Breast Cancer Research and Treatment **54:** 173–182, 1999.
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Report

Constitutive activation of pp125^{fak} in newly isolated human breast cancer cell lines

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Key words: breast cancer cell lines, pp125^{fak} activation, anchorage independent growth, focal adhesion kinase, extracellular matrix

Summary

Our laboratory has developed twelve human breast cancer cell lines from primary and metastatic sites. In this report we demonstrate that eight of eight breast cancer cell lines examined exhibit constitutively tyrosine phosphorylated and enzymatically active endogenous pp125^{fak} when grown in monolayer. The activation status of pp125^{fak} in breast cancer cells in monolayer is significantly elevated over that exhibited by normal mammary epithelial cells cultured under the same conditions. Constitutive activation of pp125^{fak} is the only characteristic so far studied that all of these breast cancer cell lines have in common. In contrast to HBC cells, tyrosine phosphorylation of pp125^{fak} in HME cells was low or absent in monolayer culture but was induced to high levels by culturing the cells in *Matrigel*. Thus tyrosine phosphorylation and activation of pp125^{fak} is a regulated process in normal mammary epithelial cells, but is constitutive in breast cancer cells. Finally, analysis of the ability of normal human mammary epithelial cells and breast cancer cell lines to grow under anchorage-independent conditions indicated that normal human mammary epithelial cells rapidly and uniformly lost viability when not substrate-attached, whereas all of the breast cancer cell lines survived for a 3-week culture period. Furthermore, a subset of the breast cancer cell lines grew to form large colonies under anchorage-independent conditions. Interestingly, pp125^{fak} activation decreased dramatically in HBC cells cultured for two weeks in suspension, suggesting that activation of this kinase is not necessary for long-term growth under anchorage-independent conditions. These results suggest that constitutive activation of pp125^{fak} results in preferential survival of human breast cancer cells under anchorage-independent conditions but that activation of pp125^{fak} is not the sole mediator of anchorage-independent colony formation.

Introduction

Attachment of mammary epithelial cells to extracellular matrix (ECM) is essential for normal tissue homeostasis. Signals sent from extracellular matrix receptors on the cell surface take part in the regulation of cell growth, differentiation, morphogenesis, and survival [1]. Attachment of human mammary epithelial (HME) cells occurs, in part, through integrins, cell surface receptors composed of heterodimers of one alpha and one beta subunit [2]. The specific combination of alpha and beta subunits expressed on the surface of cells directs their substrate specificity for attachment [1].

HME cells, when placed in a laminin-rich basement membrane, undergo growth and morphogenesis, and develop acinar structures similar to mammary ducts. By contrast, human breast cancer (HBC) cells form amorphous colonies when grown in the same matrix. Recently, Howlett, et al. [3] demonstrated that HME cells cultured in *Matrigel* in the presence of anti-β1-integrin antibodies undergo apoptosis, whereas breast cancer cells do not. These observations are similar to those made earlier by Frisch and Francis [4] with HT1080 cells and MDCK cells, and by Meredith, et al. with HME cells [5]. These data suggest that normal human mammary epithelial cells require signals derived from attachment to extracellular matrix to survive in three-dimensional culture, and that human breast cancer cells have acquired independence of these signals.

In 1992, Schaller, et al. [6] identified a protein of about 125 kDa as a putative substrate of activated pp60^{c-src}. This membrane-associated tyrosine kinase was termed focal adhesion kinase, or FAK. About the same time as Schaller, et al. identified pp125^{fak}, it was demonstrated that signaling through the fibronectin/β1-integrin receptor system caused an increase in tyrosine phosphorylation of an approximately 120 kDa protein [7]. It was later shown by Guan and Shalloway [8] that the 120 kDa protein activated by the fibronectin/β1-integrin interaction was pp125^{fak}. In fibroblasts, the *fak* protein is localized to focal adhesions [6, 9] by binding to the β1-integrin [9]. The precise intracellular localization of pp125^{fak} in HME cells has still not been clearly defined.

Since blocking β1-integrin-mediated signaling triggers apoptosis in mammary epithelial cells, the activation state of pp125^{fak} may play a role in the regulation of programmed cell death. Furthermore, dysregulated pp125^{fak} activation may protect HBC cells against apoptosis, and in that way be partly responsible for certain transformed properties of HBC cells. Evidence in support of this hypothesis came from Weiner, et al. [10], who examined the expression of *fak* mRNA in breast cancer cells and normal mammary cells *in vivo*. They found that 9 of 11 primary breast cancers contained elevated levels of *fak* mRNA as compared to benign breast tissue, and all four metastatic tumors studied had high levels of *fak* expression. Later, Owens, et al. [11] demonstrated that 88% of invasive and metastatic breast tumors studied had significantly elevated pp125^{fak} levels compared to normal tissue from the same patient. These data suggest that *fak* expression plays a role in breast cancer development and invasive potential.

Our laboratory has recently established twelve HBC cell lines from various stages of breast cancer. In the present studies, several of these lines were examined for expression and activation of pp125^{fak}. The data presented here indicate that a phenotype common to all of these breast cancer cell lines is the constitutive activation of pp125^{fak} and the ability to survive in a matrix-independent manner. This is in contrast to both MCF-10A cells and primary cultures of normal HME cells which express pp125^{fak} protein in monolayer but which require growth in *Matrigel* for tyrosine phosphorylation and activation of this kinase. Thus, constitutive activation of pp125^{fak} is a common feature of human breast cancer cell lines and may underlie their ability to survive under anchorage-independent conditions.

Materials and methods

Cell culture

The base medium for MCF-10A, SUM-44PE, SUM-102PT, and SUM-190PT cells was Ham's F12 medium supplemented with 0.1% bovine serum albumin, 0.5 μg/ml funigzone, 5 μg/ml gentamycin, 5 mM ethanolamine, 10 mM HEPES, 5 μg/ml transferrin, 10 μM T_3, 50 μM selenium, 5 μg/ml insulin, and 1 μg/ml hydrocortisone. MCF-10A and SUM-102PT cell medium was further supplemented with 10 ng/ml EGF. The base medium for HME, SUM-52PE, SUM-149PT, SUM-159PT, SUM-185PE, and SUM-1315MO2 was Ham's F12 supplemented with 5% fetal bovine serum (FBS), 0.5 μg/ml fungizone, 5 μg/ml gentamycin, and 5 μg/ml insulin. SUM-52PE, SUM-149PT, SUM-159PT, and SUM-185PE cell medium was further supplemented with 1 μg/ml hydrocortisone. HME cell medium was further supplemented with 1 μg/ml hydrocortisone, 10 ng/ml EGF, and 100 μg/ml cholera toxin. SUM-1315MO2 cell medium was further supplemented with 10 ng/ml EGF and did not contain hydrocortisone. All cell culture reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

HME cells were grown on collagen-coated tissue culture plates or in *Matrigel* (Collaborative Biomedical Products, Bedford, MA). Cells grown in *Matrigel* were plated in a 50/50 (v/v) mixture of *Matrigel* and HME medium. Cells were grown for two weeks prior to use for further experiments.

Detailed descriptions of all of the SUM cell lines can be found at http://p53.cancer.med.umich.edu/clines/ekb/ethier.html on the world wide web.

Immunoprecipitations and protein blots

Cells were lysed in a buffer consisting of 20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM Na_3VO_4, 1 mM PMSF, 2 μg/ml aprotinin, and 50 μg/ml leupeptin. Protein concentrations were equalized using the Lowry method [12]. Equal amounts of protein were either loaded on 7.5% SDS-PAGE or immunoprecipitated with α-pp125^{fak} (Catalog no. F15020, Transduction Labs, Lexington, KY). Antibody was incubated with lysate for 1 h at 4°C. The immune complex was bound to protein A/G beads (Catalog no. IP10X, Calbiochem, Cambridge, MA) for 40 min 4°C. Immunoprecipitates were washed two times with phosphate buffered saline (PBS) containing 1% Triton X-100, two times with PBS containing 0.5% Triton X-100, and two times with PBS. Læmmeli sam-

ple buffer [13] was added and the samples were boiled. Equal amounts were loaded onto 7.5% SDS-PAGE. Separated proteins were blotted to PVDF membrane and probed with either α-pp125^{fak} or α-Ptyr$_{4G10}$ (Catalog no. 05-321, Upstate Biotechnology, Inc., Lake Placid, NY). These experiments were performed at least twice.

Kinase assays

α-pp125^{fak} immunoprecipitates were washed twice in lysis buffer and once in kinase assay buffer (50 mM Tris–HCl, pH 7.4, 10 mM MnCl). Immunoprecipitates were mixed with prewarmed kinase assay buffer containing 20 μM ATP, 10 μCi γ-^{32}P-ATP, and 0.5 μg/μl poly(Glu : Tyr)$_{4:1}$ (Catalog no. P-0275, Sigma Chemical Co., St. Louis, MO). The reactions were allowed to proceed for 20 min at 30°C, stopped with the addition of Læmmeli sample buffer, and boiled. Reactions were then separated on 10% SDS-PAGE, blotted to PVDF, and probed for pp125^{fak} to be sure each sample had equivalent amounts of the kinase. Blots were then exposed to film −80°C for 2 h to 2 days and quantitated by densitometry. This was repeated twice.

Soft agar assays

Six-well dishes were coated with a 1:1 mix of the appropriate 2× medium for the cell line being studied and 1% Bactoagar. Cells were plated at 1×10^3, 1×10^4, and 1×10^5 cells per well in a mixture of appropriate medium and 0.3% Bactoagar. Cells were fed three times per week for 3 weeks, stained with 500 μg/ml *p*-iodonitrotetrazolium violet (Catalog no. I-8377, Sigma Chemical Co., St. Louis, MO) overnight, counted, and photographed. Each cell line was plated in duplicate in three separate dilutions. Colonies were counted for each duplicate of each dilution and averaged together. This experiment was repeated twice.

Methyl cellulose plating

Six-well dishes were coated with 1:1 mix of the appropriate 2× medium for the cell line being studied and 1% Bactoagar. Cells were plated at 1×10^6 cells per well in a 1:1 mix of the appropriate 2× medium for the cell line being studied and 1% methyl cellulose (Catalog no. M-7027, Sigma Chemical Co., St. Louis, MO). Cells were fed three times per week for 2 weeks, retrieved, and replated. This experiment was performed in duplicate.

Results

Human breast cancer cell lines

Our laboratory has now established 12 human breast cancer cell lines from primary and metastatic breast cancer specimens. All of the HBC cell lines established thus far express luminal cytokeratins, are aneuploid, and exhibit distinct areas of gene amplification and gene loss. Descriptions and characterizations of some of these cell lines, including SUM-44PE, SUM-52PE, SUM-16LN, SUM-102PT, have been published previously [14–17]. In addition, data on the SUM-149PT and SUM-159PT cell lines have been published as part of a larger study on Stat3 activation in human breast cancer cells [18]. Finally, genetic analysis of the entire panel of breast cancer cell lines, which includes both Southern analysis and comparative genomic hybridization studies, have recently been completed (submitted for publication).

Table 1 lists the current panel of breast cancer cell lines (the SUM-16LN cell line is no longer available),

Table 1. Molecular characteristics of SUM HBC cell lines

Cell line	Oncogene Amp[a]	EGFR exp	p53 (IHC)
SUM-44PE[b]	FGFR-1[c] cycD1[d] c-myc[d]	−	+(c)[e]
SUM-52PE	FGFR-1[c] FGFR-2[c] cycD1[d]	−	+(c)
SUM-102PT	none	+++	–
SUM-149PT	none	+++	+(n)
SUM-159PT	c-myc[d]	++	+(n)
SUM-1315MO2	none	+	+(n)
SUM-185PE	none	+	NE
SUM-190PT	erb B-2[c] cycD1[c]	+	+(n)
SUM-206cwn	none	+	+(n)
SUM-225cwn	erb B-2[c] c-myc[d]	−	NE
SUM-229PE	none	+++	NE

[a]Oncogenes examined by Southern blot: erbB-2, c-myc, Prad-1, FGFR-1, 2, 4.
[b]PT = primary tumor; MO2 = mouse xenograft, second transplant generation; PE = pleural effusion; CWN = chest wall nodule.
[c]High level amplifications detected by Southern blot.
[d]Low level amplifications (2–3 fold) detected by FISH.
[e](c) = cytoplasmic staining; (n) = nuclear staining; NE = not examined.

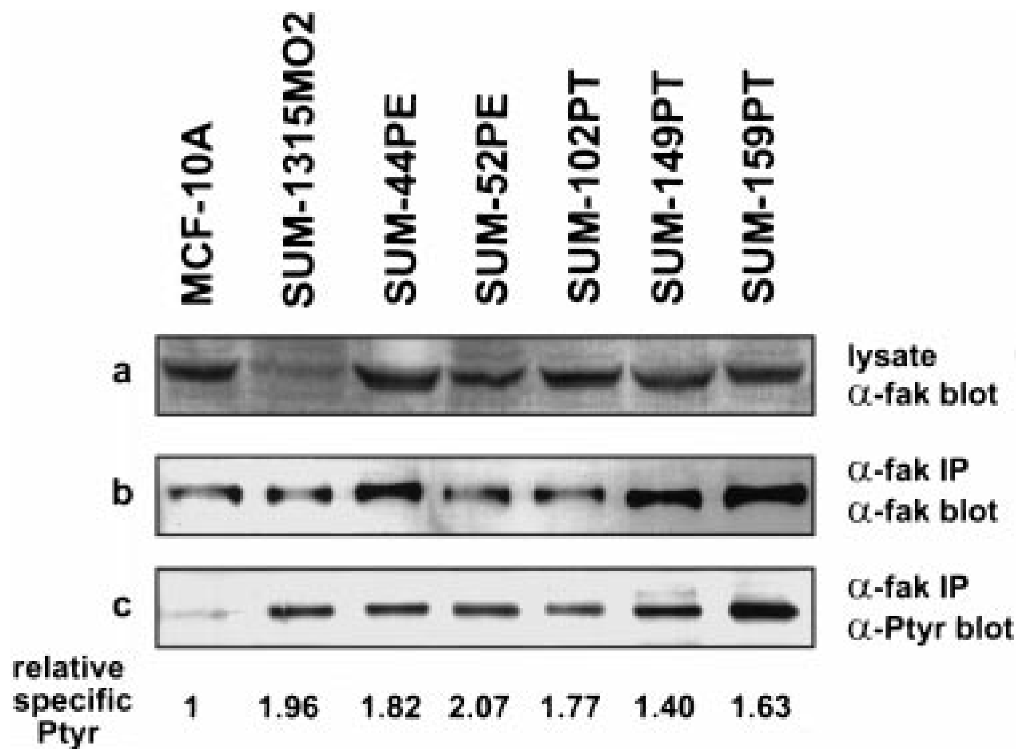

Figure 1. HBC cell lines contain increased pp125^{fak} tyrosine phosphorylation. Proteins from whole cell lysates were separated on SDS-PAGE, blotted to PVDF, and probed with α-pp125^{fak} antibody (a). pp125^{fak} was immunoprecipitated from control and HBC cells. Immunoprecipitated proteins were separated on SDS-PAGE, blotted to PVDF, and probed with α-pp125^{fak} antibody (b) or with α-Ptyr$_{4G10}$ (c). Relative specific amounts of tyrosine-phosphorylation pp125^{fak} are indicated. Relative specific tyrosine-phosphorylation was determined by quantitation of the western by densitometry, setting the amounts of pp125^{fak} and of tyrosine phosphorylation relative to the amount in MCF-10A, then normalizing the amount of phosphotyrosine to the amount of pp125^{fak} in each lane.

indicates the type of specimen from which each cell line was derived, and provides a brief summary of the cellular and molecular characteristics of each cell line. As can be seen from the table, cell lines with amplifications of the oncogenes frequently implicated in breast cancer development, including ERBB-2, Cyclin-D1 (PRAD-1), c-MYC, FGFR-1, and FGFR-2, are present in this panel. Also, some of these breast cancer cell lines express high levels of EGFR in the absence of gene amplification. One cell line, SUM-44PE, expresses high levels of estrogen receptor (D. Zajchowski, S. Ethier, unpublished observations). Finally, these cell lines exhibit a range of p53 phenotypes. Thus, this panel of human breast cancer cell lines is representative of the molecular heterogeneity observed in uncultured breast cancer specimens.

HBC cell lines have increased pp125^{fak} *activity*

Weiner, et al. and Owens, et al. [10, 11] demonstrated that human breast cancers express elevated levels of *fak* mRNA and of pp125^{fak} protein compared to normal breast tissue. Based on these observations, we sought to determine if our HBC cell lines express elevated levels of *fak* protein compared to normal proliferating HME cells. At the time this study was initiated, only eight of the current 11 cell lines had been established and fully characterized and only those are included in this study.

Western blot analysis of cell lysates obtained from HBC cell lines, MCF-10A cells, and primary HME cells demonstrated that all of these cells express similar levels of pp125^{fak} protein (Figures 1a and 3b). Thus, proliferating HME cells, unlike normal resting mammary epithelium *in vivo*, express significant levels of pp125^{fak}. To compare the activation status of pp125^{fak} in HBC cells to normal HME cells, western blots of pp125^{fak} immunoprecipitates were probed with antiphosphotyrosine antibodies or with anti-*fak* antibodies. Figure 1 shows that all of the HBC cell lines examined had increased levels of tyrosine phosphorylated pp125^{fak} compared to MCF-10A cells. In order to quantitate the difference in the levels of ty-

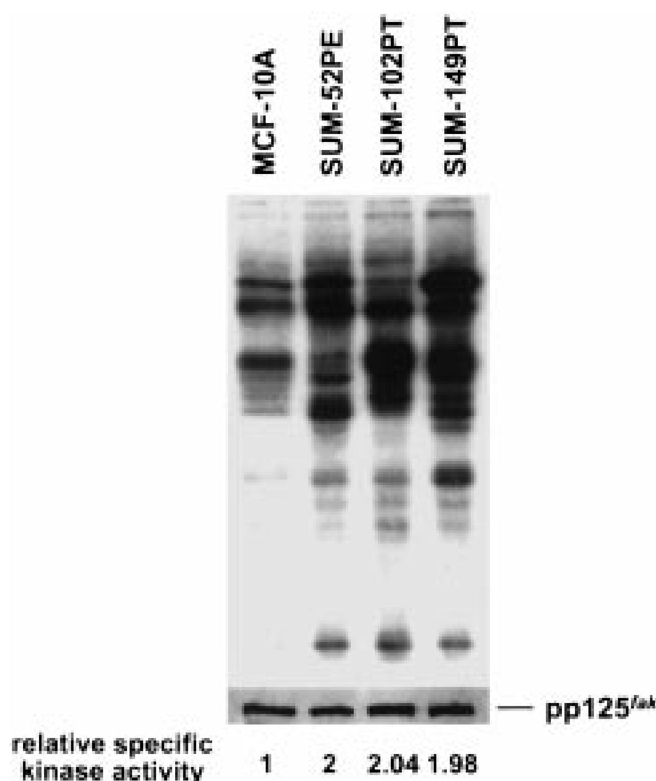

Figure 2. HBC cell lines contain increased pp125^{fak} activity. pp125^{fak} was immunoprecipitated from control and HBC cells and subjected to *in vitro* kinase assays using poly(Glu:Tyr)$_{4:1}$ as a substrate. Reaction products were separated on 10% SDS-PAGE and blotted to PVDF membrane. The blots were probed with α-pp125^{fak} antibody, to obtain the relative amounts of pp125^{fak} in each reaction, then subjected to autoradiography. Both the kinase assay and the anti-*fak* blot are shown. The relative specific kinase activity is indicated.

rosine phosphorylated pp125^{fak} in the different HBC cell lines to that found in MCF-10A cells, densitometry analysis was performed comparing the phosphotyrosine band in each cell line with that in MCF-10A cells and then normalizing that result to the overall amount of pp125^{fak} that was present in the immunoprecipitate. This analysis yielded the relative specific increase in tyrosine phosphorylated pp125^{fak} in each cell line as compared to MCF–10A cells. The results indicate that all the breast cancer cell lines examined had approximately 1.5–2.0 fold increased levels of tyrosine phosphorylated pp125^{fak} compared with MCF-10A cells.

To confirm that pp125^{fak} enzymatic activity was higher in HBC cells than in MCF-10A cells, *in vitro* kinase assays were performed. In this assay, pp125^{fak} immunoprecipitates were tested for their ability to phosphorylate the artificial substrate poly-glu-tyr. The results shown in Figure 2 confirm the increased enzymatic activity of pp125^{fak} in HBC cells compared to MCF-10A controls (Figure 1). Once again, quantitation and normalization of the kinase assay demonstrated a two-fold increase in pp125^{fak} kinase activity in HBC cells compared to MCF-10A cells.

To examine the expression and activation of pp125^{fak} in non-immortalized HME cells and to determine if pp125^{fak} present in these cells could be activated by stimulation with extracellular matrix, primary cultures of reduction mammoplasty derived HME cells were cultured either in monolayer or in the basement membrane matrix *Matrigel*. The cells were cultured using a medium that we previously showed supports the growth of normal HME cells of the luminal lineage [14]. HME cells grown in monolayer exhibited a characteristic epithelial cell cobblestone appearance, whereas the same cells grown in *Matrigel* grew into three-dimensional structures exhibiting ductal branching and formation of acini (Figure 3a). Antiphosphotyrosine-probed Western blots of pp125^{fak} immunoprecipitates derived from HME cells cultured under both conditions revealed a dramatic increase in levels of tyrosine-phosphoryplated pp125^{fak}

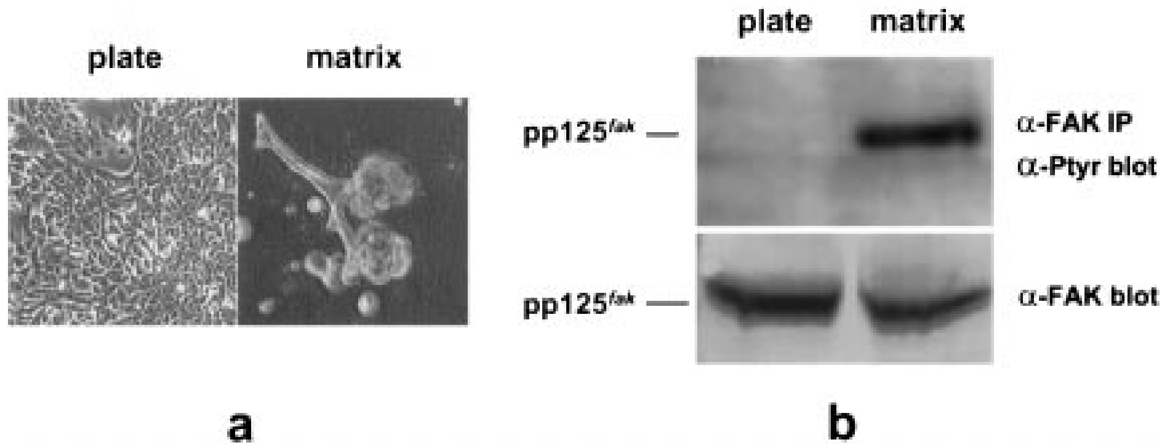

Figure 3. (a) HME cells exhibit differentiated morphology when grown in basement membrane. HME cells were grown either on collagen-coated tissue culture plates or in the laminin-rich basement membrane *Matrigel*. (b) HME cells grown in basement membrane contain higher levels of $pp125^{fak}$ activity than HME cells grown in two-dimensional culture. $pp125^{fak}$ was immunoprecipitated from cell lysates of HME cells grown on tissue culture plates or in *Matrigel*, separated on SDS-PAGE, blotted to PVDF, and probed with α-$Ptyr_{4G10}$ antibody. Whole cell lysates from the same cells before immunoprecipitation were separated on 7.5% SDS-PAGE and probed with α-$pp125^{fak}$ to show that the same amount of $pp125^{fak}$ was present under both growth conditions.

in cells grown in *Matrigel* as compared to cells grown in monolayer culture (Figure 3b). In fact, tyrosine phosphorylated $pp125^{fak}$ was undetectable in HME cells grown in monolayer. Thus, in HME cells, $pp125^{fak}$ tyrosine phosphorylation is strictly the result of interaction with the basement membrane proteins or other *Matrigel*-associated factors, in a manner that does not occur in monolayer.

In summary, these experiments demonstrated that $pp125^{fak}$ is expressed in proliferating normal and neoplastic breast epithelial cells. However, tyrosine phosphorylation and enzymatic activity of this kinase is undetectable in primary HME cells in monolayer culture, is slightly elevated in MCF-10A cells, and is activated to higher levels still in all HBC cell lines examined.

HBC cells containing activated $pp125^{fak}$ *survive under anchorage-independent conditions*

A hallmark of cellular transformation is the ability of cells to produce colonies in an anchorage-independent manner [19]. In addition, previous work has implicated $pp125^{fak}$ in the ability of epithelial cells to survive and grow in soft agar [20]. In order to assess the ability of our HBC cell lines to grow under anchorage-independent conditions, several of the cell lines, as well as HME cells and MCF-10A cells, were grown for 3 weeks in soft agar suspension. The results shown in Figures 4 and 5 indicate that some of the HBC cells formed large progressively growing colonies in agar, whereas cells from other lines formed small colonies or remained as single cells. By contrast, HME and MCF-10A cells not only did not form colonies in agar, but after the 3 week culture period, no viable cells

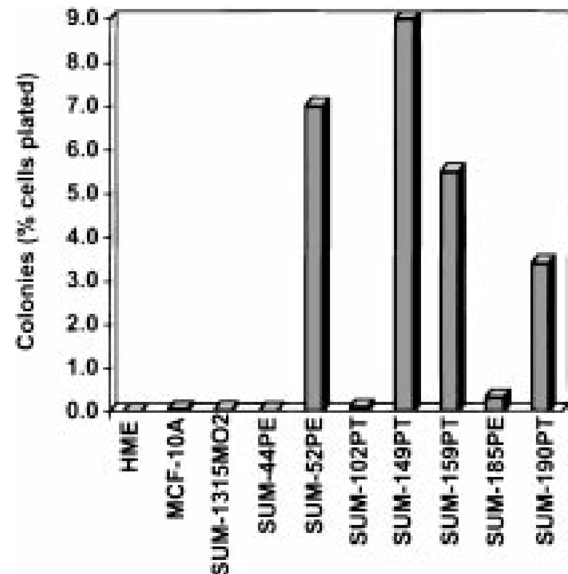

Figure 4. Anchorage-independent growth assay of HBC cell lines and normal HME cells. Control cell lines and HBC cell lines were grown in 0.3% agar for 3 weeks, stained with the viable stain *p*-iodonitrotetrazolium violet, and colonies greater than 2 mm were counted. Each cell line was plated in duplicate in three separate dilutions. Colonies were counted for each duplicate of each dilution and averaged together.

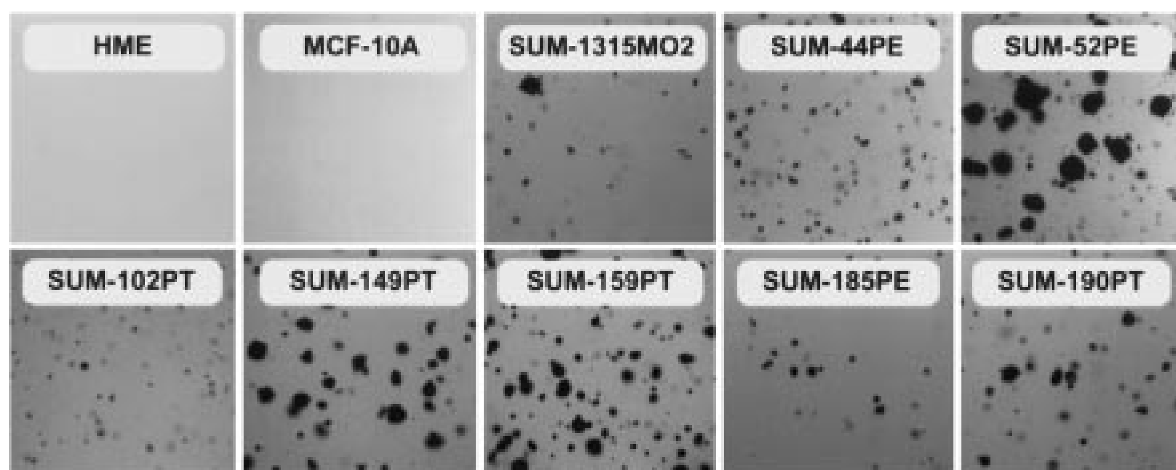

Figure 5. Growth of HME and HBC cells in soft agar. Control cell lines and HBC cell lines were grown in 0.3% agar for 3 weeks, stained with the viable stain ρ-iodonitrotetrazolium violet. Each cell line was plated in duplicate in three separate dilutions.

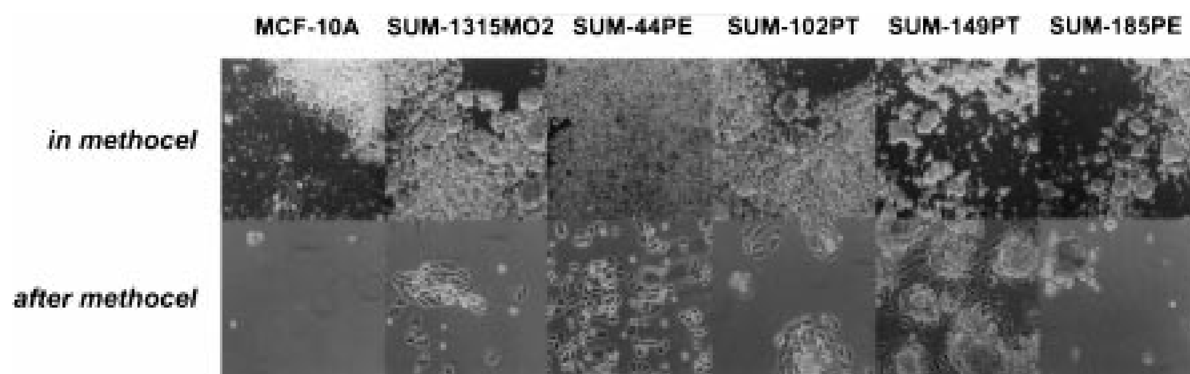

Figure 6. The HBC cells that survived in small colonies in soft agar are viable. MCF-10A, SUM-149PT, and HBC cells that formed small colonies in soft agar were plated in methyl cellulose, retrieved, and replated. Representative replatings are shown.

could be detected in these culture wells. Comparison of the activation status of $pp125^{fak}$ with the ability of a particular cell line to form large colonies in soft agar did not demonstrate a direct correlation between these two phenotypes. However, the observation that all HBC cells appeared to survive in agar, whereas MCF-10A and HME cells did not remain viable under these conditions, suggested that $pp125^{fak}$ activation plays a role in matrix-independent survival of HBC cells, if not growth potential in agar.

To confirm that the HBC cells that only formed small colonies in soft agar were indeed viable, cells from these lines were cultured for 2 weeks in suspension culture in methyl cellulose, then retrieved and replated in monolayer. As controls, MCF-10A cells, which appeared to lose viability in soft agar, and SUM-149PT cells, which formed large colonies in agar, were also grown in methyl cellulose. Over the first few days in methyl cellulose, MCF-10A cells formed small aggregates. However, after 2 weeks in suspension culture, these aggregates did not appear viable, and retrieving and replating the cells did not result in any cell attachment. The HBC cells that formed small colonies in soft agar formed similarly sized colonies when grown in methyl cellulose. When these cells were recovered from the suspension culture and placed in monolayer, they attached and resumed proliferating. As expected, SUM-149PT cells formed large colonies in methylcellulose that, when replated, attached and grew rapidly (Figure 6). These results indicate that all of the HBC cell lines tested did, indeed, remain viable under anchorage-independent conditions, whereas MCF-10A and HME cells rapidly lost viability.

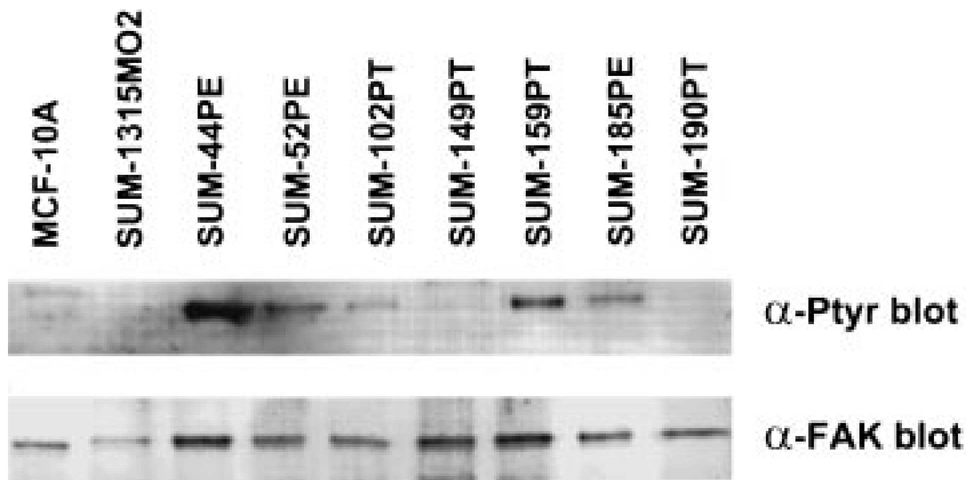

Figure 7. Suspended HME cells rapidly lose pp125^{fak} activation whereas suspended HBC cells do not. HME and HBC cells were plated on tissue culture plastic. One week later, cells were either lysed after trypsinization, or pp125^{fak} activation was determined by anti-pp125^{fak} immunoprecipitation and then anti-phosphotyrosine Western blot. The amount of pp125^{fak} was determined by probing identical blots with anti-pp125^{fak}.

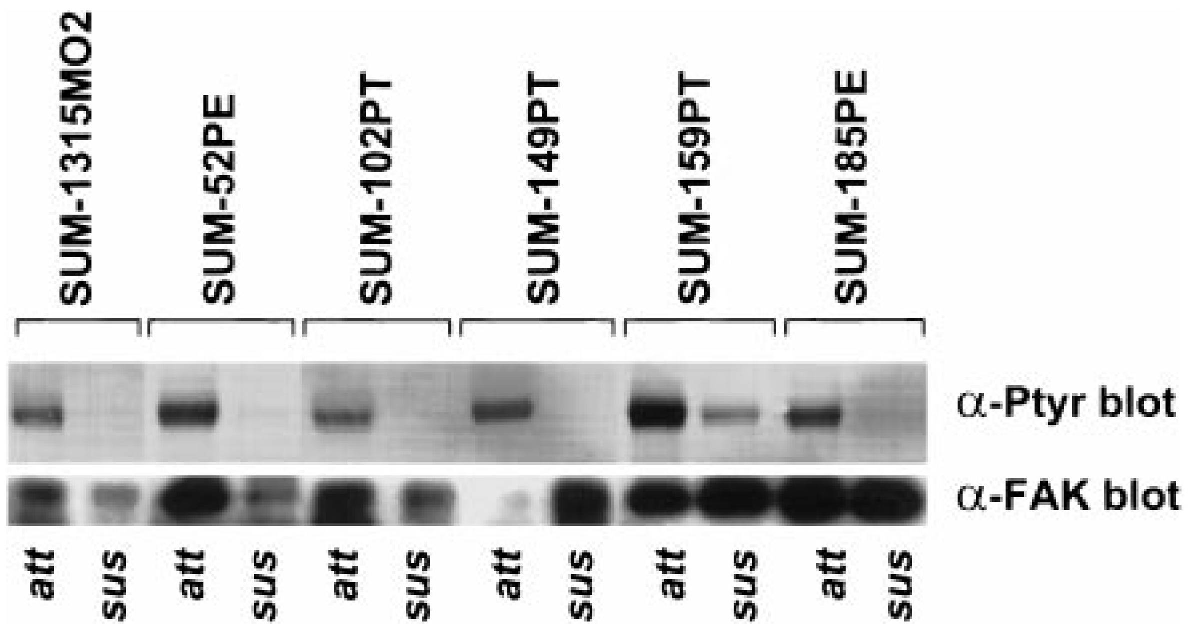

Figure 8. HBC cells maintained in a semi-solid medium lose pp125^{fak} activation. HBC cells were plated on tissue culture plastic or in methyl cellulose and maintained for two weeks. Cells were retrieved and lysed. pp125^{fak} activation was determined by anti-pp125^{fak} immunoprecipitation and then anti-phosphotyrosine Western blot. The amount of pp125^{fak} was determined by probing the same blot with anti-pp125^{fak}.

Extended survival and growth of HBC cells in suspension is independent of pp125^{fak} *activation*

To examine further the role of pp125^{fak} activation in anchorage-independent survival and growth of our HBC cell lines, experiments were carried out to measure pp125^{fak} tyrosine phosphorylation in HBC and MCF-10A cells after being released from monolayer culture, and after extended growth in methyl cellulose. The data in Figure 7 show that tyrosine phosphorylated pp125^{fak} was undetectable in MCF-10A cells and in three of the eight HBC cell lines examined after the cells had been released from the substratum. Thus, in MCF-10A and some HBC cells, tyrosine

phosphorylated pp125^{fak} is rapidly lost upon release from substrate. Interestingly, after extended growth of HBC cells in suspension culture, none of the HBC cell lines examined exhibited tyrosine phosphorylated pp125^{fak}. Thus, regardless of whether HBC cells grow to form large or small colonies in suspension, tyrosine phosphorylated pp125^{fak} was not detected in these cells after extended growth in suspension culture.

Taken together, these results suggest that the high levels of activated pp125^{fak} detected in lysates of HBC cells grown in monolayer culture were the result of interaction of the cells with the substratum in a manner which does not occur in normal HME cells. These results also suggest that in these HBC cell lines, the ability to survive for extended periods of time and to grow in suspension culture are independent of the activation status of pp125^{fak}.

Discussion

The interaction of the extracellular matrix (ECM) with epithelial cells in influencing cell growth and morphogenesis has been studied in both rodent and mammalian cells for some time. ECM is deposited by the epithelial cells and stromal fibroblasts [1]. Activation of specific signaling pathways via interactions of epithelial cell surface molecules with ECM are required for three-dimensional growth of normal human and rodent mammary epithelial cells [1]. HME cells grown in three-dimensional culture in the basement membrane *Matrigel* form structures reminiscent of fully differentiated alveoli *in vivo*. This is in contrast to HME cells grown on tissue culture plates, which have the characteristic epithelial cell cobblestone appearance. Howlett et al. [3] demonstrated the importance of the β1-integrin signaling pathway for human mammary epithelial cells grown in three-dimensional culture. Blockage of β1-integrin's interactions with the extracellular matrix using an anti-β1-integrin antibody caused the cells to undergo apoptosis. In contrast, HBC cells grown under the same conditions did not organize into differentiated structures in this basement membrane matrix and did not undergo apoptosis when exposed to the anti-β1-integrin antibody.

It is known that β1-integrin binding to ECM activates a pathway that results in activation of pp125^{fak} [8]. pp125^{fak} is a cytoplasmic tyrosine kinase [6] that binds β1-integrin in the cytosol through a sequence in its carboxy terminus [9]. It was previously shown in primary uncultured human breast cancers that *fak* mRNA and protein levels are elevated relative to surrounding normal mammary epithelia [10, 11]. This suggests that expression and activation of pp125^{fak} is an important aspect of HBC growth, particularly since these cells are often not in contact with normal substrate molecules [21]. The data obtained in our experiments support this hypothesis. We found that normal HME cells require interactions with components of *Matrigel* in order to activate endogenous pp125^{fak} (Figure 4). By contrast, all of the HBC cell lines examined had constitutively activated endogenous pp125^{fak} when grown in monolayer. The HBC cell lines developed in our laboratory represent the range of molecular diversity seen in clinical breast cancer specimens. Each of the breast cancer cell lines developed in our lab has a unique constellation of molecular alterations, and, until now, no single characteristic has been observed uniformly in all of the cell lines. The data presented here indicate that pp125^{fak} activation is a common characteristic of these HBC cell lines.

There is suggestive evidence in the literature that pp125^{fak} activation is required for anchorage-independent growth of transformed cells [22]. Our data indicate that constitutive pp125^{fak} activation may be important for survival in the absence of matrix-derived signals but is not sufficient for formation of large colonies in soft agar. This interpretation is based on the observation that HME cells failed to survive in soft agar (Figures 5 and 7), whereas the HBC cell lines with activated pp125^{fak} survived under these conditions. However, only a subset of the HBC cell lines formed large colonies in soft agar and none of the HBC cells maintained high levels of tyrosine phosphorylated pp125^{fak} after extended growth in suspension culture. Thus, activation of pp125^{fak} appears not to be required for anchorage-independent growth of HBC cells. Rather, the results of our experiments suggest that the tyrosine phosphorylation and activation of pp125^{fak} that we observed in our breast cancer cell lines is the result of interactions of HBC cells with the substrate when the cells are in monolayer culture. Similar activation of pp125^{fak} did occur in HME cells in monolayer culture but did occur when the cells were grown in *Matrigel*. Thus, aberrant cell substrate interactions that occur in HBC cells but not HME cells may result in abnormal activation of pp125^{fak}. The observation that our HBC cell lines have uniformly lost expression of β4-integrins, while maintaining expression of β1-integrins (data not shown), suggests the possibility that alterations in integrin expression underlie the abnormal activation of pp125^{fak} in HBC cells. Further work will be required to better understand the relationship between integrin expression and pp125^{fak} activation in HBC cells.

Acknowledgements

KWI was supported in part by Cancer Biology training grant no. T32 CAO 9676

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