BMC Cancer

Research article

Recruitment of focal adhesion kinase and paxillin to β1 integrin promotes cancer cell migration via mitogen activated protein kinase activation

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Abstract

Background: Integrin-extracellular matrix interactions activate signaling cascades such as mitogen activated protein kinases (MAPK). Integrin binding to extracellular matrix increases tyrosine phosphorylation of focal adhesion kinase (FAK). Inhibition of FAK activity by expression of its carboxyl terminus decreases cell motility, and cells from FAK deficient mice also show reduced migration. Paxillin is a focal adhesion protein which is also phosphorylated on tyrosine. FAK recruitment of paxillin to the cell membrane correlates with Shc phosphorylation and activation of MAPK. Decreased FAK expression inhibits papilloma formation in a mouse skin carcinogenesis model. We previously demonstrated that MAPK activation was required for growth factor induced in vitro migration and invasion by human squamous cell carcinoma (SCC) lines.

Methods: Adapter protein recruitment to integrin subunits was examined by co-immunoprecipitation in SCC cells attached to type IV collagen or plastic. Stable clones overexpressing FAK or paxillin were created using the lipofection technique. Modified Boyden chambers were used for invasion assays.

Results: In the present study, we showed that FAK and paxillin but not Shc are recruited to the β1 integrin cytoplasmic domain following attachment of SCC cells to type IV collagen. Overexpression of either FAK or paxillin stimulated cancer cell migration on type IV collagen and invasion through reconstituted basement membrane which was dependent on MAPK activity.

Conclusions: We concluded that recruitment of focal adhesion kinase and paxillin to β1 integrin promoted cancer cell migration via the mitogen activated protein kinase pathway.

Background

Integrin extracellular domains bind extracellular matrix molecules such as type IV collagen and laminin while the cytoplasmic domains bind specific components of the actin cytoskeleton [1]. Integrin-extracellular matrix interactions also activate signaling cascades such as mitogen activated protein kinases [2]. Integrin binding to extracel-lular matrix or integrin crosslinking increases tyrosine phosphorylation of focal adhesion kinase (FAK) [3]. FAK consists of a central catalytic domain and amino and car-boxyl terminal non- catalytic domains. A focal adhesion targeting sequence within the carboxyl terminus is required for localization to focal adhesions [4]. FAK undergoes autophosphorylation on a single tyrosine

residue which creates a binding site for SH2 containing proteins. Inhibition of FAK activity by expression of its carboxyl terminus decreases cell motility, and cells from FAK deficient mice also show reduced migration [5,6].

Paxillin is a focal adhesion protein which is also phosphorylated by a number of stimuli on tyrosine [2]. Paxillin interacts with FAK at two sites in the amino and carboxyl termini which are not required for targeting paxillin to focal adhesions [7]. A mutant paxillin protein lacking both FAK binding sites exhibits reduced tyrosine phosphorylation [8]. FAK recruitment of paxillin to the cell membrane correlates with Shc phosphorylation and activation of the MAPKs ERK and JNK [9]. However JNK phosphorylation is not dependent on FAK nor on phosphatidylinositol 3-kinase (PI3K) activity.

Transformation of FAK deficient fibroblasts with the v-Src oncogene promotes cellular motility equal to that of FAK re-expression [10]. However, these cells are not invasive and required intact FAK expression to produce this phenotype. Decreased FAK expression inhibits papilloma formation in a mouse skin carcinogenesis model [11]. These studies indicate that FAK has a key role in tumorigenesis and invasion. We previously demonstrated that PI3K, ERK, and JNK activation were required for growth factor induced in vitro migration and invasion by human squamous cell carcinoma (SCC) lines [12,13]. These studies led us to hypothesize that FAK and paxillin may mediate signals from the substratum via ERK in order to regulate cell migration. In the present study, we show that FAK and paxillin but not Shc are recruited to the β1 integrin cytoplasmic domain following attachment of SCC cells to type IV collagen. This recruitment correlated with increased cellular migration on this substratum. Overexpression of either FAK or paxillin stimulated cancer cell migration on type IV collagen and migration through reconstituted basement membrane which was dependent on ERK activity. We concluded that recruitment of focal adhesion kinase and paxillin to β1 integrin promoted cancer cell migration and invasion via the mitogen activated protein kinase pathway.

Methods
Cell culture and stable transfection
SCC4 and SCC25 cells were cultured in Dulbecco's modified Eagle medium (DMEM), 10% fetal bovine serum, 40 μg/ml gentamicin at 37°C in a humidified atmosphere of 5% CO_2. Cells were plated on tissue culture plastic dishes or commercially available plates coated with type IV collagen (Becton Dickinson). Some cells were pretreated with blocking antibodies to β1 or β4 integrins (Invitrogen) for 1 hour prior to plating [14,15]. Other cultures were deprived of attachment by plating on uncoated petri dishes in media containing 1.6% methylcellulose. Cells

were transfected with 5 μg expression vector for FAK or paxillin (kindly provided by Dr. Thomas Parsons) or neomycin resistance plasmid alone using Lipofectamine reagent according to manufacturer's recommendations (Invitrogen). Cells were selected in 400 μg/ml G418 for 14 days. Resistant clones were picked for expansion and characterization. FAK and paxillin expression was determined by western blot.
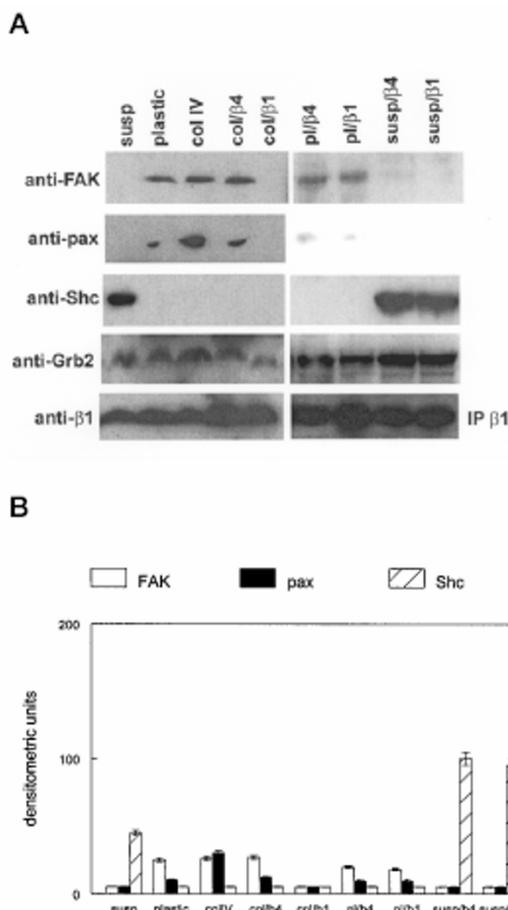
Immunoprecipitation
Cultures were lysed in 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 1% Nonidet P-40, 10% glycerol, 1 mM NaF, 0.1 mM sodium orthovanadate, and protease inhibitors for 30 minutes at 4°C. Lysates were centrifuged at 10,000 × g for 10 minutes and anti-β1 integrin antibody (Invitrogen) was incubated with the supernatants for 1 hour at 4°C. Antigen-antibody complexes were precipitated with protein A/G agarose beads for 1 hour at 4°C. Samples were boiled in Laemmli buffer for 3 minutes, separated by SDS-PAGE, and blotted to polyvinylidene difluoride membranes. Blots were incubated with anti-FAK, paxillin (Transduction Laboratories), Shc, and Grb2 (Santa Cruz Biotechnology) antibodies to determine relative association with β1 integrin. Blots were then incubated with anti-β1 integrin antibody to ensure equal amounts of immunoprecipitated protein in each lane. Bands were analyzed by laser densitometry and the numerical data subjected to t test.

Western blot
75 μg total cellular protein was separated by SDS-PAGE on 10% resolving gels under denaturing and reducing conditions. Separated proteins were electroblotted to PVDF membranes according to manufacturer's recommendations (Roche Molecular Biochemicals). Blots were incubated with antibodies to human FAK, paxillin, ERK1, activated ERK1 (Transduction Laboratories), or β1 integrin (Invitrogen) for 16 hours at 4°C. After washing in Tris buffered saline containing 0.1% Tween 20 (TBST, pH 7.4), blots were incubated for 30 minutes at room temperature with anti-IgG secondary antibody conjugated to horseradish peroxidase. Following extensive washing in TBST, bands were visualized by the enhanced chemiluminescence method (Roche Molecular Biochemicals). Bands were analyzed by laser densitometry and the numerical data subjected to t test.

Migration and invasion assays
Cells were plated at confluence on plastic in the presence or absence of type IV collagen for 24–48 hours. Some cells were pretreated with blocking antibodies to β1 or β4 integrins for 1 hour prior to plating. Other cultures were treated with 50 μM PD98059, a selective inhibitor of ERK activation. The dishes were scored with a pipet tip across the center of the plate. The number of cells which
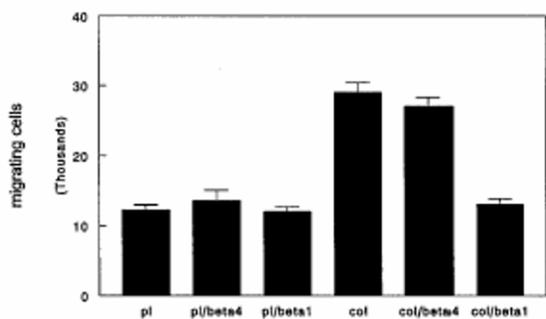
A

B

Figure 1
FAK and paxillin immunoprecipitate with β1 integrin follow-ing cell attachment to type IV collagen. SCC25 cells were plated in semisolid media (susp), on tissue culture plastic (pl), or on type IV collagen (col) for 2 hours. Some cultures were preincubated with antibodies which block attachment by β1 or β4 integrin subunits. β1 integrin was immunoprecipitated (IP β1) from cell lysates and western blotted using the anti-bodies indicated at left. These experiments were performed three times with similar results. Representative blots are shown. (B) Densitometric quantitation of blots in Fig. 1A.

migrated into the blank area of the dish during the speci-fied time period was determined by counting of 10 ran-dom fields using phase contrast microscopy. For invasion assays, 2×10^5 cells were plated in triplicate into commer-cially available Matrigel invasion chambers (Becton Dick-inson). Cells which migrated through the reconstituted basement membrane after 24 hours were fixed in metha-nol, stained with hematoxylin, and counted.

Results

Integrin binding to extracellular matrix increases associa-tion of the intracellular domain with FAK and paxillin [3,7]. To determine if FAK and paxillin could interact with the β1 integrin subunit in human SCC lines, we immuno-precipitated this receptor from SCC4 and SCC25 cells cul-tured in suspension, plated on tissue culture plastic, or attached to type IV collagen. As shown in Fig. 1A and 1B, 4 fold more FAK protein associated with β1 integrin sub-unit when cells were plated on both plastic and type IV collagen compared to anchorage deprived cells (p < 0.00001). Preincubation with a blocking antibody to β1 integrin but not β4 integrin inhibited the association of FAK with β1 integrin when cells were plated on type IV collagen. Paxillin was also recruited to the β1 integrin complex when cells were plated on plastic (2 fold, p < 0.03) or type IV collagen (5 fold, p < 0.00001) but not when the cells were anchorage deprived. Incubation with blocking antibody to β1 integrin also inhibited the associ-ation of paxillin with the integrin subunit in cells plated on type IV collagen (5 fold, p < 0.03). Blocking antibodies failed to inhibit FAK or paxillin recruitment when cells were plated on plastic, indicating that the antibody effec-tively blocked attachment to type IV collagen. However the blocking antibodies did not inhibit cell attachment since the cells could also adhere to the tissue culture plas-tic dishes via charge interactions. In contrast, the 52 kd Shc isoform was detected in the β1 integrin complex only when cells were anchorage deprived. Shc association was not detected in plated cells regardless of substratum or antibody preincubation (p < 0.05). Grb2 associated with β1 integrin under all conditions, indicating that the observed changes in FAK, paxillin, and Shc binding were specific. Similar results were obtained using the SCC4 cell line. These results indicated that FAK and paxillin were recruited to β1 integrin when cells were attached to sub-stratum while Shc was associated with the receptor in anchorage deprived cells.

FAK deficient cells are less migratory than their normal counterparts [6], and our data indicated that attachment to type IV collagen increased paxillin association with β1 integrin when compared to plastic. To determine if FAK and paxillin association with β1 integrin correlated with increased migration, we plated SCC4 and SCC25 cells on plastic or type IV collagen with or without preincubation with integrin blocking antibodies. As shown in Fig. 2, attachment to type IV collagen produced a greater than 2 fold increase in the number of cells which migrated into a blank area of the culture dish. This migration was mark-edly inhibited by preincubation with blocking antibody to β1 integrin but not to β4 integrin. Integrin blocking antibodies had no effect on cellular migration on plastic, indicating that the antibodies effectively blocked attach-ment to extracellular matrix. Similar results were obtained

Figure 2
Type IV collagen stimulates β1 integrin dependent migration of SCC25 cells. SCC25 cells were plated on plastic (pl) tissue culture dishes or type IV collagen (col) coated plates. Some cultures were preincubated with antibodies which block attachment by β1 or β4 integrin subunits. The number of cells which migrated into the blank area of the plate within 24 hours was counted. These experiments were performed three times with similar results. Error bars indicate SEM.

with SCC4 cells. These results indicated that FAK and paxillin recruitment to β1 integrin correlated with increased migration on type IV collagen.

FAK overexpression has been associated with increased tumor invasion and lymph node metastasis in clinical cases of human cancer [16]. To determine if FAK and paxillin overexpression produced increased in vitro migration of cancer cell lines, we stably transfected each expression vector into SCC4 and SCC25 cells. FAK and paxillin proteins were up to 20 fold overexpressed in these stable clones (Fig. 3A,3B; p < 0.05). Both FAK and paxillin overexpression effectively induced migration in SCC25 cells (Fig. 3C). FAK overexpression induced migration by 6 fold while paxillin overexpression increased motility by 3 fold when cells were plated on plastic dishes. Migration was further enhanced when cells were plated on type IV collagen. Migration was induced 7 fold when FAK overexpressing cells were plated on type IV collagen compared to controls. Paxillin overexpressing cells were also more migratory on type IV collagen than on plastic; however there was no additive effect of type IV collagen attachment in these clones. Blocking antibody to β1 integrin inhibited migration of FAK and paxillin overexpressing clones on type IV collagen but not on plastic. Blocking antibody to β4 integrin had no effect on cell migration on either substratum. We were unable to obtain stable clones expressing the 52 kD Shc isoform due to terminal differentiation of these clones (see Discussion). Similar results were obtained with SCC4 cells. These results indicate that FAK and paxillin overexpression promote migration of human
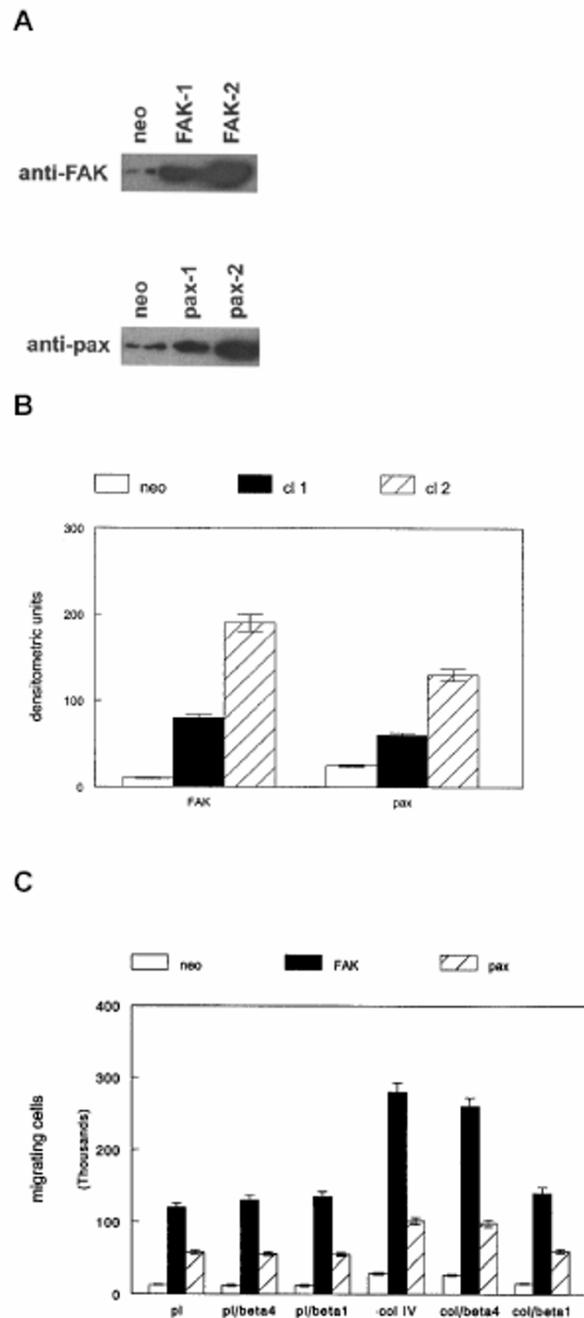
SCC lines which is enhanced by β1 integrin attachment to a relevant substratum.

FAK recruitment of paxillin to the cell membrane results in activation of the MAPK pathway [9]. To determine if the MAPK pathway contributed to FAK and paxillin induced migration, we plated these clones on type IV collagen coated dishes and treated the cells with PD98059, a selective upstream inhibitor of ERK1 activation. PD98059 treatment reduced ERK1 activation by up to 3 fold as determined by western blot using an anti-phosphoERK1 antibody (Fig. 4A,4B; p < 0.003). Treatment of FAK and paxillin overexpressing clones with PD98059 inhibited migration on type IV collagen by 75% (Fig. 4C). However treatment with this inhibitor had little effect on basal migration of control clones, which correlated with our published work [13]. Four to six fold more FAK and paxillin overexpressing cells were able to invade through reconstituted basement membrane in the Matrigel invasion assays (Fig. 4D) which correlated with the migration experiments. MEK inhibition with PD98059 also inhibited invasion by FAK and paxillin overexpressing clones. These results indicated that MAPK inhibition could decrease FAK and paxillin induced migration and invasion by human SCC cells.
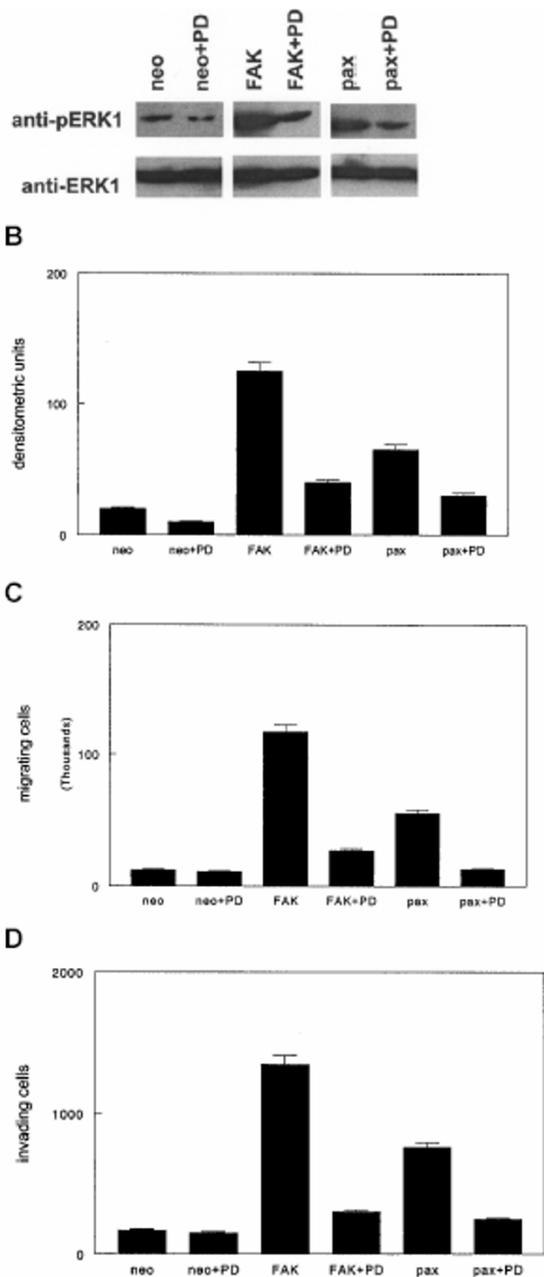
Discussion
The results of this study demonstrated that FAK and paxillin were recruited to the β1 integrin subunit when cells were plated on plastic or type IV collagen. However, the 52 kD isoform of the Shc adaptor protein was present in the complex only when cells were maintained under anchorage deprived conditions. Stratified squamous epithelial cells ultimately undergo terminal differentiation when deprived of attachment [17], and 52 kD Shc overexpression was sufficient in our study to produce terminal differentiation of cells plated on plastic tissue culture dishes. These results suggest that localization of 52 kD Shc to integrin signaling complexes may regulate terminal differentiation of stratified squamous epithelial cells following loss of basement membrane attachment. Shc is known to regulate growth factor receptor induction of the MAPK pathway [18]; it will be interesting to determine if MAPK inhibition prevents anchorage deprived terminal differentiation of stratified squamous epithelial cells.

In this context, FAK has been shown to suppress anchorage deprived cell death in MDCK cells [19]. The kinase activity and autophosphorylation site of the protein is required for this effect. FAK inhibition by overexpression of the carboxyl terminal domain causes loss of adhesion and cell death in human breast cancer lines [20]. Similarly, anchorage independent breast cancer cells undergo apoptosis when FAK function is disrupted. Interestingly, the FAK carboxyl terminus has no effect on adhesion or

Figure 3
FAK and paxillin promote SCC migration on type IV collagen in a $\beta 1$ integrin dependent manner. (A) Overexpression of FAK and paxillin in SCC25 cells. FAK and paxillin expression vectors were stably transfected into SCC25 cells as described in Materials and Methods. FAK and paxillin expression was determined in neomycin resistant control (neo), FAK overexpressing (FAK-1, -2), and paxillin overexpressing (pax-1, -2) clones by western blot using anti-FAK and anti-paxillin (anti-pax) antibodies. This experiment was repeated with additional independently isolated clones with similar results. Representative blots are shown. (B) Densitometric quantitation of blots in Fig. 3A. (C) FAK or paxillin (pax) overexpressing clones or neomycin resistant control cells (neo) were plated on plastic (pl) tissue culture dishes or type IV collagen (col) coated plates. Some cultures were preincubated with antibodies which block attachment by $\beta 1$ or $\beta 4$ integrin subunits. The number of cells which migrated into the blank area of the plate within 24 hours was counted. These experiments were performed three times with similar results. Error bars indicate SEM.

Figure 4
FAK and paxillin induce SCC migration via activation of the mitogen activated protein kinase ERK1. (A) Neomycin resistant control (neo), FAK overexpressing, and paxillin overexpressing (pax) clones were treated with the MEK inhibitor PD98059 (PD) or vehicle as described in Materials and Methods. Relative phosphorylated ERK1 and total ERK1 expression was determined by western blot using anti-phosphoERK1 (anti-pERK1) and anti-ERK1 antibodies. This experiment was repeated with additional independently isolated clones with similar results. Representative blots are shown. (B) Densitometric quantitation of blots in Fig. 4A. (C) FAK or paxillin (pax) overexpressing clones or neomycin resistant control cells (neo) were plated on plastic tissue culture dishes and treated with the MEK inhibitor PD98059 (PD) or vehicle as described in Materials and Methods. The number of cells which migrated into the blank area of the plate within 24 hours was counted. (D) FAK or paxillin (pax) overexpressing clones or neomycin resistant control cells (neo) were plated into Matrigel invasion chambers and treated with the MEK inhibitor PD98059 (PD) or vehicle. The number of cells which migrated through the reconstituted basement membrane were fixed, stained, and counted. These experiments were performed three times with similar results. Error bars indicate SEM.

viability of normal mammary epithelial cells. FAK has been shown to activate JNK which then colocalizes at focal adhesions in primary fibroblasts [21]. This activation occurs via a Ras/Rac1/Pak1/MKK4 pathway. In the presence of serum, survival signals are also transduced by PI3K and Akt. These studies suggest that FAK is also required for survival signaling from the extracellular matrix. Our future studies will examine this property of FAK in human SCC lines.

Our results demonstrated that FAK and paxillin induced migration and invasion of SCC lines was dependent on ERK activity. In colon cancer cell lines, cell attachment to collagen or laminin stimulates phosphorylation of FAK and paxillin and activates ERK1 [22]. FAK inhibition reduces attachment dependent ERK1 activation. ERK1 inhibition reduced migration of colon cancer cells [23], and inhibited growth factor induced migration in SCC lines used in the present study. FAK inhibition also disrupted growth factor stimulated migration of human lung cancer cells [24]. These studies highlight an important role for ERK signaling in mediating collagen induced migration and invasion of human cancer cell lines. FAK expression has been correlated with tumor invasion and lymph node metastasis of cancer cells [16]. In this study, FAK overexpression was detected in 59% of esophageal cancer pathology specimens. This overexpression was associated with decreased cellular differentiation, late clinical stage, increased depth of invasion, poor survival rate, and lymph node metastasis. Future clinical studies may examine FAK inhibition as potential antitumor therapy.

Conclusions
FAK and paxillin were recruited to β1 integrin when cells were attached to substratum while Shc was associated with the receptor in anchorage deprived cells. FAK and paxillin recruitment to β1 integrin correlated with increased migration on type IV collagen. FAK and paxillin overexpression promote migration of human SCC lines which is dependent on β1 integrin attachment to its physiologic substratum. MAPK inhibition could decrease FAK and paxillin induced migration and invasion of human SCC cells.

List of abbreviations
ERK extracellular signal regulated kinase

FAK focal adhesion kinase

Grb2 growth factor receptor bound protein 2

JNK jun N-terminal kinase

PI3K phosphatidylinositol 3-kinase

SCC squamous cell carcinoma

Competing interests
None declared.

Authors' contributions
DLC designed the study, supervised cell culture, and drafted the manuscript. AO performed transfections, immunoprecipitation, and western blot experiments. Both authors read and approved the final manuscript.

Acknowledgments

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Pre-publication history

The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-2407/4/18/prepub