (Gibco, Carlsbad, CA) for 10 min. After rinsing with water, antigen retrieval was performed by placing slides inside a container filled with 1X Dako Target Retrieval Solution (S1699) (Dako Corporation, Carpinteria, CA) and incubating for 20 min in a steamer at 100ºC. The slides were taken out of the steamer, and allowed to cool for 20 min in the antigen retrieval solution. After rinsing with water, avidin/biotin block was performed for 15 min each using an avidin/biotin blocking kit (SP-2001) (Vector Laboratories, Burlingame, CA). Nonspecific binding sites were then blocked by incubating slides in 1X Phosphate Buffer Saline with 0.05% Tween-20 (PBST)

and 0.3% Casein. Sections were then incubated with a rat monoclonal primary antibody 439-9B (Chemicon, Temecula, CA) for the integrin β4 subunit, rat IgG (negative control) at 5 μg/ml, diluted in antibody diluent solution (S3022) (Dako Corporation), or antibody diluent only for 1 hr at room temperature. After rinsing three times for 10 min with PBST, slides where incubated for 30 min with 10 μg/ml of a rat biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) in 1X DPBS and detected with an indirect streptavidin-biotin immunoperoxidase technique using Universal DakoCytomation Labeled Streptavidin-Biotin® 2 System, Horseradish Peroxidase and developed with 3,3'-diaminobenzidine (DAB) (Dako Corporation). Sections were then counterstained with Mayers Hematoxylin, rinsed with water, dehydrated and coverslipped with Cytoseal 60 Mounting Media (Richard-Allan Scientific, Kalamazoo, MI).

Immunohistochemical analysis

Expression patterns were analyzed using standard pathological criteria and scored using a semi-quantitative method based on the intensity and localization of immunostaining. Normal, pancreatic adenocarcinoma and chronic pancreatitis sample areas were scored from 0-3 depending on the staining intensity: 3 = very intense staining (cytoplasmic and membranous); 2 = moderate staining (cytoplasmic and membranous); 1 = staining only at the basal surface interface with the basement membrane; and 0 = no staining. PanIN lesions, PanIN 1A (n=104), PanIN 1B (n=74), PanIN 2 (n=19), and PanIN 3 (n=8), were identified in samples from UTMB, following the guidelines previously described (Hruban et al., 2001), which are summarized on the left side of Figure 2.2. PanINs were identified in 1 case of normal pancreas, 14 cases of chronic pancreatitis and 20 cases of pancreatic adenocarcinomas. Endothelial cells and cells in the peripheral nerves served as internal positive controls (Feltri et al., 1994; Hiran et al., 2003); absence of staining in these cells was used as criteria to remove a sample from the study. Images were captured with a Nikon

Digital Sight DS 5M color camera adapted to an upright Nikon Microscope Optiphot-2.

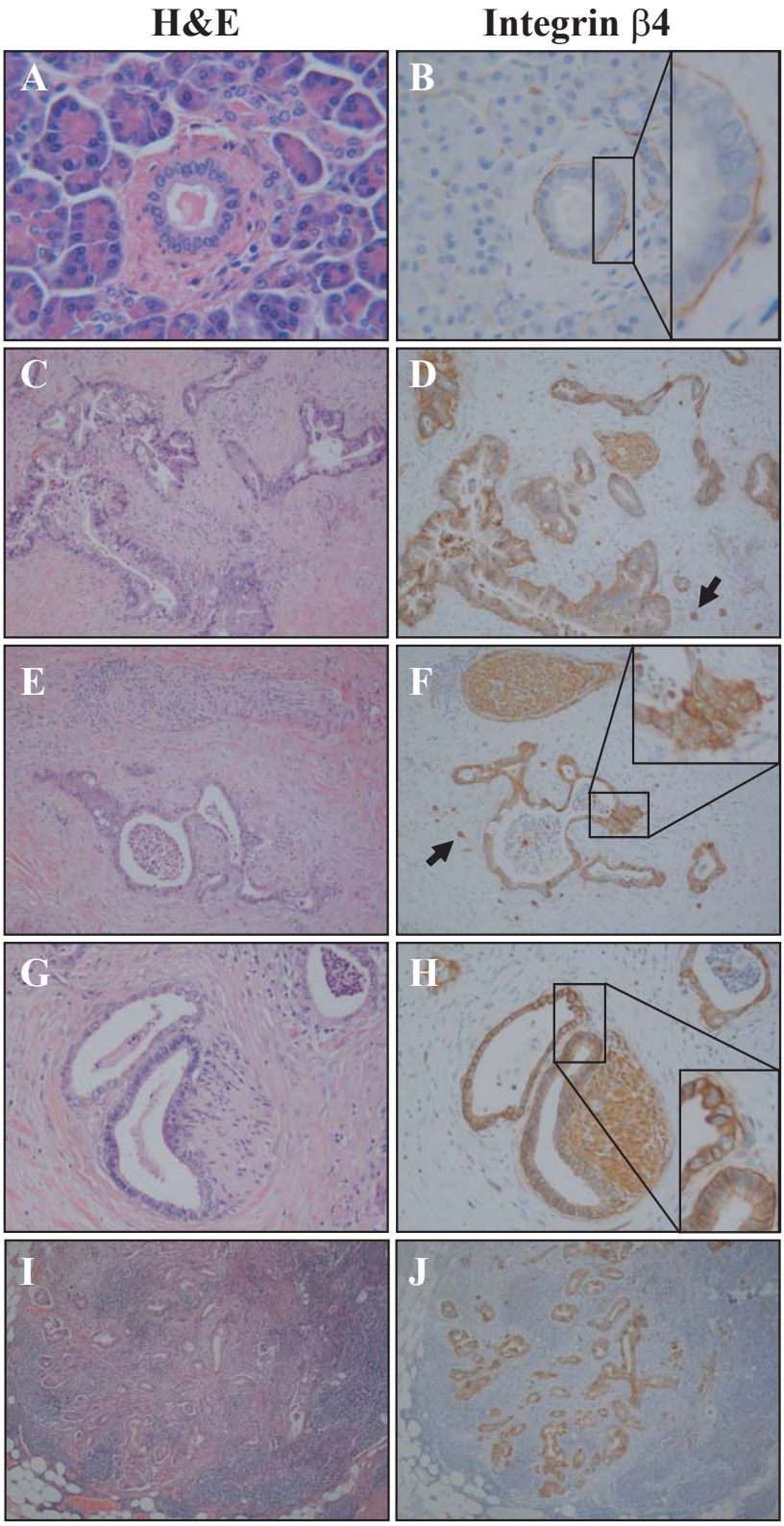
Statistical analysis

Staining data were analyzed using Sigma Stat 2.03 software statistical program (Systat Software, Point Richmond, CA). The nonparametric Mann-Whitney Rank Sum Test was used to evaluate statistical significance differences between the immunohistochemical staining intensity of normal pancreas, pancreatic adenocarcinoma and chronic pancreatitis cases.

Results

Integrin α6β4 expression and localization is altered in pancreatic adenocarcinomas compared to normal pancreas

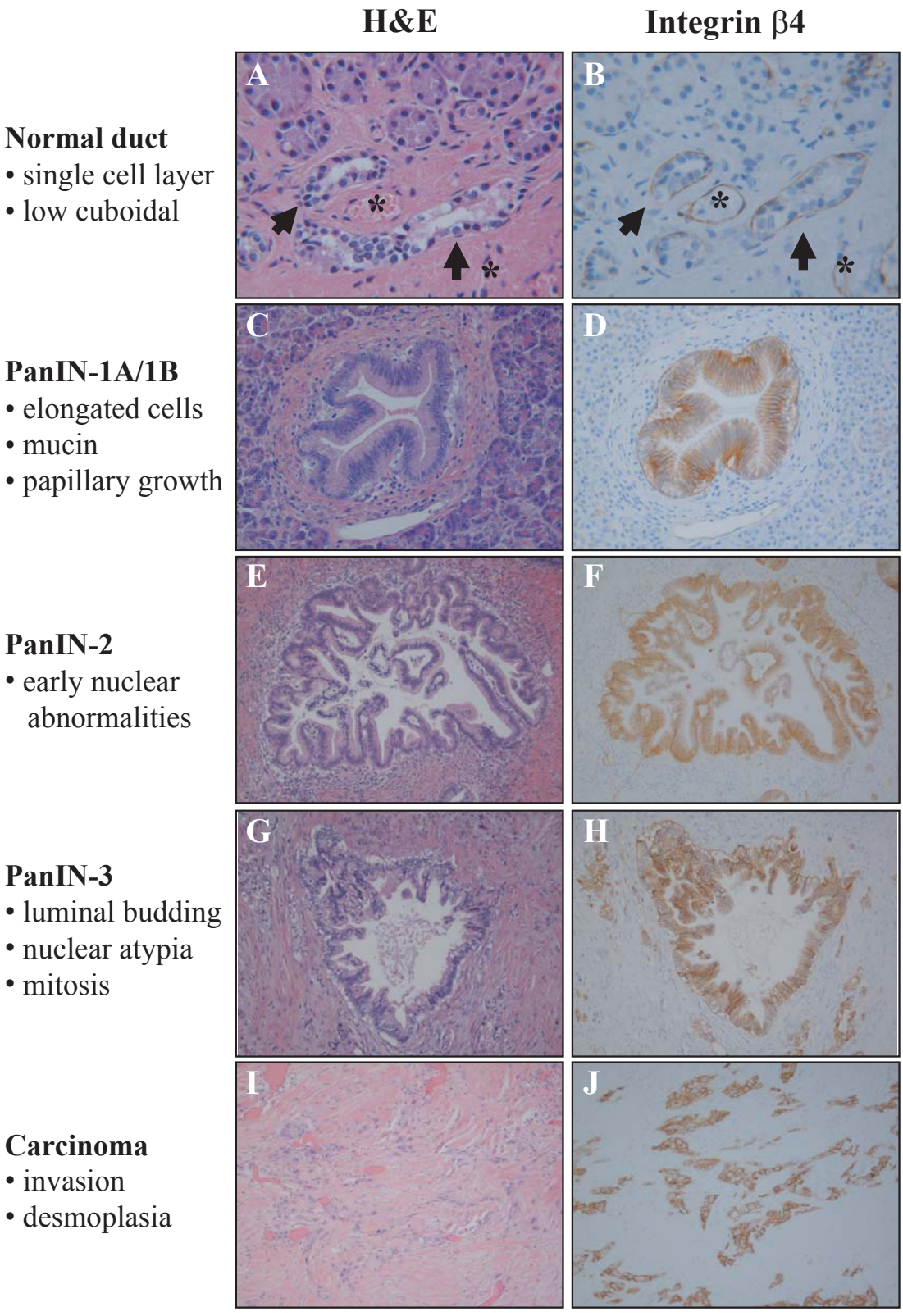
Integrin α6β4 expression is increased in several types of invasive and metastatic carcinomas including breast, colon, thyroid, gastric, bladder and squamous carcinomas and has been shown to be associated with an invasive phenotype (Mercurio and Rabinovitz, 2001). Therefore, due to the high rate of tumor dissemination associated with pancreatic adenocarcinoma, I hypothesized that integrin α6β4 would be overexpressed in the pancreatic cancer cells. To test this hypothesis, I performed immunohistochemistry for the integrin β4 subunit in 4 cases with histological normal pancreas and 35 uninvolved normal tissues from pancreatic resections for cancer. Since the integrin β4 subunit is known to only associate with the integrin α6 subunit, the staining for the β4 subunit is indicative of integrin α6β4 expression. As an internal positive control, I used staining of the integrin β4 subunit associated with Schwann cells in the peripheral nerves (Fig. 2.1H) and endothelial cells (Fig. 2.2B, asterisks) which have been reported to express this integrin previously (Feltri et al., 1994; Hiran et al., 2003). The ductal epithelial cells of these normal pancreatic tissues (97%, n=39) showed expression of integrin α6β4 where it preferentially localized at the cellular interface with the basement membrane (Fig. 2.1B, and Fig. 2.2B). Interestingly, the


H&E
Integrin β4
A
B
C
D
E
F
G
H
I
J

cells within the pancreatic acini did not display any immunoreactivity for the β4 integrin subunit; however, cells localized at the base of the acinar epithelium stained positive for integrin α6β4. These cells are likely endothelial or stellate cells associated with the acini as myoepithelial cells are not understood to exist in the pancreatic acini.

In contrast, pancreatic adenocarcinoma showed overexpression of the integrin α6β4 in 92% (104/113) of the cases examined compared to normal pancreas ($p=0.002$) or normal uninvolved tissue ($p<0.001$) when compared to chronic pancreatitis samples. Notably, the localization of the α6β4 integrin in pancreatic carcinoma cells was no longer restricted to the basal surface, but also extended to the apical and lateral sides of the cell membrane as well as within the cytoplasm (Fig. 2.1D, 2.1F, 2.1H, and 2.1J and Fig. 2.2J). A similar pattern of expression was noted in pancreatic cancer that had invaded into the peripheral nerves (Fig. 2.1H) or lymph nodes (Fig. 2.1J). Of the 20 pancreatic adenocarcinomas for which I had ample tissue to analyze (UTMB cases), six cases demonstrated perineural invasion and six cases had lymph node metastasis. In all of these cases, tumor cells displayed overexpression and altered localization of the β4 integrin subunit; cytokeratin staining was

Figure 2.1. (previous page) Expression of integrin β4 in normal human pancreas and pancreatic adenocarcinomas. Tissue sections were immunostained for the integrin β4 subunit (B, D, F, H, J) and their respective serial sections with H&E (A, C, E, G, I). IgG as well as secondary only controls were performed and showed no immunoreactivity (data not shown). A and B show representative serial sections containing normal pancreas were positive staining is found in the basal surface at the interface with the basement membrane of epithelial cells in the ductal component of the pancreas (x400 original magnification). C – J show representative areas of cancers where expression of integrin β4 is seen upregulated when compared to normal pancreas and it localized to the basal, apical and lateral sides of the membrane as well as within the cytoplasm of cancer cells (C-F, x100; G-H, x200; I-J, x40). G and H show pancreatic carcinoma with perineural invasion in which Schwann cells (also known to express integrin α6β4 and serve as an internal positive control) and pancreatic cancer cells stained for the integrin β4 subunit. I and J show pancreatic adenocarcinoma lymph node metastasis. Insets in B, F, and H show the difference in expression and localization of the integrin α6β4 between normal and pancreatic cancer cells. Arrows (D and F) show invading cancer cells, identified by the integrin β4 staining.


H&E
Integrin β4
Normal duct
• single cell layer
• low cuboidal
PanIN-1A/1B
• elongated cells
• mucin
• papillary growth
PanIN-2
• early nuclear abnormalities
PanIN-3
• luminal budding
• nuclear atypia
• mitosis
Carcinoma
• invasion
• desmoplasia
A
B
C
D
E
F
G
H
I
J

performed to confirm the epithelial nature of cells (data not shown). Insets in Figure 2.1B, 2.1F, and 2.1H demonstrate the clear distinction in localization and expression of integrin α6β4 between normal pancreas and pancreatic adenocarcinoma cells. In addition, single invading cells can be distinguished in the stromal environment by their intense integrin β4 subunit staining (Fig. 2.1D, 2.1F, arrows), which are difficult to identify by H&E staining. Cytokeratin staining was performed on serial sections to confirm the epithelial nature of cells invading the stromal environment of the tissues (data not shown). For all cases, IgG and /or secondary biotinylated antibody only controls were performed and no immunoreactivity was observed (data not shown).

The summary of patients analyzed is listed in Table 1. The majority of patients with pancreatic adenocarcinoma presented with high staining intensity of integrin α6β4. In contrast, normal or normal uninvolved tissue displayed low (basal surface only) or no staining. Notably, I found that the antibody used in this study was sensitive to the antigen retrieval method used which is affected by the way tissues are fixed after resection. For this reason, several specimens were not included on the study due to an absence of staining in control cells within the specimen. Most cases that showed no staining or moderate expression levels of the integrin β4 subunit were from the tissue microarrays for which there was no internal control to assess the quality of the tissue. Therefore, the percentage of pancreatic adenocarcinomas with high α6β4 expression may be higher considering that

Figure 2.2. (previous page) Expression of integrin β4 in the PanIN Progression Model. Tissue sections containing representative normal pancreas (A and B), PanIN lesions (C-H) and carcinoma (I and J) were immunostained for the integrin β4 subunit (B, D, F, H, J) and their respective serial sections with H&E (A, C, E, G, I). As in Figure 1, A and B show normal pancreatic ducts where the staining for integrin β4 subunit is only seen surrounding the basement membrane of the epithelial ducts (arrows) (x400). Endothelial cells serve as an internal control for the stain quality (asterisks). C - H show representative PanIN lesions as describe in the left side of the figure which summarizes the criteria used in (Hruban et al., 2001) (C-D, x200; E-H, x100). G and H shows invasive carcinoma adjacent to PanIN-3. I and J show a representative sample of a patient specimen with invasive pancreatic carcinoma (x100).

100% (n=20) of the pancreatic adenocarcinomas from my institution demonstrated intense staining. Overall, my results strongly suggest that overexpression and redistribution of the integrin α6β4 is a prominent feature of pancreatic adenocarcinoma.

Table 1. Integrin β4 subunit expression in pancreatic tissues

	Staining Intensity [1] Percentage of Cases				
Diagnosis	**0**	**1**	**2**	**3**	
Normal Pancreas (n=4)		100			
Chronic Pancreatitis (n=14)		93	7		P=0.871 vs Normal
Pancreatic Adenocarcinoma (n=113)	2	6	29	63	P=0.002* vs Normal P<0.001* vs Chronic Pancreatitis
Normal uninvolved tissue of Pancreatic adenocarcinoma cases (n=35)[2]	3	97			
Chronic Pancreatitis area within Pancreatic adenocarcinoma cases (n=15) [2]		93	7		
Total Cases n=131					

[1] Staining intensity criteria are described in the Material and Methods section.

[2] Pancreatic adenocarcinoma cases that had normal uninvolved areas and/or chronic pancreatitis areas.

* Statistical significance using the Mann-Whitney Rank Sum Test

Integrin α6β4 expression is elevated and altered in PanIN lesions

PanIN lesions are considered precursors of pancreatic adenocarcinomas (Hruban et al., 2001). Therefore, I analyzed the expression pattern of integrin α6β4 in PanIN lesions to determine if this integrin could play a role in pancreatic cancer development. Hence,

I identified PanINs of grades 1-3 in most of the pancreatic adenocarcinomas and chronic pancreatitis tissues analyzed from my institution based on established criteria (Hruban et al., 2001). These criteria are summarized on the left side of Figure 2.2. Here, I found that lesions as early as PanIN 1A showed moderate to high expression as well as altered localization of the integrin α6β4, similar to the pattern noted in pancreatic adenocarcinomas. Interestingly, this pattern of expression was maintained throughout the established PanIN progression model (Fig. 2.2 A-J and Table 2). In addition, I was able to identify several transitional lesions in which cells displaying a columnar morphology and increased mucin characteristic of PanIN 1A/1B were adjacent to low cuboidal epithelium of a normal duct. These transition regions reveal that the dramatic upregulation and altered localization of integrin α6β4 subunit are associated with the first morphological changes that characterize PanIN lesions (Fig. 2.3). Therefore, these data suggest that increased expression and redistribution of the integrin α6β4 in pancreatic adenocarcinomas is an early and prominent event in the development of pancreatic cancers that is associated with the first discernable step in pancreatic tumor progression.

Table 2. Integrin β4 subunit expression in Pancreatic Intraepithelial Neoplasia Lesions

		Staining Intensity [1] Percentage of Lesions		
Type of Lesion	**Number of lesions identified**	**1**	**2**	**3**
PanIN 1A	104		25	75
PanIN 1B	74		3	97
PanIN 2	19			100
PanIN 3	8			100

[1] Staining intensity criteria are described in the Material and Methods section.

Only cases from UTMB were included in this analysis, due to the ample tissue available in these samples.

Integrin α6β4 expression in chronic pancreatitis is similar to normal pancreatic ducts

Chronic pancreatitis is an inflammatory disease of the pancreas that has similar histological characteristics as pancreatic adenocarcinoma, which can make the distinction of the two diseases challenging. To investigate whether integrin β4 subunit staining could

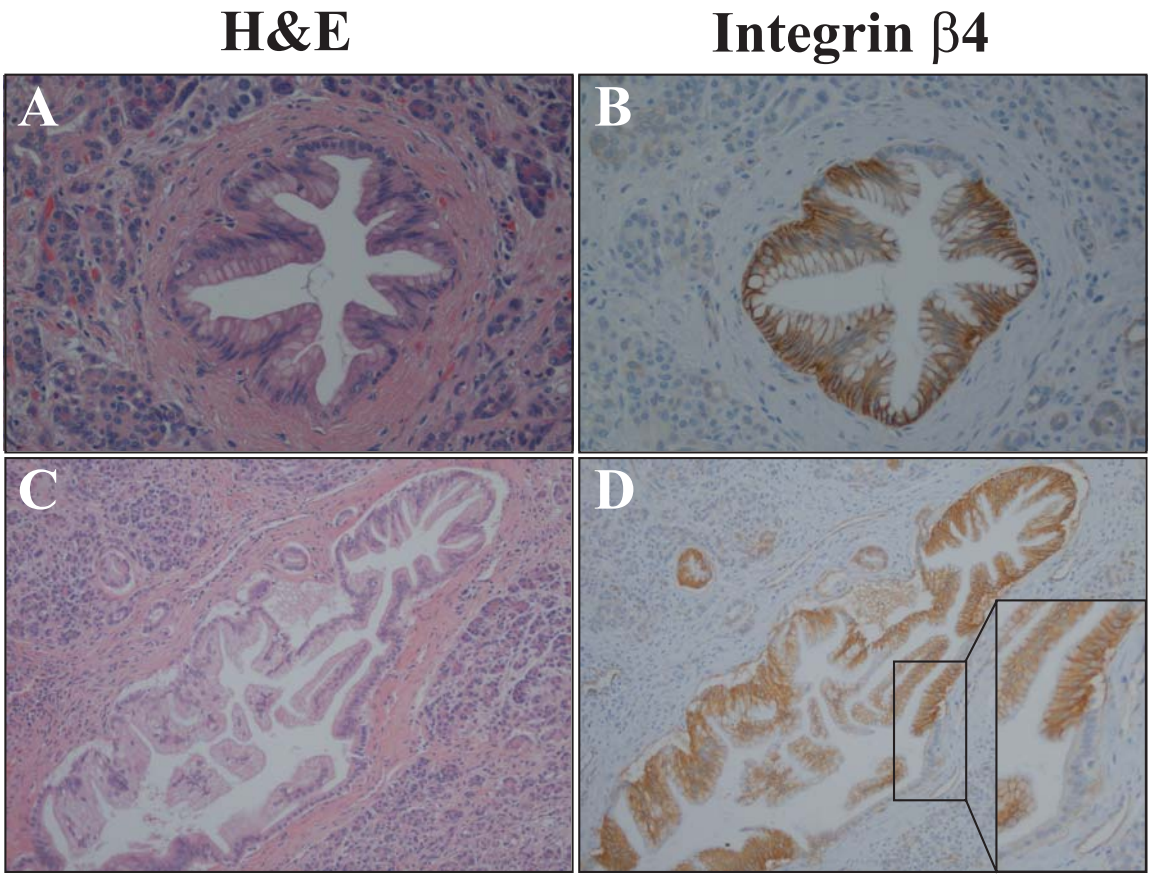


Figure 2.3. Expression of integrin β4 in PanIN lesion showing residual normal duct. Immunostaining for the integrin β4 subunit (B and D) and respective serial section stained with H&E (A and C). Panels A and B show a PanIN-1A/1B lesion showing an area of residual normal low cuboidal epithelium (x200). Transitional area in B shows the dramatic redistribution and overexpression of the integrin β4 subunit in early PanIN lesion when compare to the normal cells. Panels C and D shows an example of a PanIN-2 lesion that also show areas with residual normal low cuboidal epithelium. Inset in panel D show the dramatic change in the integrin β4 subunit expression and localization as the epithelium changes from normal to preneoplastic (x100).

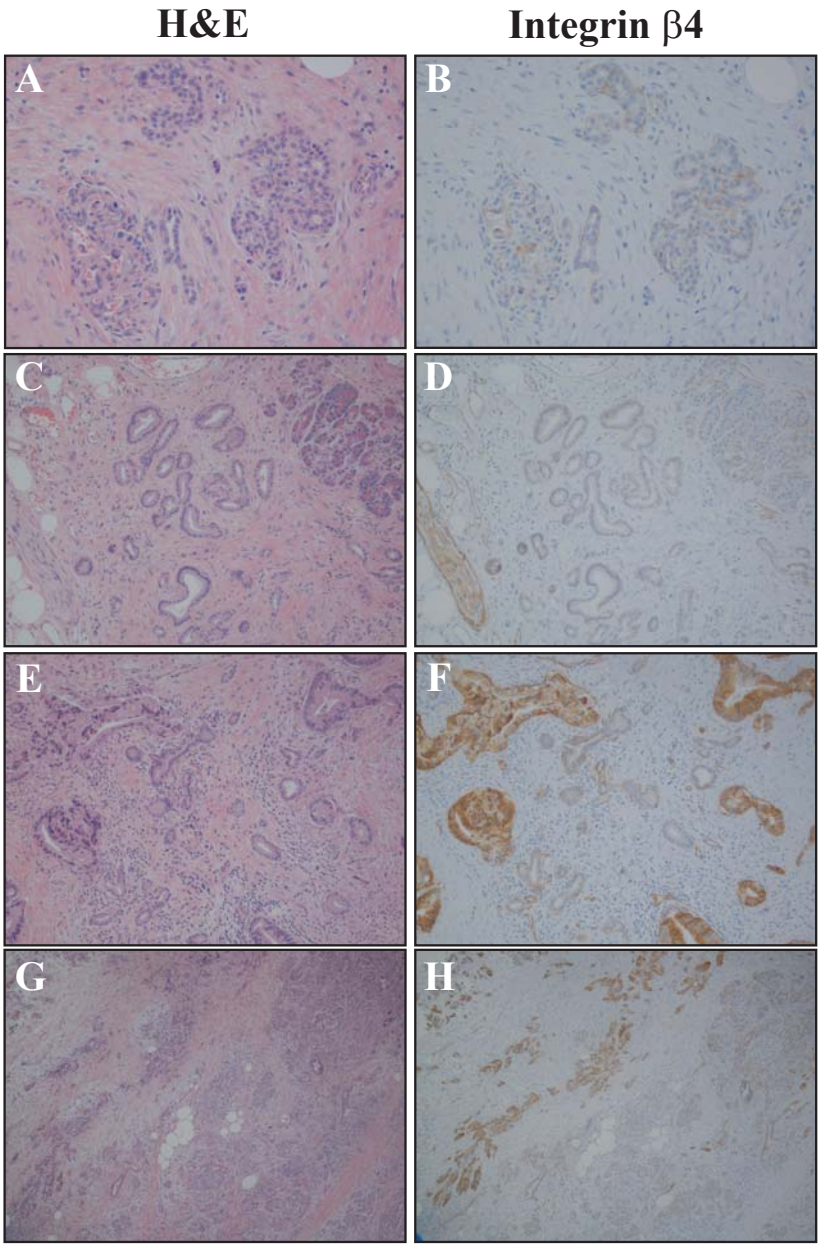


Figure 2.4. Expression of integrin β4 in a patient with pancreatic cancer and pancreatitis. Tissue sections of a patient diagnosed with chronic pancreatitis were immunostained for the integrin β4 subunit (B) and the respective serial section with H&E (A). (A-B, x200) shows an area of chronic pancreatitis for which the integrin β4 staining resembles the normal pancreas. C and H show sections of cases that presented with both pancreatic adenocarcinoma and chronic pancreatitis. (C-D, x100) show chronic pancreatitis area only where the staining pattern resembles that of normal pancreas. (E-F, x100 and G-H, x40) show an area of cancer and chronic pancreatitis in the same area at different magnifications.

distinguish pancreatic cancer from chronic pancreatitis, tissue sections from 14 patients with the final diagnosis of chronic pancreatitis were analyzed for integrin α6β4 expression. In addition, areas of chronic pancreatitis identified within pancreatic adenocarcinoma samples (n=15) were analyzed. As shown in Figure 2.4B and 2.4D, integrin β4 staining in chronic pancreatitis samples 93% (13/14) resembles the staining pattern of normal pancreatic ducts (p=0.871) where the integrin α6β4 staining is restricted to the basal plasma membrane (Fig. 2.1B and 2.2B). The quantification of these observations is shown in Table 1. Interestingly, when pancreatic adenocarcinoma and chronic pancreatitis were found in the same patient specimen, the dramatic overexpression and altered localization of integrin α6β4 present within the cancer nicely distinguished the cancer from chronic pancreatitis (Fig. 2.4F and 2.4H). These results demonstrate that the integrin α6β4 expression pattern noted in pancreatic adenocarcinomas is consistently and dramatically different from chronic pancreatitis.

Discussion

Pancreatic adenocarcinomas are highly aggressive and metastatic cancers that are thought to arise from noninvasive neoplastic precursor PanIN lesions (Kloppel and Luttges, 2004). A novel finding in my study is that the upregulation and altered localization of the integrin α6β4 occurs early in pancreatic adenocarcinoma tumor progression, (i.e. PanIN 1A lesions). Here, I find that integrin α6β4 is overexpressed in all 205 of the PanIN lesions identified. Interestingly, I was able to identify PanIN lesions juxtapositioned to residual normal low cuboidal epithelium (Fig. 2.3). The changes in α6β4 integrin expression and distribution were noted at the interface where the abrupt transformation between normal and preneoplastic lesions occurs. This change within the transitional areas provides evidence that this integrin is overexpressed at an early stage in pancreatic adenocarcinoma development and it may be upregulated as a result of, or directly contribute to, the initiation

of PanIN lesions that may eventually develop into an invasive carcinoma. Interestingly, another major finding here is that the α6β4 integrin expression and altered localization associated with PanIN lesions is sustained during pancreatic cancer development and is associated with pancreatic adenocarcinomas. In this study, I show that the integrin α6β4 is dramatically overexpressed in most of the pancreatic adenocarcinoma samples analyzed (92%, 104/113) when compared to normal pancreas and chronic pancreatitis samples. Therefore, my data suggest that the integrin α6β4 is likely involved in cancer development due to the prevalence of its overexpression in the earliest preneoplastic lesions and its persistence through tumor progression.

Multiple studies have correlated integrin α6β4 with tumor progression, predominantly with the later stages of progression where cells acquire the ability to invade and metastasize. In bladder cancers, the integrin α6β4 staining pattern correlated with the histological stage and grade of tumors (Mialhe et al., 1997) while in colorectal cancers, the integrin α6β4 expression was found to be increased predominantly in less differentiated cancers (Falcioni, 1994). Integrin α6β4 also is expressed at the invasive fronts in association with its ligands laminin-1 and laminin-5 in gastric cancers (Tani et al., 1996) where it is suggested to be involved in the invasion process. In pancreatic cancer, prior studies using gene microarray analysis and immunohistochemistry demonstrated that the β4 integrin subunit was upregulated when compared to normal pancreas and pancreatitis tissues (Crnogorac-Jurcevic et al., 2003; Gleason et al., 2005; Logsdon et al., 2003) as well as associated with cases that presented with lymph-node metastasis (Nakamura et al., 2004). Furthermore, the potential role of the α6β4 integrin in metastasis is supported by studies in papillary thyroid carcinoma which suggested that the increased expression of the integrin β4 subunit in cancer lesions could play a role in the development of lymph node metastasis (Kitajiri et al., 2002). Certainly, the *in vivo* data coupled with extensive *in vitro* data suggest that the α6β4 is associated with the invasiveness and metastatic potential of late stage cancers

(Falcioni, 1994; Kitajiri et al., 2002; Mercurio and Rabinovitz, 2001; Mialhe et al., 1997; Tani et al., 1996). In my study, the 6 pancreatic adenocarcinoma cases that were associated with lymph node metastasis displayed overexpression of the integrin β4 subunit in cancer cells (Fig. 2.1 I-J). When pancreatic cells were found invading the peripheral nerves, the invading cancer cells also overexpressed integrin α6β4. I find that the integrin α6β4 is upregulated and redistributed early during pancreatic tumor progression, and persists through progression to lymph node metastasis. Therefore, my findings suggest that, unlike carcinomas of the breast, bladder, colon, stomach and thyroid, increased expression of integrin α6β4 in pancreatic adenocarcinomas occurs early rather than later in progression.

Interestingly, the expression pattern of integrin α6β4 in most of the pancreatic adenocarcinomas studied, changes from basal membrane localization in normal pancreatic ductal cells to where it now also localized to the cytoplasm, and the apical and lateral regions of the cell membrane. This pattern of expression is similar to that noted in squamous cell carcinomas and adenocarcinomas of the lung, where the integrin α6β4 was found to stain the plasma membrane and the cytoplasm of tumor cells (Mariani Costantini et al., 1990). The cytoplasmic staining seen in most of the cancers that overexpressed the β4 integrin subunit is likely due to the excess synthesis of this chain since there are no known signaling functions associated with cytoplasmic pools of integrin subunits. Integrins require dimerization of the α and β subunits to be transported to the plasma membrane where they function as extracellular matrix receptors and signal transducers. Therefore, it is likely that the β4 subunit is synthesized in excess and resides in the endoplasmic reticulum until paired with newly synthesized α6 subunit prior to transport to the plasma membrane. These observations are in good agreement with early studies on integrins and pancreatic carcinomas that reported the α6 integrin subunit to be diffusely distributed on the surface of carcinoma cells and to be present at the RNA level in pancreatic cancer cell lines and pancreatic tumor extracts (Lohr et al., 1996; Weinel et al., 1995) . Therefore, it

is the plasma membrane-associated integrins that are expected to function in adhesion and signaling.

How the integrin α6β4 contributes to pancreatic adenocarcinoma development could be explained by the ability of integrin α6β4 to interact with and facilitate signaling from several molecules understood to be upregulated in pancreatic cancers. Evidence shows that cooperation between the α6β4 integrin and activated forms of Ras can lead to the formation of human squamous cell carcinomas (Dajee et al., 2003). Importantly, activating K-Ras mutations are present in virtually all pancreatic adenocarcinomas and are suggested to play a role in tumor initiation due to its activation in early PanIN lesions (Moskaluk et al., 1997). Moreover, recent mouse and rat models have shown the importance of Ras mutations in pancreatic adenocarcinoma development (Hingorani et al., 2003; Ueda et al., 2006). The hepatocyte growth factor receptor, c-Met, is another molecule of interest found to be upregulated in early PanIN lesions and pancreatic adenocarcinomas (Di Renzo et al., 1995; Yu et al., 2006). c-Met is known to cooperate with integrin α6β4 to enhance the migratory and invasive potential of cancer cells *in vitro* (Chung et al., 2004; Trusolino et al., 2001). Furthermore, the tumorigenic potential of c-Met in cancer cells *in vitro* has been attributed to its cooperation with the integrin α6β4 (Bertotti et al., 2005). The integrin α6β4 has also been shown to promote the signaling of transcription factors such as NFAT-1 in carcinoma cells (Jauliac et al., 2002). NFAT-1 was found to be amplified in pancreatic cancers (Holzmann et al., 2004) which could lead to the regulation of pro-invasive genes, such as seen in breast carcinomas with NFAT-mediated upregulation of autotaxin (Chen and O'Connor, 2005). Furthermore, NFAT-1 can also induce the expression of cyclooxygenase-2 (Yiu and Toker, 2006) which is known to be overexpressed in pancreatic tumors (Okami et al., 1999). The association of the α6β4 integrin and known cooperating oncogenes such as Ras and c-Met and regulation of transcription factors such as NFAT-1 in early tumor precursor lesions, suggests that integrin α6β4 could cooperate and/or activate these factors

to drive the development and progression of pancreatic adenocarcinomas and contribute to the invasive and metastatic phenotype of these cancers. Therefore, I suggest that early expression of α6β4 in PanIN lesions can predispose pancreatic adenocarcinomas to become among the most invasive and metastatic cancers. Certainly, future studies are needed to determine the cooperation of integrin α6β4 with these factors and to elucidate how they facilitate the invasion and metastasis of pancreatic cancer cells.

A common challenge for clinicians with a patient that has a pancreatic mass is to determine if it is pancreatic cancer or chronic pancreatitis, as the two diseases might present with similar symptoms and histological characteristics. This distinction is crucial for treatment and management of each disease. Fine needle aspiration cytology (FNAC) obtained during endoscopic ultrasonography (EUS) has been employed for diagnosing pancreatic masses; however, it is useful as a positive predictive but not as a negative predictor since cancer could be missed during sampling (Balthazar, 2005). Even with FNAC, it can be difficult to determine if a lesion is cancer, preneoplastic or pancreatitis. Here, I show that 93% (13/14) of the chronic pancreatitis samples and 100% (15/15) of the chronic pancreatitis areas found within the patients with pancreatic adenocarcinomas resembled the integrin α6β4 expression pattern of normal pancreas. Moreover, when chronic pancreatitis was present in pancreatic adenocarcinoma samples, I was able to distinguish the areas of pancreatic cancer easily as the integrin α6β4 staining pattern highlighted the areas of cancer in a particular pancreatic tissue, even when it was not apparent with H&E staining. I suggest that the staining pattern of integrin α6β4, in conjunction with other markers and histological characteristics, could aid in distinguishing pancreatic cancer from chronic pancreatitis as well as detect early lesions of pancreatic cancer.

In summary, this study shows that the integrin α6β4 is upregulated in pancreatic cancers and that its localization is altered, where it is no longer restricted to the basal surface and can be found in the cytosol, and on the basal, lateral and apical sides of the plasma

membrane. These events occur early in the progression of pancreatic cancer development starting at the PanIN-1A stage. Furthermore, this study shows that the altered staining pattern of integrin α6β4 in pancreatic adenocarcinoma tissues can help identified cancer areas in the background of chronic pancreatitis. My study is in agreement with mounting evidence that the integrin α6β4 plays a major role in pancreatic adenocarcinomas. Certainly, further studies are needed to understand the mechanisms involved in the induction of the integrin β4 subunit expression and redistribution, as well as how it contributes to cancer progression.

CHAPTER THREE: INTEGRIN α6β4 PROMOTES THE MIGRATION AND INVASION OF PANCREATIC CANCER CELLS THROUGH THE UPREGULATION OF TIAM-1 AND SUBSEQUENT ACTIVATION OF RAC

Introduction

Pancreatic adenocarcinoma is the fourth leading cause of cancer-related death in the United States (Jemal et al., 2006) and its poor prognosis relates to a high incidence of tumor cell invasion and metastasis. In order for tumors to become metastatic, they must follow a series of steps that included: detachment from the primary tumor by invasion through the basement membrane, embolism, intravasation, transport, arrest at a distant site, attachment, extravasation, response to the microenvironment and tumor cell proliferation (Fidler, 1990). Interestingly, integrin molecules are involved in more then one of these steps, making them good candidates to study and understand some of the basic mechanisms involved in the metastatic process. My studies, as well as others, have shown that the pro-invasive and pro-metastatic integrin α6β4 is selectively over expressed in pancreatic adenocarcinomas (Crnogorac-Jurcevic et al., 2003; Cruz-Monserrate et al., 2007; Gleason et al., 2005; Logsdon et al., 2003; Nakamura et al., 2004) when compared to normal pancreas and chronic pancreatitis. This overexpression occurs at an early stage in pancreatic cancer progression (Cruz-Monserrate et al., 2007).

Integrins regulate many cellular functions such as tumor development, cell migration and invasion (Guo and Giancotti, 2004). Because of its role tumor migration and invasion of other tumors, this work focused on the α6β4 integrin. The β4 integrin subunit is unique among the integrin β subunits in that its cytoplasmic domain is 1000 amino acids longer than the other integrins and it can only associate with the α6 integrin subunit extracellular domain (Hemler et al., 1989). The α6β4 integrin is primarily expressed on

the basal surface of most epithelia where it functions as an adhesion receptor to maintain epithelial structure and integrity through the anchoring of the epithelium to its underlying basement membrane via the formation of hemidesmosomes (Borradori and Sonnenberg, 1999). Moreover, the α6β4 integrin is found to be increased in several types of invasive and metastatic carcinomas with increased levels of expression correlating with a highly invasive and motile phenotype (Mercurio and Rabinovitz, 2001) as well as reduced patient survival (Grossman et al., 2000). In adenocarcinomas, the α6β4 integrin is released from the hemidesmosomes and associates with the actin cytoskeleton (Rabinovitz et al., 1999) were it can contribute to an invasive phenotype by its cooperation with growth factor receptors (Lipscomb and Mercurio, 2005) and signaling from molecules such as PI3-K (Shaw et al., 1997), Akt (Bachelder et al., 1999), ErbB-2 (Gambaletta et al., 2000), ErbB-3 (Folgiero et al., 2007), NFAT (Jauliac et al., 2002), NFkB (Mainiero et al., 2003), IRS1-2 (Shaw, 2001), MAPK (Dans et al., 2001), cAMP-specific phosphodiesterase (O'Connor et al., 1998), Rac1 (Zahir et al., 2003), and RhoA small GTPases (O'Connor et al., 2000).

The involvement of the α6β4 integrin in the invasive phenotype of multiple carcinomas and upregulation of this integrin in pancreatic adenocarcinomas prompted me to investigate *in vitro* how the α6β4 integrin may contribute to pancreatic cancer cell migration and invasion. Therefore, the goals of this study were: 1) to see if the integrin α6β4 contributes to the migration and invasion phenotype in pancreatic cancer cell lines and 2) to assess some of the mechanism of how integrin α6β4 contributes to the migratory and invasive phenotype in these cells.

Material and Methods

Cell Culture and Antibodies

MiaPaCa-2, Panc-1, (from America Type Culture Collection, ATCC) and Panc-1 subclones were cultured in Dulbecco's modified Eagle's medium (DME) high glucose

with 10% Fetal Plex (Gemini Bio-Products, West Sacramento, CA), 1% L-glutamine, 1% penicillin, and 1% streptomycin (GIBCO BRL, Gaithersburg, MD). Suit-2 (obtained from Dr. Takeshi Iwamura, Miyazaki Medical College, Miyazaki), BXPC-3, and ASPC-1 (obtained from ATCC) cells were cultured in RPMI 1640 medium containing 10% Fetal Plex serum plus 1% L-glutamine, 1% penicillin, and 1% streptomycin. The antibodies used include: anti-β4 integrin subunit, 505 rabbit polyclonal (Arthur Mercurio, University of Massachusetts, Worchester) and 439-9B rat monoclonal (Chemicon, Temecula, CA); GoH3 rat monoclonal anti-α6 integrin subunit (Santa Cruz Biotechnologies, Santa Cruz, CA); C-28 rabbit polyclonal anti-c-Met (Santa Cruz Biotechnologies); mouse monoclonal anti-Rac-1 antibody (BD Biosciences, San Jose, CA); rabbit polyclonal anti-Tiam-1 antibody (Bethyl Laboratories, Montgomery, TX), rat monoclonal anti-tubulin (Chemicon, Temecula, CA), and AC-15 mouse monoclonal anti-β-actin (Sigma Aldrich, St. Louis, MO).

Immunoblotting

Cells were lysed with RIPA buffer (150 mM NaCl, 0.5 mM EGTA, 0.1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 50 mM Tris-HCl, pH 7.4) containing 15 µg/ml protease inhibitor cocktail (Sigma Aldrich) and 1 mM phenylmethanesulfonyl fluoride (PMSF). Whole cell lysates were resolved on a 7.5% or 15% polyacrylamide gel and then transferred to a nitrocellulose membrane. Membranes were blocked with 5% milk for at least 30 min and probed with the indicated primary antibodies overnight at 4°C. After washing with TBS-Tween (20 mM Tris·HCl, pH 7.6, 14 mM NaCl) containing 0.1% Tween 20), membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibody. Membranes were developed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnologies, Rockford, IL) or Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA).

Fluorescence activated cell sorting (FACS)

Cells were harvested using 0.05% trypsin-EDTA and washed three times with serum-free medium containing 250 μg/ml heat inactivated BSA (medium/BSA). Cells were then incubated with primary antibody or IgG only for 1hr at 4°C and washed three times with 1X Dulbecco's Phosphate Buffer Saline, (DPBS; pH 7.4) (Gibco, Carlsbad, CA) containing 250 μg/ml heat inactivated BSA, (DPBS/BSA). Cells were then incubated with Cy2-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA) and analyzed on a FACS-Scan (Becton-Dickinson, Franklin Lakes, NJ). Relative fluorescence from the IgG only samples were subtracted from the samples treated with primary antibody.

Migration and Invasion assays

Cells were trypsinized, rinsed three times with medium/BSA, and resuspended in medium/BSA at 5 x 10^5 cells/ml. Transwell membranes (6.5 mm diameter, 8μm pore size; Corning, Corning, NY) were coated with 15 μg/ml of laminin-1 (Trevigen, Gaithersburg, MD) for 30 min at 37°C and then washed three times with medium/BSA for chemotaxis studies. For invasion assays, 7.3 μg of Collagen I (Vitrogen/PureCol, Leimuiden, The Netherlands) were diluted in cold water and dried onto the upper surface of each transwell chamber membrane. Matrix was reconstituted with medium without serum for one hour prior to use. HGF (50 ng/ml, unless otherwise indicated; Peprotech, Rocky Hill, NJ) diluted in medium/BSA was added to the bottom chamber as a chemoattractant. Cells (50,000) were added to the upper chamber and allow to migrate for 4 hrs or invade for 6 hrs at 37°C. Non-migrating or non-invading cells were removed with a cotton swab from the top chamber. Cells remaining in the bottom were fixed with 100% methanol, stained with 1% crystal violet in 2% ethanol and quantified visually from four random fields from each membrane using bright-field optics. Values for triplicate membranes are reported as a mean number of cells per mm^2 +/- the standard deviation of the mean.

RNA extraction and Real Time PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized using 5µg of RNA and the SuperScript™ III First-Strand Synthesis System (Invitrogen) as per the manufacture's recommendations. Real-Time PCR was performed at the Real-Time PCR Core facility, Sealy Center for Cancer Cell Biology, UTMB. I used Applied Biosystems inventoried 20X assay mixes (P/N 4331182) of primers and TaqMan MGB probe (FAM™ dye-labeled) for the target gene (TIAM1, Hs00180075_ m1) and pre-developed 18S rRNA (VIC™-dye labeled probe) TaqMan® assay reagent (P/N 4319413E) for endogenous control. For relative quantitation of gene expression, real time PCR was performed with 40 ng cDNA for both target gene and endogenous control. Using universal PCR master mix reagent kit (P/N 4304437). The cycling parameters for real time PCR were: Uracil-DNA-Glycosylase activation 50ºC for 2 min, AmpliTaq activation 95ºC for 10 min, denaturation 95ºC for 15 seconds and annealing/extension 60ºC for 1 min (40 cycles) on ABI PRISM® 7000 Sequence Detection System. Duplicate CT values were analyzed in Microsoft Excel using the comparative $C_T(\Delta\Delta C_T)$ method as described by the manufacturer (Applied Biosystems, Foster City, CA). The amounts of targets ($2^{-\Delta\Delta CT}$) were obtained by normalizing to endogenous reference (18s rRNA) and relative to a calibrator (one of the experimental samples).

Small interference RNA (siRNA) electroporation

Suspended cells (3×10^6) were electroporated with or without 200 nM of the indicated siRNA (300 V, 500 µF) with the Gene Pulser Xcell System (BioRad Laboratories, Hercules, CA) and then plated in normal growth medium. Cells were given fresh medium after 24 hr and harvested after 48 hr. Control non-targeting siRNA (non) or siRNAs designed to target the integrin β4, integrin α6, Rac-1 and Tiam-1 were SMARTPools (Dharmacon, Chicago, IL) containing four siRNA sequences. For rescue experiments, cells were electroporated

as described above; 48 hr later, cells were transfected with 10 μg of a Tiam-1 full length construct (John Exton, Vanderbilt University, Nashville, TN) using 30 μl Lipofectamine 2000 (Invitrogen). The following day, cells were assayed for migration and invasion.

Clone selection, transfections, and stable cell lines

The parental Panc-1 cells were sorted for the integrin β4 subunit by FACS analysis (Becton-Dickinson, Franklin Lakes, NJ). High and low integrin β4 expression cell populations were isolated and further subcloned by dilution cloning. Individual clones of the sorted population including Panc-1/2G6 low integrin β4 expresser; Panc-1/3D7 and Panc-1/ZCM high integrin β4 expressers were further identified. To isolate the Panc-1/Δcyt clone, Parental Panc-1 cells were transfected with a mutated β4 cDNA that lacked the cytoplasmic domain with the exception of four amino acids distal to the transmembrane sequence (β4-Δcyt (Clarke et al., 1995)) (Arthur Mercurio, University of Massachusetts, Worchester), using Lipofectamine 2000 (Invitrogen), selected with Genecitin (2 mg/ml) (G418; Invitrogen) and subcloned by dilution cloning. Expression of integrin β4 in cell lines and Panc-1 subclones were confirmed by immunoblotting and FACS.

Rac Activity Assays

Suspended cells (1 x 10^6) were plated on laminin-1 (15 μg/ml, Trevigen) coated plates in medium/BSA and allowed to attach and spread for 2 hr. Plated cells were then left untreated or treated with HGF (50 ng/ml HGF) for 5 min, then harvested with Rac lysis buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1% Nonidet P-40, 10% glycerol, 2 mM $MgCl_2$, 15 μg/ml protease inhibitor cocktail, and 1mM PMSF). Cell extracts were clarified by centrifugation, and 10% of the total volume was taken from the supernatant to compare total Rac content. The remaining extract was combined with 2 volumes of binding buffer (25 mM Tris, pH7.5, 1 mM dithiothreitol, 40 mM NaCl, 30 mM $MgCl_2$, 0.5% Nonidet P-40) and the bacterially produced Rac/Cdc42 binding domain of Pak (PBD)-GST fusion

protein (Rick Cerione, Cornell University) coupled to glutathione beads and incubated for 30 min at 4 °C. Beads were then rinsed three times with binding buffer and eluted in 2X Laemmli sample buffer. The aliquots of total cell extracts and the eluents from the PBD beads were analyzed for bound Rac1 molecules by Western blotting analysis.

Statistical Analysis

All experiments were repeated at least three times and data were reported as mean ± S.E. The statistical significance of differences between groups was calculated using a two-tailed *t* test ($P < 0.05$ was taken as significant) with the Sigma Stat 2.03 software statistical program (Systat Software, Point Richmond, CA).

Results

Expression of integrin α6β4 correlates with the ability of pancreatic cancer cell lines to chemotax towards HGF.

Integrin α6β4 expression has been positively correlated with the invasive and migratory phenotype of cancer cells and is increased in several types of invasive and metastatic carcinomas including colon, thyroid, breast, gastric, bladder, pancreas, and squamous carcinomas (Cruz-Monserrate et al., 2007; Gleason et al., 2005; Logsdon et al., 2003; Mercurio and Rabinovitz, 2001). Due to the high tumor dissemination rate and early overexpression of integrin α6β4 associated with pancreatic adenocarcinomas (Cruz-Monserrate et al., 2007; Lillemoe et al., 2000), I hypothesized that pancreatic cancer cell lines with increased levels of integrin α6β4 expression would migrate at a higher rate than cells with low levels of integrin α6β4. To test this hypothesis, I assessed the cell surface expression of both the integrin α6 and β4 subunits by FACS analysis (Fig. 3.1A) in five pancreatic cancer cell lines. I found that MiaPaca-2 and Panc-1 cells contained very low levels of cell surface α6β4 integrin while ASPC-1, Suit-2, and BXPC-3 cells each had

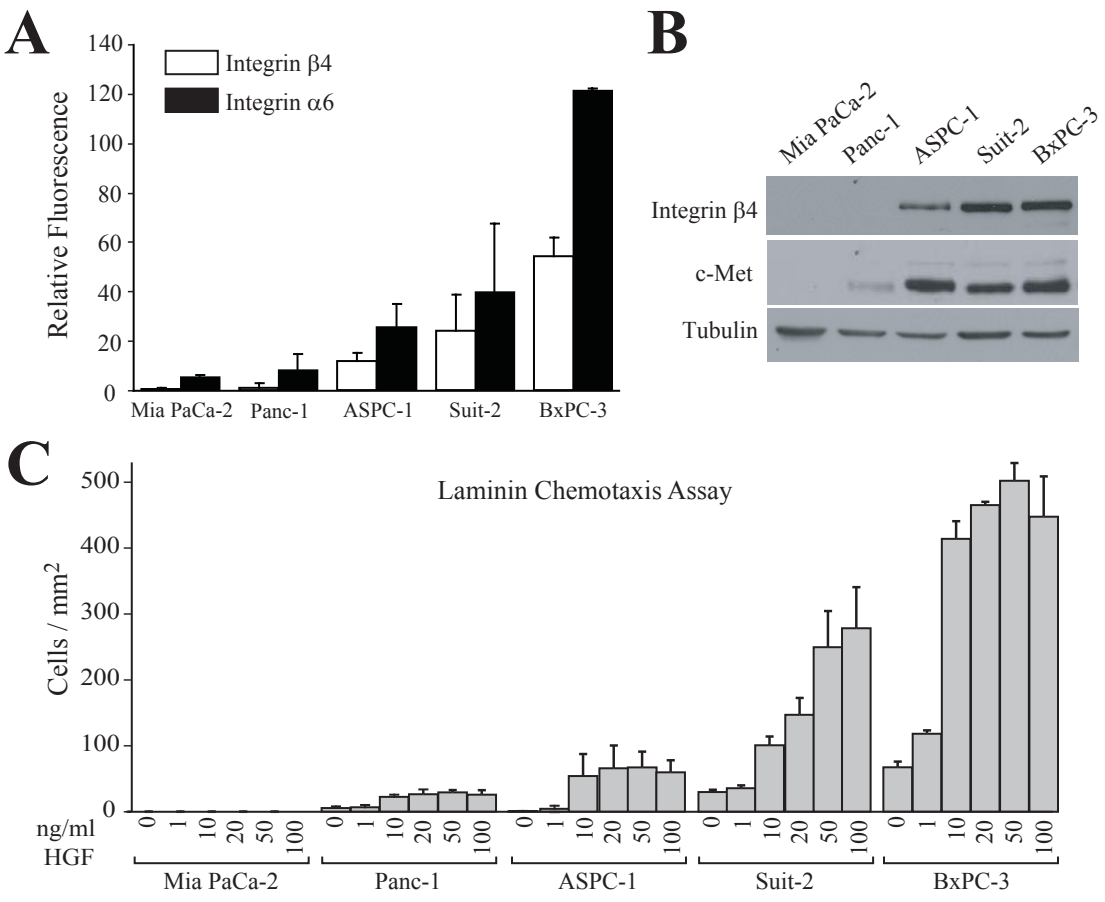


Figure 3.1. Integrin β4 expression in pancreatic cancer cell lines correlates with chemotactic efficiency towards HGF. (A) Select pancreatic cell lines, as noted, were assessed for both the α6 and β4 integrin subunit cell surface expression by FACS. Values represent average mean fluorescence and standard deviation of triplicate determinations. (B) Total cell lysates (80 μg) from pancreatic cancer cell lines were immunoblotted and probed for expression of integrin β4 subunit, c-Met and tubulin (loading control). (C) Pancreatic cancer cell lines were assayed for migration using a Transwell chamber assay toward increasing concentrations of HGF as described under "Materials and Methods". Values reported represent the mean number of cells migrated per mm^2 ± standard deviation obtained from triplicate determinations.

increasingly high levels. Analysis of whole cell lysates for total expression of integrin β4 by immunoblot analysis yielded similar results (Fig. 3.1B). Next, Transwell chemotaxis assays were performed using HGF as the chemoattractant since its receptor c-Met is known to cooperate with the integrin α6β4 to increase motility and invasion of cells in other tumor models (Chung et al., 2004; Trusolino et al., 2001). Here, I found that the integrin α6β4 expression levels correlated well with the ability of cells to chemotax towards HGF on laminin-1, a ligand of integrin α6β4 (Fig. 3.1C). Interestingly, the rate of HGF-induced migration, in cells that expressed c-Met does not correlate with the amount of c-Met expressed (Fig. 3.1B). Moreover, increasing amounts of HGF concentrations resulted in increased motility of Suit-2 and BXPC-3 cells which did not follow a bell-shape curve which is characteristic of receptors that are endocytosed after treatment with increasing concentrations of ligand. Overall, these results indicate that integrin α6β4 expression positively correlates with the ability of pancreatic cancer cells to chemotax towards HGF on a laminin substrate.

Downregulation of α6β4 integrin expression decreases HGF-stimulated chemotactic migration and invasion of pancreatic cancer cells

To more definitely assess the importance of α6β4 integrin expression in pancreatic cancer cell motility, I utilized synthetic siRNAs (21 nucleotide RNA duplex oligonucleotides that are homologous to a desired gene and reduce their expression) designed to target the α6 and β4 subunits to reduce expression of the α6β4 integrin in ASPC-1 cells. After 48hrs of siRNA treatment against either the α6 or β4 subunit, the cell surface expression level of the α6β4 integrin was decreased resulting in reduced HGF-stimulated chemotactic migration and invasion (Fig. 3.2). Similar results were obtained with Suit-2 cells; however, these results were less consistent likely due to the high expression level of this integrin.

To further confirm the positive role of integrin α6β4 in the motile and invasive phenotype of pancreatic cancer cell lines, I isolated clones from the parental Panc-1 cell

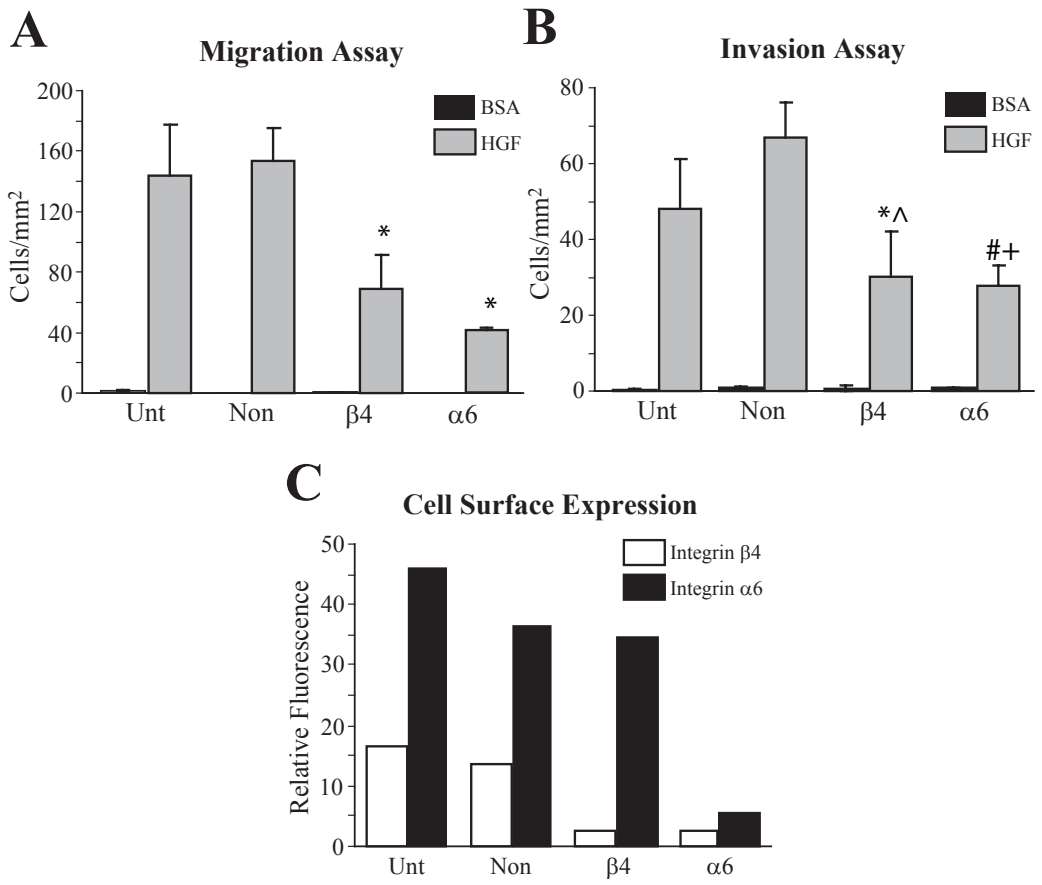
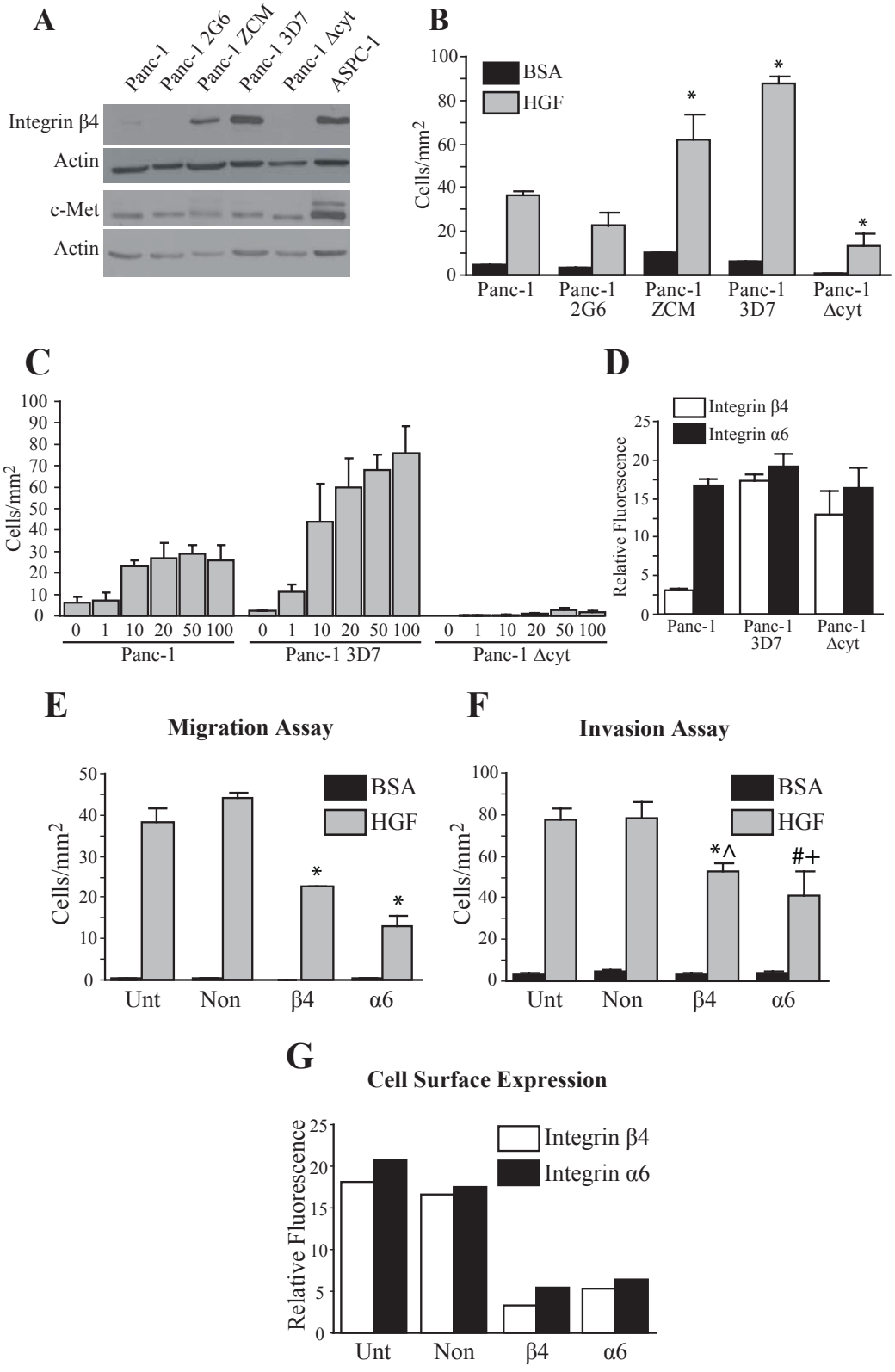


Figure 3.2. Downregulation of integrin α6β4 decreases chemotactic migration and invasion of ASPC-1 cells towards HGF. ASPC-1 cells (3 x 10^6) were electroporated only (untreated, "unt") or electroporated with 200 nM siRNA specific for the integrin subunits β4 or α6, or a non-targeting (non) control as indicated and analyzed 48hrs after siRNA treatment for chemotactic migration (A) (*P<0.001 compared to unt and non) and invasion (B) (*P=0.02 compared to unt; ^P<0.001 compared to non; #P=0.004 compared to unt; +P<0.001 compared to non) towards medium/BSA only or 50ng/ml HGF. (C) Integrin α6β4 cell surface expression determined by FACS as performed in Figure 3.1A to confirmed integrin reduction.

line, which endogenously expresses low levels (Panc-1/2G6), or high levels of integrin α6β4 (Panc-1/ZCM and Panc-1/3D7) but have similar levels of c-Met (Fig. 3.3A). The Panc-1/3D7 clone had the highest level of integrin α6β4 followed by the Panc-1/ZCM, parental Panc-1 and Panc-1/2G6 (Fig. 3.3A). Next, I assessed the ability of these cell lines to migrate on laminin-1 towards HGF and found that the levels of expression of the integrin α6β4 correlated with motility (Fig. 3.3B). In order to test the effects of signaling transduced from the integrin α6β4 cytoplasmic domain on motility, I stably transfected the Panc-1 parental cell line with a mutant β4 cDNA which lacked the cytoplasmic domain tail except for the four amino acids distal to the transmembrane sequence (Clarke et al., 1995) (Panc-1/β4-Δcyt). Expression of this construct was determined by FACS because the antibody used in this study for immunoblot analysis (505 rabbit polyclonal) recognizes the cytoplasmic domain of integrin β4 and therefore will not show a band by western blot (Fig. 3.3A, 3.3D). Shown in Figure 3.3B and 3.3C, cells stably transfected with β4-Δcyt construct had a lower migration rate towards HGF than other Panc-1 clones. Next, I treated the Panc-1/3D7 cells with siRNAs targeting the α6 and β4 integrin subunits. Similar to the ASPC-1 cells, both α6 and β4 subunits were found to be required for migration and invasion towards HGF and were effectively downregulated (Fig. 3.3E-G). Taken together, these results further confirm the involvement of the integrin α6β4 in the migratory and motile phenotype of pancreatic cancer cell lines.

Rac-1 activity is required for HGF stimulated migration and invasion of pancreatic cancer cells.

The integrin α6β4 cooperates with growth factor receptors and affects multiple signaling components that contribute to the increase migratory and invasive phenotype of cancer cells (Bon et al., 2007; Lipscomb and Mercurio, 2005). Among the molecules activated by the integrin α6β4 are PI3-K (Shaw et al., 1997) and the small GTPase Rac-1 (Zahir et al., 2003). In order to better understand signaling pathways involved in migration


A
Panc-1
Panc-1 2G6
Panc-1 ZCM
Panc-1 3D7
Panc-1 Δcyt
ASPC-1
Integrin β4
Actin
c-Met
Actin
B
BSA
HGF
Cells/mm2
Panc-1
Panc-1 2G6
Panc-1 ZCM
Panc-1 3D7
Panc-1 Δcyt
C
Cells/mm2
0 1 10 20 50 100
Panc-1
Panc-1 3D7
Panc-1 Δcyt
D
Integrin β4
Integrin α6
Relative Fluorescence
Panc-1
Panc-1 3D7
Panc-1 Δcyt
E
Migration Assay
BSA
HGF
Cells/mm2
Unt
Non
β4
α6
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Invasion Assay
BSA
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Cell Surface Expression
Integrin β4
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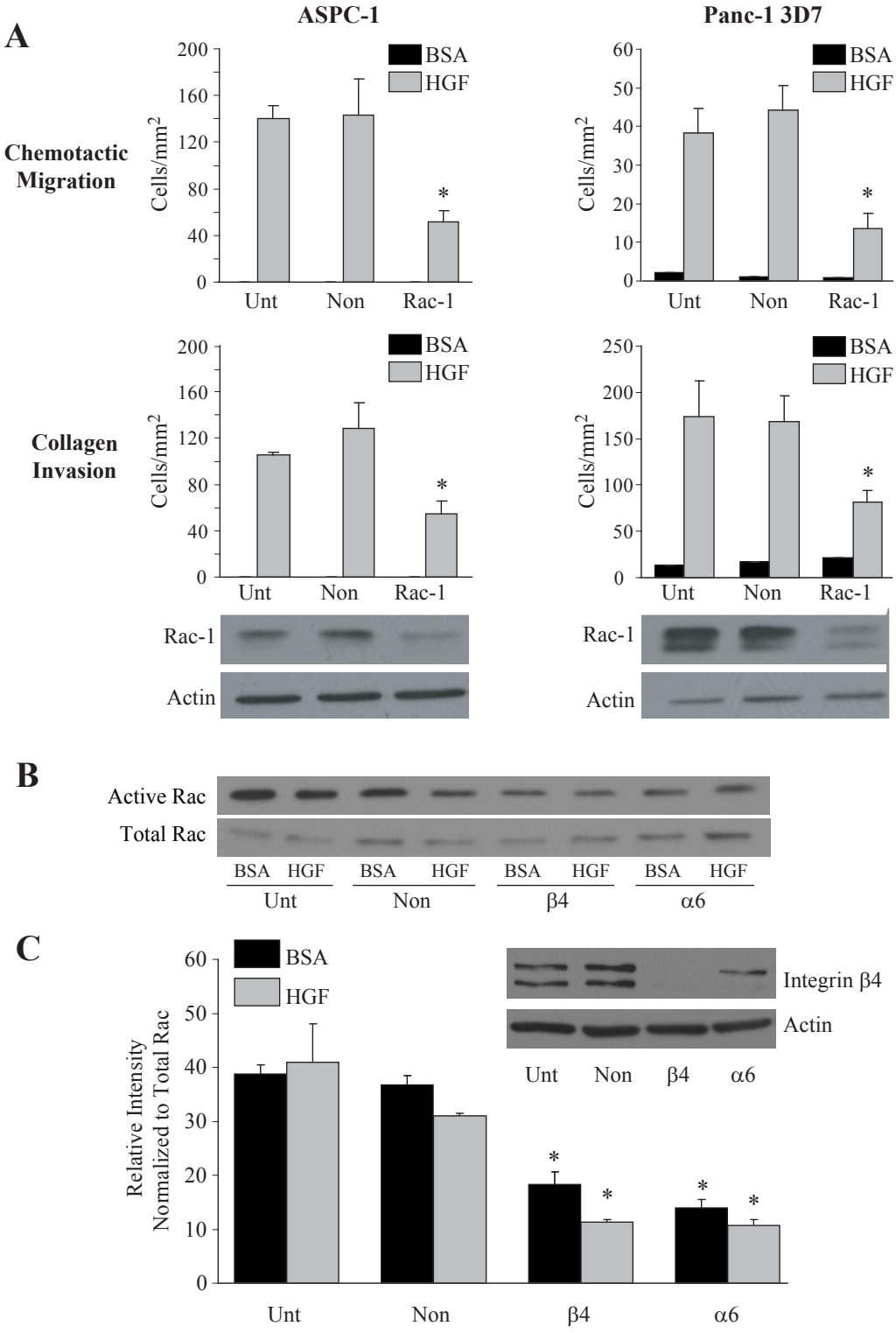
β4
α6

and invasion of pancreatic cancer cells that are dependent on α6β4 integrin expression, I focused first on the involvement of Rac-1 in these processes. Rac-1 can enhance tumor motility and invasion in culture (Keely et al., 1997) and is overexpressed in 70% of pancreatic cancer tumor samples (Crnogorac-Jurcevic et al., 2001). To test the involvement of Rac-1 in pancreatic cancer cell motility and invasion, ASPC-1 and Panc1/3D7 cells were treated with Rac-1 specific siRNAs to downregulate the expression of Rac-1 prior to assessing the cells' ability to migrate and invade towards HGF. I found that Rac-1 siRNA treatment reduced the migration and invasiveness of both cell lines towards HGF (Fig. 3.4A). Next, I assessed Rac-1 activity using a PAK domain GST-pull down assay after Panc-1/3D7 cells were treated with integrin α6 and β4 subunit siRNAs. My results indicate that basal levels of Rac-1 activity were high in these cells (Fig. 3.4B). Upon treatment with α6 or β4 siRNAs, however, basal and HGF-stimulated Rac-1 activity was decreased (Fig. 3.4B-C). Similar results were found with the ASPC-1 cell line (data not shown). Together these data suggest that Rac-1 is required for the motility and invasion and that expression of the integrin α6β4 promotes activation of Rac-1 in pancreatic cancer cells.

To further understand the mechanisms of how integrin α6β4 induces activation of Rac-1 to promote cell migration and invasion, I then investigated the possible contribution of PI3-K activation downstream of integrin α6β4. I found that PI3-K activity was critical

Figure 3.3. (previous page) Integrin α6β4 expression is required for the migration and invasion of pancreatic cancer cell lines in-vitro. (A) Total cell lysates (80 μg) from indicated cell lines were immunoblotted and probed for integrin β4 subunit, c-Met, and actin (loading control) expression. (B) Pancreatic cancer cell lines were assayed for migration towards medium/BSA only or 50 ng/ml HGF. *P<0.001 compared to Panc-1. (C) Panc-1, Panc-1/3D7 (high integrin β4 expression), and Panc-1 β4-Δcyt cells were assayed for migration towards increasing concentrations of HGF. (D) The level of integrin α6β4 expression from the cells used in (C) was confirmed via FACS. (D) Panc-1/3D7 cells (3×10^6) were treated as described in Figure 3.2 and assayed for chemotactic migration (*P <0.001 compared to unt and non) and invasion (F) towards medium/BSA or 50 ng/ml HGF (*P = 0.002 compared to unt; ^P = 0.006 compared to non; #P<0.001 compared to unt; +P=0.002 compared to non). (G) Integrin α6β4 cell surface expression determined by FACS to confirmed downregulation.


A
ASPC-1
Panc-1 3D7
Chemotactic Migration
Collagen Invasion
BSA
HGF
Cells/mm2
Unt
Non
Rac-1
Rac-1
Actin
B
Active Rac
Total Rac
BSA HGF BSA HGF BSA HGF BSA HGF
Unt
Non
β4
α6
C
Relative Intensity Normalized to Total Rac
Integrin β4
Actin
Unt
Non
β4
α6

for the migration and invasion of various pancreatic carcinoma cell lines toward HGF, as determined using the PI3-K inhibitors wortmannin and LY294002 (Fig. 3.5). However, I was unable to link the α6β4 integrin to the activation of PI3-K through either integrin clustering or reduction of the integrin α6β4 expression by siRNA alone or in conjunction with HGF treatment (data not shown). I did find however, that PI3-K activity levels as determined by tyrosine phosphorylation of the p85α subunit or phosphorylated AKT were high in all the HGF treated cells but were not dependant on the integrin α6β4 expression but rather on c-Met expression levels (Fig. 3.6). These data suggests that integrin α6β4-dependent activation of Rac-1 is not the result of integrin α6β4-dependent PI3-K activity.

Tiam-1 is upregulated by the integrin α6β4 and is the required exchange factor to activate Rac-1 and promote migration and invasion of pancreatic cancer cells.

Having established a link between the expression of integrin α6β4 and Rac-1 activation, I next sought to determine molecules that could link them to promote increase motility and invasion. To determine a panel of candidate molecules, I performed Affymetrix gene chip analysis on the Panc-1 clones and found that many genes were regulated by the expression of integrin α6β4 by more than two fold (95% confidence level). Among these

Figure 3.4. (previous page) Rac-1 is required for the chemotactic migration and invasive potential towards HGF. ASPC-1 and Panc-1/3D7 cells (3 x 10^6) were electroporated only (unt) or electroporated with 200 nM siRNA specific for Rac-1 or a non-targeting (non) control as indicated and analyzed 48hrs after siRNA treatment for chemotactic migration and invasion (A) towards medium/BSA or 50 ng/ml HGF. *P<0.001 compared to unt and non treated cells. Immunoblot of cell lysates was used to confirmed downregulation of Rac-1 for the ASPC-1 cells (A lower left) and Panc-1/3D7 cells (A lower right). (B) Panc-1/3D7 cells (3 x 10^6) were electroporated only (unt), treated with 200 nM siRNA specific for the integrin subunits β4, α6, or a non-targeting (non) control as indicated. After 48 hrs of siRNA treatment, cells were plated on laminin-1 and left untreated or treated for 5 min with 50 ng/ml HGF. Cell lysates were then analyzed for Rac-1 activity (using the PBD assay as described under "Materials and Methods"). (C) Densitometric analysis of three different exposures of immunoblots from (B) of activated (PAK-associated) Rac-1 divided by 10% of the total cellular Rac-1. *P<0.001 compared to unt and non, BSA and HGF. Insert shows immunoblot of integrin β4 to confirm target downregulation and actin as loading control.

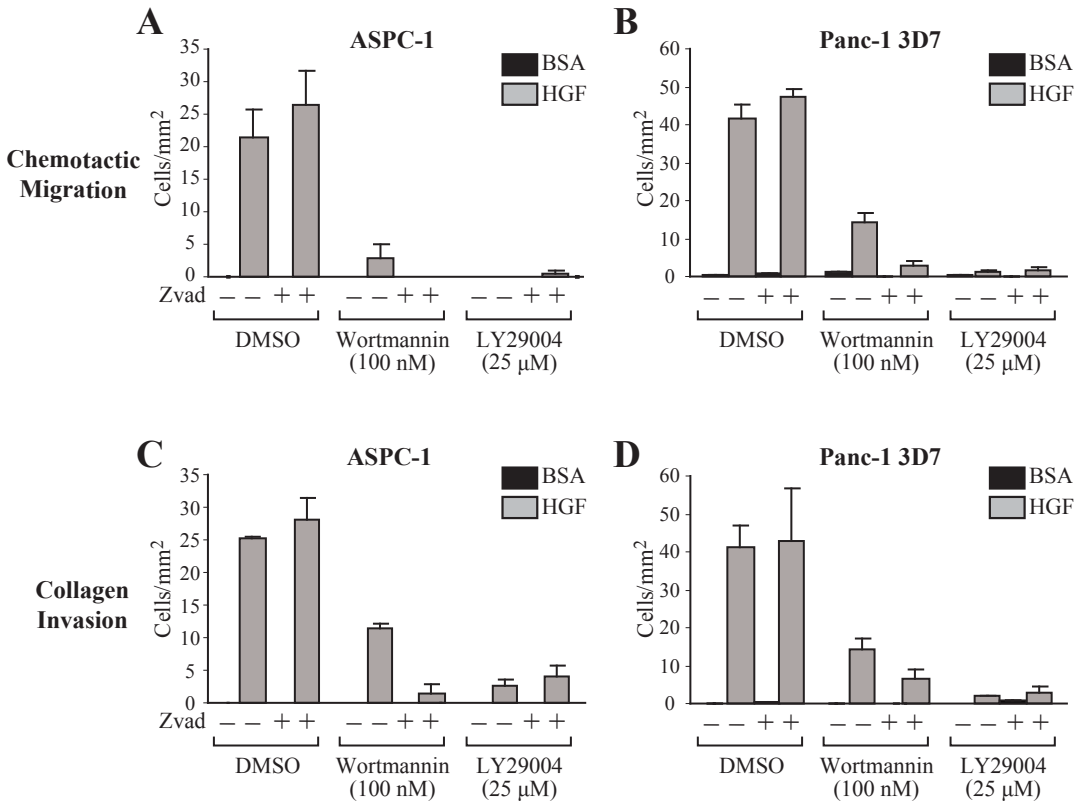


Figure 3.5. PI3-K is required for chemotactic migration and invasion towards HGF. ASPC-1 (A,C) and Panc-1/3D7 clone (B,D) cells were incubated with DMSO, LY294002, and Wortmannin for 30 min at room temperature with and without ZVad (a caspase inhibitor) and then allowed to migrate (A-B) towards HGF for four hours and invade (C-D) for 6 hours towards HGF as in Figure 3.4.

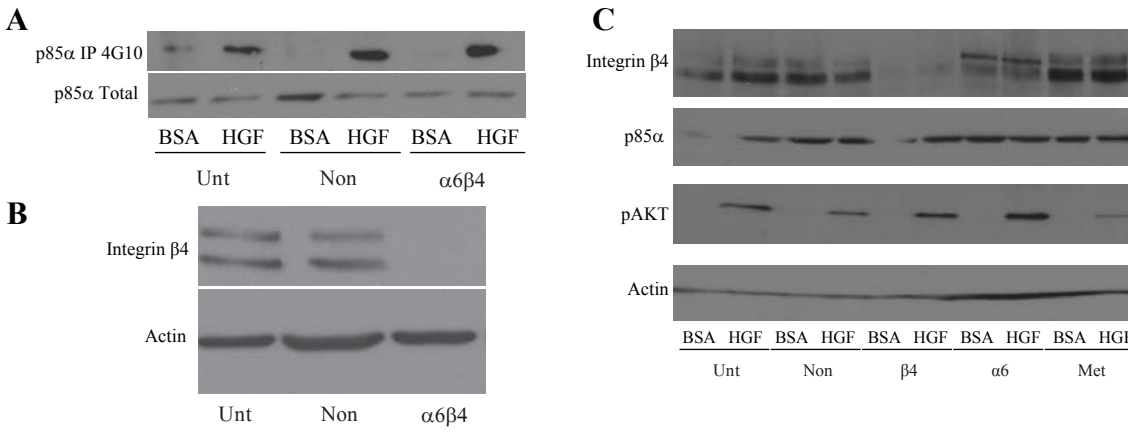


Figure 3.6. Integrin α6β4 expression is not required to activate PI3-K. Panc-1/3D7 cells (3 x 10^6) were electroporated only (unt) or electroporated with 200 nM siRNA specific for α6 and β4 or a non-targeting (non) control as indicated. After 48hrs of siRNA treatment. Cells were plated on laminin-1 and left untreated or treated for 5 min with 50 ng/ml HGF. Cell lysates were then analyzed for p85α phophostyrosine activation by immnoprecipitation with an anti-phosphotyrosine antibody (clone 4G10, that recognizes tyrosine-phosphorylated proteins) and then immunoblotted with a p85α antibody (A). (B) Immunoblot of integrin β4 to confirm target downregulation and actin as loading control. (C) Panc-1/3D7 cells treated with indicated siRNAs as in (A), cell lysates were probed with the indicated antibodies.

genes, the guanine nucleotide-exchange factor (GEF), T lymphoma invasion and metastasis (Tiam-1) was upregulated 2-fold in cells with the highest integrin α6β4 expression (data not shown). Since Tiam-1 is a Rac-specific GEF and has been implicated to play a role in cancer cell migration, invasion and tumor progression (Minard et al., 2004), I investigated the expression of Tiam-1 in various pancreatic cancer cell lines and examined its ability to activate Rac-1. Using Real Time PCR, I found that Tiam-1 was increased in three of the four pancreatic cancer cell lines (Panc-1/3D7, Suit-2 and BXPC-3) which overexpressed the α6β4 integrin (Fig. 3.7A). In addition, I found that the Panc-1/Δcyt cells, which are unable to signal through the β4 subunit, did not expressed Tiam-1 (Fig. 3.7A).

I next investigated if Tiam-1 expression was required for HGF-stimulated chemotactic migration and invasion and if it was the exchange factor responsible of promoting Rac-1 activity downstream of integrin α6β4. In order to understand the role

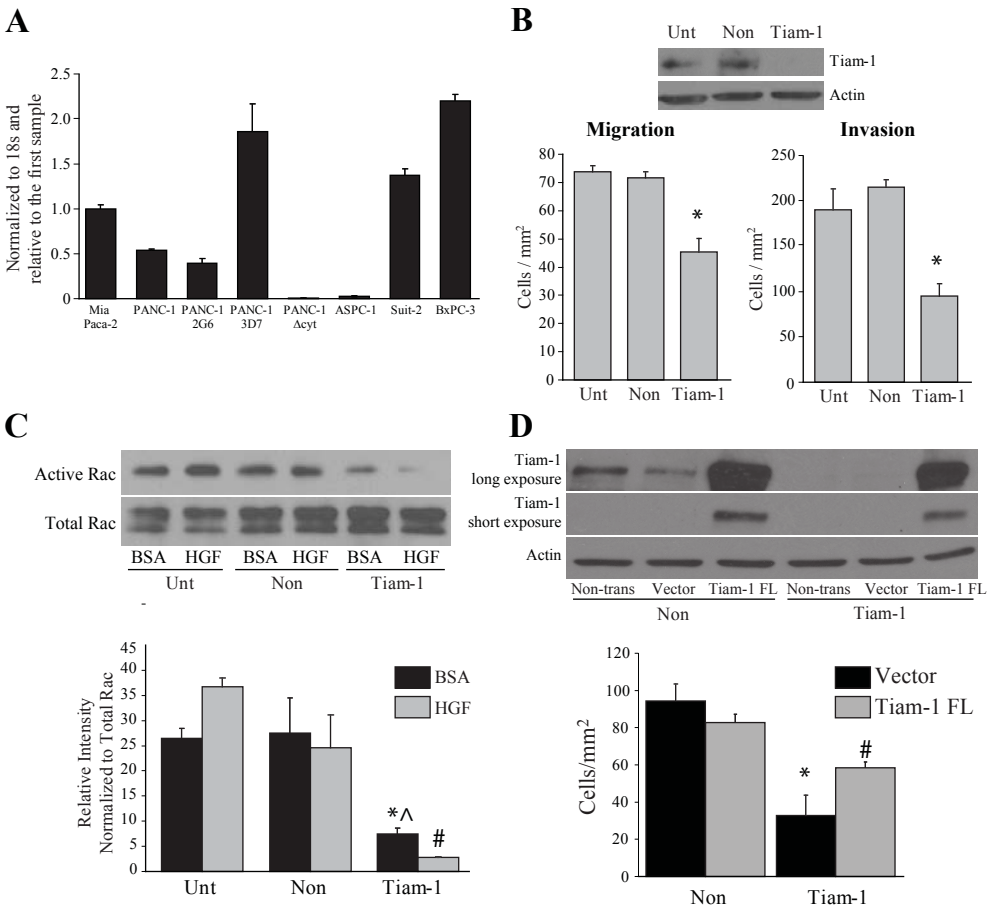


Figure 3.7. Tiam-1 expression is required for Rac-1 activity, migration, and invasion towards HGF of most pancreatic cancer cell lines that have high levels of integrin α6β4. (A) cDNA from pancreatic cancer cell lines were assessed for Tiam-1 expression via Real Time PCR as described in the Materials and Methods. (B, C) Panc-1/3D7 cells (3×10^6) were electroporated only (unt), treated with 200 nM siRNA specific for Tiam-1, or a non-targeting (non) control as indicated and analyzed 48 hrs after siRNA treatment for chemotactic laminin-1 migration and collagen invasion towards 50 ng/ml of HGF; *P<0.001 compared to unt and non treated cells (B) and for Rac-1 activity as performed in Figure 3.6 (C). Graph in (C) shows densitometry analysis from two different blot exposures; *P<0.001 compared to unt/BSA; ^P=0.003 compared to non/BSA; #P<0.001 compared to unt and non HGF. Immunoblot of Tiam-1 for siRNA target confirmation and actin as loading control (B). (D) Panc-1/3D7 cells were either treated with 200 nM siRNA specific for Tiam-1, or a non-targeting (non) control as indicated. After 48hrs of siRNA treatment, cells were either left non-transfected (non-trans), or transfected with vector only or a full length Tiam-1 construct. After 48hr of transfection cells were assessed for their ability to migrate towards HGF on laminin-1 coated transwells (bottom panel) as in (B). Immunoblot of Tiam-1 at different exposure times for transfection and siRNA target confirmation and actin as loading control (top panel). *P<0.001 compared to non/vector and non/Tiam-1 FL; #P=0.001 compared to Tiam-1 vector.

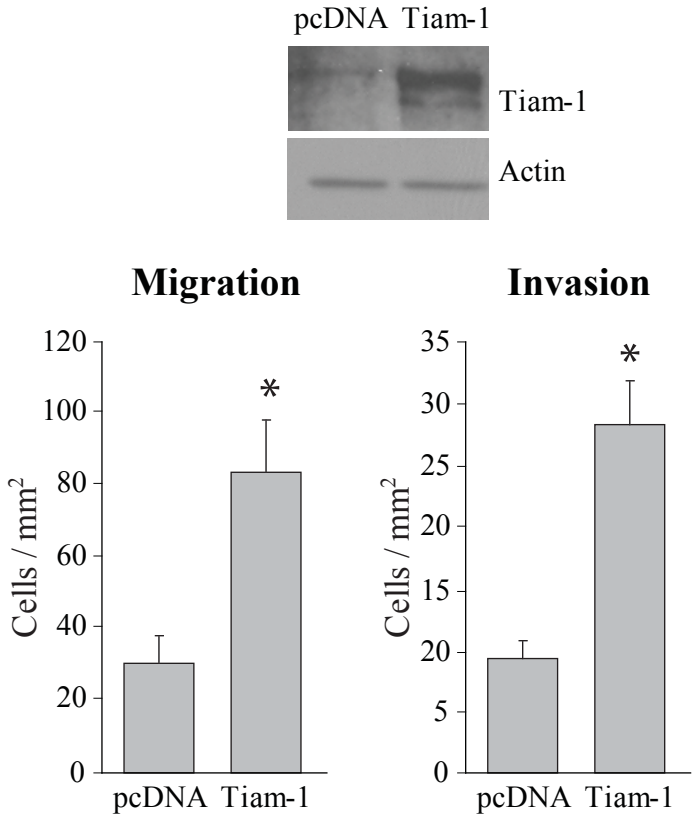


Figure 3.8. Exogenous Tiam-1 promotes migration and invasion of pancreatic cancer cells. Panc-1 2G6 cells (low integrin β4 expression) were transiently transfected with a full length construct of Tiam-1 and then assessed for chemotactic migration on laminin-1 and collagen invasion towards 50ng/ml of HGF. *P<0.001 compared to pcDNA transfected cells. Top panel shows immunoblot of Tiam-1 to confirm transfection of full length Tiam-1 construct and actin as loading control.

of Tiam-1 in migration and invasion, Panc-1/3D7 cells were treated with non-targeting or Tiam-1-specific siRNAs. After 48hrs, the ability of the cells to migrate and invade towards HGF was assessed. My results show that Tiam-1 siRNA treatment reduced the migration and invasion of Panc-1/3D7 cells towards HGF (Fig. 3.7B). I then monitored Rac-1 activity using a PAK domain GST-pull down assay and found that treatment with Tiam-1 specific siRNA also decreased Rac-1 activity (Fig. 3.7C). Moreover, the migratory phenotype that was suppressed with Tiam-1 siRNA treatment was rescued by the transfection of a full length construct of Tiam-1 (Fig. 3.7D). Furthermore, Panc-1/2G6 cells (low integrin β4

expressers) transiently transfected with a full length Tiam-1 construct displayed an increase in migration and invasion compared to control (vector only) transfected cells (Fig. 3.8). Interestingly, I did not observe expression of Tiam-1 in ASPC-1 cells suggesting that there is likely more then one exchange factor that can lead to the phenotype observed in this study. Overall this data indicates that Tiam-1 expression correlates well with α6β4 integrin expression and that this exchange factor is required for activation of Rac-1 as well as HGF induce chemotactic migration and invasion of some pancreatic cancer cells.

Discussion

The integrin α6β4 is associated with carcinoma progression and subsequent metastasis of many types of carcinomas by its ability to enhance motility and invasiveness of cancer cells (Lipscomb and Mercurio, 2005). However, the role of this integrin in pancreatic cancers has remained under explored. My studies from Chapter two and other groups have shown that the integrin α6β4 is overexpressed in pancreatic cancers when compared to normal pancreas and chronic pancreatitis (Crnogorac-Jurcevic et al., 2003; Cruz-Monserrate et al., 2007; Gleason et al., 2005; Logsdon et al., 2003; Nakamura et al., 2004). In addition, this overexpression and altered localization from the interface with the basement membrane, occurs early in pancreatic adenocarcinoma tumor progression (Cruz-Monserrate et al., 2007). In the current study, I extended my observations of the role of α6β4 integrin in pancreatic cancers. I demonstrate using siRNAs to knockdown the integrin α6 and β4 subunits that this pro-migratory and pro-invasive integrin facilitates HGF-stimulate motility and invasion of pancreatic cancer cells *in vitro*. Moreover, my data supports other studies demonstrating that signaling through the α6β4 integrin and c-Met cooperate to promote motility and invasion of cancer cells (Chung et al., 2004; Trusolino et al., 2001).

Cancer cells can acquire a motile and invasive phenotype as a result of the many

cellular mechanisms whose functions are affected and their ability to interact with the tumor microenvironment. These interactions can promote the activation of aberrant signaling pathways which often promote the activity of the Rho family of Ras-related GTP-binding proteins. Among this proteins are Rho, Rac, and Cdc42, molecules that can control actin cytoskeleton organization and result in the regulation of cell invasion, motility, adhesion and polarity (Rossman et al., 2005). In pancreatic tumors, Rac-1 expression was reported to be important because it was overexpressed in 70% of tumor samples (Crnogorac-Jurcevic et al., 2001). Moreover, Rac-1 can enhance tumor cell migration and invasion in culture (Keely et al., 1997) and is among the molecules that are activated downstream of integrin α6β4 (Zahir et al., 2003). Activation of Rac-1 mediates migration and invasion by eliciting actin polymerization at the plasma membrane to produce lamellae and membrane ruffles (Ridley, 2001). In this study, I report that Rac-1 is required for pancreatic cancer cells HGF-induced cell motility and invasion and demonstrate using siRNA that expression of the integrin α6β4 promotes activation of Rac-1 in these cells. Furthermore, I highlight a novel mechanism by which integrin α6β4 could affect activation of Rac-1 to induce motility and invasion of pancreatic cancer cells. This mechanism involves the ability of integrin α6β4 to upregulate the expression of the Rac-1 specific GEF, Tiam-1 (Fig. 3.9).

The activity of small GTPases such as Rac are tightly regulated by GEFs and GTPase-activating proteins that transition GTPases between an inactive state (GDP-bound) and active state (GTP-bound). Currently some GEFs have been identified to serve as oncogenes. Among those are Vav1, LARG, Bcr and Tiam-1 (Bassermann et al., 2002; Denicola and Tuveson, 2005; Perrot et al., 2002; Rossman et al., 2005). Interestingly, I discovered that the expression of integrin α6β4 in pancreatic cancer cell lines correlated well with the expression of the GEF Tiam-1 known to selectively activate the Rho family GTPase Rac (Michiels et al., 1995). Tiam-1 was originally identified as an invasion and metastasis inducing gene by proviral tagging, in combination with *in vitro* selection for

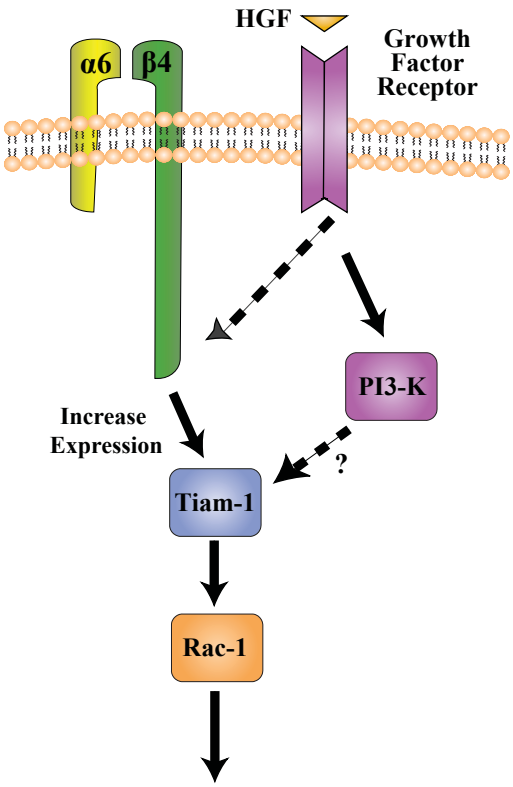


Figure 3.9. Diagram of mechanism by which integrin α6β4 could promote migration and invasion of pancreatic cancer cells. Increase α6β4 integrin upregulates expression of the GEF Tiam-1, which can then activate Rac-1 and promote HGF-induced migration and invasion. c-Met can promote PI3-K activation, but could also transtactivate integrin β4.

invasiveness in T lymphoma cells (Habets et al., 1994). But the role of Tiam-1 in cell migration, invasion, and metastasis is not limited to T lymphoma, as this exchange factor is involved in promoting tumor progression in other cancers such as colorectal, breast, colon, and lung cancers, as well as Ras-induced skin tumors (Minard et al., 2004). In this study, I found that expression of Tiam-1 was required for pancreatic cancer cells to activate Rac-1 and promote HGF-induced migration and invasion in a cell line with high levels of integrin α6β4. Furthermore, in cells which are less motile and invasive and contain low expression levels of both integrin α6β4 and Tiam-1, exogenous expression of Tiam-1 resulted in an increase in both HGF-induced migration and invasion. I also demonstrated that the cytoplasmic tail of the integrin α6β4, known to be responsible for the signaling that can be transduced from this integrin in other cancer models (Shaw et al., 1997; Trusolino et al., 2001), is required for expression of Tiam-1 since the Panc-1/Δcyt clone lacked expression of Tiam-1 and is unable to migrate after HGF stimulation.

It is interesting to note that the ASPC-1 cells which contain high expression levels of integrin α6β4 and can activate Rac-1, lacked expression of Tiam-1. These data suggests that more then one exchange factor could be responsible for Rac-1 activation by the integrin

α6β4 to promote the migratory and invasive phenotype observed. One candidate could be Vav1 as it was shown to play a role in pancreatic cancer tumorigenesis (Fernandez-Zapico et al., 2005) but others like Sos, Pix, Trio and Tiam-2 could also be involved as these are other GEFs known to activate Rac. One possibility for the discrepancy in Tiam-1 expression could be the origin from which these pancreatic cancer cell lines were derived since the ASPC-1 cells were isolated from a metastasis to the ascites (Chen et al., 1982) and the Panc-1 cells were isolated from a primary tumor of the head of the pancreas (Lieber et al., 1975). Moreover, GEF expression could be cell type specific. For example, in breast cancer cell lines other GEFs besides are involved in promoting Rac activity (unpublished observation). Taken together, these suggests that there is much to learn about the mechanisms of how integrin α6β4 effects migration and invasion of cancer cells especially how it can affect GEF expression and subsequent activation.

Activated mutant K-Ras is one of the first events in pancreatic adenocarcinomas that occurs at a frequency of around 80-100% (Klimstra and Longnecker, 1994). Interestingly, signaling induce from this type of oncogenic Ras can have different effects on Tiam-1 expression which can in turn affect tumor development and progression. For example, effective initiation of chemically induced epidermal tumors by oncogenic Ras *in-vivo* requires Tiam-1 expression and Rac signaling. However, oncogenic Ras signaling can also promote a decrease in Tiam-1 expression (Zondag et al., 2000) that correlates with malignant tumor progression (Malliri et al., 2002). Therefore, if oncogenic Ras signaling has the potential to downregulate Tiam-1 expression and promote malignant progression, then other GEFs must be utilized to induce migration and invasion of cells and/or other signaling pathways could be activated to increase Tiam-1 and compensate for the Ras-induced Tiam-1 loss. Since my results show that integrin α6β4 can upregulate Tiam-1 expression *in vitro* and this integrin is also overexpressed at an early stage during pancreatic adenocarcinoma progression (Cruz-Monserrate et al., 2007), then my study

suggests that increased expression of integrin α6β4 in pancreatic cancer cells could restore Tiam-1 expression after its possible downregulation as a result of oncogenic Ras signaling. Interestingly, recent studies have linked Ras and integrin α6β4 where Ras was shown to stimulate expression of the α6β4 integrin (Yoon et al., 2006b) and under serum depleted conditions α6β4 integrin was able mediate Ras activity of breast cancer cells (Yoon et al., 2006a). Taken together, these findings suggest the possibility of a potential cooperation between integrin α6β4 and Ras signaling in pancreatic adenocarcinomas that could contribute to its aggressive nature. However, further studies would be required to understand how integrin α6β4 and Ras signaling could cooperate to promote progression of pancreatic adenocarcinoma.

The most studied signaling molecule activated by the α6β4 integrin is PI3-K, whose activation is important for carcinoma invasion (Shaw et al., 1997). In order to activate PI3-K, integrin α6β4 is sought to synergize with specific growth factor receptors such as ErbB2 and c-Met as well as other molecules like the insulin receptor substrates that contain a PI3-K biding motif (Lipscomb and Mercurio, 2005). In this study, as reported for other cancers, I found that PI3-K activation was required for migration and invasion of pancreatic cancer cells with high expression levels of integrin α6β4 as determined using PI3-K specific drug inhibitors. Nevertheless, I found that PI3-K activation after HGF-stimulation was not dependant on α6β4 integrin expression levels in the pancreatic cancer cell lines tested. One possible explanation for these results could be due to the techniques used in this study. As reported previously (Lipscomb et al., 2003) downregulation of integrin α6β4 using transient transfection of synthetic siRNAs is a challenge, as the levels of knockdown are not 100%. This suggests that even low levels of integrin α6β4 remaining on the cell surface after siRNA treatment, coupled with the ability of this integrin to amplify signaling due to its cooperation with other growth factors could be enough to activate PI3-K. Furthermore, PI3-K activity required to promote motility and invasiveness of pancreatic carcinoma cells

could be induced by HGF/c-Met signaling (Fig. 3.9) and/or oncogenic K-Ras signaling rather then from integrin α6β4. Both HGF/c-Met and oncogenic K-Ras signaling pathways are know to induced PI3-K activity (Campbell et al., 2007; Nakanishi et al., 1999).

In summary, this study reports the novel finding that integrin α6β4 upregulates the expression of the Rac-specific GEF, Tiam-1 in pancreatic cancer cell lines. Furthermore, I show evidence that expression of Tiam-1 and integrin α6β4 enhance HGF-induced migration and invasion which was dependent on Rac-1 activity. Therefore I suggest a new mechanism for the integrin α6β4 to induce migration and invasion of cancer cells that is dependent on Tiam-1 expression and subsequent Rac-1 activation. This study in pancreatic cancer provides an important extension of the numerous *in vitro* studies that highlight the contribution of the integrin α6β4 expression and related signaling to induce migration and invasion of cancer cells.

CHAPTER FOUR: SUMMARY AND FUTURE DIRECTIONS

Summary

Despite extensive clinical efforts, mortality of patients with pancreatic adenocarcinomas is still high. This is oftentimes because most patients do not develop symptoms until after the cancer has metastasized at which time treatment is not efficacious. In addition to this, many patients are not correctly diagnosed until months or even years after first developing symptoms. Consequently, understanding the genetic and cellular changes that contribute to the development and malignant progression of pancreatic adenocarcinomas is vital. This knowledge will help in the development of early diagnostic markers and drugs to treat patients at different stages of the disease and/or help prevent the carcinoma from developing or progressing to an advanced stage. Although clinical progress has been relatively slow, our current knowledge of the genetic and cellular events that lead to an infiltrating adenocarcinoma has grown and a model of pancreatic adenocarcinoma progression has emerged (Hruban et al., 2001). The basis of this model is the formation of proliferative PanIN lesions that together with commonly found genetic alterations can lead to an advanced disease. However, much is still unknown about how these genetic and cellular alterations progress. A better understanding of these lesions as well as the basic mechanisms of cell migration and invasion offers the best hope for treating pancreatic adenocarcinoma.

Because clinical studies support the positive correlation between integrin α6β4 expression with multiple metastatic cancers (Mercurio and Rabinovitz, 2001), the overall goal of this dissertation was to study the significance of integrin α6β4 expression and signaling in pancreatic adenocarcinoma that contribute to increased cell migration and invasion. *The hypothesis of this dissertation was that expression of and signaling from integrin α6β4, in cooperation with HGF stimulation facilitated cell migration and invasion*

of pancreatic adenocarcinomas.

To test this hypothesis, I first determined if expression of the pro-invasive integrin α6β4 was related to pancreatic adenocarcinoma tumor progression using patient samples (Chapter 2). I found that integrin α6β4 was upregulated in PanIN lesions (as early as PanIN-1A) and pancreatic adenocarcinomas when compared to normal pancreas and chronic pancreatitis. In addition, localization of integrin α6β4 was altered, so that it was no longer restricted to the basal surface. Rather integrin α6β4 was found in the cytosol, and on the basal, lateral and apical sides of the plasma membrane. Overexpression of this integrin at an early stage in pancreatic adenocarcinomas suggests that α6β4 integrin could contribute to the development and progression of this cancer to an advanced stage. The ability of α6β4 integrin to cooperate, activate and/or promote expression of many factors that can contribute to the dissemination of carcinomas and the fact that many of those factors are known to be present in pancreatic adenocarcinomas could help explain why pancreatic adenocarcinomas are so metastatic. However, further studies are needed to understand the mechanisms involved in the induction of integrin α6β4 expression and redistribution, as well as how it contributes to pancreatic adenocarcinoma progression.

Unfortunately, of all cancers, pancreatic adenocarcinomas are among the most difficult for pathologists to diagnose. Histological features are similar between benign reactive glands of chronic pancreatitis and an infiltrating gland of well-differentiated pancreatic adenocarcinoma that distinguishing the two diseases can be extremely difficult. Therefore, the risks of a misdiagnosis are high. Interestingly, in this dissertation I found that the staining pattern of integrin α6β4 could clearly distinguish cancerous areas from chronic pancreatitis. In particular, the staining of integrin α6β4 highlighted cancerous areas even when cancerous regions were not apparent upon H&E staining, the standard method used by pathologists. This suggests that the staining pattern of integrin α6β4, in conjunction with other known markers and histological characteristics, could allow pathologists to

identify and diagnose pancreatic adenocarcinoma versus chronic pancreatitis, as well as be useful for the detection of early lesions associated with pancreatic cancer.

To further understand the relevance of the integrin α6β4 expression in pancreatic adenocarcinomas and test the hypothesis of this dissertation, I assessed if the expression of this integrin facilitated HGF-induce migration and invasion using pancreatic cancer cell lines *in vitro*. Consistent with the hypothesis, I found that expression of integrin α6β4 enhanced the ability of pancreatic cancer cell lines to migrate towards HGF. I also demonstrated that when the expression levels of this integrin were reduced, the cell lines ability to migrate and invade towards HGF were also reduced.

Over the years, the role of integrin α6β4 in many cell processes such as adhesion, survival, migration and invasion has become evident. This is particularly true in migration and invasion of carcinoma cells as I also demonstrated in this work on pancreatic cancer. Despite this knowledge, much is unknown about the mechanisms of how this integrin can promote such events. To help address how integrin α6β4 could contribute to the migration and invasion of cancer cells I explored mechanisms of how the α6β4 integrin could contribute to the invasive and migratory phenotype in my system. Among the most studied mechanisms to date for which integrin α6β4 can promote invasion of carcinoma cells is the PI3-K–Rac-1 pathway (Shaw et al., 1997). Since I found that Rac-1 expression and activity were required for HGF-induced migration and invasion and Rac activation was dependent on integrin α6β4 expression, I then explored the possibility of the involvement of PI3-K activation downstream of integrin α6β4. Contrary to what is known about the ability of this integrin to activate PI3-K and promote invasion of carcinoma cells (Shaw et al., 1997), I did not find activation of PI3-K to be dependent on integrin α6β4 expression, although both proteins were required for migration and invasion. This finding suggested that α6β4 integrin must activate or control other signaling molecules to promote Rac-1 activity and therefore migration and invasion. Therefore, I next focused my attention on

other molecules upstream of Rac. Preliminary gene array studies using the pancreatic cancer cell lines develop in this study (Panc-1 low β4 expressers vs. high β4 expressers) suggested that integrin α6β4 could increase the expression of the Rac-specific GEF, Tiam-1. I confirmed this gene array data by Real Time PCR and found that the expression of Tiam-1 was upregulated in most of the pancreatic cancer cell lines with higher levels of integrin α6β4 expression. In addition, I showed that Tiam-1 expression was required for HGF-induced migration and invasion that was dependent on Rac-1 activity. This dissertation therefore provides evidence of a new mechanism by which integrin α6β4 induces some of the steps towards tumor progression that are dependent on Tiam-1 expression and subsequent Rac-1 activation. This study confirmed my hypothesis that expression of and signaling from integrin α6β4, in cooperation with HGF stimulation, facilitates progression, cell migration and invasion of pancreatic adenocarcinomas. These results suggest that blocking integrin α6β4-induced signaling could serve as a therapeutic target to decrease migration and invasion of cancer cells in tumors. However, developing therapies targeted to block integrins that are expressed by epithelial tumor cells and other types of cells will certainly pose a challenge and thus we need to learn more about the α6β4 integrin-related cell signaling mechanisms in tumors so that better targeted therapies may be created for cancer patients.

Future Directions

How does the integrin α6β4 regulate GEFs expression and/or activation?

Integrin α6β4 can regulate the expression of multiple proteins at the level of transcription and translation. For example, the motility factor autotaxin in breast carcinoma cells is upregulated by integrin α6β4 dependent activation of the transcription factor NFAT-1 (Chen and O'Connor, 2005). Among other transcription factors regulated by the integrin α6β4 are NFAT-5 (Jauliac et al., 2002), NFkB (Mainiero et al., 2003), c-Jun, and STAT-3

(Guo et al., 2006). Furthermore, integrin α6β4 can also regulate proteins, such as VEGF, ErbB3 and ErbB2, at the level of translation which is dependent on the eukaryotic translation initiation factor 4E (Chung et al., 2002; Folgiero et al., 2007; Yoon et al., 2006a). In this study, I found that the integrin α6β4 was able to increase expression of the Rac guanine exchange factor Tiam-1. How integrin α6β4 controls the expression of this protein is subject to further studies. It will be interesting to explore which method (transcription or translation) this integrin uses to control Tiam-1 expression. Furthermore, integrin α6β4 could also be regulating other properties of Tiam-1, such as its activity and/or localization, two mechanisms critical for function and control of this protein to activate Rac and control migration and invasion events. Tiam-1 can also be regulated by other mechanisms which include phosphorylation by tyrosine kinases, Ca^{2+} calmodulin-dependent kinase II (CaMKII) or related kinases, interaction with PI3-K product PtdIns(3,4,5)P3, binding to cell surface molecule CD44 or cytoskeletal protein ankyrin, as well as direct binding to activated Ras (Minard et al., 2004). Therefore, there is the possibility that integrin α6β4 could contribute directly or indirectly in some of these events to activate Tiam-1. Since the second part of this dissertation focused on HGF-related migration and invasion, there is also the possibility that the tyrosine kinase receptor c-Met could be contributing to the activation of Tiam-1. The only study thus far that has linked HGF-induced signaling and Tiam-1 involves the ability of HGF to decrease thrombin-induced endothelial permeability by Tiam-1-mediated activation of the Rac pathway and by Tiam-1-Rac-dependent inhibition of the Rho pathway (Birukova et al., 2007). However, no mechanisms were provided by Birukova et al. as to how HGF-induced signaling controlled Tiam-1 activation. Thus, it will be interesting to learn if the integrin α6β4 cooperation with c-Met could contribute to the activation of the Tiam-1-Rac pathway to control migration and invasion events.

What other mechanisms do the integrin α6β4 control to promote migration and invasion?

The mechanism of how the integrin α6β4 switches from an adhesive receptor in the hemidesmosomes to a signaling receptor that promotes migration and invasion of cancer cells is an important topic of investigation. However, most of the studies and findings related to the migration and invasion of cells as a result of integrin α6β4 signaling have focused on other carcinoma models like breast and colon. In this dissertation I have provided evidence of the importance of this integrin in pancreatic adenocarcinoma progression, cell migration and invasion. I described in Chapter three a novel mechanism of how integrin α6β4 could promote migration and invasion of pancreatic carcinoma cells. However, more studies are needed as many other signaling pathways are likely involved given that Tiam-1 was not expressed in all of the integrin α6β4 positive cell lines studied. To further investigate signaling from this integrin a better *in vitro* model needs to be developed. In particular, most of the conclusions from this study rely on the use of transient transfection of siRNAs, a technique which has many limitations. One of the limitations is that the small oligos of siRNA only provide down-regulation of the integrin α6β4 for a short period of time which does not allow sufficient time for us to observe any of the long-term effects resulting from lack of integrin α6β4 expression. Since the α6β4 integrin affects other proteins at the level of transcription by the upregulation of transcription factors, it suggests that it must take some time to shut down all of the signaling molecules that can be controlled by this integrin. Therefore, I propose the development of stable siRNA cell transfectants using a commercially available hairpin siRNA construct proven to be effective in downregulating the target of interest. Once β4 positive cell lines are confirmed for the expression of the shRNA construct with subsequent decreased levels of integrin α6β4, then gene array and proteomic studies comparing parental, vector control, shRNA β4, shRNA α6 expressing cells may provide a better insight of the molecules and signaling pathways affected as a

result of integrin α6β4 expression. With this information, understanding of some of the mechanisms and signaling pathways involved that are specific for integrin α6β4-related effects in pancreatic adenocarcinoma may be elucidated. Furthermore, these cells could be useful for studying integrin α6β4 *in-vivo*. For these studies, stably transfected cells could be injected orthotopically into the mouse pancreas where tumor growth and metastasis can be analyzed to help answer the question of whether expression of integrin α6β4 is required for growth and metastasis of pancreatic tumors *in-vivo*.

What is the role of integrin α6β4 in pancreatic adenocarcinoma tumor formation and progression?

The results from my study suggest that integrin α6β4 could be implicated in tumor progression by its early overexpression and redistribution in PanIN lesions. In order to investigate *in vivo* the role of integrin α6β4 in tumor progression of pancreatic adenocarcinoma a mouse model should be developed. To accomplish this goal I suggest that mice be developed where we can induce the expression of the integrin β4 subunit to a specific cell type in the mouse pancreas at a given time. Therefore, I suggest that overexpression of the integrin β4 subunit gene be targeted to the ductal epithelial cells of the pancreas under the control of a tetracycline-inducible transgene. These mice could help us understand tumor progression and metastasis in two ways; first they will let us to know if integrin α6β4 is involved in pancreatic tumor formation and if so, we could assess how closely it recapitulates the human disease (eg. PanIN lesions and invasive carcinoma). Second, these mice could be crossed with an established mouse model of pancreatic adenocarcinoma to examine if integrin α6β4 over-expression could accelerate the progression of the disease. An example of a mouse model that could be used for this aim is the one where endogenous expression of activating mutation of K-Ras^{G12D} was targeted to the progenitor cells of the mouse pancreas (Pdx1-Cre/LSL- K-Ras^{G12D}). This model was shown to recapitulate the human disease in which it induces pancreatic adenocarcinoma

formation from PanIN lesions to an invasive and metastatic disease (Hingorani et al., 2003).

Does the 3D model of growing cancer cells lines *in vitro* provide a better model for the study of pancreatic cancers?

Although we have learned much about cancer biology from mouse models, whether mouse models accurately recapitulate human cancers remains to be fully validated (Rangarajan and Weinberg, 2003). Therefore, cell culture models remain an important complement to *in vivo* studies using mouse models. Most studies using cancer cell lines rely on two-dimensional (2D) cell culturing methods where cells grow as a monolayer on culture plates. This method of growing cells does not allow cells to form their physiologic 3D organizational structure. In the breast cancer field, scientists have developed a method of growing cells in 3D that more accurately recapitulate the way most epithelial cells grow *in vivo* (Debnath et al., 2003). This method has been successfully applied to study different aspects of cancer biology, such as resistance to apoptosis, with much success (Weaver et al., 2002). Interestingly, in addition to the studies described in this dissertation, I was also able to adapt this 3D culturing method to grow normal ductal pancreatic epithelial cells and pancreatic cancer cells (Fig. 4.1). This 3D culture methodology potentially could be of great benefit in pancreatic cancer studies because cells are simulated to grow more closely to their normal physiologic environment. In addition, studying integrins using 3D culturing methods vs. 2D might be of greater physiological relevance, because the composition of integrin adhesions and integrin signaling might be different between the two models (Zahir et al., 2003). Furthermore, studies using breast carcinomas grown in 3D have revealed that integrin α6β4 has the ability to mediate hemidesmosome formation and a polarized phenotype, which can lead to promote resistance to apoptosis from chemotherapeutic drugs (Weaver et al., 2002). Since pancreatic adenocarcinoma treatment tends to fail due to the cancers ability to acquire chemotherapeutic resistance, and I show that integrin α6β4 is

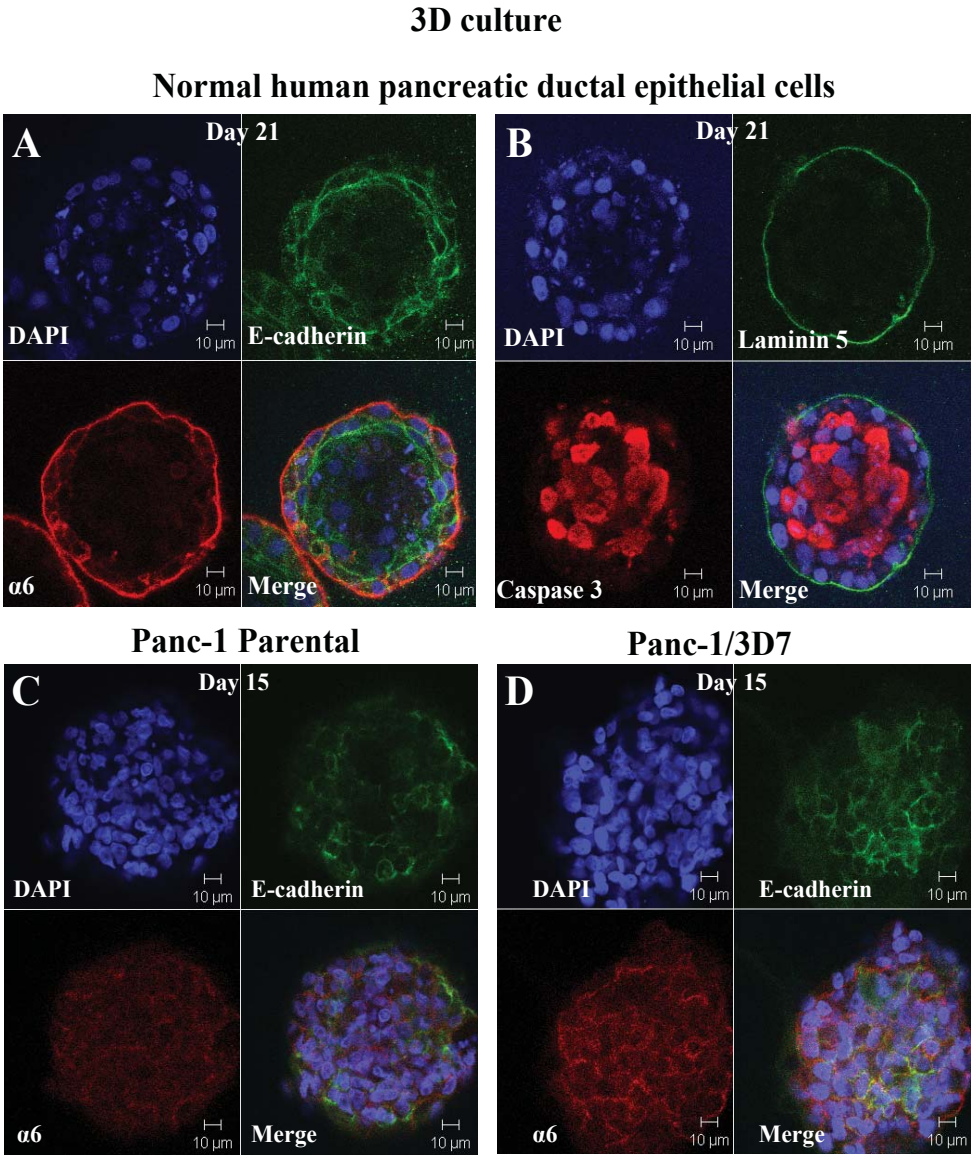


Figure 4.1. Pancreatic cells grown in 3D. Normal human pancreatic ductal epithelial cells (A-B), Panc-1 Parental (C) and Panc-1/3D7 (D) cells were seeded onto a glass chamber previously coated with 40µl of chilled Matrigel a reconstituded basement membrane. Cells were overlaid with dilute Matrigel and cultured for 15-21 days. Medium with diluted (2%) matrigel was changed every 4 days. This 3D cell culturing method was modified from (Debnath, et al., 2003). Cells were fixed and stained for integrin α6 (hemidesmosomal marker), E-cadherin (cell junctions marker), Laminin-5 (basement membrane marker), or active caspase-3 (apoptosis marker) as indicated. Nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI). (A-B) Normal Pancreatic ductal cells grown as acini structures in 3D with a define basemembrane and a hollow lumen in the middle formed by cells that underwent apoptosis. (C-D) Panc-1 cancer cells grown in 3D forming spheres with no apparent lumen.

overexpressed in this cancer, it will be interesting to use this 3D culturing method to study the role of integrin α6β4 in the chemotherapeutic resistance of this cancer.

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VITA

Zobeida Cruz Monserrate was born in September 19, 1978 to Carmen Julia Monserrate Collazo and Ramón Cruz Baéz in Hato Rey, Puerto Rico and has been married to Carmelo Mateo for almost 7 years. Zobeida attended the Lincoln Military Academy High School and subsequently The University of Puerto Rico-Humacao, graduating in 2000, Magna Cum Laude with a Bachelor of Science in Coastal Marine Biology. Soon after, she joined the laboratory of Dr. Ernest Hamel at the National Cancer Institute, National Institute of Health in Frederick MD, where she worked for two years as a predoctoral fellow researching the interactions of antimitotic drugs with tubulin. While at NIH she was accepted to take graduate level courses at The Johns Hopkins University. In 2002 Zobeida moved to The University of Texas Medical Branch at Galveston and joined the Cell Biology graduate program. After completing the First-Year Basic Biomedical Science Curriculum and rotating in various laboratories within the Sealy Center for Cancer Cell Biology she joined the laboratory of Dr. Kathleen O'Connor. During her 1st year of graduate school Zobeida applied for an NIH Predoctoral Fellowship Award (F31) which was awarded that same year in September 2003. She has received multiple travel awards to attend and present her work at national meetings. Some of her awards and honors include; in 2005 she received the honor of been selected to attend the AACR Edward A. Smuckler Memorial Workshop Class for which only 100 students are selected nationwide every year. In 2006 she was awarded the Rose and Harry Walk Research Award, which recognizes a graduate student at UTMB who has made significant contributions towards a better understanding of mechanisms leading to significant pathologic changes in the human organism and the Who's Who Among Students in American Universities & Colleges. In addition, her work has received many awards as best poster presentation and recently poster of distinction at the 2006 Joint Meeting of the American Pancreatic Association/International Association of Pancreatology in Chicago. Zobeida gained teaching experience while at the O'Connor Lab via helping rotation students and a SURP student who was recognized with the Dean's Award at the end of the summer as a result of her work under the guidance of Zobeida.

Education

B.A., Coastal Marine Biology May 2000, University of Puerto Rico-Humacao

Publications

Articles

Cruz-Monserrate, Z., Qiu, S., Evers, B. M., and O'Connor, K. L. Upregulation and redistribution of integrin α6β4 expression occurs at an early stage in pancreatic adenocarcinoma progression. Modern Pathology, 20: 656-667, 2007.

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Cruz-Monserrate Z and O'Connor KL. Integrin α6β4 promotes the migration and invasion of pancreatic cancer cells through the upregulation of Tiam-1 and subsequent activation of Rac-1. To be submitted to Cancer Research.

Abstracts

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2. Cruz-Monserrate Z., Qiu S, Evers BM and O'Connor KL. Integrin α6β4 Contributions in Pancreatic Carcinoma. 2006 Joint Meeting of the American Pancreatic Association/International Association of Pancreatology, Chicago, Illinois, November 1-4, 2006.

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6. Cruz-Monserrate Z. and O'Connor KL. Integrin α6β4 contributions to an invasive phenotype in pancreatic carcinoma. American Society for Cell Biology 45th Annual Meeting. San Francisco, CA, Dec. 10-14, 2005.

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