Title

Loss of the [beta]$_1$ integrin subunit in F9 teratocarcinoma cells reveals modulation of [alpha][subscript v] family integrin receptor affinity by [beta]$_1$, diminished metastatic capacity, and defects in random migration

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Loss of the β_1 Integrin Subunit in F9 Teratocarcinoma Cells Reveals Modulation of α_V Family Integrin Receptor Affinity by β_1, Diminished Metastatic Capacity, and Defects in Random Migration.

by

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DISSERTATION

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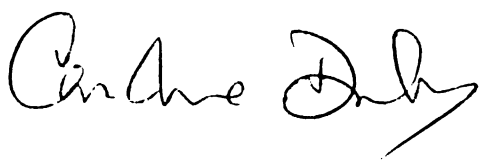
ABSTRACT

Loss of the β_1 Integrin Subunit in F9 Teratocarcinoma Cells Reveals Modulation of α_V Family Integrin Receptor Affinity by β_1, Diminished Metastatic Capacity, and Defects in Random Migration.

Integrin heterodimers mediate cell interactions with extracellular matrix and other cells. Two major integrin families: α_V and β_1, have been identified on the basis of their ability to partner with several β subunits or several α subunits, respectively. To distinguish the roles of these families, the β_1 subunit was mutated by gene targeting in F9 teratocarcinoma stem cells, thereby eliminating the function of all β_1 associated heterodimers in these cells and their parietal endoderm (PE) differentiated counterparts. The function of α_V family integrins was preserved in these cells (named TKO). Deletion of β_1 integrins eliminated adhesion of TKO and TKO-PE cells to, and migration on, collagens, laminin and fibronectin. Furthermore, TKO-PE cells were unable to organize a fibrillar fibronectin matrix. In contrast, adhesion of TKO and TKO-PE to vitronectin was not only preserved, but substantially enhanced. Surprisingly, however, migration by TKO cells on vitronectin was suppressed. The altered adhesion and migration properties of TKO translated to reduced ability of TKO to metastasize from the spleen to the liver in vivo. Several possible mechanisms were tested that might explain how loss of β_1 integrins, which do not recognize vitronectin in these cells, leads to enhanced adhesion of TKO and TKO-PE to vitronectin. No differences were found in the levels of α_V family integrins on control F9, F9-PE, TKO or TKO-PE. In addition, each α_V family member expressed on F9 made the

same percentage contribution to the overall adhesion to vitronectin as it did in TKO cells. To determine whether the intrinsic affinity of the α_v integrins for vitronectin was altered in TKO cells, adhesion assays were conducted at 4°C so as to compare initial binding strength independently of post-attachment strengthening. In this assay, TKO and TKO-PE still showed enhanced binding to vitronectin. Thus, loss of β_1 integrins results in an increased affinity of α_v family integrins for their major substrate, vitronectin. This is a novel example of transdominant inhibition of one integrin by another. Such integrin cross-talk may be regulated by cytoplasmic proteins that bind to the β_1 subunit specifically. In the absence of β_1 , this signaling mechanism would be eliminated.



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LIST OF ABREVIATIONS

ECM	extracellular matrix
EM	electron microscopy
ES	embryonal stem
F9	the F9 teratocarcinoma cell line
FCS	fetal calf serum
Fn	fibronectin
ILK	integrin linked kinase
Ln	laminin
PTHrP	parathyroid hormone related peptide
PTHrPR	parathyroid hormone related peptide receptor
PE	parietal endoderm
TKO	β_1 null cells derived from F9 cells
TKOβ_1	TKO cells stably expressing murine β_{1A}
TW	transwell
Vn	vitronectin

Chapter 1

INTRODUCTION AND GOALS

Living cells receive a constant profusion of signals from the environment. The behavior of each cell; the decision to divide or not to divide, to synthesize, to secrete, to migrate, to stay still, to differentiate, to continue living or to die, is the net result of the interaction of signals from within and without the cell. In vivo, most cells live surrounded by a complex, information-rich mixture of different proteins and proteoglycans known as the extracellular matrix (ECM). The primary cellular receptors for the ECM are a family of proteins known as integrins.

Integrins are transmembrane αβ heterodimers that are expressed in all metazoan cells (Hynes and Zhao 2000). The extracellular domain of the integrin heterodimer binds to ECM proteins and receptors on the surface of other cells. The cytoplasmic domain binds to the actin cytoskeleton, making a physical link between the cell and its environment. Through this connection, integrins transmit mechanical force and biochemical signals. The number of known integrin family members is over 20, and almost all cells express several simultaneously (Hynes 1987). Some integrin heterodimers bind to a single ligand; others recognize several different ligands. Of the integrins expressed by a given cell, there are often several which can bind to the same ligand. However, there is a growing body of evidence that each integrin assembles a unique

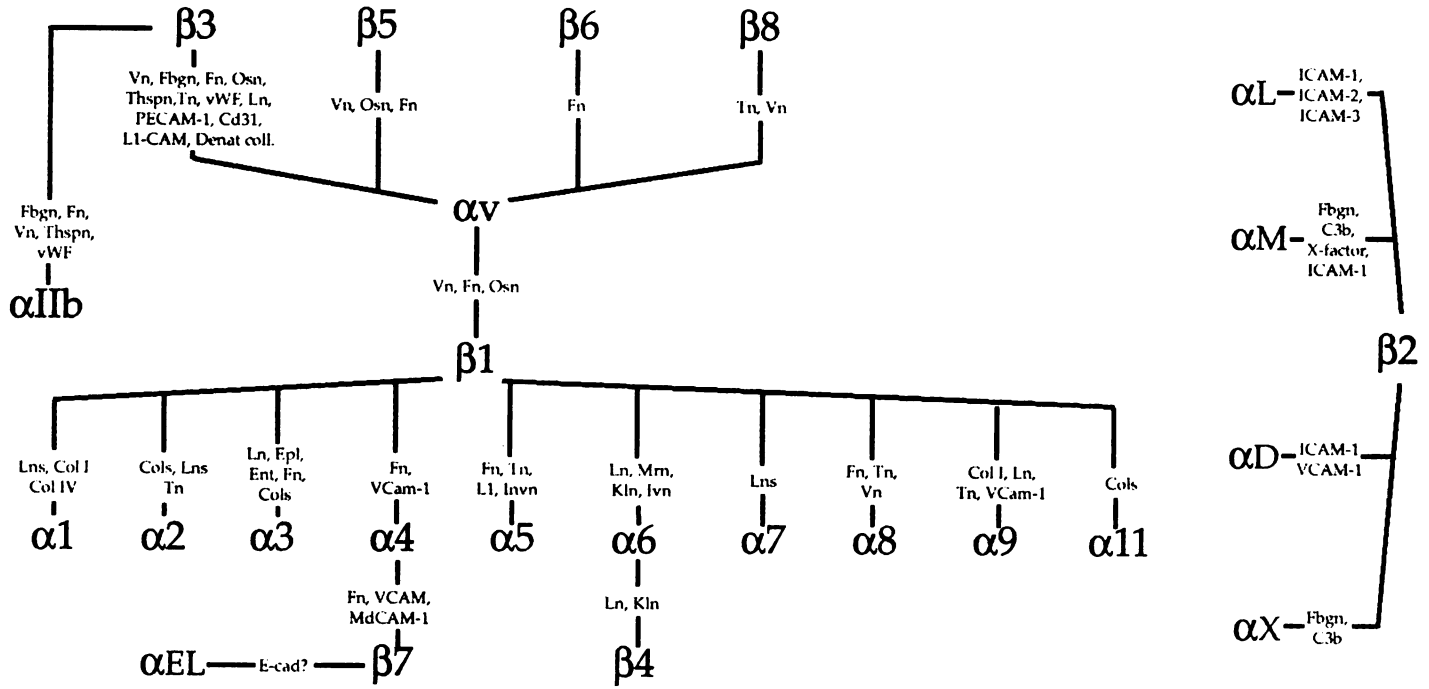
combination of cytoplasmic and membrane proteins that trigger different signaling cascades (Giancotti and Ruoslahti 1999).

At any given moment the cell is bound to several different ECM molecules via a set of different integrins (Hynes 1987). Normally there are also unbound integrins in the membrane. In this complex situation it is difficult to discern the contribution of each different integrin and it has been useful to examine the effects of deleting individual integrins. In 1993, Stephens et al. were the first to publish work on a targeted deletion of the entire β_1 family of integrins (Stephens, Sonne et al. 1993). This knockout decreased the number of known integrins expressed by the F9 teratocarcinoma cell line from **7** ($\alpha_6\beta_4$, $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_\varsigma\beta_1$, $\alpha_V\beta_3$ and $\alpha_V\beta_5$) to **3** ($\alpha_6\beta_4$, $\alpha_V\beta_3$ and $\alpha_V\beta_5$).

Integrin Structure

Both parts of the integrin heterodimer are type-I transmembrane glycoproteins and all known integrins are heterodimers. At the moment there are 17 α subunits and 8 β subunits known (figure one). Although 17 α subunits and 8 β subunits could theoretically associate to give 136 different integrin heterodimers, the actual number is closer to 23. Most α subunits can associate with only 1 β subunit. An exception to this rule is the α_V chain. α_V is "promiscuous" and forms heterodimers with 5 different β chains. Integrin heterodimers can be divided into families based on common subunits. My research concerns the β_1 and α_V integrin families.

Figure 1: Vertebrate Integrins



The primary structure of β integrin chains consists of a large extracellular domain containing 4 repeats of a forty amino acid cysteine-rich domain. The cytoplasmic tail of most β chains is short; generally less than 50 residues. α chains all contain seven homologous polypeptide repeats in the extracellular domain, the most N-terminal three or four of which contain cation binding sites with sequence homology to EF hands. Some α subunits contain a 180 amino acid insert between the second and third EF hand repeat. This insert is called an I domain and is homologous to the collagen binding domain found in von Willebrand factor. Additionally, some α chains are posttranslationally cleaved to give a 25-30 kD transmembrane chain disulfide-bonded to a larger, wholly extracellular chain. Integrin α_v is cleaved and does not contain an I domain. The cytoplasmic tails of α chains are also quite short, ranging from 40-60 amino acids. Neither α nor β cytoplasmic tails contain regions with any known enzymatic activity.

Electron micrographs of purified integrin heterodimers show a globular head formed by the extracellular domains of both chains, from which the transmembrane and cytoplasmic domains protrude as two parallel stalks (Carrell, Fitzgerald et al. 1985; Kelly, Molony et al. 1987; Nermut, Eason et al. 1991). To date, no one has been able to crystallize an intact heterodimer, so the secondary and tertiary structures of integrins are not completely resolved. At the moment there are many contradictions in the available data. As I discuss later in this chapter, integrin heterodimers appear to have numerous affinity and activity changes that coincide with changes in conformation, so there are probably many

shapes to each integrin. Figure 2 is a compromise, representing data on 2° structure, α-β interaction, ligand binding and ion binding known for integrin $\alpha_{iib}\beta_3$. The α_{iib} chain, like α_v, is post-translationally cleaved and does not contain an I domain.

Both the α and the β subunits contribute to the ligand-binding capabilities of integrins. It is not known whether the binding site is one continuous surface or several separate binding pockets. There is experimental evidence for both hypotheses (Loftus and Liddington 1997; Calvete 1999). The N-terminal amino acids of non-I-domain integrin α subunits are predicted to fold into a β propeller domain built by seven four-stranded β-sheets arranged in a torus around a pseudosymmetry axis (Springer 1997). In this model the Ca^{++}-binding sites and the ligand-binding site are on opposite sides of the torus. See figure 3.

One common motif in all the integrin ligands studied thus far is an invariant carboxylate residue, prominently displayed (Jones, Harlos et al. 1995). The residue is usually an aspartic acid, but sometimes it's glutamic acid. This carboxylate residue is thought to complete the coordination sphere of a metal ion upon binding. However, if the metal ion binding sites are on the opposite side of the structure, the ligand will have to penetrate quite deeply into the proposed propeller domain in the α chain.

Much of the metal ion binding hypothesis is derived from studies of crystallized I-domains.

Figure 2: Integrin Structure

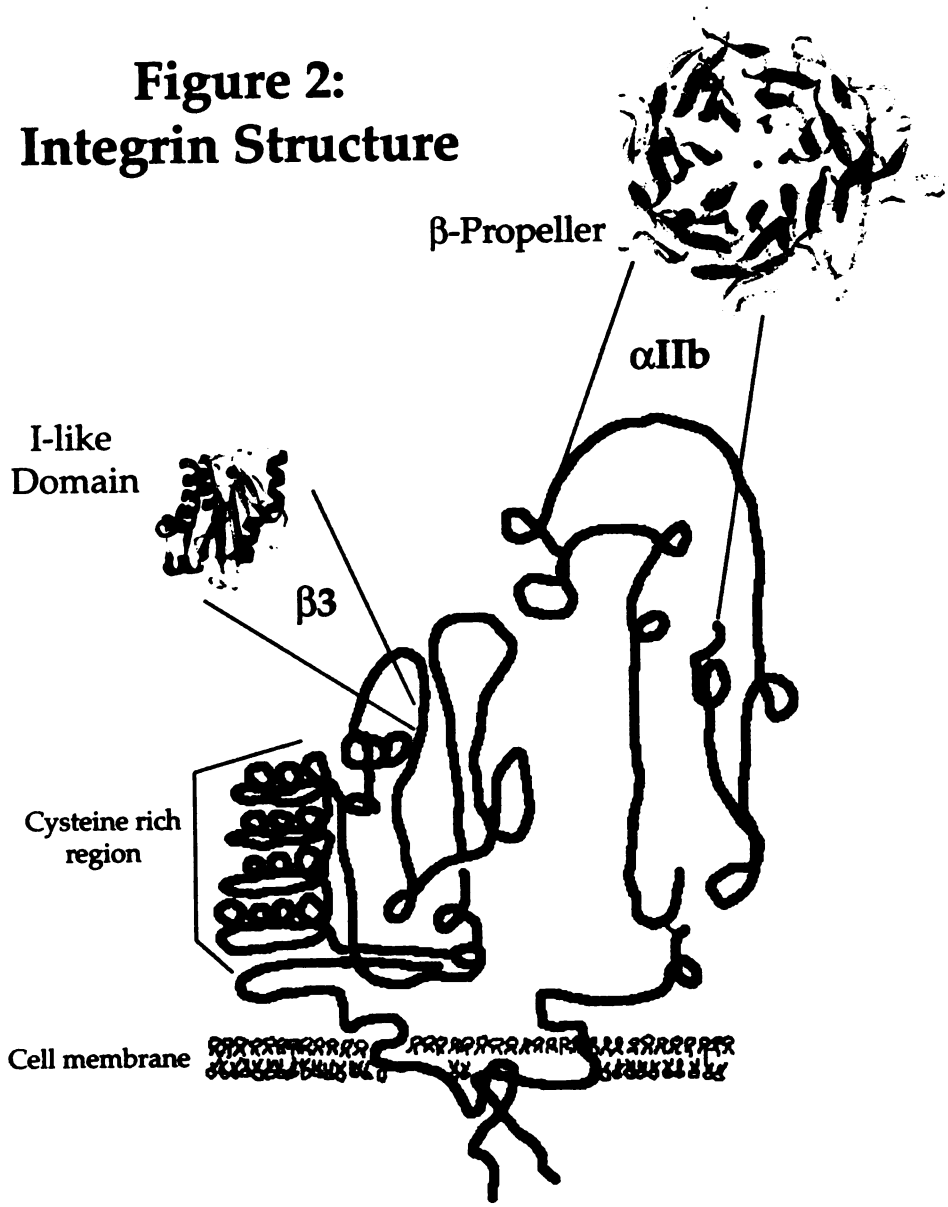
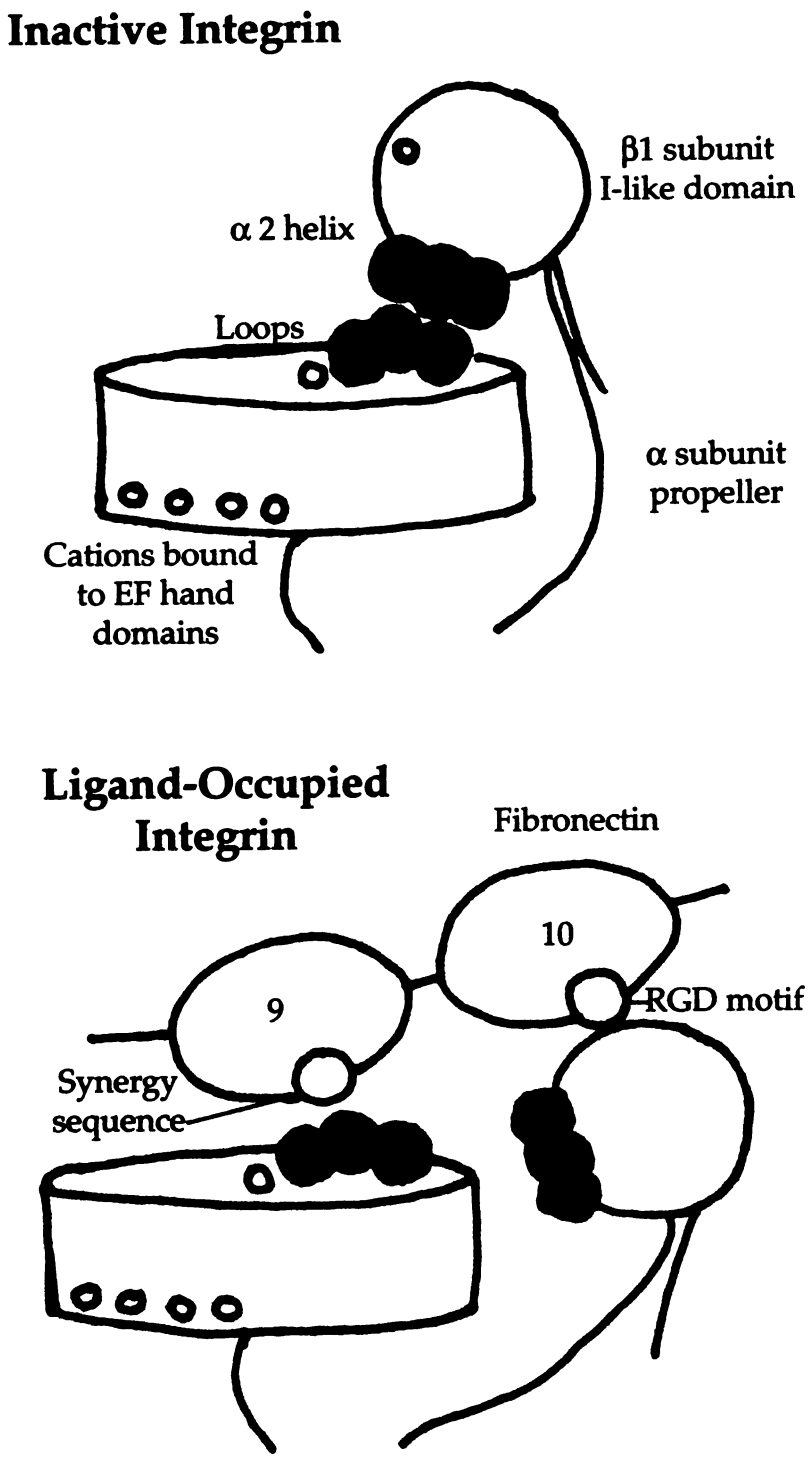


Figure 2: Adapted from Calvete, J. 1999.

Figure 3



Adapted from Loftus and Liddington, 1997.

I-domains contain a metal ion dependent binding site (MIDAS) at the top of a β sheet that is critical for adhesive function. Although the vitronectin binding integrin α chains do not contain I-domains, a domain similar to it, an "I-like domain", is found in β chains and is involved in ligand binding also. When these sequences in β_1 are exchanged for those of β_3, the ligand binding specificity of $\alpha_V\beta_1$ changes to that of $\alpha_V\beta_3$ (Takagi, Kamata et al. 1997). The position of the I-like domain in the β subunit, relative to the propeller domain of the α chain is proposed to regulate integrin affinity (See figure 3).

Strategies for Regulating Cell Adhesion

The process of integrin-mediated cell adhesion is complex and can be regulated in many ways. A brief overview of the sequence of events believed to comprise integrin mediated cell adhesion will help us understand how regulation occurs.

Let us begin with expression at the cell surface. Integrins are released from the endoplasmic reticulum as mature heterodimers of 1 α and 1 β chain. There are no enzymatic cleavages or other post-expression modifications needed for the heterodimer to function. Integrin β_1 has at least 4 splice variations (Altruda, Cervella et al. 1990; Languino and Ruoslahti 1992; Baudoin, Van der Flier et al. 1996). Integrins β_{1B} and β_{1C} are only found in humans, are unable to localize to focal contacts, and are believed to play roles in regulating adhesion and cell growth (Balzac, Belkin et al. 1993; Balzac, Retta et al. 1994; Meredith, Takada et al. 1995). Integrin β_{1D} is found in differentiated muscle tissue in all vertebrates.

F9, TKO, and TKOβ_1 cells express solely the β_{1A} splice variant and it is this isoform that this thesis addresses.

The cell can regulate adhesion at the level of integrin expression. By changing the quantity of a given integrin at the cell surface, cell adhesion can be increased or decreased. This strategy is often adopted during differentiation. In fact, like their counterparts in a developing embryo, parietal endoderm differentiated F9 cells express more integrins than undifferentiated F9 cells (Burdsal, Lotz et al. 1994). TKO cells could strengthen their bond to vitronectin simply by expressing more vitronectin-binding integrins than F9 cells.

Another way to regulate adhesion at the level of synthesis is to express a new integrin. This strategy is also frequently employed during differentiation (Morini, Piccini et al. 1999). Fundamentally, differentiation requires a cell to change identity. Changing the extracellular matrix receptors with which it interacts with the world is a powerful way for the cell to effect change.

After expression the fun begins. For the purposes of discussion the formation of integrin mediated adhesion can be broken into 3 steps: ligand binding, clustering with other integrin receptors, and recruitment of signaling molecules and cytoskeletal proteins. Each of these steps can be regulated. However, it is mistake to view this series of events as a consistently linear process. The diversity of cellular processes that integrins are a part of, as well as the variety of

potential regulatory strategies ensure that different cells go about the formation of integrin adhesion differently.

The theoretical starting point for the adhesion process is a single integrin adrift in the plasma membrane. In most cells, unbound integrins are localized diffusely all over the cell surface and are assumed not to be binding to any signaling molecules or cytoskeletal proteins. Data to support these conclusions come from immunofluorescent staining of fixed cells, immunofluorescent staining of live cells using monovalent, non-function perturbing antibodies, and fluorescence recovery after photobleaching (FRAP) assays. However, there is also evidence that some unbound integrins may be anchored to the cytoskeleton (Kucik, Dustin et al. 1996). Integrin $\alpha_L\beta_M$ (LFA-1) on resting B cells is substantially less mobile within the plane of the membrane than a control membrane protein. PMA-induced activation or the addition of cytochalasin D both increase the Brownian motion of this integrin suggesting that it is restrained, by binding to the actin cytoskeleton, in resting cells. Release from the cytoskeleton upon activation suggests that the integrin-actin bond can also be part of a low affinity state for integrins. The results of this study are not entirely conclusive because the experimental design does not permit a distinction between clustered integrins and single integrins.

Not all unbound integrins are equal. Some are able to bind ligand and some are not. The ability of an integrin dimer to bind ligand is referred to as its affinity and the affinity of many integrins can be regulated. Integrin affinity regulation

was first observed in leukocytes and platelets. On platelets, surface $\alpha_{iib}\beta_3$ exists in a low affinity "resting" state until other receptors at the surface of the platelet bind to an agonist. Within seconds of agonist binding, $\alpha_{iib}\beta_3$ shifts to a high affinity state and begins to bind soluble fibrinogen and von Willebrand factor (vWF), and hemostasis proceeds. Tight and rapid regulation of $\alpha_{iib}\beta_3$ affinity has obvious charms, as both uncontrolled blood clotting and slow clotting are lethal. Similarly $\alpha_v\beta_3$, is in a resting state on the surface of many myeloid and endothelial cells. Upon stimulation, it undergoes rapid activation and mediates migration and adhesion (De Nichilo and Burns 1993; Stern, Savill et al. 1996; Trusolino, Serini et al. 1998). An explicit demonstration of $\alpha_v\beta_3$ activation regulation, using a patch engineered ligand mimetic antibody, was published recently [Pampori, 1999 #1].

Thus far, binding of β_1, β_2 and β_3 integrins have been shown to be regulated at the level of affinity. There is some evidence that the affinity of β_5 and β_5 integrins can also be regulated, while it appears that β_4 integrins are not regulated in this way. The difference between a high affinity integrin and a low affinity integrin appears to be a matter of conformation. Fluorescence resonance energy transfer studies, using monoclonal antibodies bound to extracellular domains of $\alpha_{iib}\beta_3$, show that increase in affinity is associated with a change in the relative positions of the subunits (Sims, Ginsberg et al. 1991).

Based on modeling of the binding domains of $\alpha_{iib}\beta_3$, a proposed model for activation consists of the β_3 I domain shifting away from the center of a β

propellor domain in the α subunit (Loftus and Liddington 1997). This shift would reveal binding sites in both the α and β subunits. Integrin mutations that affect affinity include two single amino acid substitutions in β_3, one causing constitutively high affinity of the β_3 heterodimer (Kashiwagi, Tomiyama et al. 1999), one abolishing activation by agonists (Blystone, Lindberg et al. 1995). The constitutively activating mutation is in the cysteine-rich extracellular stem region of β_3 and may alter the extracellular conformation directly. The other is a mutation in a tyrosine in the cytoplasmic tail and is believed to block association with intracellular signals generated by agonist binding.

No discussion of integrin affinity is complete without mention of the antibodies specific for high affinity conformations. In 1985, Shattil et al published a paper about PAC-1, an antibody specific for high affinity $\alpha_{iib}\beta_3$ (Shattil, Hoxie et al. 1985). PAC-1 binds to human platelets after agonist-induced activation and competes with fibrinogen and vWF for binding of $\alpha_{iib}\beta_3$. It, and other antibodies like it, behaves like a legitimate ligand. It binds to the ligand-binding site of integrins in the high affinity conformation. These antibodies can most accurately be referred to as "ligand mimetic" (Bazzoni and Hemler 1998). Sadly there are no known ligand mimetic antibodies for murine β_1, β_3, or β_5.

There is another group of antibodies that are specific for high affinity integrins. This group binds to epitopes outside the ligand-binding site that are revealed after ligand and/or cation binding. They were originally referred to as "ligand induced binding site" or LIBS antibodies. However it was later discovered that

divalent cation binding can also induce some of the same epitopes and ligand binding isn't necessary in all cases. Different heterodimer partners affect the formation of the epitope and change the cation effects. To denote these findings (Bazzoni et al. 1998), this class of antibodies can also be called "cation/ligand induced binding site" or CLIBS antibodies. 9EG7 is the best characterized CLIBS antibody available that recognizes murine integrins. It binds β_1.

CLIBS antibodies do not block integrin function or compete with ligand to bind to integrins. They recognize high affinity integrins "post facto": after the integrin has bound ligand (Puzon-McLaughlin, Kamata et al. 2000). Judging by intrinsic fluorescence and hydrodynamic studies done on $\alpha_{iib}\beta_3$ (Parise, Helgerson et al. 1987), it appears that ligand binding involves a partial unfolding of the receptor and exposes a set of epitopes different from those of an empty integrin in high affinity conformation. Binding of CLIBS antibodies can stabilize the ligand-bound conformation and increase the effective affinity of some integrins. Because of this antibodies are sometimes called activating antibodies. This is inaccurate, as they do not induce the shift from low affinity to high affinity conformation.

As students of chemistry know, all binding processes are theoretically reversible. The binding of high affinity state integrin to ligand is a biphasic event. K_D of the initial stage varies between 4.5uM and 10 nM (Orlando and Cheresh 1991; Faull, Wang et al. 1996; Pfaff, Du et al. 1999). After a short time binding stabilizes and is no longer practically dissociable (Orlando and Cheresh 1991). The transition to

the second stage is also conformation dependent and can be induced both by extracellular and intracellular interactions.

After binding, integrins cease their solitary ways. They aggregate or "cluster" with other integrins, with proteins in the membrane and with intracellular proteins. The enhancement of the integrin-ligand bond due to clustering of the integrins and recruitment of intracellular proteins, especially elements of the actin cytoskeleton, is known as "avidity". The difference between affinity (attraction) (Faulkes 1956)), and avidity (eagerness) is semanticly subtle. In the literature avidity is used to describe both the effects of increased local concentrations of integrin (Hato, Pampori et al. 1998) alone and the effects of attachment of the integrins to the cytoskeleton alone (Blystone, Williams et al. 1997). Some use it to refer to both (Jones, Harlos et al. 1995) and many authors simply avoid using the term entirely (Pujades, Alon et al. 1997; Jenkins, Nannizzi-Alaimo et al. 1998).

The confusion has roots in biology as well as semantics. Under physiological conditions, integrin clustering and the recruitment of intracellular and membrane proteins often happen simultaneously (Hynes 1987; Miyamoto, Teramoto et al. 1995). However, it is useful to separate the processes experimentally, as it is theoretically likely that each contributes a discrete quantum of strength to the cell-substrate bond. Clustering or oligomerization increases the local concentration of integrins and decreases the chance of the cell

losing contact with the ligand. If one integrin lets go of the ligand, there is another integrin within binding distance.

Hato et al. recently published an elegant exploration of the contribution of integrin clustering (Hato, Pampori et al. 1998). They grafted repeats of the FK506 binding protein, FKBP12, onto the COOH-terminus of α_{IIb} and used a synthetic, membrane permeable FKBP12 dimerizing protein (Amara, Clackson et al. 1997) to cluster the $\alpha_{IIb}\beta_3$ heterodimers. Ligand binding was assessed with ligand mimetic Fab fragments. Their results show that clustering mediates a modest (approximately 20% of maximal binding) increase in cell binding that is additive to the greater increase caused by a CLIBS antibody that stabilizes the ligand-bound conformation of $\alpha_{IIb}\beta_3$. They also note the recruitment of FAK and Syk, due to integrin clustering alone.

The modest 20% increase in cell binding attributable to integrin clustering is somewhat surprising to those who are familiar with the literature on lymphocyte binding. In vivo, induction of integrin clustering, through binding to polyvalent extracellular matrix molecules, has a profound effect on lymphoid cell adhesion. Platelets and lymphoid cells do not recognize soluble fibrinogen, fibronectin or vitronectin until agonist binding stimulates them. However, they will bind to these ligands quite readily when the ligands are in a polymerized form that induces clustering mechanically (Halvorson, Coligan et al. 1996)

Fibrinogen, fibronectin and vitronectin can self-assemble into multivalent polymers at sites of vascular damage and leakage (Brown, Guidi et al. 1999). In ECM polymers, integrin-binding sites are in close proximity (Mosesson, Siebenlist et al. 1995) and the same rebinding and chelation effects that are seen with integrin clustering apply. In work by Stupak et al., $\alpha_v \beta_3$ mediated lymphoblast cell line binding to polymerized fibrin is equal to PMA stimulated binding to soluble, monomeric fibrinogen (Stupack, Storgard et al. 1999). Without PMA, the cells don't recognize the monomeric ligand at all. Binding to polymeric ligand also recruits Syk kinase in this system.

When a cell in suspension attaches to a surface, the connection of integrins to the actin cytoskeleton can be seen as a change in cell shape. The typical suspended cell is spherical. If it is able to attach to the substrate via integrins, and if culture conditions support normal metabolic processes, the cell will deform, changing from a sphere into something between a hemisphere and a thin sheet. The exact shape and degree of flattening depend on the cell type and the substrate. Classically, this process is referred to as cell spreading and it is actin dependent.

The connection of the cytoskeleton to integrins provides the lion's share of the cell-substrate bond strength. Integrins and clusters of integrins can and do tear out of the membrane of living cells. Some rapidly migrating cells seem not to bother with conserving or recycling their integrins. They leave trails of protein rich plaques behind them as they go. These plaques stain positively for integrins, vinculin, and numerous other proteins that complex with integrins at the

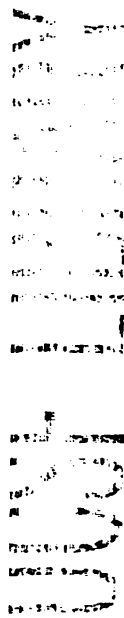
membrane, but they do not contain actin (Lauffenburger and Horwitz 1996). It appears that the cells break the contact between the cytoskeleton and the integrins and just discard the integrins. This seems like a wasteful and traumatic way of moving about and it may be an artifact of culturing the cells on artificial 2 dimensional matrices in vitro. However, it is sustainable over the hours, and sometimes days, that the cells are maintained in culture.

By contrast, anyone who has cultured adherent cells knows that detaching a spread cell from matrix without breaking the integrin-matrix bond tears the entire cell apart. The strength of the integrin-ligand bond can be measured, either with purified integrin and ligand, or by measuring the force it takes to pull an unspread cell off the matrix and dividing that force by the number of integrins in the area of contact (Lotz, Burdsal et al. 1989). The strength of the integrin-actin bond has not yet been measured. It isn't practical to do using whole cells, because cells don't tear neatly at the integrin-actin juncture. The experiment hasn't yet been done in vitro, with purified components, because we don't yet know exactly how integrins are connected to actin.

β cytoplasmic tails are necessary to link integrins to the actin cytoskeleton (Chrzanowska-Wodnicka and Burridge 1992), but the linkage is not direct. Clustered integrins typically co-localize with actin in zones of close apposition of the cell membrane to the ECM. In some cells they form large, easily visualized plaques. These zones are known variously as focal contacts, focal adhesions, matrix adhesions and fibrillar adhesions. For the purposes of this study I will

use the term focal adhesions to refer to sites at the substratum where integrins and actin co-localize, and the term fibrillar adhesion, to refer to sites where integrins and actin co-localize with fibrillar ECM components.

Not all cells form discernable focal or fibrillar adhesions. They are most frequently seen in fibroblast cells and fibroblastic cell lines, and are in some ways an artifact of cell culture. Focal contacts are useful to the researcher because they are large and can be visualized with fluorescent light microscopy. Migrating cells and transformed cells are less likely to form large focal contacts. F9 cells do not form many focal contacts. They are more likely to form distinctive fibrillar contacts and those only after extended culture. The limited number of focal contacts and the small size of the cells make it somewhat more difficult to determine how integrins and signaling proteins interact during the early stages of cell binding.



There are a large number of proteins that also localize to focal and fibrillar adhesions. At least 17 proteins are known to bind to the cytoplasmic tails of various β integrins (Pfaff, Liu et al. 1998; Liu, Thomas et al. 1999). 4 of them: talin, filamin, α-actinin, myosin and skelemin, bind filamentous actin too (Horwitz, Duggan et al. 1986; Otey, Pavalko et al. 1990; Loo, Kanner et al. 1998; Reddy, Gascard et al. 1998). Hypothetically any of these proteins could link integrins to the actin cytoskeleton. There are also two other groups of proteins: one that binds both to integrin cytoplasmic tails and to actin binding proteins (ex: paxillin (Schaller, Otey et al. 1995)), and another that binds both to actin and to

integrin binding proteins (ex: vinculin (Menkel, Kroemker et al. 1994; Bubeck, Pistor et al. 1997)).

What is confusing to the scientist is, no doubt, useful to the cell. The proteins at focal adhesions transduce mechanical force and transmit signals in and out of the cell. There is ample evidence that the complexes of proteins at focal adhesions vary with the integrins that are involved and with cell type (Calderwood, Zent et al. 1999; Liu, Thomas et al. 1999). Likewise, the constituents of the complexes vary with time (Miyamoto, Teramoto et al. 1995; Miyamoto, Katz et al. 1998), and depend on the location and function of the complex (Zamir, Katz et al. 2000).

Talin was the first cytoplasmic protein shown to bind to integrins directly. A closer look at this molecule demonstrates the complications and possibilities present in the integrin-cytoskeleton juncture. Talin is composed of 2 ~270kD subunits arranged in an anti-parallel homodimer. It is a major structural component of focal adhesions, along with vinculin and actin. Each chain consists of an N-terminal 50kDa globular head domain, that includes a 200 amino acid region homologous to the erzrin, radixin and moesin (ERM) family or proteins, and a 220kDa, C-terminal rod domain that contains a ILWEQ actin binding domain (McCann and Craig 1997). Talin contains binding sites for actin, vinculin, focal adhesion kinase (FAK), phospholipids, and the transmembrane protein layilin (Borowsky and Hynes 1998).

When integrins cluster, talin is one of the first proteins to co-localize with them. Talin localization isn't dependent on tyrosine phosphorylation or actin polymerization (Miyamoto, Teramoto et al. 1995). Talin-null ES cells exhibit extensive membrane blebbing and defects in cell adhesion and spreading. They cannot assemble focal adhesions or actin stress fibers and display reduced surface expression of integrins and disrupted golgi export (Priddle, Hemmings et al. 1998). Taken together, these results suggest that talin is involved in surface expression of integrins and is required for the formation of focal adhesions and actin stress fibers. When talin-null ES cells are differentiated they form no organized tissues and only two morphologically distinct cell types. However, these cell types express normal levels of β_1 integrin and are capable of spreading and forming stress fibers and focal adhesion-like plaques. This suggests that in some cell types talin isn't essential for normal expression of integrins and formation of focal contacts and stress fibers.

Although β integrin tails are necessary and sufficient to link the heterodimer to the cytoskeleton, the α chains play a role too. In contrast to β subunits, α cytoplasmic tails show little sequence homology, except for the membrane proximal KXGFFKR sequence. This suggests that each tail plays a unique role in integrin function. Each α subunit is also highly conserved among different species, which also indicates that they are important for integrin function (Hynes 1987; Sastry and Horwitz 1993).

The first α mediated effects to be discovered were negative. Truncation of the α_1, α_4 or α_{IIb} cytoplasmic tail increases β tail mediated interactions with the cytoskeleton (Chrzanowska-Wodnicka and Burridge 1992). More recently Kieffer et al reported that f-actin binds directly to the α_2 cytoplasmic tail (Kieffer, Plopper et al. 1995). Removal of the last 5 amino acids from the C-terminus of the tail disrupts this binding. Liu et al. have also discovered an interaction between paxillin, an intracellular adaptor protein commonly found in focal adhesions, and the cytoplasmic tail of α_4 (Liu, Thomas et al. 1999). Calreticulin, a luminal endoplasmic reticulum calcium-binding protein binds to the KXGFFKR motif common to all α chains. Calreticulin deficient cells have severe defects in integrin dependent cell adhesion, and the defects can be rescued by expression of calreticulin.

Integrins as Signaling Molecules

Thus far I have discussed integrins primarily in their role as structural molecules, linking the cytoskeleton to the extracellular matrix. This is only half of the story, if that. Integrins aren't just fastening devices. They transmit signals from the environment into the cell and from the cell out to the environment. As I mentioned earlier, the affinity of integrins can be regulated by signals from within or without the cells. Integrins are now known to be connected with numerous signaling networks. As this thesis does not deal primarily with signaling, I refer my readers to Giancotti and Ruoslahti's succinct review in volume 285 of Science (Giancotti and Ruoslahti 1999). In this section I'll discuss

some of the signaling proteins integrins appear to interact with, some proteins that bind exclusively to β_1 and β_3, and some findings that are unique to F9 cells.

Integrins are neither kinases nor phosphatases. They do the work of transmitting signals by organizing complexes of other proteins, some of which are enzymaticly active. In recent years it has become increasingly apparent that the linear models of signal transduction that arose in classical biochemical studies of metabolism don't reflect physiological reality. Instead, proteins associate into large networks where they are subject to control by many interdependent events. While location is not quite as important in signal transduction as it is in real estate, it is very important. The location of individual proteins within a network and the location of signaling networks within the cell is critical to function and regulation.

The cell membrane is one of the most important organizing sites for networks within the cell and integrins are a key focus for these networks. As I've discussed, integrin binding can be regulated and the formation of integrin-ligand bonds, integrin clusters and integrin-actin bonds are distinct processes. The formation of a signaling complex around integrins begins when the integrins aggregate. Clustering without ligand binding, induced by divalent antibodies that don't interfere with ligand binding, leads to rapid recruitment of Syk kinase in monocytes and platelets (Lin, Rosales et al. 1995) and of FAK and tensin in human foreskin fibroblasts (Miyamoto, Teramoto et al. 1995; Miyamoto, Katz et al. 1998). These associations are independent of actin polymerization or

phosphorylation. When phosphorylation and actin polymerization are allowed to proceed naturally, artificially induced integrin clustering also leads to co-localization of cortactin, pp120, GAP, PLC-γ, PI 3-kinase, PTP-1D, c-Src, Fyn, Csk, RhoA, Rac 1, Grb 2, SOS, Ras, Raf 1, MEKK, MEK 1. ERK 1, ERK 2, and JNK in fibroblasts. Actual ligation of the integrins can cause the addition of growth factor receptors to the complex.

Interestingly, the identity of the proteins recruited by different receptors and organizing molecules is not always unique. Many individual signaling proteins, like Src-family tyrosine kinases, small GTPases (Rac, Rho, cdc 42), phosphatidylinositol kinases, mitogen activated kinases (MAPKs) and transcription factors, are activated by multiple, structurally diverse receptor families to effect distinct biological results. In the realm of integrin signaling, many of the enzymaticly active proteins that appear to bind to integrins, like FAK and ILK, will bind to most or all known integrins. However, there are some proteins that appear to interact specificly with particular integrins, and some that demonstrate limited ranges of preference. These proteins are of particular interest as we try to understand the mechanisms that underlie the apparent inhibition of vitronectin binding by integrin β_1.

There are 2 proteins known to associate exclusively with the β_1 subunit: integrin cytoplasmic domain associated protein (ICAP) and CD98. Both bind to the cytoplasmic tail and neither will bind to the cytoplasmic tails of other β or α integrin subunits (Chang, Wong et al. 1997; Zent, Fenczik et al. 2000). ICAP is

phosphorylated upon integrin binding and appears to regulate chemotaxis (Zhang and Hemler 1999). CD98 is a type II transmembrane glycoprotein that is involved in amino acid transport and cell fusion CD98was discovered in a screen for proteins that modulate the ability of the β_{1A} cytoplasmic tail to block integrin activation in a specific cell line (Fenczik, Sethi et al. 1997; Zent, Fenczik et al. 2000). CD98 overexpression overcomes the inhibition of integrin activation by the β_{1A} tail.

There is one protein believed to interact exclusively with the β_3 subunit (Shattil, O'Toole et al. 1995). β_3-endonexin binds to the membrane proximal NITY sequence in the β_3 cytoplasmic tail (The corresponding sequence in β_1, NPXY, doesn't support β_3-endonexin binding.) (Eigenthaler, Höfferer et al. 1997). β_3-endonexin expression can increase the affinity of $\alpha_{iib}\beta_3$ (Kashiwagi, Schwartz et al. 1997). It also binds to cyclin A and may be a link in the network connecting integrins to control of cell cycle progression (Ohtoshi, Maeda et al. 2000). Additionally there is a recently discovered protein, TAP 20, that interacts with the β_5 subunit (Tang, Gao et al. 1999). As with any discovery, time and experimentation will tell whether these proteins are truly exclusive to one integrin subunit or another. One of the first supposedly exclusive integrin-binding proteins, integrin associated protein (IAP), was recently found to associate with a β_1 family integrin, as well as the originally discovered β_3 integrins (Chung, Wang et al. 1999).

Studies of the signaling specific to parietal endoderm differentiated F9 cells suggest that some signaling networks are more important in PE F9 cells than others. F9 cells are a useful in vitro model system for the formation of extraembryonic endoderm. In vivo, the extraembryonic endoderm forms sequentially: first the primitive or visceral endoderm forms on the side of the inner cell mass that faces the blastocoel, and then the parietal endoderm cells differentiate and migrate out from the visceral endoderm along the inner surface of the blastocoel (See figure 4). The inner cell mass marker antigen is SSEA-1 (stage specific embryonic antigen-1) (Solter and Knowles 1978). Visceral endoderm cells lose SSEA-1, begin to express α-fetoprotein (α FP), polarize and organize a basement membrane. Parietal endoderm cells are motile, null for SSEA-1, and express tissue vimentin and plasminogen activator (tPA).

These differentiation processes, and the concomitant shifts in marker expression can be reproduced in vitro by the sequential addition of trans-retinoic acid and dibutyryl cyclic AMP (dbcAMP) to F9 cells in culture (Strickland and Mahdavi 1978; Strickland, Smith et al. 1980). DbcAMP is a membrane permeable form of cyclic AMP. Parathyroid hormone (PTH) or parathyroid hormone related peptide (PTHrP) can substitute for the dbcAMP in the differentiation of visceral endoderm to parietal endoderm (Chan, Strewler et al. 1990; van de Stolpe, Karperien et al. 1993). Expression patterns of PTHrP and the PTH/PTHrP receptor suggest that this molecule does the job in vivo (van de Stolpe, Karperien et al. 1993; Karperien, van Dijk et al. 1994; Karperien, Lanser et al. 1996).

Fig 4: F9 Teratocarcinoma Cells

Undifferentiated

Analogous to inner cell mass cells
Express stage specific embryonic antigen #1

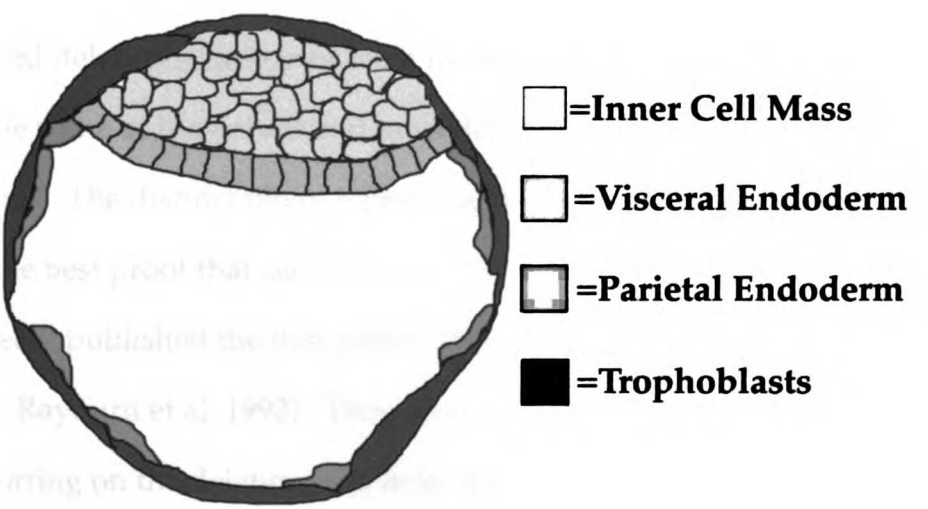
Visceral Endoderm Analogues

Grown in suspension, with retinoic acid
For an epithelial layer with tight junctions
Express α-feto protein

Parietal Endoderm Analogues

grown on a substrate with retinoic acid and dibutyryl cyclic AMP
Express thrombomodulin, vimentin and tPA
Highly motile

Blastocyst



PTH and PTHrP bind to a common receptor that couples to at least 2 G proteins, G_s and G_q, and activates the adenylate cyclase and phospholipase Cβ signal transduction pathways (Jüppner, Abou-Samra et al. 1991; Abou-Samra, Jüppner et al. 1992). Ligand binding of this type 1 PTH/PTHrP receptor causes elevation of intracellular cAMP levels and inhibits Ras and ERK activity (Verheijen and Defize 1995; Verheijen and Defize 1997; Verheijen, Wolthuis et al. 1999). This suggests that the Fyn/Shc pathway and some connections in the FAK network may not be especially active in PE F9 cells. Likewise, the Src mediated portion of the FAK network may be especially important. Felsenfeld et al. show that, in Src null fibroblasts, integrin mediated connections between vitronectin and the cytoskeleton are inhibited, while connections between fibronectin and actin are not affected (Felsenfeld, Schwartzberg et al. 1999).

Effects of Integrin Subunit Knockouts

Targeted deletion of integrins from transgenic mice and cell lines continues to provide some of the richest and most definite information about the role of integrins. The distinct phenotypes observed in the transgenic knockouts have been the best proof that each integrin has a unique function in vivo. In 1993, Yang et al published the first paper, about the targeted deletion of α_5 in mice (Yang, Rayburn et al. 1993). They were followed within the year by Stephens et al reporting on the deletion of β_1 from the F9 teratocarcinoma cell line (Stephens, Sonne et al. 1993). Deletions of many other integrins have followed (Fässler, Georges-Labouesse et al. 1996).

The knockout of integrin β_1 in F9 cells reduced cell adhesion to β_1 specific ligands, suppressed cell migration, and disrupted morphological differentiation. However, the tissue specific differentiation marker expression that F9 cells are capable of was not effected. Visceral endoderm (VE) and parietal endoderm (PE) specific genes were expressed normally, in response to differentiation factors used in cell culture. Disruption of differentiation at a morphological level was quite profound. VE cells didn't organize a coherent basement membrane, and PE cells lost much of their normal motility. These finding suggest that while the absence of β_1 integrin cannot disrupt the expression of a gene program, the physical contribution of β_1 integrin to development is essential.

In mice, deletion of β_1 results in inner cell mass failure and peri-implantation lethality. Knockouts of β_1 in other cell lines and tissues have resulted in other losses of normal function, including defects in fibronectin matrix organization (Wennerberg, Lohikangas et al. 1996), basement membrane assembly and laminin-1 expression (Sasaki, Forsberg et al. 1998), and impaired metastasis, invasion (Stroeken, van Rijthoven et al. 1998), and migration (Hirsch, Iglesias et al. 1996). Analysis of mutants of the β_1 cytoplasmic tail, expressed in β_1 null fibroblastic cells revealed that threonines 788 and 789 are essential for activation of β_1 and that tyrosines 783 and 789 are necessary for optimal migration and appear to regulate the assembly of actin stress fibers (Sakai, Zhang et al. 1998; Wennerberg, Fässler et al. 1998).

Goals of the Present Study

The F9 β_1 null cell line, named TKO, binds to vitronectin with greater affinity than F9 cells. It appears that the presence of β_1 integrin decreases vitronectin binding by F9 cells. The goals of this study were:

1. To determine whether the increase in vitronectin binding is exclusive to the undifferentiated TKO or extends to parietal endoderm differentiated cells as well.

2. To reintroduce β_{1A} to the TKO line to test the dependence of the null phenotype on the original deletion of β_1.

3. To analyze the motility defect in TKO cells and its consequence in vivo.

4. To examine the affects of β_1 deletion on ECM organization in parietal endoderm differentiated F9 and TKO cells.

5. To discover the mechanisms underlying the increase in vitronectin binding in TKO cells.

Chapter 2

Materials and Methods

Antibodies

Ha 2/11 Armenian hamster IgM monoclonal anti-rat β_1, grown from cells given to the Damsky laboratory by Donna Mendrick, and concentrated by ammonium sulfate precipitation as described in "Antibodies, a laboratory manual" (Harlow and Lane 1988). Other antibodies were obtained as follows: 9EG7 monoclonal hamster anti-mouse β_1 integrin from Pharmingen Co., AB1930 rabbit polyclonal anti-human α_v cytoplasmic tail from Chemicon Co., a polyclonal rabbit anti-β_1 cytoplasmic tail from the Randall Kramer laboratory at UCSF, Rb8275 polyclonal anti-human β_3 cytoplasmic tail from the Mark Ginsberg laboratory at Scripps, #4377 polyclonal rabbit anti-human β_5 cytoplasmic tail from the Louis Reichardt laboratory at UCSF, rabbit monoclonal anti-mouse β_5 from the Dean Sheppard laboratory at UCSF, polyclonal anti-human β_8 from the Steve Nishimura laboratory at UCSF, and polyclonal anti-human fibronectin from Telios Co..

Cell Culture

Undifferentiated F9, TKO and TKOβ_1 cells were maintained in an atmosphere of 7% CO_2, in DMEM medium with 5.4g/liter glucose, with 10% heat denatured fetal calf serum, 2mM glutamine, 100ug/ml streptomycin, 100units/ml penicillin, and 86.4μM beta mercaptoethanol. To differentiate the cells into

parietal endoderm they were maintained for a minimum of 48 hours in 50% DMEM, 50% Hams F-12, with glutamine, pen/strep, 1mM dibutyryl cyclic adenosine monophosphate (Sigma #D 0627), and $1x10^{-7}$M trans retinoic acid. This medium is referred to as PE differentiating medium.

Retransfection with β_1 and selection of positive clones.

35 mm wells of 30% confluent TKO cells were transfected with the pECEβ_1 plasmid using Lipofectamine (Gibco) at 4 ul Lipofectamine per well. The pECEβ_1 construct was generated by inserting the XbaI-SalI fragment of the full-length murine β_1 integrin cDNA into a PECE derived plasmid containing the PGK promoter and a puromycin resistance gene. 2 days after transfection, selection was imposed with 3uM puromycin. At 20 days the surviving cells were screened by FACS for β_1 staining using the HA 2/11 antibody. The puromycin resistant population was mixed, with approximately 40% of the cells positive for β_1. The resistant population was plated at low dilution and 10 clones expressing β_1 at 89%-110% of the level of the parental F9 line were pooled to form the TKOβ_1 cell line.

Immunoprecipitation

Cells were detached with Gibco dissociation medium, triturated to break up groups of cells, brought up in PBS, and centrifuged. Aliquots of cell suspension were counted. The pellets were suspended in PBS 1mg/ml sulfo-NHS-LC biotin (Pierce #21335) at $1x10^7$ cells per ml. The cells were incubated with the biotin on

ice for 30 minutes, with occasional gentle swirling. The biotin was washed away with 2 changes of PBS 50mM glycine and the labeled cell pellets lysed in lysis buffer (100mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, with 50uM $Na_2 MoO_4$, 1mM $Na_3 VO_4$, 1mM NaF, aprotinin 25ug/ml or 1TIU/ml, 1 mM PMSF, 5-50 ug/ml leupeptin and 25-50 ug/ml pepstatin) at $1x10^7$ cells per ml. The lysate was incubated on ice for 30 min with occasional vortexing, then centrifuged in a microcentrifuge at 14,000 rpm for 15 min. The supernatants were decanted into fresh tubes and pre-cleared by incubating with protein A sepharose (Pharmacia) for 1 hr at 4°C on a nutator. The beads were discarded and the protein concentration of each sample measured by BCA (bicinchoninic acid) protein assay kit from Pierce (#233335; (Smith, Krohn et al. 1985)). 100ug of protein in 500ul lysis buffer was the standard starting point for integrin immunoprecipitations. Antibody was added and the sample was nutated at 4°C overnight. The immune complexes were captured on protein A sepharose during one hour of nutation at 4°C. The beads were spun down and the supernatant was reserved for further rounds of immunoprecipitation as needed. The pellet was washed first in fresh lysis buffer, 2 washes of 10 minutes, then in 100mM Tris pH 7.4, 1M NaCl, 2 washes of 10 minutes, and finally in 100mM Tris pH 7.4, 150mM NaCl. The pellets were then suspended in 80ul of non-reducing sample buffer and boiled for 5 minutes. 25 ul of this sample was loaded per lane in a 7.5% SDS acrylamide gel. The gels were run until the 65kDa molecular weight marker was at the bottom. The separated proteins were transferred to nitrocellulose. The nitrocellulose blots were blocked in PBS 5% BSA for 1 hour, washed 2 times briefly, and incubated with horseradish peroxidase labeled

streptavidin at 1:4000 in PBS for 45 minutes. The excess streptavidin was washed off with 3 10 minute washes in PBS 0.1% Tween 20, and the blots were developed with ECL solution and visualized on Amersham ECL film.

Bod Outgrowth Assay

Bods are bodies of cells formed by growing F9 cells in standard petri plates with no coating. The cells can't adhere to the plate, but they do adhere to one another and grow in suspension, forming aggregates. For these experiments the cells were grown 4 days in parietal endoderm differentiation medium. On the 4^{th} day, the bods were aspirated one by one, and plated on tissue culture treated plates that were previously coated with ECM molecules at 10ug/ml and blocked with 1% BSA. The bods adhered to the plates, and cells migrated out from the bods, onto the surface of the plate. The diameter of the bod or the width of the margin of migrating cells was measured using a stage micrometer.

Transwell Motility Assay

Filter membranes were coated on both sides with ECM proteins at 10ug/ml for 1 hour at 37°C or overnight at 4°C. The membranes were then blocked by incubating in PBS/1% BSA for 30 min at 37°C. Cells were detached using Gibco Dissociation Buffer, triturated to produce a single cell suspension, and counted. $1.2x10^5$ cells in 100ul 50% DME/50% Hams F-12/2%FCS were pipetted into each filter capsule and the capsules were placed in 24 well plate wells that were previously filled with 600ul of the same medium. The cells were allowed to

settle naturally and incubated at 37°C for 4 to 48 hours. After incubation the cells were removed from the top surface of each membrane by rubbing with a cotton swab saturated with PBS. Then the cells on the bottom of the membranes were fixed and stained with the Fisher Leukostat kit, the membranes were cut out of the capsules with a scalpel and mounted in Cytoseal (Stephens Scientific). The cells were counted by photographing 5 fields per sample through a 40X DIC objective on a Zeiss Axiovert 35 microscope, developing the photos and counting the images of the cells with a mechanical tallying pen.

Intrasplenic injection into mice

The cells were injected into nude mice following the protocol published in Stroeken et al. (Stroeken, van Rijthoven et al. 1998). . Briefly, $1x10^6$ cells were suspended in a minimal amount of PBS and injected, through the cleansed abdominal wall, into the spleen of an anaesthetized nude mouse.

Immunostaining

Cells were differentiated by 3 days culture in PE medium. 12 mm coverslips were coated with the specified ECM molecules at 10ug/ml for 1 hour at 37°C, or overnight at 4°C, and blocked for 30 minutes in 1% BSA at 37°C. Cells were plated at low density and allowed to grow for 4 days in PE medium. They were then rinsed 2 times with room temperature PBS and fixed in PBS 4% paraformaldehyde (made fresh) for 10 minutes at room temperature. The fixative was aspirated and the cells were washed 2 times with PBS, permeablized

with 0.1% Triton X-100 in PBS for 10 minutes at room temperature, and washed once with PBS. I used 50ul per coverslip of part A biotin/streptavidin blocking solution (Vector Laboratories) with 0.5% BSA and 1% normal serum from the animal that the secondary antibodies were made in, incubated for 10 minutes, aspirated part A, added 50 ul of part B and incubated for 10 minutes. Part B of the blocking solution was aspirated and, without washing the coverslips, the primary antibodies were added. The coverslips incubated overnight at 4° in a moist chamber. The next day, the primary antibody was washed off with three 10 minute changes of PBS and gentle shaking. Biotinylated secondary antibodies were added at a 1:1000 dilution, incubated 45 minutes at room temperature and then washed thrice in PBS. To visualize, the streptavidin conjugated tertiary elements; rhodamine phalloidin and Hoechst were added for a final incubation of 15-30 minutes. The coverslips were washed three times more in PBS, mounted in Vector mounting medium, sealed with nail polish, rinsed, and viewed.

Shear Stress Adhesion Assay

Flat bottomed 96 well Costar tissue culture plates were incubated with 50 ul of a 10ug/ml solution of purified human vitronectin (purified as described in (Yatohgo, Izumi et al. 1988)) or bovine fibronectin (Sigma F 4759) for 1 hr at 37°C or overnight at 4°. After incubation they were blocked with 1% BSA (Sigma A 7906) for 30 min at 37°C and washed 2X with 200ul PBS. F9, TKO and TKOβ_1 cells were labeled with calcein AM (Molecular Probes C-3100) at 2.5 ug/ml in DME for 20 minutes at 37°C. Labeled cells were washed 1X with calcium magnesium free PBS, 0.4% EDTA, incubated in Cell Dissociation Buffer (Gibco

13151-014) for 20 min, and shaken to free the cells from their substrate. The cells were centrifuged and resuspended in DME 0.1% BSA at $6x10^5$ cells per ml. The prepared wells were filled with 200 ul of DME 0.1% BSA with or without inhibitory antibodies and peptides. 50 ul of cell suspension was put into each experimental or control well. Standard wells consisted of a 2 fold dilution series of the same 50 ul aliquots. The plates were incubated at 37° for 60 minutes and then the experimental and control wells were washed with PBS in a Molecular Devices microplate washer set at slow speed. The media was gently aspirated from the standards wells with a multichannel pipettor. All wells were filled with 50ul 0.2% Triton-X 100, and the entire plate was frozen at -20°. The plates were then thawed, and read on a Cytofluor II fluorescence multiwell plate reader (Perseptive BioSytems) at 485/535 nM. Data were expressed as number of cells in well or as percentage of control. Values shown are means (+/-SD) for quadruplicate samples.

Centrifugal Force Adhesion Assay

Dynatech Immulon B plates were coated with the desired ECM molecules, as in the centrifugation adhesion assay, and blocked with 0.1% BSA for 30 min at 37°. After washing the wells 2-3X with PBS, the experimental wells and the BSA coated negative control wells were filled with 200ul medium (50/50 DME/Hams F-12, 0.1% BSA) and the standard wells with 50 ul. The plates were chilled plates on ice. The cells were labeled with 5ug calcein AM (Molecular Probes) per ml for 20-30 minutes at 37°C, washed twice with cold medium and plated at $6x10^5$ cells per well. The plates were covered with tape and the cells were spun onto the

plates at 1,000 rpm (~175G) for 1 minute. Afterwards, I turned them upside down and spun for 10 minutes at 2,000 rpm (~690G). The tape was removed and the medium gently aspirated. The wells were then filled with 100ul 0.1% Triton X-100 in ddH_20. The standards were plated and allowed to bind at 37°C. After they spread, I aspirated their medium very gently and filled the wells with 100 of a ul 0.1% TX-100 solution in ddH_20. The plates were frozen, thawed, and read on a Cytofluor II fluorescence multiwell plate reader (Perseptive BioSytems) at 485/535 nM. Data were expressed as number of cells in well or as percentage of control. Values shown are means (+/-SD) for quadruplicate samples.

Immunoblotting

Cell lysates were loaded on SDS PAGE gels of 6-8.5% acrylamide, depending on the proteins in question. A Hoeffer minigel apparatus was used to run the gel and the proteins were transferred to nitrocellulose using a Biorad wet transfer or semi-dry transfer apparatus. The nitrocellulose was blocked using 1-5% BSA in PBS for 30 minutes to overnight. Then the antibodies were applied in PBS/1% BSA and incubated overnight. The blots were washed three times, for 10 minutes each, in PBS/0.1% Tween 20, then biotinylated secondary antibodies were diluted in PBS/0.1% Tween 20, applied and incubated for 1 hour. The blots were washed 3 X 10 minutes in PBS/0.1% Tween 20, and a HRP streptavidin reagent was added, incubated 30 minutes, and washed off as before. I used Amersham ECL reagents to develop the blots.

Video Microscopy

Video microscopy was done on a Nikon inverted microscope with a 40X Fluor objective and a TRITC excitation/emission filter. Images were captured with a Hamamatsu ccd video camera model XC-77. The camera was controlled and the images were collected and analyzed using Universal Imaging Image and Metamorph software. The coverslips were placed in a stage incubator heated by circulating water to maintain an even 37° C temperature. pH was maintained by the use of 25mM HEPES in the medium and a 5% CO_2 gas mixture blown over the medium. Evaporation was controlled by floating a layer of silicon oil over the top of the medium. After differentiation for 3-4 days in PE medium with 10% FCS, the cells were labeled for 30 minutes with octadecyl rhodamine (Molecular Probes catalogue # O-246) at 1:1000. Then the cells were rinsed 3X in PE 2% FCS and maintained in PE medium with 2% FCS and 25mM HEPES for the duration of the experiments. Single cells were tracked migrating either in low density culture or migrating into a wound in a high density monolayer.

Chapter 3

Loss of β_1 Integrin in Parietal Endoderm Differentiated F9 Cells Affects Adhesion, ECM Organization, Migration In Vitro and Metastasis In Vivo.

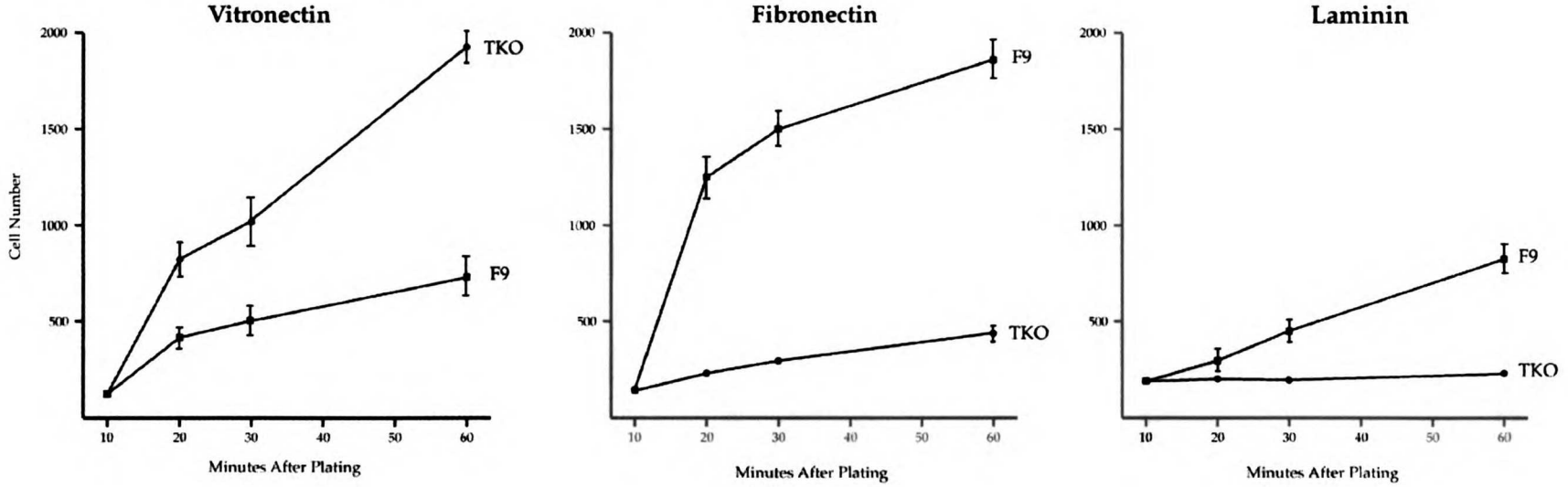
Previous studies of the β_1 null F9 cell line (named TKO) revealed the expected loss of adhesion to β_1-specific ligands like collagen and laminin (Stephens, Sonne et al. 1993). TKO cells were found to express the same α_v family integrins as F9 cells, including $\alpha_v\beta_3$, a receptor that recognizes numerous ligands (see figure 1), including fibronectin and vitronectin. Despite expressing a potential receptor for fibronectin, TKO cells do not bind to fibronectin. Another striking finding in the TKO cells a sharp increase in binding to vitronectin. When the TKO cells were differentiated into the parietal endoderm analogue (by addition of retinoic acid and dbcAMP to the medium), they were not motile. The goals of the present study were to study the mechanisms behind the increase in vitronectin binding and the loss of motility in the absence of β_1 expression. We chose to do all of our studies in parietal endoderm (PE) differentiated cells because this offered a chance to also look for connections between the mechanisms regulating adhesion and motility. PE cells also can organize fibronectin into polymerized fibrils, giving us another readout for the function of TKO integrins.

Parietal endoderm differentiated TKO cells also display increased adhesion to vitronectin.

We were curious, generally, whether the binding profile of parietal endoderm differentiated F9 and TKO cells resembled that of the undifferentiated cells and, specificly, whether the increase in vitronectin binding observed in undifferentiated TKO cells was seen in PE differentiated TKO cells as well. In these assays we evaluated binding as the cells spread, using shear stress applied to the cells with a mechanical plate washer. Figure 5 compares binding to fibronectin, vitronectin and laminin. The concentration of substrate used in this assay, 10ug/ml, and the time at which the binding was tested, 60 minutes, gave optimal binding for the cells. The results parallel those seen with undifferentiated cells (Stephens, Sonne et al. 1993). The strongest binding seen is that between F9 cells and Fn. The bond between TKO and Fn is negligible. Next strongest is the bond between TKO cells and Vn. F9 cells do form a distinct bond with Vn, but it is weaker than that between TKOs and Vn. F9 cells also bind to laminin and this bond is comparable in strength to their bond with Vn. TKO cells do not bind to Ln at all.

Binding assays on multiple concentrations of vitronectin (figure 6) show a consistent 1.75-3-fold difference between the number of TKO cells binding to Vn and the number of F9 cells. It is also apparent that the concentration of Vn necessary for binding of a given number of F9 cells is 9-27 fold greater than is necessary to bind the same number of TKO cells.

Figure 5: Graph of the number of PE differentiated cells binding to 10ug/ml substrate. Binding was challenged by shear stress, data points represent the mean of 5 fields photographed with a 40X objective. Error bars are standard deviation from the mean.



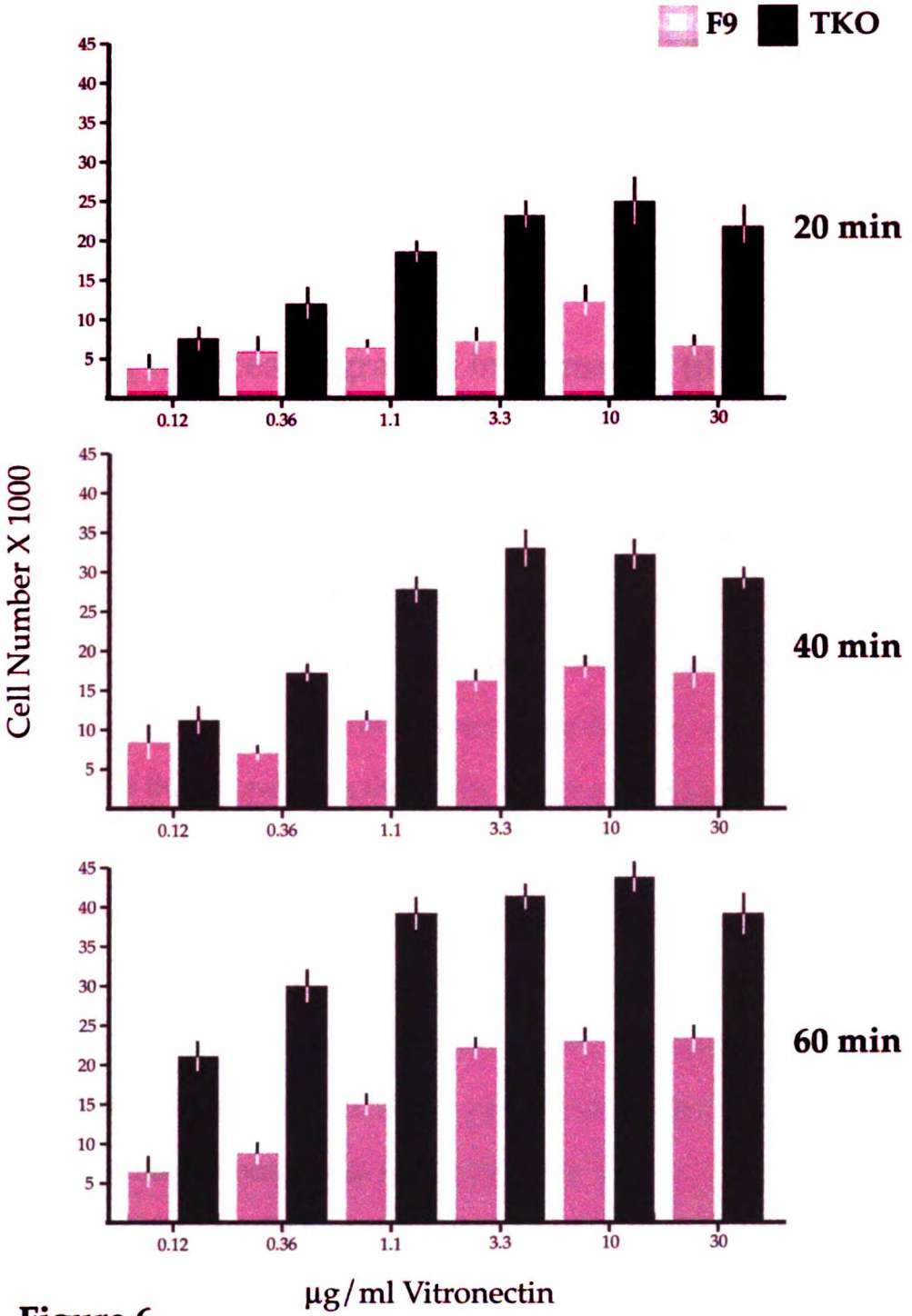


Figure 6

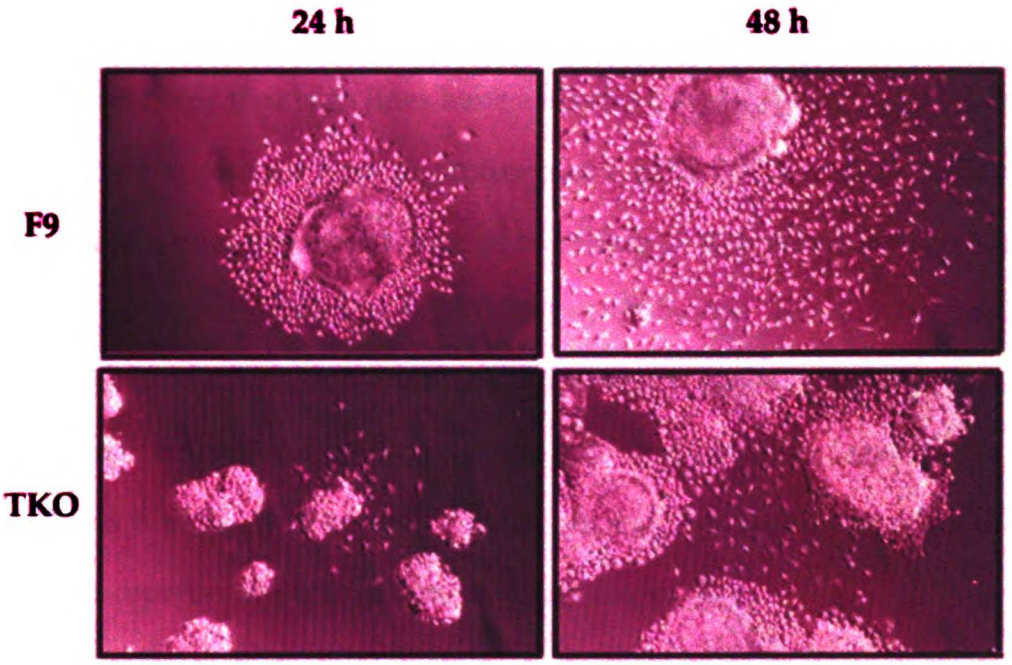
Graph of the number of PE differentiated F9 and TKO cells bound to 6 different concentrations of Vn after 20, 40 and 60 minutes incubation. Adhesion is measured with shear stress assay. Bars are the mean of 8 wells in 2 separate experiments. Error bars are standard deviation.

For example, at 60 minutes after plating, an average of 22,000 TKO cells bind per well at a coating concentration of 0.12 ug/ml Vn. At that time point, it takes 3.3ug/ml of Vn to support the binding of approximately 22,000 F9 cells. Interestingly, both cell lines appear to reach maximum binding for a given time period at 3.3ug/ml Vn.

Loss of β_1 reduces migration in transwell filter motility assays

Loss of integrin β_1 has dramatic effects on cell motility as well as cell adhesion. In the original studies of parietal endoderm differentiated TKO cells, Stephens et al grew bods in standard medium, plated the undifferentiated bods on defined substrates in parietal endoderm differentiating medium and cultured them for 5 days. Under these conditions there was almost no outgrowth from the TKO bods (Stephens, Sonne et al. 1993). When I began to work with the F9 and TKO system, I wanted to shorten the time this assay took so I handled the cells differently. I grew the bods of cells in PE medium for 3 days, then plated them on defined substrates and cultured them in more PE medium for 1-3 days. In effect, I differentiated the bods before plating them. This change in culture medium resulted in outgrowth of PE cells from the bod within 12 hours of plating and produced PE TKO cells that migrated out and away from the bods, unlike what was seen in the Stephens paper. figure 7. The TKO cells still didn't migrate as far from the bods as the F9 cells, but they did migrate.

Figure 7: PE F9 and TKO Bod Outgrowths

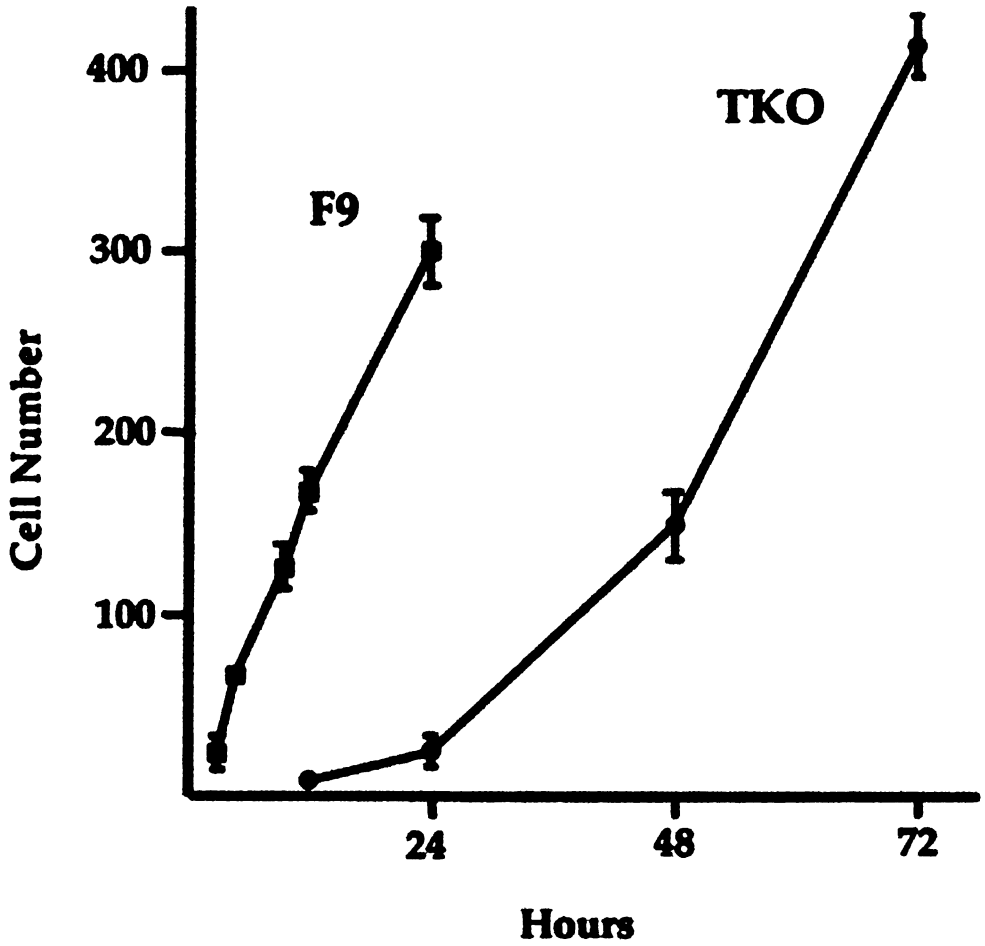


Phase micrographs of outgrowth of F9 and TKO PE bods plated on fibronectin coated substrates. Bods grown for 4 days in PE differentiation medium were plated on Fn coated 35mm dishes in PE medium, 10% FCS, and photographed at the timepoints indicated. The TKO bods took longer to bind to the substrate, and the outgrowth of PE cells was less extensive, but there were cells that moved out and away from the halos of cells immediately surrounding the bods.

To further analyze the motility defect I did a series of experiments using the modified Boyden chamber to assess random migration. The cells were plated on filter membranes that had been coated on both sides with extracellular matrix proteins and the filter capsules were immersed in wells containing the same medium that the cells were plated in. These assays reveal that TKO cells took 12-24 hours longer to reach the bottom of the membrane than the F9 cells did (figure 8). Scanning electron micrographs of the cells at the bottom of the filter membranes show that both lines have a similar morphology (figure 9). In response to suggestions from my colleagues that the PE TKO cells were passing through the membrane under force of crowding, rather than by migration, I tried the assay with undifferentiated (UD) F9 and TKO cells. The UD TKO cells did not appear on the bottom of the membrane until 72 hours (figure 14A). UD cells divide faster than PE cells (discussion with L. Grabel) so the crowding hypothesis is unlikely to be true. This result suggests that the motility seen in PE TKO cells represents a true, albeit slow, cell migration.

To determine whether this delay was due to a change in the speed of cell migration or to delays in the onset of cell migration, I then performed monolayer wound healing assays and time-lapse video microscopy of sparsely plated single cells. Under the conditions prevailing in time-lapse video microscopy and monolayer wound healing, I was unable to detect any migration of TKO cells whatsoever (figures 10 and 11), even after 24 hours. It has been shown that extracellular matrix concentration can inhibit cell motility (Huttenlocher, Ginsberg et al. 1996).

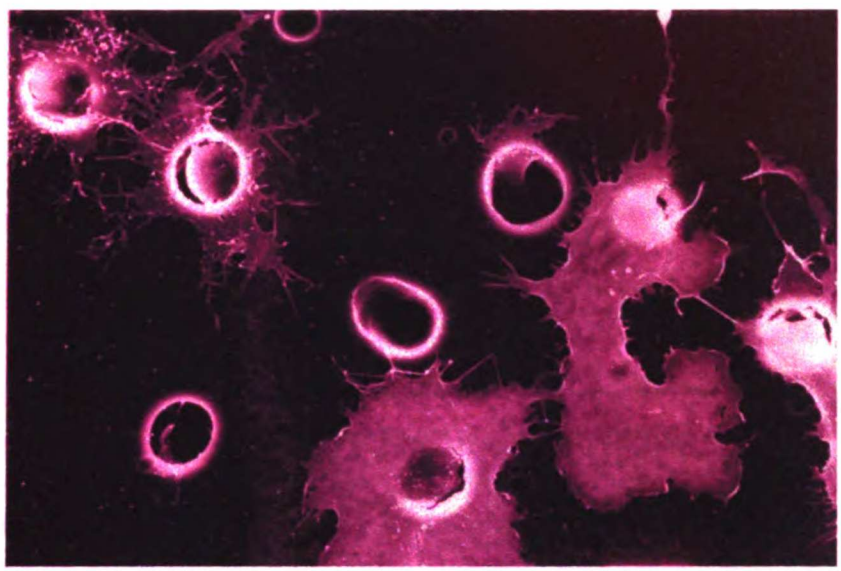
Figure 8: Transwell Filter Migration Assay on Fn Coated Membranes



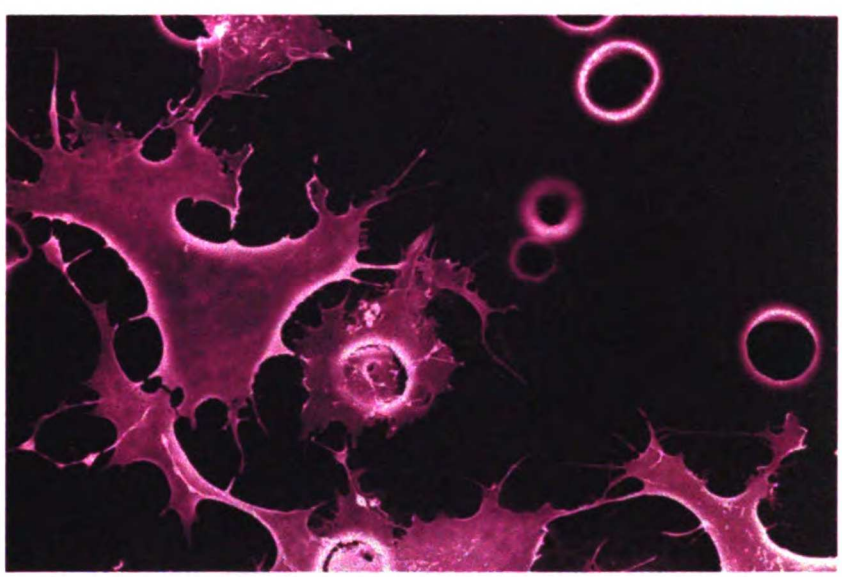
Graph of the number of cells on the bottom of Transwell filter membranes. Cells were cultured, seeded in the filter capsules, harvested and counted as described in the Methods section. Timepoints for F9 were 2, 4, 8, 12 and 24 hours. After 24 hours the F9 cells on the bottom of the membranes were too numerous to count. TKO cells were counted at 12, 24, 48 and 72 hours. Cell numbers are averages of 5 40X fields. This data is representative of 4 experiments.

Fig 9: EM of Bottom of Transwell Filter

F9
8 h

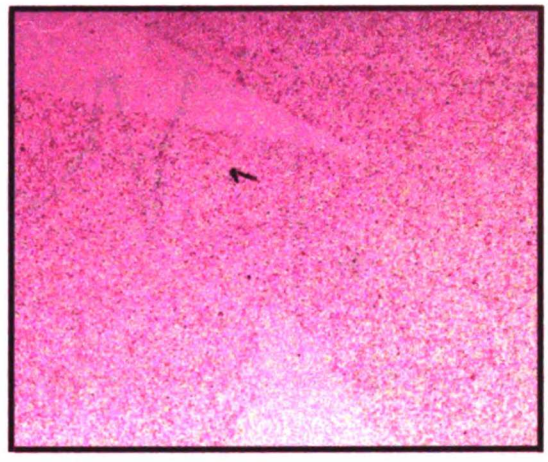


TKO
12 h

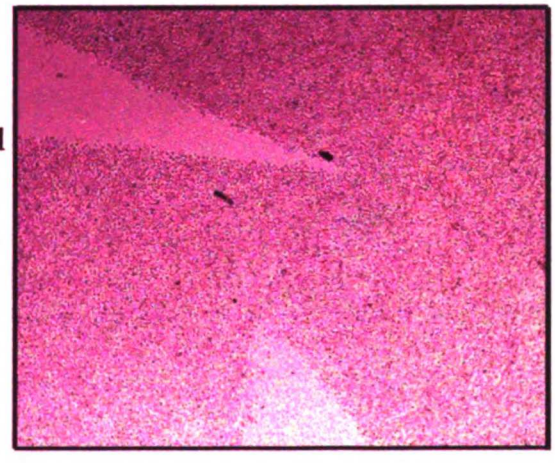


Scanning electron micrograph of cells migrating through the 8m holes in a Vn coated filter membrane, viewed from below. Both cell lines have the filopodia and lamellapodia typical of parietal endoderm.

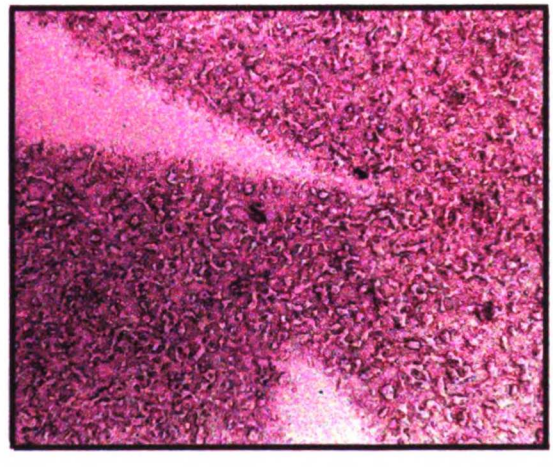
Figure 10: Wound Healing of PE TKO Cells on Vitronectin



4 h



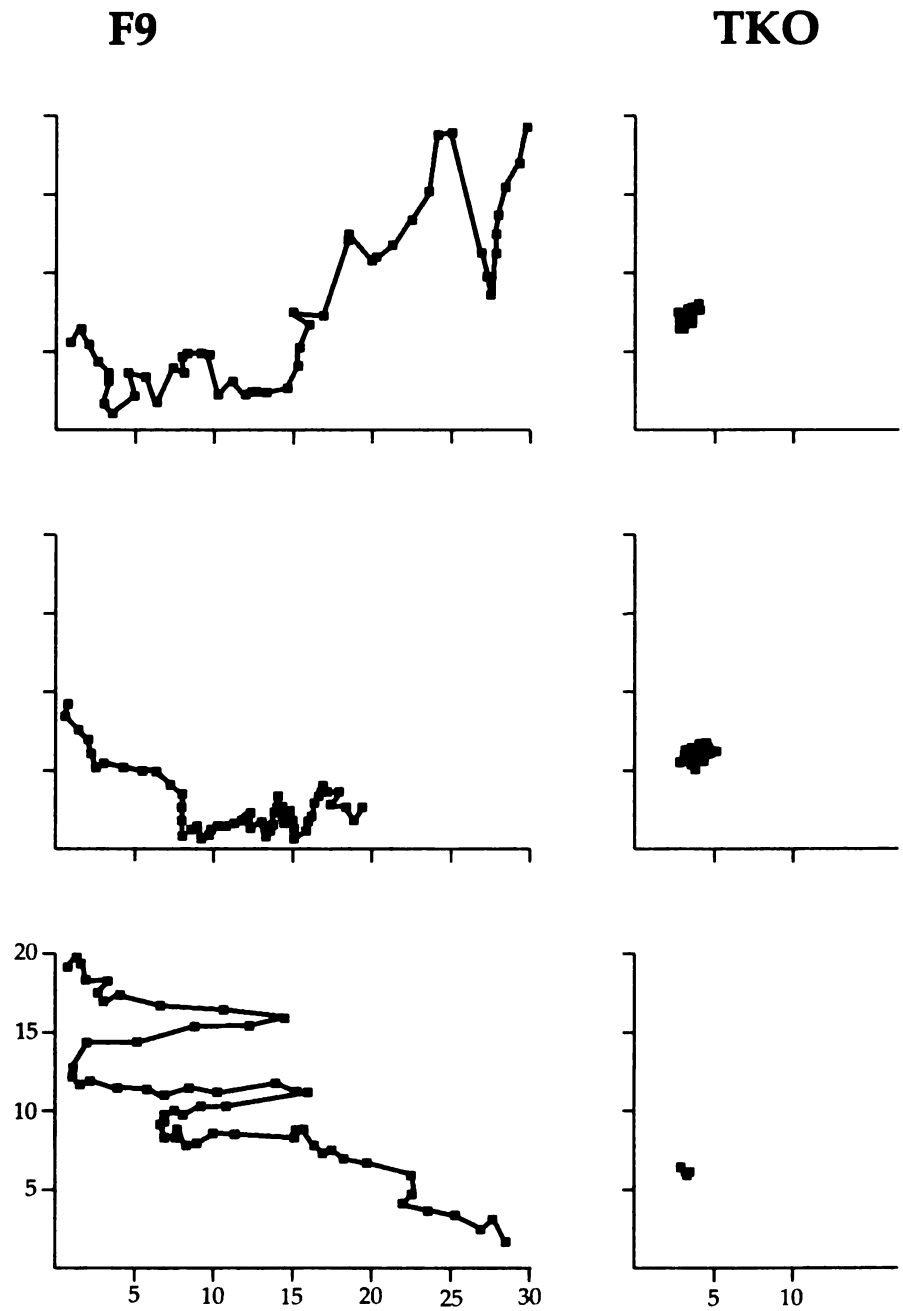
24 h



48 h

Phase contrast photos of PE TKO cells not migrating into a wound in the mololayer. Substrate coated with 1mg/ml Vn.

Figure 11



3 hour timelapse video of single cells plated at low density. Distances are given in mM. All graphs are scaled equally.

However, I found no concentration of vitronectin or fibronectin, either high, or low that supported the motility of TKO cells in wound healing or video microscopy assays. Under the same conditions, F9 cells migrated at speeds between 10 and 30 um per hour (figure. 11). The concentrations of substrate used in the video microscopy studies were varied between 1 and 10 ug/ml, however no correlation between substrate concentration in this range, and speed of migration was observed. These concentrations were chosen based on the adhesion assays shown in figure 6.

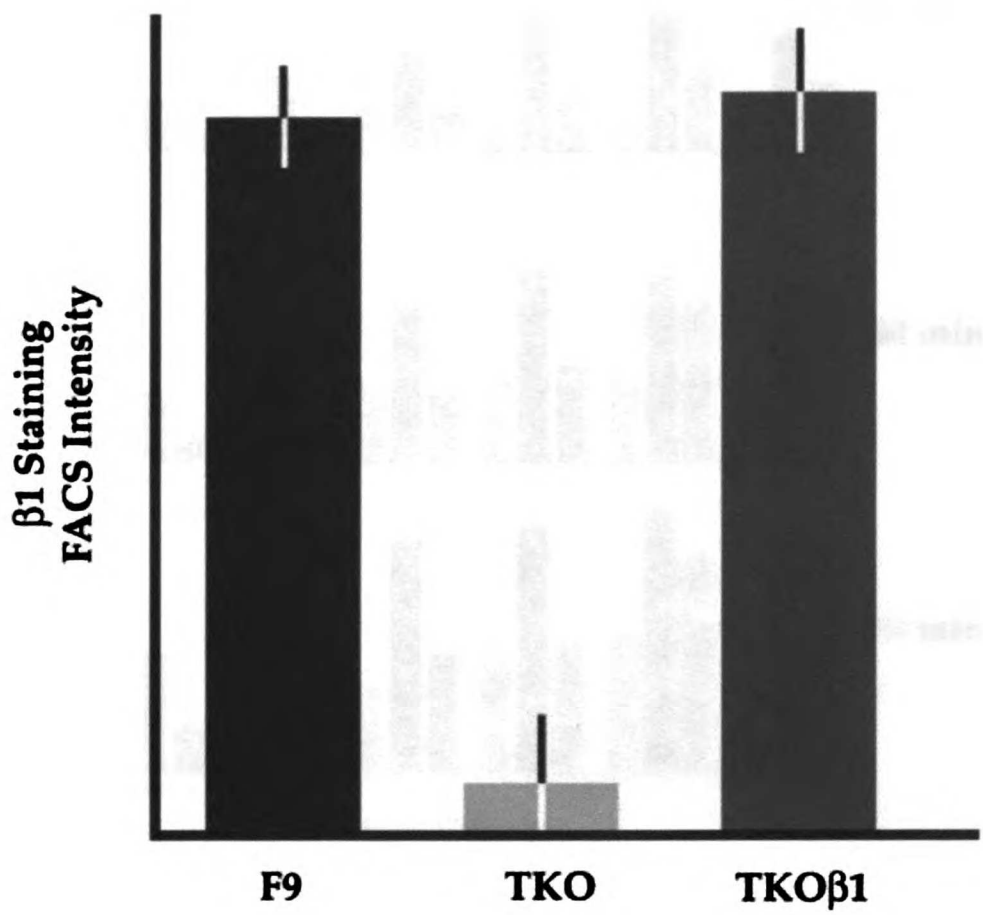
The difference in results between the bod outgrowth and modified Boyden chamber assays and the wound healing and video microscopy assays suggests that there may be factors secreted by the TKO cells themselves that promote a slow migration in the absence of integrin β_1. It is possible that the TKO cells secrete a migration inducing factor when they are cultured in multiple layers, as can occur in the modified Boyden chamber. To address this question we investigated the effects of PDGF, TGFβ, HGF, FGF, PTHRP and PMA using the bod outgrowth assay, and the effects of PMA and lysophosphatidic acid, using the transwell filter assay. In the bod outgrowth assay, PMA, at 50 and 100ng/ml, and HGF, inhibited cell adhesion and eliminated cell migration (data not shown). PDGF, TGFβ, FGF and PTHRP had no effects on migration (data not shown). It should be noted that there are no chemotactic factors for F9 cells known. In the transwell filter assays, we placed the PMA or lysophosphatidic acid in both the upper and lower chambers of the transwell apparatus, thus assaying random motility rather than chemotaxis.

We also tried transfecting the cells with a myristylated FAK expression construct, then examining migration with the transwell filter assay. Myristylation would cause FAK to localize to the plasma membrane. The localization of FAK is regulated in part by its activation state (Schlaepfer, Hauck et al. 1999). Placing it in proximity to integrins without regard to activation state could support the formation of signaling complexes in the absence of integrin β_1. The transfected cells did not migrate to the underside of the filter membrane any faster than the controls. Thus it appears that relocalization of FAK is not sufficient to support migration in TKO cells. Results of studies on FAK in GD25 cell support this hypothesis since loss of β_1 or mutation of β_1 cytoplasmic tyrosines affected activation of FAK, but not localization.

Retransfection with β_1 restores wildtype adhesion and motility

Until I reintroduced integrin β_1 to the TKO cells, we could not eliminate the hypothesis that the TKO phenotype was due to a random mutation coincidental with the targeted deletion of β_{1A}. This was a fundamental control for the system. Using lipofectamine, I introduced a plasmid containing murine β_1 and a puromcyin resistance gene, under control of the phosphoglycerol kinase promoter, into TKO cells. Cells resistant to puromycin were screened by immunofluorescent staining with an antibody to β_1.

Figure 12:
Integrin β1 Expression



FACS of cells stained with antibody to integrin β1. TKOβ1, the TKO cells re-transfected with β1A, express β1 at levels equal to the parental F9 cells.

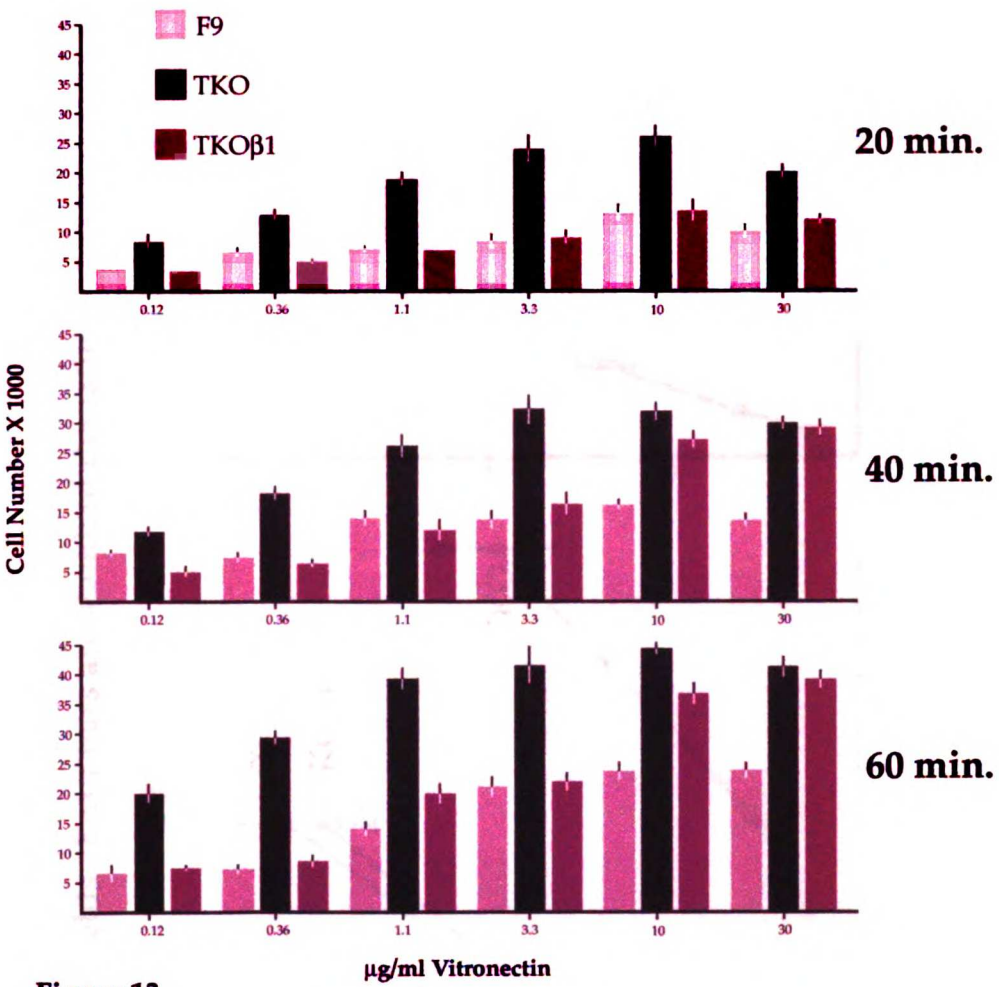


Figure 13
Graph of the number of PE differentiated F9, TKO and TKOβ1 cells bound to 6 different concentrations of Vn after 20, 40 and 60 minutes incubation. Adhesion is measured with shear stress assay. Bars are the mean of 8 wells in 2 separate experiments. Error bars are standard deviation.

Figure 14: Migration of PE F9, TKO and TKOβ1 cells through Fn and Vn coated transwell filters. TKOβ1 cells move like F9 cells. Points represent the average of 5 40x microscope fields. Error bars are standard deviation.

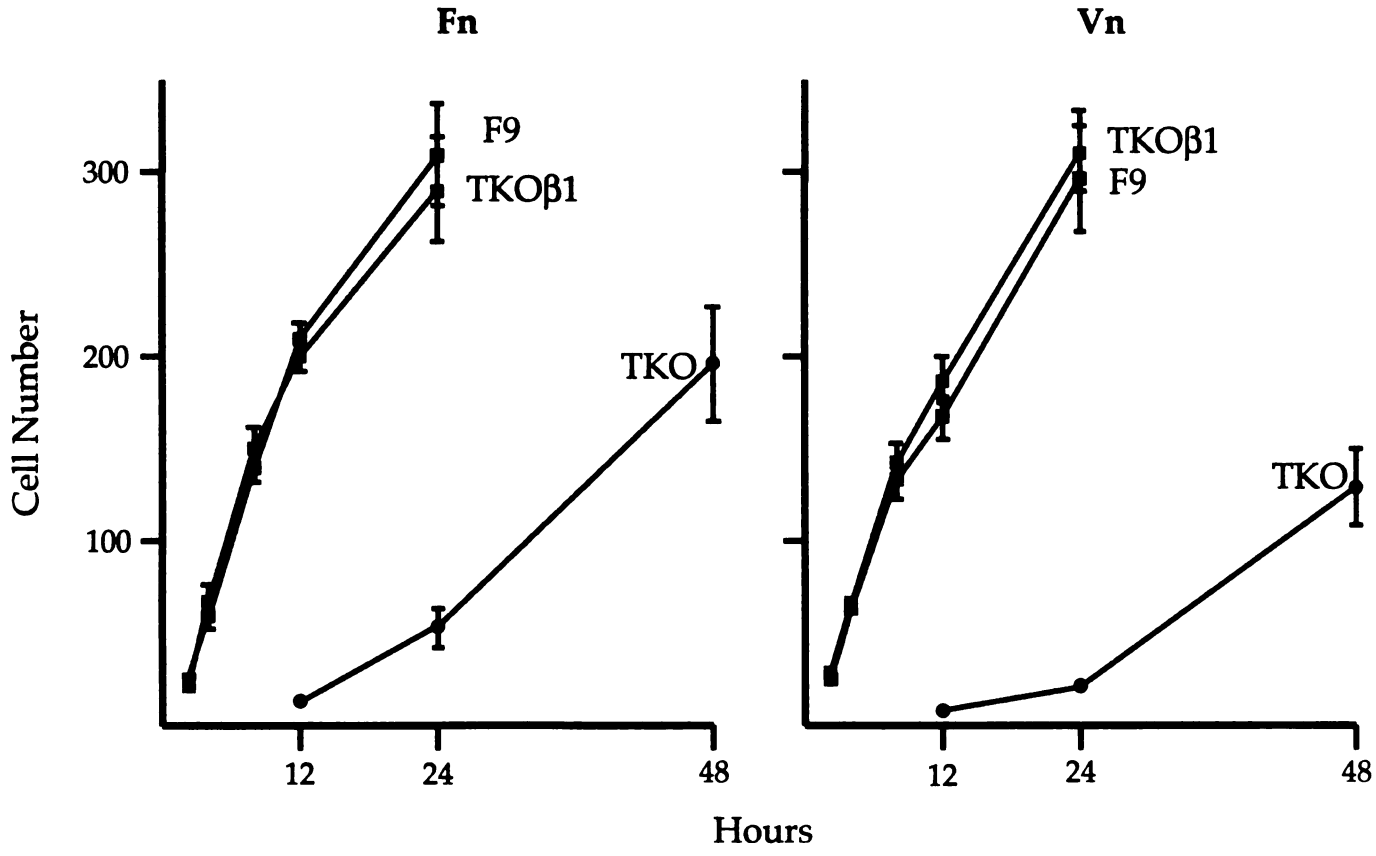
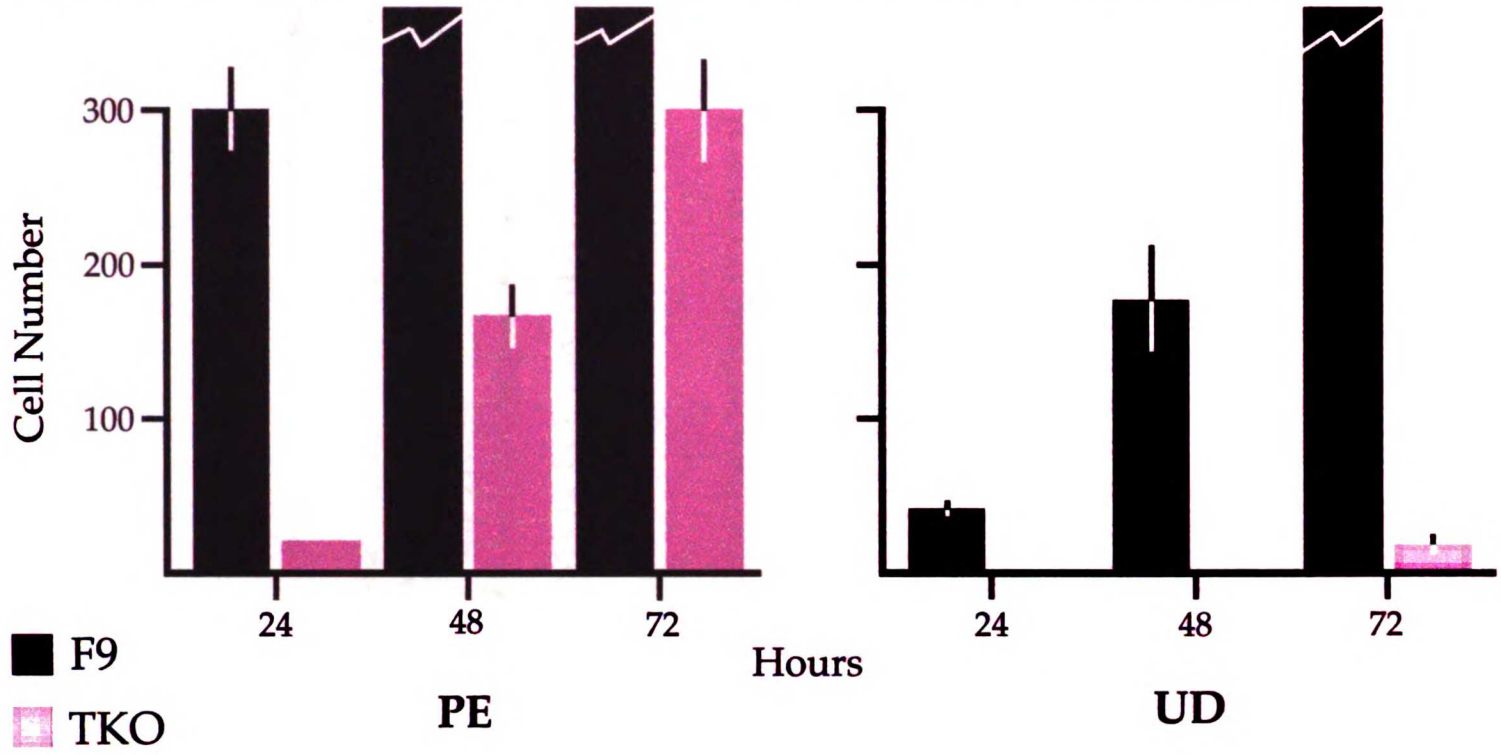


Figure 15
Migration of PE and UD F9 and TKO cells through Fn coated Transwell filter membranes. Slashed columns represent cell numbers over 500 and too high to be counted accurately. Note that PE TKO cells migrate to the bottom of the membrane by 24 hours, and UD TKO cells don't show until 72 hours. Error bars are S.D..



A positive line was isolated and cloned by single-cell dilution. TKO cells stably re-transfected with murine integrin β_{1A} express between 89% and 110% of the quantity of β_1 expressed by F9 cells, as assayed by FACS (Figure 12). TKOβ_1 cells migrate on fibronectin like F9 cells (Figure 14). Their binding to vitronectin is similar to that of F9 cells at low concentrations of vitronectin and at early timepoints (Figure 13). However, at substrate concentrations over 3.3 ug/ml and times over 20 minutes the number of TKOβ_1 cells bound to Vn approaches that of the TKO cells. This deviation from the F9 phenotype suggests that formation of the integrin-cytoskeleton bond in the TKOβ_1 cells proceeds differently than it does in the F9 cells. However, the majority of the findings show that the TKO phenotype is caused by the loss of β_1 integrin.

Loss of β_1 Reduces Fn and Ln Matrix Organization.

F9 and TKOβ_1 cells organize fibronectin into matrices characterized by short fibrils and intensely staining protein aggregates (figure 16 and 17). Matrix staining is most intense between cells in multilayered cell aggregates, and seldom observed around isolated cells. Unlike the GD25 cells, that are able to use their $\alpha_v\beta_3$ integrins to organize a fibronectin matrix when allowed extra time, TKO cells appear unable to organize fibronectin into fibrils even after protracted periods of time.

Figure 16: Fibronectin matrix is organized into short fibrils by PE F9 and TKOβ1 cells. PE TKO cells do not organize Fn into fibrils. Fn is visualized by immunofluorescent staining with antibody to Fn. Actin is visualized with rhodamine phalloidin. Cells were cultivated for 5 days.

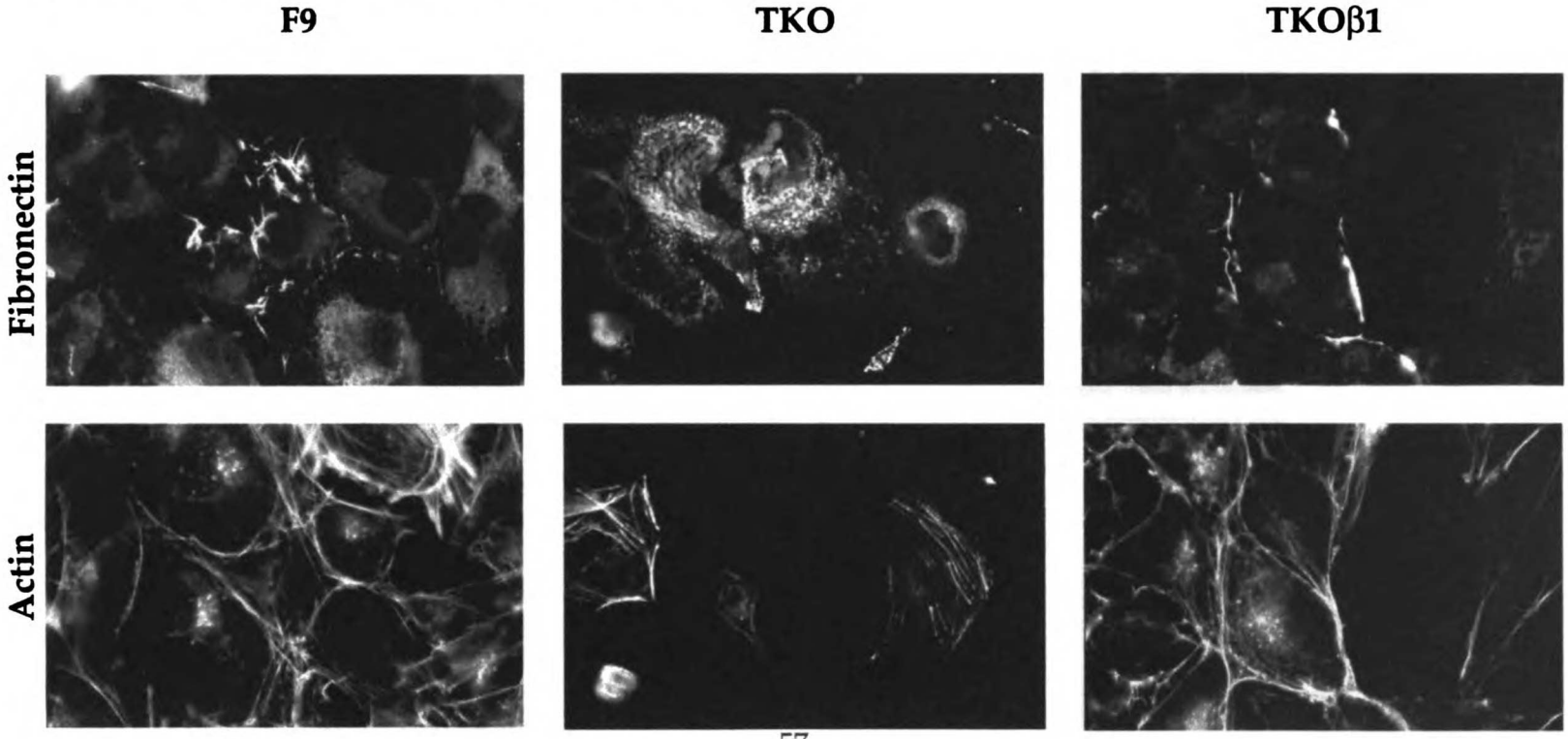


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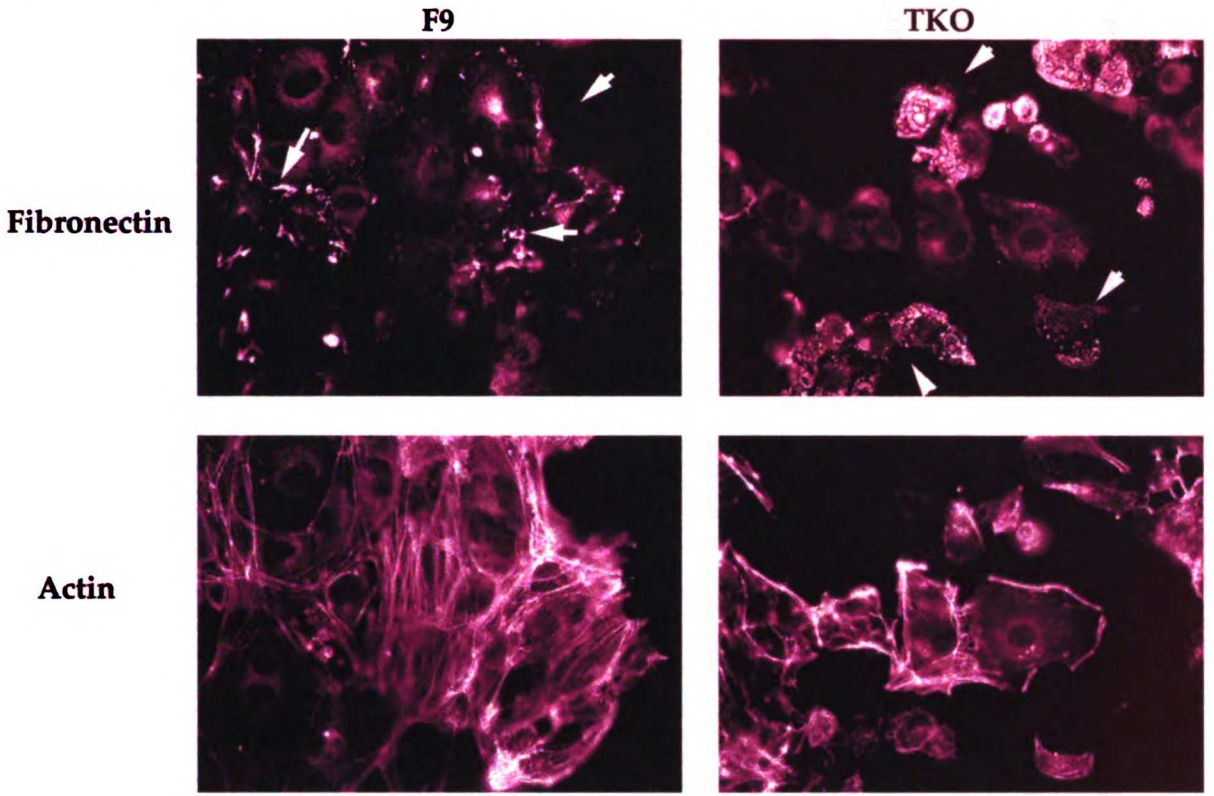
Figure 17: Fibronectin matrix is deposited in areas of high cell density by F9 cells. TKO cells deposit fibronectin in areas of low cell density and areas peripheral to the cells.

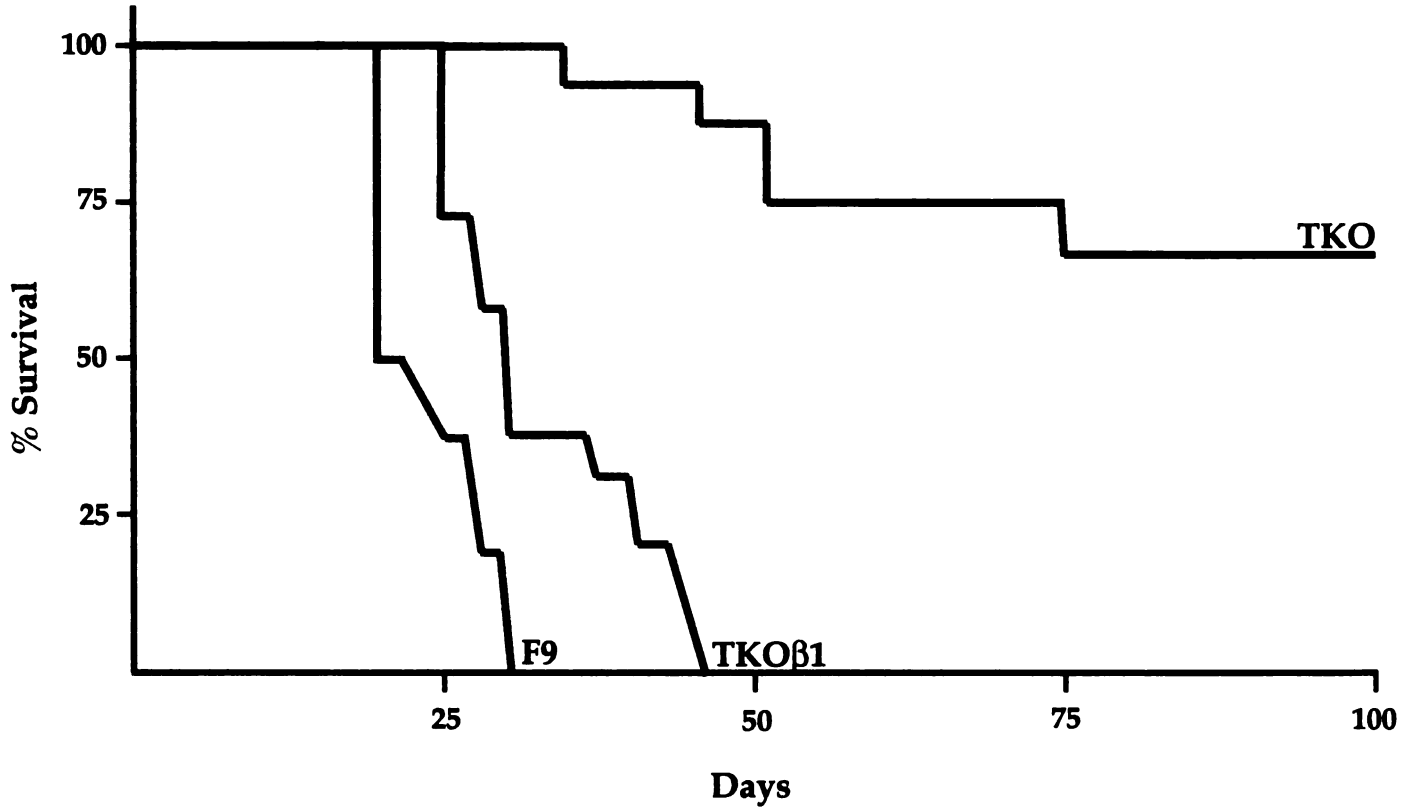


The lack of fibril organization by TKO cells is consistent with the primary role of $\alpha_5\beta_1$ integrin in fibronectin matrix organization (Wennerberg, Lohikangas et al. 1996) and the fact that cells binding to vitronectin exhibit decreased levels of cell surface matrix assembly sites (Pankov, Cukierman et al. 2000). We can assume that the TKO cells are binding to a vitronectin matrix since they show so little binding to fibronectin and don't express any receptors for laminin or collagens. The TKO "matrix" is very different from F9 or TKOβ_1. There are no fibers or dense aggregates surrounding TKO cells and very little matrix staining between layers of TKO cells. Instead, the most prominent fibronectin staining is in mottled pools around and under isolated cells (Figure 16 and 17, see arrows in figure 17). We would have liked to examine the vitronectin matrix and compare the structures produced by the three different cell lines, but were unable to do so because there was no antibody available for immunofluorescent staining of murine vitronectin.

Loss of β_1 eliminates liver invasion after intrasplenic infection of cells.

In experiments done in collaboration with the Ed Roos laboratory at the Netherlands Cancer Institute in Amsterdam, the metastatic capacity of F9, TKO and TKOβ_1 cells was analyzed. The cells were injected into the spleens of nude mice and the mice were assessed for survival and the development of spleen and liver tumors. F9 and TKOβ_1 injected mice began to die at 10 days and mortality was absolute by 21 days (figure 18).

Figure 18: Survival of mice injected intrasplenicly with 1x106 cells. Injection with F9 or TKOβ1 cells correlates with early mortality. Injection with TKO cells does not correlate with early mortality.



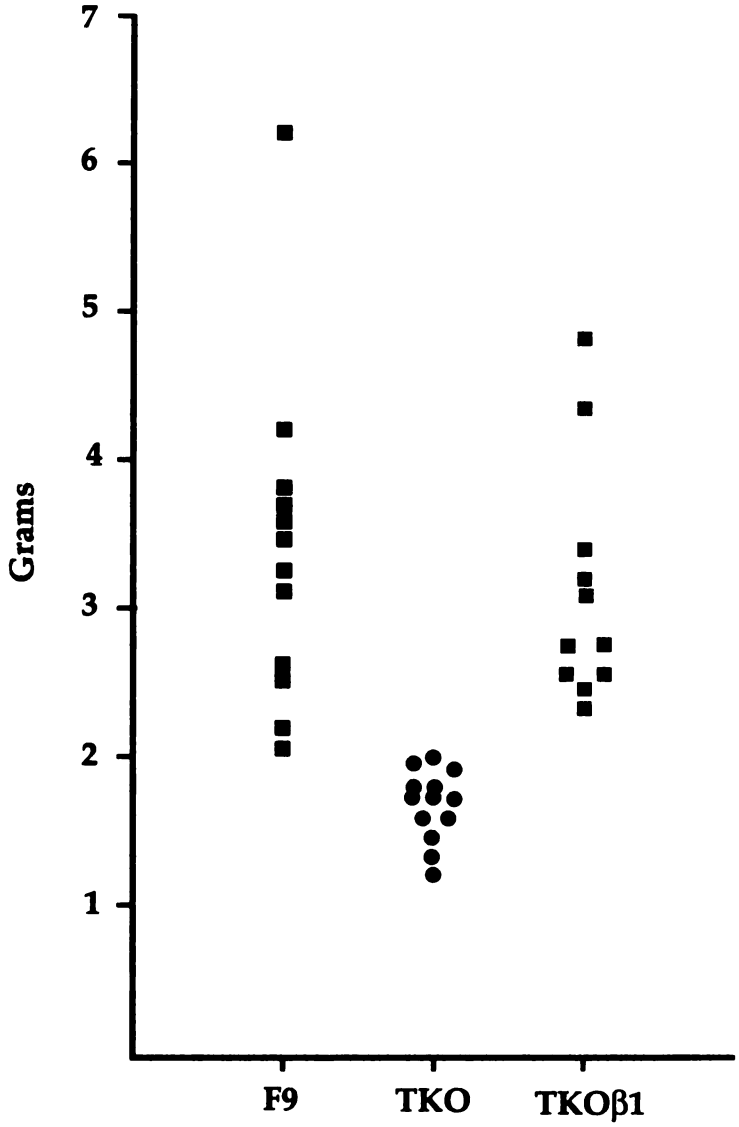


Figure 19:
Weight in grams of livers taken from nude mice 20 days after intrasplenic injection of 1x106 F9, TKO, or TKOβ1 cells. Increase in liver weight indicates the presence of tumor loci and correlates with mortality.

The majority of the TKO injected mice survived to 42 days. All 3 lines formed small tumor foci in the spleens however, only F9 and TKOβ_1 lines invaded the liver. The size and number of the tumor foci can be assessed by the weight of the livers taken from these mice (figure 19). The liver metastases are probably responsible for the mortality. Metastasis to the liver requires cells to invade the hepatic endothelium (Kemperman, Driessens et al. 1995). These data support the hypothesis that β_1 integrin is required for this invasion.

Summary of results in chapter 3.

To summarize, the results presented in this chapter support 5 distinct conclusions. First, parietal endoderm differentiated TKO cells display the same enhanced binding to vitronectin, relative to F9 cells, as undifferentiated TKO cells do. Second, the loss of β_1 has a significant negative impact on the motility of the TKO cells. They are unable to migrate in wound healing assays or when plated at low density, and they are unable to metastasize from the spleen to the liver in vivo. However, third, TKO cells are not completely incapable of migration. In bod outgrowth and transwell filter assays they do migrate in a limited fashion. Fourth, in this cell line β_1 integrins are required for the formation of fibronectin fibrils. And fifth, the TKO phenotype is caused by the loss of integrin β_1.

Chapter 4

Regulation of Adhesion to Vitronectin in F9 and TKO Cells

α_v Family Expression is not Upregulated

TKO, F9 and TKOβ_1 cell lines all express α_v, β_3 and β_5 integrins. None of the lines express β_6 or β_8 (β_6 data not shown, β_8 figure 20). Stephens et al. demonstrated that undifferentiated F9 and TKO cells express α_v, β_3 and β_5 at equal levels (Stephens, Sonne et al. 1993). Quantitative immunoprecipitation of integrins from PE differentiated F9, TKO and TKOβ_1 cell shows that α_v and β_5 are expressed at equal levels in all the cell lines (Figure 21). β_3 expression is 8% higher in TKO cells ($p=0.05$ over 5 experiments). Although this difference between the cell lines is consistent and significant it is not likely to account for the 1.75 to 3-fold difference in the number of cells that bind to vitronectin.

β_1 Family Integrins Play a Limited Role in F9 and TKOβ_1 Binding to Vitronectin.

Both β_1 family and α_v family integrins can bind to vitronectin. In F9 and TKOβ_1 cells, $\alpha_v\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are all potential vitronectin receptors. To assay the potential contribution of these integrins to vitronectin binding, we did cell adhesion assays in the presence of a function-perturbing antibody to mouse β_1.

Figure 20: Immunoblot for integrin β8. Control lysates from C6 and SW480β8 cells show β8 staining. Lysates from PE F9, TKO and TKOβ1 cells do not.

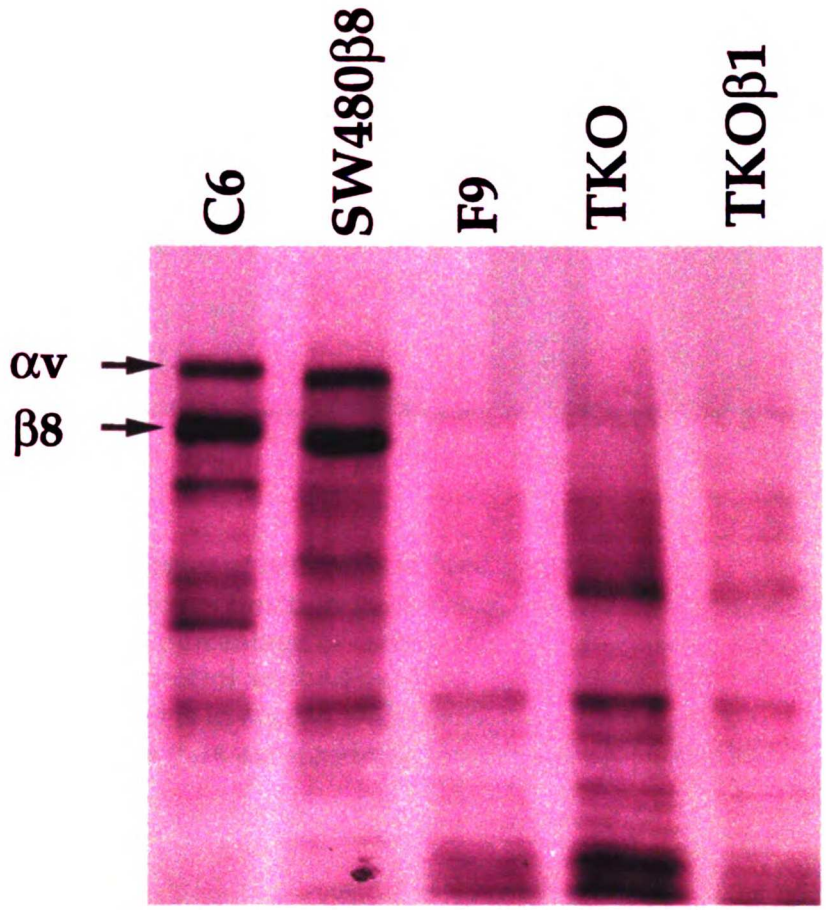
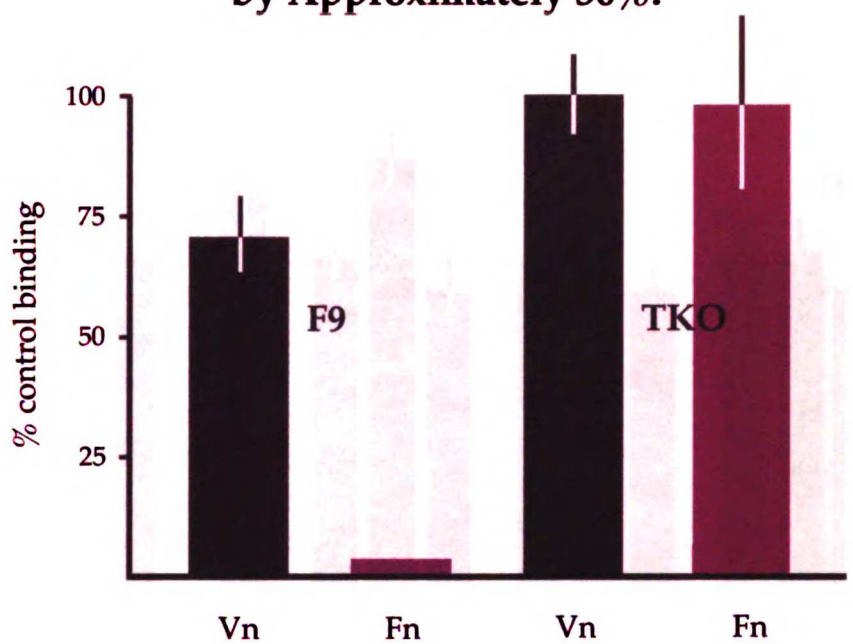


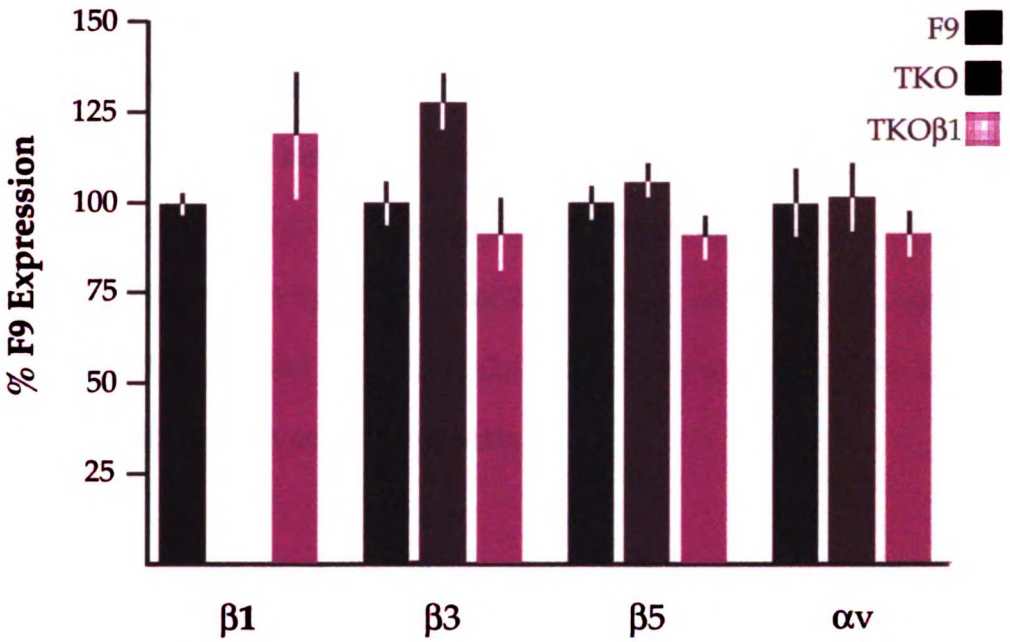
Figure 21

Antibody to β1 Inhibits F9 Binding toVitronectin by Approximately 30%.



Graph of number of cells able to bind to substrate in the presence of function-perturbing antibody to integrin β1, expressed as percentage of cells bound to substrate in the presence of control antibodies. Each bar represents the mean of 4 wells and is in accord with the results of 3 separate assays. Error bars are standard deviation from the mean.

Figure 22:
Expression levels of integrin subunits assayed by quantitative immunoprecipitation. Loss of β1 does not lead to a significant increase in the expression of other integrin subunits.



The antibody, Ha2/11 is a hamster IgM raised against β_1 expressing rat glomerulus cells. It recognizes functional epitopes on β_1 (Mendrick, Kelly et al. 1995).

Cells were plated in the presence of the antibody, allowed to attach and spread for 60 minutes, and the adhesion was challenged by a mechanically controlled washing process (see methods). The results of these assays show that quantities of antibody sufficient to completely block F9 binding to fibronectin, reduce F9 binding to vitronectin by 30% (figure 22). Neither anti-KLH IgG, grown and concentrated in parallel with the Ha2/11 aby nor purified murine IgM affected binding to fibronectin or to vitronectin.

F9 and TKOβ_1 cell adhesion to fibronectin is β_1 mediated and approximately twice as strong as binding to vitronectin (see figure 5). At antibody concentrations sufficient to completely block binding to fibronectin, we saw a 30% inhibition of binding to vitronectin. This result shows that the contribution of all β_1 integrins to F9 and TKOβ_1 binding is, at most, 30% β_1 family and the remaining 70% of the binding can be attributed to either $\alpha_v\beta_3$ or $\alpha_v\beta_5$.

All 3 Lines Use the Same α_v Family Integrins to Bind Vitronectin

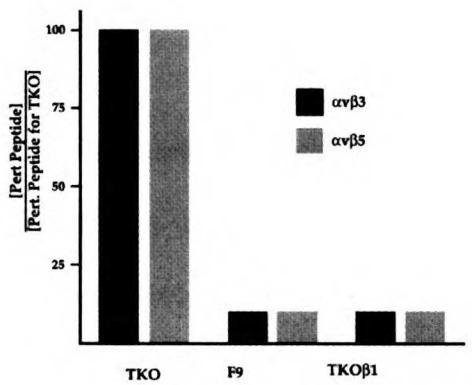
Because TKO, F9 and TKOβ_1 cells appear to be operating primarily with the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors, we wondered if the difference in cell binding was

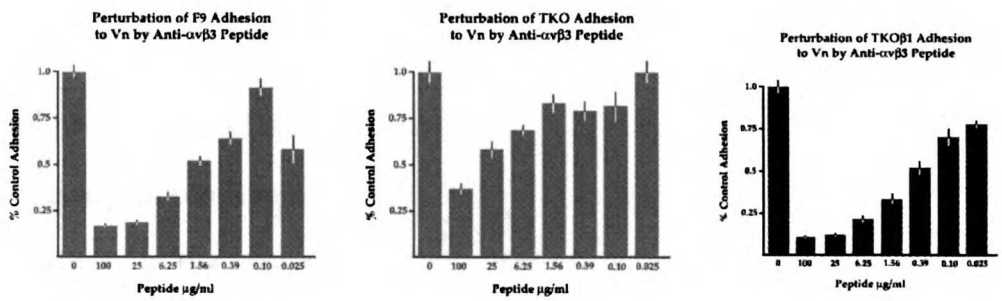
caused by a shift in the integrins used to bind Vn. If β_1 and non-β_1 expressing cells used different α_v family integrins and if these different integrins had different affinities for vitronectin, that might explain the binding differences. Because there are no function-perturbing antibodies to murine $\alpha_v\beta_3$ or $\alpha_v\beta_5$ we used RGD-related peptides provided by Telios Co. They gave us two peptides, one, 32M (RGDD[tBuG]C-NH_2), that blocked $\alpha_v\beta_3$ binding, and one, 5U (GPenFRGDSFCA), that blocked $\alpha_v\beta_5$ binding. The peptides were not entirely specific. In solid-phase assays, using purified ligands, the concentration of 32M sufficient to inhibit Vn binding to $\alpha_v\beta_3$ by 50% (IC_{50}) is 0.6nM. The IC_{50} of 32M for $\alpha_v\beta_5$ and Vn is 14nM, a difference of approximately 20-fold. For 5U, the IC_{50} for Vn and $\alpha_v\beta_3$ is 2.7 mM and for Vn and $\alpha_v\beta_5$ is 46 nM, a difference of approximately 60-fold.

To measure the ability of these reagents to block binding, we again measured the amount of peptide necessary to reduce cell binding to 50% of control. Because the two peptides do not have the same IC_{50}' for their respective favored integrins, we couldn't compare these numbers directly. Instead we compared the ratio of the IC_{50} for TKO and Vn to the IC_{50} for F9 and TKOβ_1 and Vn for each peptide. The ratio represents the difference between the amount of peptide required to perturb adhesion of the β_1 expressing cells and the amount required to perturb adhesion of the β_1 null cells (Figure 23).

In both cases, the IC_{50} for TKO and Vn was higher than the IC_{50} for F9 or TKOβ_1 and Vn.

Figure 23: Perturbation of adhesion to Vn by peptides specific for αvb3 or αvβ5 integrins, assayed by shear stress adhesion assay. The ratio of the concentration of peptide necessary to inhibit binding by the cell line of interest to the concentration of peptide necessary to inhibit binding of TKO cells, reveals that F9 and TKOβ1 cells use the same αv family integrins as TKO cells.





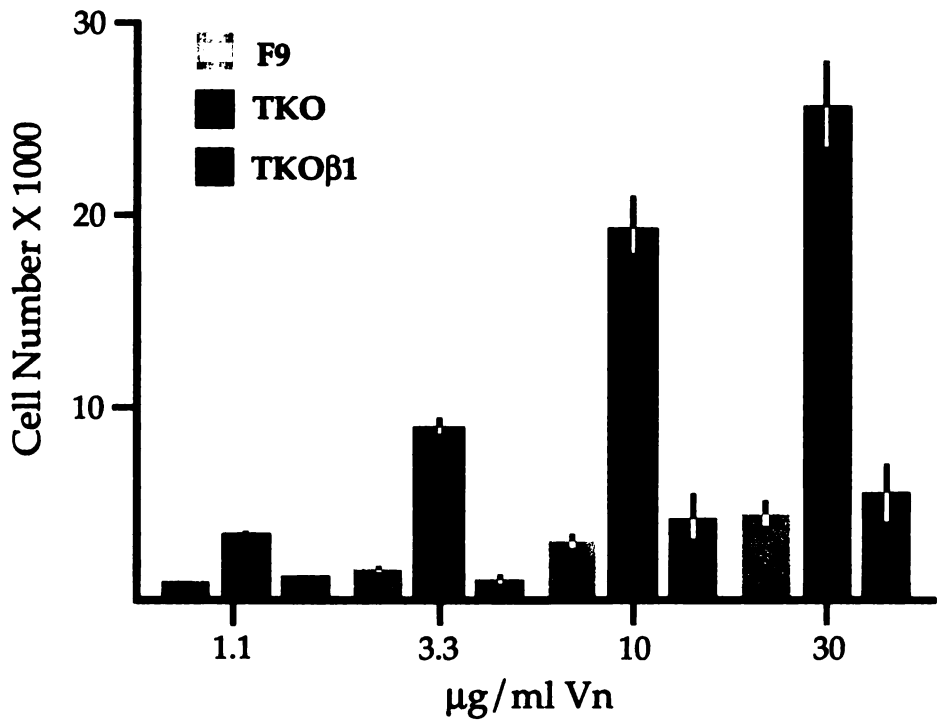
This is to be expected because the overall bond strength between TKO cells and Vn is higher. What is interesting is that the ratio between IC_{50} β_1 and IC_{50} β_1 null is the same for both peptides. If the β_1 and β_1 null cells used different α_V family integrins to bind to Vn this would not be the case. If, for example, F9 and TKOβ_1 used $\alpha_V\beta_3$ to bind Vn, and TKO used $\alpha_V\beta_5$, then the anti-$\alpha_V\beta_5$ peptide would be more effective against F9 and TKOβ_1 than the anti-$\alpha_V\beta_3$ peptide. The $\alpha_V\beta_3$ blocking peptide and the $\alpha_V\beta_5$ blocking peptide were equally effective in blocking F9 and TKOβ_1 binding, supporting the hypothesis that the cells do not favor one of these integrins over the other in binding to vitronectin.

It must be noted that this experiment does not define which of the candidate α_V family integrins the 3 cell lines use, only that they use the same ones. To distinguish between $\alpha_V\beta_3$ and $\alpha_V\beta_5$ will require either agents that act at equal concentrations, or agents that are entirely specific for a given heterodimer.

Adhesion to Vitronectin is enhanced prior to cell spreading

After examining the contributions that upregulation of integrin expression, β_1 family binding, and integrin switching might make to the phenomenon of increased cell binding to vitronectin, we chose to study binding prior to cell spreading. In the preceding assays, adhesion was analyzed as the resistance of spreading cells to shear forces at 37°C. In the following series of assays we examined the resistance of unspread cells to gravitational force at 0-4°C.

Figure 24
Graph of the number of PE differerentiated F9, TKO and TKOβ1 cells bound to 4 different concentrations of Vn. Adhesion is measured at 0°C by the centrifugal force assay. Bars are the mean of 8 wells in 2 separate experiments. Error bars are standard deviation.



By performing the assays at 0-4°C, and by applying the challenge to adhesion immediately after plating, we minimize the contribution of actin cytoskeleton binding, and inhibit signaling events, as discussed in the introduction (McClay, Wessel et al. 1981). This allows a more direct assessment of the affinity state of the integrins. As shown in figure 24, the increased binding of TKO cells to vitronectin is evident in the earliest stages of binding. The number of F9 and TKOβ_1 cells that bind to vitronectin is 4-5 times fewer than the number of TKO cells. These data suggest that the difference in vitronectin binding is due to changes in the affinity of α_v family integrins for vitronectin.

Summary of chapter 4 results.

In summary, I tested the F9, TKO and TKOβ_1 cells for regulation of integrin binding at the level of expression, for integrin switching and for changes in integrin affinity. There was no significant change in the level of expression of α_v, β_3 or β_5. TKO cells do not express novel vitronectin receptors, and there is no apparent change in the identity of the α_v family receptors that the 3 lines use to bind vitronectin. The β_1 family receptors do participate in vitronectin binding, but total blockade of β_1 function reduces F9 vitronectin binding by only 30%. Thus, F9 binding to vitronectin is mediated primarily by α_v family integrin receptors. Finally, the difference in cell binding at low temperature and short incubation time strongly suggests that the vitronectin receptors on TKO cells are in a higher affinity state than those on F9 and TKOβ_1 cells.

Chapter 5

Conclusions, Discussion and Future Directions

We have reintroduced murine integrin β_1 to the TKO β_1 knockout cell line and analyzed the vitronectin binding, motility, and metastatic phenotypes of the PE differentiated F9, TKO and TKOβ_1 cell lines. Six major conclusions can be reached from the results of our work.

1) The increase in vitronectin binding seen in undifferentiated TKO cells is also seen in parietal endoderm differentiated TKO cells.

2) Loss of integrin β_1 in F9 cells leads to a complete loss of the ability to form fibronectin fibrils.

3) Migration of parietal endoderm differentiated TKO cells is significantly reduced, but not eliminated.

4) Metastasis to the liver, of cells injected into the spleen, and the mortality associated with this metastasis are dependent on expression of integrin β_1 in these cell lines.

5) The TKO adhesion and motility phenotypes are due solely to the loss of integrin β_1.

6) The increase in vitronectin adhesion seen in TKO cells correlates with an increase in initial affinity and suggests that transdominant inhibition of α_v family integrins by the β_1 integrin subunit occurs in F9 and TKOβ_1 cells.

The increase in vitronectin binding seen in undifferentiated TKO cells is also seen in parietal endoderm differentiated TKO cells.

Our discovery, that enhanced vitronectin binding prevails in parietal endoderm differentiated TKO cells as well as undifferentiated TKO cells, was important for a number of reasons. Initial studies of the TKO cells and other β_1 null cell lines supported the idea that there is a distinct division between the processes of morphogenesis and gene expression in differentiation. Somehow there is a boundary between the signals contributed by integrins and "hard-wired" differentiation pathways. This boundary permits the expression of differentiation markers in the absence of morphogenic change and input. The fact that modulation of Vn binding by integrin β_1 occurs in both differentiation states suggests that the apparent affinity modulation of α_v family integrins by β_1 is independent of the differentiation cues in these cells.

Our finding that enhanced Vn binding also occurs in PE differentiated TKO cells also permitted the concomitant study of motility and cell adhesion. Although motility is associated exclusively with the PE differentiated cells, it is possible

that the phenomena of enhanced adhesion to Vn and loss of motility are mechanistically related.

Loss of integrin β_1 in F9 cells leads to a complete loss of the ability to form fibronectin fibrils.

Location and organization are just as important in the extracellular matrix as they are within the cell. The extracellular matrix contains information not only in the elements it is composed of, but in the way those elements are arranged relative to one another. A relatively well studied example of this principle is the polymerization of fibronectin into fibrils. When soluble fibronectin is added to cells it first binds to the cell surface in a deoxycholate-soluble and reversibly bound pool. Over time the fibronectin is changed, by the cell, into a deoxycholate insoluble, polymeric fibrillar matrix (McKeown-Longo and Mosher 1984; Chen and Mosher 1996). Endogenous fibronectin can be polymerized in the same way (Lyubimov and Vasiliev 1982). Interaction with the fibrillar form of fibronectin can cause changes in cell cycle progression (Bourdoulous, Orend et al. 1998; Sechler and Schwarzbauer 1998), cell migration (Morla, Zhang et al. 1994), and actin organization (Sechler and Schwarzbauer 1997), that are different from those caused by the binding of cells to unpolymerized fibronectin.

Both $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins have been shown to support the organization of fibronectin into fibrils (Wu, Bauer et al. 1993; Yang, Rayburn et al. 1993). The loss of fibronectin fibril organization after deletion of β_1 from F9 cells indicates that

these cells do not normally use $\alpha_v\beta_3$ to organize Fn, and moreover that they do not adapt to the loss of β_1 by beginning to use $\alpha_v\beta_3$ when β_1 is lost. These results differ from those seen in the GD25 β_1 null cell line and may be traced to the ability to bind to Fn with α_v family receptors.

As can be seen in figure 5, TKO cells do not bind well to Fn. TKO cells express both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors, both of which can recognize Fn. However, depending on the assay used, TKO binding to Fn is either non-existent or very slightly above background. This suggests that, in TKO cells, neither $\alpha_v\beta_3$ nor $\alpha_v\beta_5$ can function as Fn receptors. GD25 cells are able to use α_v family receptors to bind to Fn. GD25 cells bind to Fn in numbers equal to GD25 cells that have been re-transfected with β_1 (GD25β_1 cells) (Wennerberg, Lohikangas et al. 1996). GD25 take longer than GD25β_1 to organize Fn into fibrils, but they are able to do it.

It is also possible that TKO cells may express higher levels of Fn than F9 or TKOβ_1 cells. The large unorganized pools of Fn that are seen when TKO cells are cultured at low density (figure 17) suggest that TKO cells may actually secrete more Fn than F9 or TKOβ_1 cells. GD25 cells do not upregulate the expression of Fn (Wennerberg, Lohikangas et al. 1996). This question about the TKO phenotype merits further work. Western blotting of cells and substrate dissolved in 1% SDS sample buffer would establish the absolute level of expression. To analyze the degree of Fn organization biochemically, comparison of the Fn content of supernatants and deoxycholate lysates from all 3 lines at daily

timepoints (timepoints chosen based on the apparent rate of Fn polymerization by F9 cells) is an appropriate experiment.

Migration of parietal endoderm differentiated TKO cells is significantly reduced, but not eliminated.

The motility of TKO cells also deserves further study. My results show that, while the TKO cells are certainly less motile than F9 or TKOβ_1 cells, they are able to move. It appears that TKO cells have a more restricted set of conditions for motility. I detected their motility only in the transwell filter and bod outgrowth assays, not in assays for wound healing or random migration at low cell density.

As can be seen in figure 9, the F9 and TKO cells are morphologically similar. They both have the filopodia and somewhat rounded cell bodies typical of PE cells. Because our video microscope required that we keep the cells on the microscope stage for the duration of each study, we tried the wound healing assay to see if the TKO cells might move in the more controlled environment of the incubator. The wound healing assay corroborated the results of the video studies. F9 cells fill a wound within 24 hours (data not shown); TKO cells had not migrated into the wound by 48 hours (figure 10).

These results are somewhat contradictory, yet the assays that gave positive results for motility differ from the assays that showed no motility. Transwell filter migration and bod outgrowth assays feature migration from a dense

multilayered assembly of cells into an area of low cell density. We chose to videotape the cells at low density to easily track individual cells. The monolayer wound healing assay is intermediate between the high density conditions of the transwell and bod assays and the low density of the video experiments. Rather than migrating from a multilayered group of cells, cells in the wound healing assay were given the chance to migrate out of a confluent monolayer of cells.

Most of the studies of cell migration and motility published use relatively rapidly moving cells. The TKO cells fall on the very slow end of the published spectrum. None the less, as figure 15 demonstrates, moving slowly is different from not moving. The slower, more restricted motility, seen in TKO cells, may actually have physiological relevance. In 1996, Beate Lanske et al. published the first study of the type I PTH/PTHrP receptor knock out mouse (Lanske, Karaplis et al. 1996). The most shocking discovery in these mice was that the parietal endoderm formed normally. The cells at the periphery of the visceral endoderm layer changed, began to express PE markers and migrated out along the inside of the blastocoel. Signaling through the PTH/PTHrP receptor had been thought to be essential for the formation of PE in vivo.

This sort of result is not uncommon in transgenic mice. Often mice null for proteins that appear to be essential for distinct developmental processes, based on antibody or anti-sense blockade of their function in vitro, make it though those processes in vivo just fine. The reasons for this are many and complex. However, when cell migration and shape change are involved it is possible that

the speedy movements studied in vitro aren't entirely necessary. Slow migration and movement may be sufficient.

The PTH/PTHrP receptor null mutation is an embryonic lethal, but the embryos die at day 12.5; after the PE has formed. The small size of the 8.5 and 9.5 day embryos suggests that the PE cells may not be migrating or interacting with the cell layers adjacent to them in an normal manner. This degree of development can be contrasted with that of the β_1 null embryos (Stephens, Sutherland et al. 1995). In β_1 null embryos the visceral endoderm doesn't develop normally and there is no real development of parietal endoderm. These embryos fail at approximately 4.5 days.

Later studies on ES cells from PTH/PTHrPR null mice and normal mice (Verheijen and Defize 1995) show that the receptor is absolutely required for complete PTHrP induced PE differentiation in vitro. Unlike secreted factors, receptors absent in vivo cannot be compensated for by maternal expression. However, retinoic acid alone was able to induce low level expression of the PE marker, thrombomodulin, in both cell PTHrPR-null and wild type lines. It is possible that this low level of differentiation is sufficient for the initial formation of the PE, but not enough to support maturation. It could be very instructive to compare the migration and matrix organizing abilities of these cells to TKO cells.

Metastasis to the liver, of cells injected into the spleen, and the mortality associated with this metastasis are dependent on expression of integrin β_1 in these cell lines.

Intrasplenic injection of carcinoma cells is a test for the ability to metastasize to the liver. The portal circulatory system collects venous blood from the gastrointestinal tract and routes it through the liver. The splenic vein joins with the superior mesenteric veins to form the portal vein and the portal vein drains directly into the liver. Metastasis to the liver is unlike many other metastases because the capillaries in the liver are fenestrated. The secretion of proteases is less important in hepatic metastasis than it is in tissues with contiguous capillary walls. There is no basal lamina between the endothelium and the hepatocytes. Integrin receptors for fibronectin have been demonstrated to be critical for hepatic metastasis [Kemperman, 1995 #195]. In the F9/TKO/TKOβ_1 system, the absence of receptors recognizing fibronectin is probably the reason why TKO cells do not metastasize to the liver.

The TKO adhesion and motility phenotypes are due solely to the loss of integrin β_1.

The finding that murine integrin β_{1A} restores the parental (F9) phenotype to TKO cells, was a control necessary for the interpretation of all the phenotypes observed in the F9/TKO system. The loss of enhanced vitronectin binding and the gain of binding to β_1-dependent ligands and motility in TKOβ_1 mean that the

TKO phenotype is not caused by genetic damage incidental to the gene targeting and selection processes. However, the process of making a stable β_1 positive line from the TKO cells also revealed that the TKO line is not hospitable to the introduction of new genes. Maximal transfection and electroporation rates were under 15%. These difficulties were made worse by the fact that under selection conditions TKO cells display a remarkable ability to edit introduced DNA, retaining antibiotic resistance genes and rejecting the gene of interest. Other researchers also had difficulty with both transient transfection and the formation of stable lines (personal communication with the Laura Grabel, Randall Kramer, and Rudolf Grosshedl labs). Adding an internal ribosomal entry site sequence to the construct did not improve matters.

The frequency with which the TKO cells excise the gene of interest made experiments assessing the effects of mutations in the β_1 cytoplasmic tail much more difficult. To find the TKOβ_1 cell line we screened 260 puromycin resistant clones, by immunofluorescence, to find one line that expressed β_1. At about the time I would have done the studies of β_1 cytoplasmic tail mutations, similar work was published using another β_1-null cell line, GD25 (Sakai, Zhang et al. 1998; Wennerberg, Fässler et al. 1998). These papers revealed that threonines 788 and 789 are essential for activation of β_1 and that tyrosines 783 and 795, in the NPXY motifs near the end of the cytoplasmic tail, are necessary for optimal migration and appear to regulate the assembly of actin stress fibers. The GD25 studies do not address the increase in binding to vitronectin that we observed since GD25 cells do not exhibit enhanced binding to α_v family ligands. Newer gene

introduction techniques like viral infection and Trojan peptides offer new hope for these experiments in F9 cells. (Trojan peptide are a new class of peptide reagent that, when mixed with cDNAs, are actively taken up through membrane transport pores (Ali, Joao et al. 1999).) It will be interesting, given 2 cell lines with such distinct differences in the use of similar integrins, to see if the specific tyrosines and threonines in the cytoplasmic tail of β_1 play the same roles in the F9-TKO system as they do in the GD25 system.

The increase in vitronectin adhesion seen in TKO cells correlates with an increase in initial affinity and suggests that transdominant inhibition of α_V family integrins by the β_1 integrin subunit occurs in F9 and TKOβ_1 cells.

Although TKO is the only cell line for which published data exists demonstrating enhanced binding to a ligand after deletion of β_1, there are many published accounts of interactions between different integrins expressed by the same cell. The phenomenon is referred to as "integrin crosstalk" (Blystone, Lindberg et al. 1995; Blystone, Williams et al. 1997; Porter and Hogg 1997; Blystone, Slater et al. 1999) or "transdominant inhibition" (Díaz-González, Forsyth et al. 1996). Transdominant inhibition is perhaps the most descriptive name because all the interactions thus far described are characterized by inhibition of the function of one integrin by another. Specificly, the current literature identifies sets of two integrins, one of which (the transducer), when bound to ligand, produces a signal that inhibits the activity of the other (the target integrin). Where it has

been assayed (Díaz-González, Forsyth et al. 1996; Blystone, Slater et al. 1999), the apparent mechanism by which the function of the target integrin changes is affinity modulation.

My work suggests that interaction between β_1 family integrins and α_V family integrins in F9 and TKOβ_1 cells operates in the same manner. PE TKO cells do not regulate their integrin binding at the level of expression. They do not express significantly higher levels of α_V, β_3 or β_5 subunits (figure 21). Nor do they express other known vitronectin receptors (figure 20). The binding perturbation assays, using peptides specific for $\alpha_V\beta_3$ or $\alpha_V\beta_5$ (figure 23) suggest that TKO cells use the same α_V family integrins to bind to vitronectin as F9 and TKOβ_1 cells do. This argues against integrin switching.

The ligation of a β_1 family integrin during vitronectin binding is demonstrated in the anti-β_1 antibody perturbation experiments by a 30% decrease in vitronectin binding by β_1 expressing cells (figure 21). It is possible to argue that the decrease in total vitronectin binding is due to the participation of a population of receptors with a lower affinity for vitronectin, i.e. the β_1 family receptors. However, the results of the binding assays done at low temperature (figure 24) argue against this.

At low temperature and early timepoints, as these assays were performed, the bond between the cell and the substrate is primarily the bond between the integrin extracellular domain and the substrate. The formation of connections

with the cytoskeleton and cytoskeletal remodeling are retarded at 0° C. On 30ug/ml vitronectin, at 0°C 26,604 TKO cells, 4794 F9 cells and 6063 TKOβ_1 cells bound. The difference is between 4 and 5 fold. Without significant participation by the cytoskeleton it is difficult to imagine how 30% of the binding integrins could cause a 4-5 fold decrease in adhesion (figure 24).

The β_1 blocking experiments reveal another important point. Binding of the anti-β_1 antibody doesn't enhance binding F9 or TKOβ_1 adhesion to vitronectin. Thus, blocking β_1 interaction with ligand is not a pheno-copy of the targeted deletion of β_1. This also supports our hypothesis that the decrease in vitronectin binding observed in cells with β_1 is not due to a simple competition between vitronectin receptors of different affinity. We do not know whether Ha2/11 is a ligand mimetic antibody or merely blocks ligand binding through steric hindrance (Mendrick, Kelly et al. 1995), so we can't say for sure that antibody binding has the same intracellular sequellae as vitronectin binding.

The signaling mechanisms by which trans-dominant inhibition by integrins works are just beginning to be understood. Blystone et al. published the first such report in 1999 . In human macrophages, ligation of the $\alpha_v\beta_3$ receptor inhibits $\alpha_5\beta_1$ mediated phagocytosis and migration. Ligation of $\alpha_5\beta_1$ activates the calcium- and calmodulin-dependent protein kinase II (CamKII). This activation is required for phagocytosis and migration. Simultaneous ligation of $\alpha_v\beta_3$ or expression of the β_3 cytoplasmic tail prevents the activation of CamKII. As discussed next, in the F9/TKO/TKOβ_1 system, molecules that are especially

worth studying include Src, ILK, ICAP, CD98, β_3-endonexin and PAK20. These molecules thought to associate exclusively with the cytoplasmic or transmembrane domains of β_1, β_3, and β_5 integrin subunits.

Studies of Src- fibroblasts (Felsenfeld, Schwartzberg et al. 1999) show that loss of Src causes strengthening of α_V family integrin mediated adhesion to vitronectin, and decreased spreading. Their studies, using a visual assessment of adhesion and a laser trap and microspheres coated with vitronectin to measure activation state and strengthening, showed equal levels of initial binding and activation, and greater levels of strengthening. These results are different from mine that show higher levels of initial binding, followed by consistently higher levels of adhesion. However the decreased spreading seen in the Src- cells is also seen in TKO cells (Stephens, Sonne et al. 1993). Src sequestration by β_1 is thus a possible mechanism for the inhibition of α_V function seen in TKO cells.

The kinases known to interact with integrins are logical suspects in studying the mechanism of transdominant inhibition of α_V family integrins. ILK and the α catalytic subunit of phosphatase 2A have been shown to immunoprecipitate with $\alpha_6\beta_1$ in F9 cells (Mulrooney, Foley et al. 2000). However, $\alpha_6\beta_1$ is primarily a laminin receptor and these studies were done on vitronectin and fibronectin. While PE F9 cells do secrete substantial quantities of laminin, the adhesion experiments in this study were performed in time spans too short for self-secreted ligands to be a significant factor. Generally, the proteins believed to interact exclusively with α_V family or β_1 family integrins are of particular interest

because the absence of their preferred ligand would entirely disrupt their activity. CD98, a tetraspan protein with roles in ion transport, is especially intriguing because it was discovered in a screen for factors that overcame transdominant inhibition by the β_1 tail (Fenczik, Sethi et al. 1997).

With a single exception (Simon, Nutt et al. 1997), the presence of the cytoplasmic tail of the transducing integrin is necessary and sufficient to support transdominant inhibition between integrins. In the future, expressing the β_{1A} cytoplasmic tail, without the extracellular domain, in TKO cells will allow us to definitively assess whether the increase in vitronectin binding seen in TKO cells is due to competition for vitronectin ligand between α_V and β_1 family vitronectin receptors of varying affinity for ligand and actin or whether it is due to inhibition of α_V family affinity by β_1.

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