Deoxycholic acid differentially regulates focal adhesion kinase phosphorylation: Role of tyrosine phosphatase ShP2

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Running Title: Regulation of FAK by Deoxycholic Acid

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FOOTNOTES

†The abbreviations used are: DCA, deoxycholic acid; FAK, focal adhesion kinase; ECM, extracellular matrix; MTT, tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ODN, oligodeoxynucleotide; PTPase, protein tyrosine phosphatase; PBS, Phosphate buffered saline; AOM, azoxymethane; Tyr, Tyrosine
Abstract

Environmental factors, including dietary fats, are implicated in colonic carcinogenesis. Dietary fats modulate secondary bile acids including deoxycholic acid (DCA) concentrations in the colon, which are thought to contribute to the nutritional-related component of colon cancer risk. Here we demonstrate, for the first time, that DCA differentially regulated the site-specific phosphorylation of focal adhesion kinase (FAK). DCA decreased adhesion of HCA-7 cells to the substratum and induced dephosphorylation of FAK at Tyrosine-576/577 (Tyr-576/577) and Tyr-925. Tyrosine phosphorylation of FAK at Tyr-397 remained unaffected by DCA stimulation. Interestingly, we found that c-Src was constitutively associated with FAK and DCA actually activated Src, despite no change in FAK-397 and an inhibition of FAK-576 phosphorylation. DCA concomitantly and significantly increased association of tyrosine phosphatase ShP2 with FAK. Incubation of immunoprecipitated FAK, *in vitro*, with GST-ShP2 fusion protein resulted in tyrosine dephosphorylation of FAK in a concentration dependent manner. Antisense oligodeoxynucleotides directed against ShP2 decreased ShP2 protein levels and attenuated DCA induced FAK dephosphorylation. Inhibition of FAK by adenoviral-mediated over-expression of FRNK, and gene silencing of Shp2 both abolished DCA’s effect on cell adhesion, thus providing a possible mechanism for inside-out signaling by DCA in colon cancer cells. Our results suggest that DCA differentially regulates focal adhesion complexes and that tyrosine phosphatase ShP2 has a role in DCA signaling.
Introduction

Colorectal cancer is a leading cause of cancer-related deaths in the United States. In addition to genetic factors, dietary constituents play important roles in tumor development (1, 52). In this regard, diets high in animal fat predispose to experimental colon cancer (33). Endogenous bile acids, secreted in response to dietary fats, are thought to contribute to this increased risk (1, 56, 69). The primary bile acids, cholic acid and chenodeoxycholic acid, are synthesized and conjugated to glycine and taurine in the liver and secreted into the bile to facilitate dietary fat absorption (29). Most of the bile acids are re-absorbed from the distal small intestine via the enterohepatic circulation. A small fraction (less than 5%) escapes into the colon where they are deconjugated and further metabolized by colonic bacteria. In the colon, cholic acid is converted primarily to deoxycholic acid (DCA) and chenodeoxycholic acid is metabolized to lithocholic acid. Epidemiological studies have suggested that these secondary bile acids promote colon cancer (9, 47). The notion that elevated fecal bile acids are linked to increased risk for colorectal cancer is also supported by the observation that patients with colorectal adenomas have elevated concentrations of bile acids in their blood and stool (3, 48). We have demonstrated previously that feeding rats a diet containing cholic acid promoted the development of azoxymethane (AOM)-induced colonic tumors (17).

A number of potential mechanisms have been proposed for bile acid-induced colonic tumor promotion, including: (i) increased peroxidative damage to nucleic acids (15); (ii) increased peroxidative damage to lipids and proteins (14); (iii) increased reactive nitrogen and oxygen radicals that alter REDOX-sensitive cell signaling (34). Several specific nuclear targets for bile acids recently have been identified, such as the pregnane X receptor, the farnesoid X receptor and the nuclear vitamin D receptor permitting bile acid to control distinct patterns of gene
expression (22, 38, 39). However, no cell surface receptor for bile acids has yet been identified and it is presumed that these amphipathic molecules exert their effects by perturbing plasma membrane structure and function. In this regard, DCA also causes ligand-independent transactivation of epidermal growth factor (EGF) receptor in hepatocytes, leading to signaling through mitogen-activated protein kinase pathways (55, 70).

Focal Adhesion Kinase (FAK), a cytoplasmic kinase localized in focal adhesions, at which cells make close contacts with the extracellular matrix (ECM) through integrins, is one of the most prominently tyrosine-phosphorylated proteins in many cell types in response to cell adhesion (51). Engagement of integrins and other adhesion receptors can induce activation of FAK (5), which leads to phosphorylation of several tyrosine residues through autophosphorylation and recruitment of Src (63). Each of the FAK tyrosine residues is implicated in generating a distinct signal, FAK Tyr-397 in recruiting Src, PI-3 kinase and p130Cas to focal adhesions; FAK Tyr-576 and -577 in up-regulating FAK kinase activity and FAK Tyr-925 in activating the Ras-MAPK pathway (61). FAK has been implicated in cell motility (63), apoptosis and anchorage-independent growth (20). Because these are key processes by which a transformed cell becomes invasive and metastatic, FAK may be intimately involved in colonic carcinogenesis. Studies have demonstrated over-expression of FAK as colon tissues become transformed and develop into primary, invasive carcinomas (7). Despite the well-characterized roles of FAK in focal adhesion formation, the function of this signaling component in the actions of DCA has never been studied. Here we report, for the first time, that DCA regulates FAK by selectively dephosphorylating distinct tyrosine residues through the recruitment of tyrosine phosphatase ShP2 and there is compelling functional evidence that secondary bile acids may regulate membrane signaling.
Materials and Methods

Materials: DCA, fibronectin, laminin, poly-l-lysine, collagen-I, BSA, crystal violet and anti-β-actin antibodies were obtained from Sigma-Aldrich (St. Louis, MO). Protein A/G plus agarose, polyclonal anti-FAK, anti-Src, monoclonal anti-ShP2, and anti-phosphotyrosine antibodies (PY-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cell Signaling provided phosphospecific FAK-925, -576/577 and Src-418 polyclonal antibodies. Phosphospecific FAK-397 was obtained from Biosource (Camarillo, CA). Three 20-mer oligodeoxynucleotides were synthesized by Bio Synthesis (Lewisville, TX). Fusion protein of the phosphatase domain, comprising amino acids 224-529 of human ShP2, tagged at the N-terminus with GST was obtained from Abcam (Cambridge, MA). For Western blotting studies, electrophoretic grade acrylamide, bisacrylamide, Tris, SDS and pre-stained molecular weight markers were obtained from Bio-Rad Laboratories. Kodak supplied X-OMAT blue film for xerography. Polyvinylidene difluoride membranes (Immobilon-P) were purchased from Millipore, Inc. ECL Western blotting detection reagents, as well as horseradish peroxidase coupled anti-mouse and anti-rabbit secondary antibodies, were obtained from Amersham Biosciences. Protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Indianapolis, IN). Other reagents were of the highest quality available and obtained from Sigma, unless otherwise noted.

Cell culture: Dr. Susan Kirkland (ICRF, London UK) generously provided the HCA-7 cells, that were derived from a rectal carcinoma (42). Cells were grown in McCoy’s media containing 10% fetal calf serum and penicillin/streptomycin mixture. Pre-confluent cells (70-80% confluent) were used for experiments. Cells were treated with DCA or vehicle for the indicated times and at the indicated concentrations.
Cell viability and toxicity assays: Cell viability of DCA treated (100µM, 3hrs) HCA-7 cells was assessed by the colorimetric conversion of tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan as previously described (46). To assess cell death as a consequence of exposure to bile acid, cultured cells in 24-well plates were incubated with DCA (100µM, 3hrs). Cells were then incubated for 1 h in culture medium containing 5µg/ml propidium iodide, a cell membrane impermeant nucleic acid stain. Images of cells were obtained on a microscope equipped for phase contrast and fluorescence imaging. Total number of cells and the number of propidium iodide stained cells were counted. The average proportion of dead cells per well, calculated from three images, was considered to be a single data point.

Cell adhesion assays: 96 well plates were coated with collagen-I, fibronectin, laminin and polyl-lysine in pre-coating buffer (15mM Na$_2$CO$_3$, 35 mM NaHCO$_3$, pH 9.4) overnight at 4°C. Nonspecific binding sites were blocked with 1% BSA in PBS for 1 hr at room temperature. Serum starved HCA-7 cells were treated with DCA (100µM, 1hr), rinsed with serum free medium and harvested with 2mM EDTA in PBS. In some experiments cells were pre-incubated with serum free media containing 1mM MnCl$_2$ for 1 hr. followed by DCA treatment as described above. Recovered cells (5x10$^4$/well) were plated in triplicate onto pre-coated 96 well plates in serum free medium containing 0.1% BSA and allowed to adhere for 30 min at 37°C. Adherent cells were washed twice with PBS and fixed with 3.7% paraformaldehyde. Cells were then stained with 0.1% crystal violet in 20% methanol for 20 min. The dye was aspirated and wells washed three times with reagent grade water. The dye was extracted with 50% ethanol in 50mM sodium citrate, pH 4.5, and quantified by measuring absorbance at 550nm on ELISA reader.
**Immunolocalization:** HCA-7 cells were plated on glass slides. At 50% confluency medium was aspirated and cells were washed twice in 1 ml of sterile PBS, fixed for 15 min with 2% paraformaldehyde in PBS at 4°C. Cells were then washed 3 times with PBS and covered with 1ml of 1% glycerol in PBS for 15 min. Cells were permeabilized with 1 ml 0.2% Triton X-100 in PBS. To prevent non-specific staining, slides were blocked with 1% goat serum and incubated overnight at 4°C with phosphospecific FAK antibodies (1:1000). Next morning slides were washed twice with PBS and blocked again as described above. Cells were then incubated with goat anti-mouse rhodamine secondary antibodies (1:30) in block solution for 1 hr. After washing FITC phalloidin (1:40) in PBS was added for 30 min. Slides were mounted with coverslips using Slow Fade and Airvol and dried overnight in the dark. Fluorescent-labeled cells were then viewed with the use of a laser scanning confocal microscope. (Model LSM 510, Zeiss).

**Preparation of cytosolic cell extracts and immunoprecipitation:** Pre-confluent cells were serum starved for 18hr and then stimulated with DCA (100µM) for indicated times. Cells were lysed in a buffer containing 25 mM HEPES pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 1mM EDTA, 2% glycerol, 1mM Na₃VO₄, 50mM NaF and Roche protease inhibitors cocktail. For study of phosphatase activity, Na₃VO₄ and NaF were excluded from lysis buffer. After incubation on ice for 15 min, the lysates were passaged several times through a 25-gauge needle, and insoluble materials were removed by centrifugation at 4°C at 10,000g. The cell lysates (200µg protein) were precleared by incubation with protein A/G agarose plus for 1 hr. The lysates were then incubated with appropriate antibody (2µg for each sample) overnight at 4°C. To collect specific immune complexes, 50µl of protein A/G agarose plus beads were added and sample was incubated on a rotator at 4°C for 2 hr. The beads were washed three times with lysis buffer. In some experiments serum starved HCA-7 cells were washed twice with PBS and detached gently.
with PBS containing 2 mM EDTA. Cells were maintained in suspension in PBS for 30 min at 37°C before the addition of DCA (100µM) for the indicated time periods. Cells were then lysed and immunoprecipitated with anti-FAK antibodies as described above.

**Western blotting:** Proteins were extracted in SDS-containing Laemmli buffer and subjected to quantitative Western blotting as described previously (4). Briefly, proteins (50µg) were separated by SDS-PAGE using a 10% resolving polyacrylamide gel and electroblotted. Blots were incubated overnight at 4°C with specific primary antibodies anti-FAK (1:5000), anti-ShP2 (1:3000), and anti-Src (1:5,000), followed by 1 hr incubation with appropriate peroxidase-coupled secondary antibodies and subsequent detection by enhanced chemiluminescence.

**In vitro FAK activity assay:** FAK was immunoprecipitated from DCA treated cells with anti-polyclonal FAK antibody and washed twice with lysis buffer and once with kinase buffer (10mM Tris, pH 7.4, 10mM MnCl₂, 2mM MgCl₂, 0.02% Triton X-100). Immunoprecipitates were incubated at 30°C for 30 min with 10 µM ATP (10µci/assay) alone (FAK autophosphorylation) or 50µg poly (E4Y1) (Mr 20K-50K, Sigma) in 30 µl kinase buffer. In autophosphorylation experiments, the reaction was terminated by the addition of SDS buffer. Samples were separated on a 10% resolving gel, and the FAK band was identified by radioautography. In experiments employing the synthetic peptide substrate, the reactions were terminated by spotting the supernatant on p81 phosphocellulose strips. Strips were washed three times in 75 mM phosphoric acid and the radioactivity was quantified by Cherenkov counting. Parallel immunoprecipitates were probed for FAK abundance to ensure comparable kinase mass in samples from control and DCA-treated cells.
**Immune complex phosphatase assay:** HCA-7 cells were treated with DCA for indicated times and immunoprecipitates of ShP1 and ShP2 were prepared as described above and washed three times with cold lysis buffer. Phosphatase activity was assayed by resuspending the final pellet in a total volume of 80 µl of reaction buffer (phosphatase buffer, pH 5.5, and containing 1mg/ml bovine serum albumin, 5 mM EDTA, 10mM dithiothreitol). The reaction was initiated by the addition of p-nitrophenyl phosphate (10mM, final concentration) for 30 min at 30°C. The reaction was stopped by the addition of 0.9 ml of 1N NaOH, and the absorbance of the samples was measured at 410 nm.

**Tyrosine phosphatase activity and FAK tyrosine dephosphorylation assay:** The Tyrosine phosphatase activity of GST-ShP2 (catalytic domain) was determined by hydrolysis of p-nitrophenyl phosphate in 80µl reaction mixture containing 50mM Hepes, pH 7.0, 20mM NaCl, 1 mM DTT, 10mM p-nitrophenyl phosphate and various amounts of the enzyme at 30°C for 10 min. The reaction was stopped by the addition of 80µl of 1M NaOH and the absorbance was measured at 410 nM. One unit of tyrosine phosphatase was defined as the amount of enzyme that produces 1 A₄₁₀ unit in this assay.

For FAK-dephosphorylation assay, the FAK was immunoprecipitated from preconfluent serum starved HCA-7 cells. Immunoprecipitates were incubated with different amounts of GST-ShP2 in 50mM Hepes, pH 7.0, 20mM NaCl, 1mM DTT in 80µl reaction volume at 30°C for 10 min as described above. After the reaction, immunoprecipitates were washed three times with lysis buffer and resolved by SDS-PAGE. FAK tyrosine dephosphorylation was then examined by Western blotting with an antiphosphotyrosine antibody (Py-20).
Antisense oligodeoxynucleotides transfection of HCA-7 cells: Biosynthesis, TX, synthesized specific antisense oligodeoxynucleotides (ODNs) for human ShP2. Sequences were as follows:

Antisense, CTCCGCGATGTCATGTTCCT; Sense, GAGGAACATGACATCGCGGA; Scrambled antisense TGGGTGTGTCCAAGAGAACT. The phosphorothioate modified ODNs were resuspended in sterile water at 100µM concentrations. To transfect, cells were grown to 50-70% confluency and then incubated with 1µM of oligodeoxynucleotides with oligofectamine (Invitrogen) transfection reagent for 24 hours. A day after transfection, cells were serum starved overnight and then treated with DCA.

FRNK Transfection of HCA-7 cells: A replication-deficient adenovirus (Adv) expressing GFP-tagged FRNK was constructed as previously described (27). The Adv was propagated in human embryonic kidney-293 cells (HEK-293) and purified by double CsCl centrifugation. As a control for nonspecific effects of adenoviral infection, and Adv expressing GFP alone (Adv-GFP) was used. Multiplicity of viral infection (MOI) was assayed by viral dilution in HEK-293 cells grown on 96 well plates. HCA-7 cells (50-70% confluent) were infected (10-100 MOI, 24hr) with either Adv-GFP or Adv-FRNK. Twenty-four hours after transfection cells were serum starved overnight and then treated with or without DCA (100µM, 1hr). Fluorescent microscopy and Western blotting was used to confirm expression of these constructs in colon cells.

ShP2 siRNA Transfection of HCA-7 cells: Validated synthetic siRNAs for ShP2 were purchased from Qiagen (Valencia, CA). HCA-7 cells were grown to attain approximately 50-70% confluence. Transfection of SiRNA was performed using HiPerFect transfection reagent (Qiagen) to achieve a final RNA concentration of 5 nM. Cells were either treated with HiPerFect (mock transfection) or siRNA. Twenty-four hours after transfection cells were serum starved
overnight and then treated with or without DCA (100µM, 1hr). Cells were also lysed in lysis buffer and Western blot analysis was performed using ShP2 antibodies.

Statistics: Data were expressed as means ±SD. All statistical analyses were performed by student’s t-test. Values of p < 0.05 were considered statistically significant.

Results

DCA inhibits integrin-mediated cell adhesion: DCA inhibited cell adhesion to collagen-I (Fig. 1a), fibronectin (Fig. 1b) and laminin (Fig. 1c). Maximum inhibition was observed at 100µM concentration. Further, adhesion of untreated HCA-7 cells on collagen-I (Fig. 1d), fibronectin (Fig. 1e), and laminin (Fig. 1f) was up regulated with dose-dependent increase in matrix concentration. However, similar adhesion kinetics was not observed in DCA-treated cells. Adhesion to poly-L-lysine, which does not require integrins, was not affected by DCA-treatment (Fig. 1g). We chose to study these matrix proteins because collagen-I represents the dominant collagen of the interstitial matrix beneath the basement membrane, laminin is the dominant non-collagenous constituent of the basement membrane itself, and serum fibronectin is a primary constituent of blood clots, which often form on bleeding mucosal lesions.

Viability of HCA-7 cells after DCA treatment: Cell viability of DCA-treated cells was assessed with the MTT colorimetric assay, and propidium iodide staining. In MTT assay, mitochondrial dehydrogenases of viable cells convert MTT to purple formazan crystals. There was no change in MTT conversion in cells treated with 100µM DCA for 3 hours (negative data not shown). Total cell death was also measured using propidium iodide, which indicates membrane integrity. Viable cells exclude the dye. There was no significant cell death after DCA (100µM, 3 hrs) treatment (negative data not shown).
DCA regulates FAK phosphorylation on specific residues: Focal adhesion complexes play an important role in the modulation of cell adhesion, which involves integrins. To investigate the mechanism by which DCA inhibits cell adhesion, we examined the effects of DCA stimulation on the phosphorylation status of the FAK, which is implicated in cell adhesion. After DCA (100µM) treatment, cell lysates were immunoprecipitated with anti-FAK antibody and further analyzed by Western blotting with an antiphosphotyrosine antibody. FAK was rapidly dephosphorylated in a time dependent manner (Fig. 2a). The membranes were stripped and re-probed with the FAK antibody to ensure that similar amounts of total FAK had been precipitated from each sample. In some experiments serum starved HCA-7 cells were rinsed with PBS and harvested with 2mM EDTA in PBS. Cells were gently detached, maintained in suspension for 30 min, and subsequently stimulated with DCA (100µM) for the indicated time periods. Cell lysates were immunoprecipitated with anti-FAK antibody and further analyzed by Western blotting with an antiphosphotyrosine antibody. In these conditions we observed only a weak phosphorylation of FAK in unstimulated cells compared to adherent cells. Further, DCA treatment, in suspension, had no significant effect on FAK phosphorylation (Fig. 2b). In order to determine whether DCA effect on FAK dephosphorylation is not restricted to a single cell line we utilized HT-29 cells, which also express high levels of FAK. DCA treatment significantly reduced FAK phosphorylation in a time dependent manner (Fig. 2c). As the kinase activities of FAK are modulated by its degree of tyrosine phosphorylation, dephosphorylation of FAK may lead to reduction of its kinase activities. However, FAK immunoprecipitated from cells treated with DCA (100µM, 30 minutes) showed no reduction in vitro autophosphorylation activity compared with FAK from untreated cells (negative data not shown). However, FAK precipitated from cells
stimulated with DCA showed reduction in kinase activity towards an exogenous substrate, poly (E4Y1) (Fig. 2d).

FAK can be tyrosine phosphorylated on a number of tyrosine residues, including Tyr-397, -925 and -576/577 in response to various stimuli. To map DCA-responsive phosphorylation sites on FAK, we utilized a series of phosphospecific antibodies. DCA rapidly dephosphorylated Tyr-925 and -576/577. However, DCA had little or no effect on the phosphorylation status of Tyr-397 (Fig. 3).

Tyr-397 is the major autophosphorylation site of FAK that associates with c-Src during integrin activation. We investigated c-Src association with FAK by utilizing phospho-active Src and native Src antibodies (Fig. 4). HCA-7 cells were treated with DCA (100µM, 30 minutes), FAK was immunoprecipitated and probed for phospho-active FAK (Tyr-397) or native Src or phospho-active Src (Tyr-418). DCA treatment significantly activated c-Src as assessed by increased tyrosine phosphorylation of Src at Tyr-418 (Fig. 4a). Src was constitutively associated with FAK as detected by co-immunoprecipitation analyses and there was no further increase in Src recruitment after DCA treatment (Fig. 4a). Src-family kinase inhibitor PP2 decreased the tyrosine phosphorylation of Src (Tyr-418) and also decreased the tyrosine phosphorylation of FAK (Tyr-397), detected by co-immunoprecipitation experiments (Fig. 4b).

FAK localizes to focal adhesions: To confirm the significance of DCA mediated changes in the tyrosine phosphorylation of FAK, we examined the existence of these events in vivo. HCA-7 cells were treated with DCA (100µM, 30 min) and FAK Tyr-397, and FAK Tyr-925 (phosphorylated forms of FAK) were analyzed by dual labeling immunofluorescence using polyclonal antibodies against phosphorylated forms of FAK (red color) and FITC-conjugated
phalloidin (green color) to identify f-actin filaments. In control cells, immunostaining of FAK Tyr-925 was predominantly co-localized with actin containing focal adhesion complexes at the cell-substratum interface. However, DCA (100µM, 30 min) caused a dramatic loss of staining intensity at focal adhesions (Fig. 5). Similar results were obtained by staining cells with FAK Tyr-576/577 (data not shown). Analysis of morphology of focal contacts revealed that control cells were anchored to the substratum by mature focal adhesion complexes that encircled the entire cell. In contrast, when the cells were activated with DCA small focal adhesion points were either accumulated at one pole of the cell or partially lost. However, DCA treatment had no effect on immunostaining of FAK Tyr-397 (Fig. 5).

DCA stimulates tyrosine phosphatase activity: To explore the mechanism and significance of FAK dephosphorylation by DCA, we treated HCA-7 cells with phenylarsine oxide (PAO), an inhibitor of protein tyrosine phosphatases (PTPases) (23). Cells were then treated with DCA (100 µM) for the indicated times. FAK was immunoprecipitated and blotted for phosphotyrosine as described above. Pretreatment of cells with PAO (10µM, 10 min) blocked the dephosphorylation of FAK by DCA (Fig. 6a). To determine the potential involvement of PTPases in DCA-mediated dephosphorylation of FAK, we analyzed the effect of DCA on phosphorylation status of ShP1 and ShP2, the SH2 domain containing PTPases. DCA treatment significantly increased tyrosine phosphorylation of ShP2 but not ShP1 in a time dependent manner (Fig. 6b). This suggests a relatively early involvement of ShP2 in DCA signaling.

Tyrosine phosphorylation of ShP2 has been correlated with its activation (67). Next, we assessed whether DCA stimulated the enzyme activity of ShP1 and ShP2. Immunoprecipitated ShP1 and ShP2 from control or DCA treated cells were assayed for phosphatase activity using pNPP as a substrate. A two to three fold increase in phosphatase activity was consistently observed.
in ShP2 immunoprecipitates and significant activity was detectable as late as 30 min after DCA stimulation (Fig. 6b). However, ShP1 activity was not significantly different.

**Increased association of ShP2 with FAK upon DCA stimulation:** The rapid and specific dephosphorylation of FAK upon DCA treatment and its inhibition by PTPase inhibitors are consistent with the involvement of PTPases in this process. ShP2 is a PTPase consisting of two Src homology (SH2) domains followed by a catalytic phosphatase domain. To determine whether ShP2 is associated with FAK, FAK was immunoprecipitated and immunoprecipitates were subjected to Western blotting with anti-ShP2 antibody. Stimulation with DCA significantly increased the association of ShP2 with FAK in a time dependent manner (Fig. 7).

**ShP2 dephosphorylates FAK in vitro:** To evaluate whether ShP2 was responsible for FAK tyrosine dephosphorylation in DCA-stimulated cells, we immunoprecipitated FAK from serum starved HCA-7 cells and incubated with various amounts of a GST fusion protein of the ShP2 catalytic domain. Incubation of FAK with GST-ShP2 fusion protein resulted in tyrosine dephosphorylation of FAK in a concentration dependent manner (Fig. 8a). The GST control protein had no apparent effect on FAK dephosphorylation in vitro. These results demonstrate that FAK is a substrate of ShP2.

We next examined whether FAK tyrosine dephosphorylation by ShP2, in vitro, resulted in loss of FAK-associated Src. FAK immunoprecipitates from serum starved HCA-7 cells were incubated with GST-ShP2 or with GST as a control. The immune complex was washed to remove proteins that had been dissociated from the immune complex. The immune complex was then analyzed by Western blotting for total Src and FAK. Incubation of FAK with ShP2, in vitro, did not affect Src binding to FAK (Fig. 8b). These results demonstrate that binding of Src to FAK is independent of FAK tyrosine dephosphorylation by ShP2.
Antisense ODN directed against ShP2 decreased ShP2 protein levels and attenuated DCA induced FAK dephosphorylation: To determine whether ShP2 was necessary for DCA induced FAK dephosphorylation we used anti-sense oligonucleotides approach to inhibit endogenous levels of ShP2 protein (49). A conventional pharmacological inhibitor that selectively targets ShP2 is currently not available. The use of an antisense ODN that selectively down-regulates the levels of ShP2 has been reported. HCA-7 cells were transfected with specific ShP2 antisense ODNs for 24 hours without serum to protect stability of ODNs. The ShP2 antisense ODN but not the nonsense ODN sequence significantly decreased the expression of ShP2 protein in HCA-7 cells (Fig. 9a). Antisense ODNs did not alter expression of a closely related phosphatase ShP1 (negative data not shown). The viability of cells assessed at this time always exceeded 90% and was not different from control cells, indicating that at concentration of 1µM the ODNs were non-toxic to the cells. For FAK-dephosphorylation experiments, cells were transfected with antisense ODNs as described above. After 24 hours, cells were treated with DCA (100µM, 60 min). FAK remained phosphorylated for up to 1 hr after stimulated with DCA in cells transfected with ShP2 antisense compared to control (Fig. 9b).

Manganese partially reversed the DCA-response on cell adhesion: To determine whether the DCA effect on cell adhesion is solely on integrin confirmation, integrins were fully activated by incubating cells with manganese. The Mn^{2+} cells adhered more than the cells without Mn^{2+}. Though, as compared to control, there was a partial reversal of the DCA effect on cell adhesion, DCA treatment of Mn^{2+} cells still demonstrated a significant reduction in cell adhesion (Fig. 10).

GFP-tagged FRNK decreased the FAK expression, activation and attenuated the DCA effect on cell adhesion: To determine whether FAK expression and activation are important determinants of DCA induced loss of adhesion, we utilized adenoviral (Adv) FRNK constructs. Infection of
HCA-7 cells with Adv-GFP-tagged FRNK (10-100 MOI) demonstrated a dose-dependent decrease in FAK expression and FAK Tyr-397 activation, with a maximum effect observed at 100 MOI (Fig. 11b) Further, FRNK-infection significantly decreased adhesion of HCA-7 cells on collagen-I and fibronectin (Fig. 11c) compared to GFP-expressing cells. As predicted, DCA significantly decreased cell adhesion in Adv-GFP-infected cells (control). However, DCA had no effect on cell adhesion of FRNK-infected cells (Fig. 11c).

**ShP2 siRNA abolished the DCA effect on cell adhesion:** Finally, we were prompted to determine whether ShP2 was necessary for the DCA induced loss of adhesion. siRNA directed gene silencing in colon cells decreased the ShP2 protein expression (Inset, Fig. 12). Total FAK expression (unrelated gene) was not altered in these cells (Inset, Fig. 12). Decrease in ShP2 level resulted in slight increase in basal cell adhesion of control cells. However, cell adhesion was not inhibited by DCA in ShP2 reduced cells compared to mock transfected cells (Fig. 12).

**Discussion**

The genesis of these studies was an initial observation that exposure of cultured HCA-7 colon cancer cells to DCA reduced the cell adhesion to extra cellular matrices. In an effort to better understand this phenomenon, we examined several aspects of FAK signaling that are believed to be involved in cell adhesion. Our results indicate that physiological concentrations of DCA dephosphorylated FAK on specific tyrosine residues and PTPase inhibitors block such dephosphorylation. Our data indicate that ShP2 may be one PTPase involved in this process. These results also demonstrated that DCA stimulation triggers FAK/ShP-2 association and FAK dephosphorylation was differentially regulated. Bile acids, end product of cholesterol metabolism, participate in fat digestion in the gastrointestinal tract. Majority of bile acids present in millimolar ranges during digestion are efficiently absorbed via both active and passive mechanisms by the
small intestine, and only a small fraction of bile acid escapes from enterohepatic circulation resulting in the bile acid concentrations in the cecum being in micromolar ranges (43). Previous reports have indicated no evidence of cytotoxicity when gastric mucosal cells were exposed to DCA (50-300µM)(57). Further, total concentrations of bile acids in the aqueous phase of stool ranges from 50-300µM and DCA is the main soluble bile acid ranging from 10-200µM (68). We first established what DCA treatments would not be toxic to the cells. As measures of toxicity, we assayed the cells ability to oxidize MTT and to exclude propidium iodide. For all these measures, doses of DCA employed in these studies caused no detectable toxicity to the cells, indicating that the results observed were not due to overt cytotoxicity.

Accumulating evidence suggests that the secondary bile acids are involved in the progression of colon cancer cells to a more invasive phenotype and that this may involve reorganization of cytoskeleton architecture. Here we investigated the effects of DCA-induced early signaling on the focal adhesion proteins FAK. Our findings suggest that DCA regulates the tyrosine phosphorylation of focal adhesion proteins, but not all tyrosine sites are targets of DCA signaling. DCA-induced dephosphorylation of FAK depends on integrin arrangement, since it does not occur in cells that are in suspension.

FAK co-localizes with integrins in focal adhesions. Tyrosine phosphorylation of FAK and its catalytic activity stimulation is dependent upon integrin mediated cell adhesion. FAK contains large N- and C-terminal domains that flank the catalytic domain. At the amino-terminus of FAK is a FERM domain (41). Between the FERM and catalytic domains is Tyr-397, which is the major autophosphorylation site of FAK. On its activation, Tyr-397 is a docking site for the SH2 domains of a number of proteins including Src (12), p85 subunit of PI3K (10), phospholipase C-γ
and Grb7 (25). In DCA treated cells phosphorylation at FAK autophosphorylation site Tyr-397 was preserved, however, it induced dephosphorylation of FAK at Tyr-576, -577 and -925. Further, FAK immunoprecipitated from cells treated with DCA showed no change in autophosphorylation activity compared with FAK from untreated cells. We further investigated co-association of Src with FAK in DCA stimulated cells. Results demonstrated that Src was constitutively associated with FAK. DCA treatment significantly activated c-Src as assessed by increased tyrosine phosphorylation of Src at Tyr-418 with no further increase in Src recruitment after DCA treatment. Previous studies have demonstrated that the interaction of FAK with the Src SH2 domain renders the autophosphorylation site of FAK resistant to the action of tyrosine phosphatases (12). In our studies *in vitro* experiments also demonstrated that binding of Src to FAK is independent of FAK tyrosine dephosphorylation by ShP2. In HCA-7 cells, therefore, constitutive association of Src with FAK and activation of Src upon DCA treatment may explain preservation of the Tyr-397 phosphorylation despite Tyr-576/577 and Tyr-925 dephosphorylation in the presence of DCA, a unique FAK/Src regulation by tumor promoter bile acid. Further, the c-Src/FAK relationship appears to be reciprocal as FAK phosphorylation on Tyr-397 is required for c-Src binding to FAK SH domain. In hepatic cells, FAK/c-Src association is adhesion dependent (8), while in human colon cancer cells, increased c-Src activity disrupts adhesion (31). Similarly in our studies, increased c-Src activation by DCA may be responsible for decreased cell adhesion.

Two phosphorylation sites in the activation loop of the catalytic domain, tyr-576, and -577, are regulatory sites of phosphorylation that enhance catalytic activity. At the carboxyl-terminus of FAK is a 150-amino acid focal adhesion-targeting (FAT) domain. This domain is essential for localization of FAK to sites of cell-ECM adhesion (13). Tyr-925, in the FAT domain of FAK,
can bind to SH2 domain of Grb-2 and presents one of the mechanisms of activation of the Ras/MAPK pathway (60). DCA selectively dephosphorylated FAK at Tyr-576, -577 and -925 and reduced FAK kinase activity towards a model, exogenous substrate (E4Y1). However, FAK tyrosine phosphorylation at the Tyr-397 autophosphorylation site was preserved. The dissociation of FAK autocatalytic activity from its activity against exogenous substrates is consistent with what is known about the role of the Tyr-576/-577 phosphorylation sites in the regulation of FAK-dependent signaling (59). FAK phosphorylates itself at Tyr-397 during integrin engagement and clustering, which provides a docking site for Src family kinases to bind to FAK via their SH2 domains. Src then phosphorylates FAK at multiple sites, including Tyr-576 and -577 within the catalytic subdomain VIII present in many different protein tyrosine kinases. FAK autophosphorylation at Tyr-397 is thus required for adhesion-dependent Tyr-576/-577 phosphorylation of FAK by Src, which in turn increases FAK catalytic activity at both the Tyr-397 site, as well as towards exogenous substrates (58). Calalb et al. (6) have previously shown that Y→F mutations at Tyr-576 and -577 reduced FAK kinase activity approximately 50% against a similar, model substrate (E4Y1), which is similar to the degree of inhibition in extrinsic kinase activity observed in immunoprecipitates following mutation of the Tyr-397 autophosphorylation site. Although the physiological significance of decreased Tyr-576/-577 phosphorylation on the phosphorylation of endogenous substrates (other than FAK itself) remains unknown, ShP2-dependent dephosphorylation of Tyr-576/-577 would be expected to reduce downstream signaling, in a fashion similar to the effect of dephosphorylation or mutation of catalytic subdomain VIII sites on other protein tyrosine kinases (19).

Since tyrosine phosphorylation of FAK regulates its catalytic activity and association with other signaling molecules, dephosphorylation of tyrosine residues is potentially a very important
mechanism in regulating FAK signaling in DCA treated cells. The fact that DCA differentially dephosphorylated tyrosine residues of FAK raises the possibility that distinct protein tyrosine phosphatases may be responsible for dephosphorylation of different sites. Since different phosphorylation sites function to regulate catalytic activity and protein-protein interactions, site-specific dephosphorylation of FAK by DCA may be an effective mechanism to modulate some aspects of FAK signaling independently from others. A number of PTPase have been suggested as regulators of FAK, including PTP-PEST (62), PTP1B (35), ShP2 (44), and PTEN (24). Experiments with the tyrosine phosphatase inhibitor PAO support the involvement of tyrosine phosphatases in DCA-induced cytoskeleton signaling. DCA triggered tyrosine phosphorylation of ShP-2 and its association with FAK. To test whether ShP2 was necessary for DCA-stimulated FAK dephosphorylation, we decreased ShP2 protein levels via the use of antisense methodology. We found that antisense ODN but not control ODN significantly attenuated DCA-stimulated FAK dephosphorylation.

Conformational changes in integrins have been suggested to underlie the modulation of their affinity for ECM components. If DCA treatment did indeed inhibit cell adhesion by converting integrins into an inactive confirmation, then treatment of cells with manganese, which uniformly activates integrins by inducing an active confirmation (21), should be able to reverse the inhibitory effects. Manganese increased the adhesion of control cells, presumably by binding extracellular domains directly and altering integrin-binding affinity. Previous studies have shown that Ca\(^{+2}\) decreased colon cancer cell adhesion and that both Mg\(^{+2}\) and Mn\(^{+2}\) increased cell adhesion (18, 66). Earlier studies in several other cell types have reported that Mn\(^{+2}\) enhances the adhesive properties of many integrins (16, 64). Small changes in divalent cation concentration can substantially stimulate or inhibit integrin binding under physiological conditions (66).
Further, the affinity of integrins for their extracellular ligands can also be regulated from within the cells through intracellular signaling that may be mediated through integrin cytoplasmic domains (36). In our investigations, addition of manganese to medium didn’t completely reverse the inhibitory effects of DCA on cell adhesion, indicating a possible inside-out signaling upon DCA treatment.

Although outside-in signaling pathways from integrin to the nucleus have been well studied, inside-out pathways for cell adhesion have received little attention. Many intracellular pathways can control inside-out regulation of integrin functions (30). Such inside-out signaling could be mediated by FAK activation (32, 50). More recently, extracellular pressure promoted the colon cancer cell adhesion via actin dependent inside-out FAK and Src signals and FAK inhibition by FRNK transfection prevented the effect of pressure on cell adhesion (65). We also demonstrated that GFP-FRNK, the C-terminal non-catalytic domain of FAK that competes with FAK for localization in focal adhesions and interferes with FAK signaling, significantly inhibited FAK expression and tyrosine phosphorylation and reduced cell adhesion. However, the inhibitory effect of DCA on cell adhesion was not observed in FRNK infected cells, suggesting that FRNK and DCA interfere with cell adhesion via the same signal transduction pathway. These results also point towards a possible inside-out signaling mechanism by DCA in these cells. Further, tyrosine phosphatase ShP2 was responsible for DCA induced FAK dephosphorylation in colon cells. Moreover, SiRNA directed ShP2 reduction in these cells activated colon cancer cell adhesion and abolished the DCA effect on cell adhesion. The increased cell adhesion in ShP2 reduced cells and the absence of inhibitory effect of DCA could be due to elevated levels of phosphorylated FAK in the absence of ShP2, as observed in antisense experiments.
Several receptor tyrosine kinases have been reported to reduce tyrosine phosphorylation of FAK, including the EGFR (37), Insulin receptor (2), IGF-1R (40) and EphA2 (44). Since bile acids have no known transmembrane receptors, trans-activation of receptor tyrosine kinases by DCA may be responsible for reduced tyrosine phosphorylation and catalytic activity of FAK. In this regard, DCA has been implicated in ligand independent activation of EGFR and insulin receptor but not IGF-1R in primary rat hepatocytes (26). Further, bile acid transporters have not been identified in the colon and labeled bile acids are not taken up by colon cancer cells, in vitro, (53). It is presumed that these amphipathic molecules exert their effects by perturbing plasma membrane structure and function. DCA is known to deplete cholesterol from plasma membranes (28). A number of key signaling molecules, including EGFR and Ras, partition into cholesterol-rich lipid rafts within the plasma membrane (45, 54). Cholesterol depletion has been shown to activate EGFR and Ras signaling in other cells (11).

Our results define a new signaling pathway, originating with DCA-induced FAK dephosphorylation. In summary, our results suggest that DCA differentially regulates signaling from focal adhesion complexes through selective phosphorylation/dephosphorylation or through association of participating components and that these regulatory events may have distinct roles in diverse physiological and disease processes including inflammation, wound healing and cancer progression.
References:


**Figure Legends**

**Fig. 1. DCA inhibits integrin-mediated cell adhesion.** 96 well plates coated with 10µg/ml collagen-I (a), fibronectin (b), and laminin (c). Serum starved HCA-7 cells were treated with DCA (10-125µM, 1hr) and detached with 2mM EDTA. Cells (5x10^4/well) were then plated on matrix-coated 96 well plates and after 30 min adherent cells were stained with crystal violet. Values represent mean A_{550} of the extracted dye from triplicate wells. Results are the means ±SD of three independent experiments. *p<0.05 compared with control. Plates were also coated with serially diluted (0.05-5µg/ml) collagen-I (d), fibronectin (e), laminin (f). Control untreated (filled square) and DCA treated (100µM, 1hr, open diamond) cells (5x10^4/well) were plated and cell adhesion was calculated as described above. Results represent mean ±SD of two independent experiments in triplicate. Plates were also coated with 10µg/ml poly-l-lysine (g). Control and DCA treated (100µM, 1hr) cells (5x10^4/well) were plated and cell adhesion was calculated as described above. Results are the means ±SD of three independent experiments in triplicate.

**Fig. 2. DCA induces FAK dephosphorylation.** (a) Serum starved HCA-7 cells were stimulated with vehicle or DCA (100µM) for the indicated times and lysed. FAK was immunoprecipitated and blotted sequentially for phosphotyrosine (pY, upper panel) and, after stripping, for FAK (lower panel). The results shown are representative of three independent experiments. (b) Serum starved HCA-7 cells were detached, maintained in suspension for 30 min and subsequently stimulated with DCA (100µM) for the indicated times and lysed. FAK was immunoprecipitated and blotted sequentially for phosphotyrosine (pY, upper panel) and, after stripping, for FAK (lower panel). The results shown are representative of three independent experiments. (c) Serum starved HT-29 cells were stimulated with DCA (100µM) for the indicated times and lysed. FAK was immunoprecipitated and blotted sequentially for phosphotyrosine (pY, upper panel) and, after stripping, for FAK (lower panel). The results shown are representative of two independent experiments. (d) Reduced FAK kinase activity towards an exogenous substrate, poly (E4Y1).
Cells were stimulated with DCA (100µM) for 30 min, lysed and immunoprecipitated. FAK kinase activity towards an exogenous substrate, poly (E4Y1) was assayed as described in methods. Values were expressed as % maximal and given as Means ±SD of three independent experiments. *p<0.05 compared with control.

Fig. 3. **DCA differentially regulates tyrosine phosphorylation of selective residues on FAK.** HCA-7 cells were serum starved and treated with DCA (100µM) for the indicated times and cell lysates were analyzed by immunoblotting with site-specific phosphotyrosine antibodies against FAK. Blots were also stripped and reprobed with antibodies, which recognize total FAK. The results shown are representative of three independent experiments.

Fig. 4. **FAK is constitutively associated with c-Src.** (a) DCA activates c-Src in HCA-7 cells. Serum starved cells were treated with DCA (100µM, 30 min). FAK was immunoprecipitated and probed with antibody specific to active FAK (FAK Tyr-397) or Src (Src Tyr-418) or native Src. Blots were stripped and reprobed for total FAK. (b) PP2 inhibits Src and FAK phosphorylation. Serum starved cells were pre-treated with PP2 (30µM, 15min) and then stimulated with DCA as described above. FAK was immunoprecipitated and probed with antibody specific to active FAK (FAK Tyr-397) or c-Src (Src Tyr-418). Blots were stripped and reprobed for total FAK. The results shown are representative of three independent experiments.

Fig. 5. **DCA affects the status and localization of FAK to focal adhesions.** HCA-7 cells were treated with DCA (100µM, 30 min). Cells were fixed and FAK was analyzed by confocal microscopy after dual-labeling immunofluorescence using FITC-conjugated phalloidin (green color), as a marker actin filaments and rabbit pAb against FAK Tyr-925 and FAK Tyr-397 (red color). Yellow color indicates colocalization of actin with FAK. All images were taken with identical laser and microscope settings (1µm optical sections obtained at the cell-substratum interface). Note that in control serum starved cells FAK Tyr-925 and Tyr-397 staining co-localized predominantly to actin-containing dots. Control cells were
anchored to the substrate by mature focal adhesion points. There was a dramatic loss of staining intensity corresponding to phosphorylated form of FAK Tyr-925 and cells displayed dynamic, immature dot-like focal adhesion site remnants. However, FAK Tyr-397 staining was relatively unaffected.

**Fig. 6. DCA stimulates tyrosine phosphatase activity.** (a) Inhibition of PTPase activity diminishes FAK dephosphorylation. HCA-7 cells were pre-incubated for 10 min with DMSO (vehicle), or 10µM Phenylarsine oxide (PAO), a PTPase inhibitor. Cells were then treated with 100 µM DCA for indicated time periods. FAK was immunoprecipitated and blotted sequentially for phosphotyrosine (pY, upper panel) and, after stripping, for FAK (lower panel). The results shown are representative of three independent experiments. (b) DCA induces tyrosine phosphorylation of ShP2 in HCA-7 cells. Cells were treated with DCA (100µM) for the indicated times and cell lysates were immunoprecipitated with anti-ShP1 and -ShP2 antibodies and Western blotted for phosphotyrosine (insert). DCA stimulates enzyme activity of ShP2. Cells were treated with 100µM DCA for 30 min as described above. ShP1 and ShP2 was immunoprecipitated from total lysates and analyzed for phosphatase activity as described in methods. Phosphatase activity was expressed as the percentage of activity in the control untreated cells. Results are the means ±SD of three independent experiments. *p<0.05 compared with control.

**Fig. 7. DCA treatment increases association of ShP2 with FAK.** HCA-7 cells were stimulated with DCA (100µM) for the indicated periods of time. FAK was immunoprecipitated and probed for ShP2. The results shown are representative of three independent experiments.

**Fig. 8. ShP2 dephosphorylates FAK in vitro.** (a) FAK was immunoprecipitated from serum starved HCA-7 cells and incubated with different amounts of GST fusion protein of the ShP2 PTPase domain (ShP2-PTPase) as described under methods. After the reaction the immune complex was washed and subjected to immunoblotting analyses with anti Py-20 antibodies for tyrosine dephosphorylation of FAK (upper panel). The blot was reprobed with anti-FAK antibody (lower panel). The graph represents the average of two experiments. (b) FAK was immunoprecipitated from serum starved HCA-7 cells and
incubated with GST-ShP2 (8U ShP2-PTPase) as described above. The immune complex was washed and the analyzed by immunoblotting for c-src (upper panel) and total FAK (lower panel). The results shown are representative of three independent experiments. U, units.

**Fig. 9. Antisense ShP2 prevent tyrosine dephosphorylation of FAK.** (a) Antisense (AS) oligodeoxynucleotides directed against ShP2 decreases ShP2 protein expression. Cells were treated with 1µM ShP2 antisense (AS), sense (SS) and nonsense (NS) oligodeoxynucleotides or medium (Control, Ct) for 24 hours. ShP2 protein levels were measured by Western blotting (upper panel). Blots were also re-probed with β-actin (lower panel). (b) Antisense (AS) oligodeoxynucleotides directed against ShP2 prevent tyrosine dephosphorylation of FAK by DCA. Cells were transfected with oligodeoxynucleotides as described above and treated with DCA (100µM, 60 min). Cell lysates were immunoprecipitated with anti-FAK and probed with anti-phosphotyrosine antibodies (upper panel). Blots were also re-probed with anti-FAK (lower panel). The results shown are representative of three independent experiments.

**Fig. 10. Mn^{2+} partially prevents the DCA effect on cell adhesion.** Figure represents the adhesion of DCA treated HCA-7 cells compared with respective controls with or without 1 mM Mn^{2+}. Serum starved HCA-7 cells were treated with DCA (100µM, 1hr) with or without 1mM Mn^{2+}. After EDTA detachment, cells (5x10^4/well) were plated on 96 well plates coated with 10µg/ml collagen-I and after 30 min cell adhesion was estimated as described before. Results are the means ±SD of three independent experiments in triplicate. *p<0.05 compared with respective controls. **p<0.05 compared with cells without Mn^{2+} treatment.

**Figure 11. FAK suppression prevents the DCA effect on cell adhesion.** Replication-deficient adenoviruses (Adv) expressing GFP or GFP-tagged FRNK were propagated in HEK-293 cells and purified by double CsCl centrifugation. Multiplicity of viral infection (MOI) was assayed by viral dilution in HEK-293 cells grown on 96 well plates. Then HCA-7 cells (50-60% confluent) were infected
(10-100 MOI, 24hr) with either Adv-GFP or Adv-FRNK. (a), Fluorescent microscopy of GFP and GFP-FRNK expressing HCA-7 cells (100 MOI, 24hr) (b), Cell lysates were separated by SDS-PAGE and probed with a polyclonal antibody to the C-terminal domain of FAK (which recognizes both FAK and FRNK, upper panel) and a polyclonal antibody specific to active FAK (FAK Tyr-397, lower panel). Note a significant reduction of total FAK expression and activation at 100 MOI. (c), Twenty-four hours after infection (100 MOI) cells were serum starved, treated with DCA (100µM, 1hr) and detached with 2mM EDTA. Cells (5x10^4/well) were then plated on collagen-I or fibronectin coated 96 well plates and after 30 min adherent cells were stained with crystal violet. Results are the means ±SD of two independent experiments in triplicate *p<0.05 compared with GFP alone.

**Figure 12.** *ShP2 down-regulation by siRNA abolishes the DCA effect on cell adhesion.* Transfection of siRNA decreases ShP2 protein expression. Cells were treated with 5nM siRNA or HiPerFect transfection reagent alone for 24 hours. ShP2 protein levels were measured by Western blotting (Inset). Blots were also re-probed with total FAK to confirm any nonspecific decrease of an unrelated gene. Cells were transfected with ShP2 siRNA as described above and serum starved cells were treated with DCA (100µM, 1hr). After EDTA detachment, cells (5x10^4/well) were plated on 96 well plates coated with 10µg/ml collagen-I and after 30 min cell adhesion was estimated as described before. Results are the means ±SD of two independent experiments in triplicate. *p<0.05 compared with control.
Fig. 1
Fig. 2

(a) Ct 7.5 15 30 60

(b) Ct 7.5 15 30 60

DCA (min)

FAK activation (% Maximal)

(c) Ct 7.5 15 30 60

DCA (min)

pY - FAK

Total FAK

(d) FAK Activation (% Maximal)

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Ct

DCA
Fig. 3

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<td>Total FAK</td>
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DCA (min)
Fig. 4

(a)

(b)

Ct  Ct  DCA  DCA  

FAK - 397

Src - 418

Total Src

Total FAK

Ct  DCA  DCA  

FAK - 397

Src - 418

Total FAK

PP2

Page 37 of 45
Fig. 6
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**Fig. 7**

- **ShP2**
- **IgG**

- **DCA (min)**
Fig. 8
Fig. 9
Fig. 10
Fig. 11
Fig. 12

Cell Adhesion (% Maximal)

- Ct
- DCA
- Ct siRNA
- DCA siRNA

* Shp2
* FAK