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Loss of Tyrosine Phosphatase Dependent Inhibition Promotes Activation of Tyrosine Kinase c-Src in Detached Pancreatic Cells

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Abstract

Despite an intense focus on novel therapeutic strategies, pancreatic adenocarcinoma remains one of the deadliest human malignancies. The frequent and rapid mortality associated with pancreatic cancer may be attributed to several factors, including late diagnosis, rapid tumor invasion into surrounding tissues, and formation of distant metastases. Both local invasion and metastasis require disruption of tumor cell contacts with the extracellular matrix. Detachment of normal cells from the extracellular matrix leads to a form of programmed cell death termed anoikis. Pancreatic cancer cells avert anoikis by activation of signaling pathways that allow for adhesion-independent survival. In the present studies, cellular signaling pathways activated in detached pancreatic cancer cells were examined. We demonstrate a rapid and robust activation of Src kinase in detached pancreatic cancer cells, relative to adherent. Src autophosphorylation rapidly returned to baseline levels upon reattachment to tissue culture plastic, in the presence or absence of specific extracellular matrix proteins. Treatment of pancreatic cancer cells with tyrosine phosphatase inhibitors increased steady-state Src autophosphorylation in adherent cells and abrogated the detachment-induced increase in Src autophosphorylation. Src was found to co-immunoprecipitate with the Src Homology 2 (SH2) domain containing protein tyrosine phosphatase (SHP-2) in pancreatic cancer cells, suggesting that SHP-2 may participate in regulation of Src autophosphorylation in adherent cells. Src family kinase (SFK) dependent increases in Akt and Jun N-terminal kinase (JNK) phosphorylation were observed in detached cells, indicating the potential for Src-dependent activation of survival and stress pathways in pancreatic cancer cells that have detached from the extracellular matrix.

Keywords

carcinoma; focal adhesion kinase; p130 CAS; paxillin; anoikis; cell stress

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a devastating illness, currently ranked as the fourth leading cause of cancer-related death in the United States. Despite years of intense research, the five-year survival rate stands at less than 5% [1]. The reasons for this grim statistic are multi-fold and include a lack of obvious symptoms during the early stages of the disease, rapid acquisition of tumor resistance to standard treatment options (radiation and chemotherapy), and rapid tumor progression to an invasive and metastatic phenotype [2–4].

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Approximately 80% of pancreatic tumors have metastasized or are not amenable to surgery at the time of diagnosis [4]. The current standard of care for pancreatic cancer patients is treatment with the nucleoside analog gemcitabine, which only offers a modest survival benefit [5]. It is clear that there is an urgent need for improved treatment strategies and novel molecular targets to combat this deadly malignancy.

Several studies in recent years have focused on the role of the non-receptor tyrosine kinase c-Src in pancreatic cancer progression and metastasis. C-Src (Src) is a ubiquitously expressed 60 kDa member of the Src family of non-receptor tyrosine kinases. Src family kinases are essential components of a variety of signal transduction pathways, regulating diverse cellular activities [6–8]. Visser and co-workers demonstrated elevated Src expression and kinase activity in pre-neoplastic pancreatic lesions in a rat model [9]. Lutz and co-workers later observed elevated expression and activity of Src in pancreatic cancer tissue samples and cell lines, relative to normal pancreatic tissue and cells, respectively [10]. Many cell surface receptors and adhesion proteins have been demonstrated to require Src family kinases for signal transduction, including the epidermal growth factor receptor (EGFR) [11], the insulin-like growth factor receptor (IGFR) [12], c-Met [13], cholecystokinin 2 receptor (CCK2R) [14,15], carcinoembryonic antigen cell adhesion molecule 6 (CEACAM6) [16,17], and integrins (alpha(v)beta(3), alpha(v)beta(5), and beta(1)) [18,19]. Src is a key regulator of multiple aspects of pancreatic cancer cell biology, including cell proliferation [12,20], anchorage-independent growth [18], adhesion to extracellular matrix components [21], invasion [22], production of pro-angiogenic cytokines (vascular endothelial growth factor (VEGF) and interleukin (IL)-8) [11,12,23], and resistance to gemcitabine and 5-fluorouracil (5FU)-mediated cellular cytotoxicity [24–26]. Multiple *in vivo* studies have demonstrated that inhibition of Src expression or kinase activity abrogates the angiogenesis and metastasis of orthotopic pancreatic tumors and the growth of human pancreatic tumor xenografts in mouse models [20,25,27–30]. Taken together, these data indicate the potential value of Src as a novel therapeutic target for the treatment of pancreatic cancer.

Our previous studies have focused on the importance of Src activation for progression and metastasis of human pancreatic cancer. A hallmark characteristic of cancerous and, especially, metastatic cells is their ability to survive and proliferate in the absence of attachment to the extracellular matrix. Upon detachment from the extracellular substratum, cells cease dividing and eventually undergo a form of programmed cell death known as anoikis [31]. In many cancer model systems, including colon [32], prostate [33], lung [34], and osteosarcoma [35], Src activation plays a key role in allowing detached cancer cells to subvert anoikis, yet mechanisms of this activation remain poorly understood. In the present studies, we have examined the regulation of Src autophosphorylation in adherent and detached pancreatic cancer cells. We have demonstrated a rapid and sustained increase in Src autophosphorylation upon detachment of pancreatic cancer cells from the extracellular substratum. Furthermore, we present data herein suggesting that the increase in Src autophosphorylation may be due in part to alleviation of protein tyrosine phosphatase-mediated downregulation of Src autophosphorylation. Src activity in detached cells was shown to be important for activation of Akt and Jun N-terminal Kinase (JNK), suggesting the importance of Src or Src family kinases for activation of survival and stress pathways in detached pancreatic cancer cells. Together, these data suggest at least one mechanism by which Src may contribute to the metastatic progression of human pancreatic cancer cells.

MATERIALS AND METHODS

Cell Culture and Reagents

The PANC-1 and L3.6pl cell lines were a generous gift from Dr. Isaiah Fidler (University of Texas-M. D. Anderson Cancer Center, Houston, TX). The Hs766T, and BxPC3 cell lines were obtained from ATCC (ATCC, Manassas, VA). The L3.6pl cell line was selected for its ability to form liver metastases when grown in nude mice. L3.6pl cells were derived from a repeated cycle of injecting COLO 357 pancreatic cancer cells into the pancreas of nude mice, selecting liver metastases, and re-injecting these into the pancreas [36]. Cells were maintained in DMEM or MEM supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 2 mM L-glutamine (Gibco Life Technologies, Grand Island, NY), and 0.6% penicillin/streptomycin (Gibco).

Plasmids

Plasmid-encoded RNA interference (RNAi) sequences were utilized to downregulate SHP-2 expression in pancreatic cancer cells through the Invitrogen BLOCK-iT POL II miR RNAi expression vector system (Invitrogen, Carlsbad, CA). Pre-validated oligonucleotides (Hmi414198 PTPN11 and Hmi414199 PTPN11) harboring sequences designed to produce microRNA (miRNA) specific for the human SHP-2 gene (PTPN11), as well as non-targeted control oligonucleotides, were obtained from Invitrogen (sequences available through the manufacturer). Single stranded oligonucleotide pairs were annealed and ligated into the pcDNA 6.2-GW+EmGFP vector as per the manufacturer's protocol. Plasmids encoding the Hmi414198 PTPN11 and Hmi414199 PTPN11 sequences were designated SHP-2 RNAi 1 and SHP-2 RNAi 2, respectively. Pre-validated RNAi oligonucleotide sequences (s11507 and s11508) targeting the protein tyrosine phosphatase 1B (PTP1B) gene were also obtained from Invitrogen. These sequences are referred to as PTP1B RNAi 1 (s11507) and PTP1B RNAi 2 (s11508) throughout the text. All plasmids were transfected via Lipofectamine (Invitrogen). RNAi oligonucleotides were transfected via Lipofectamine RNAiMAX (Invitrogen). All transfections were carried out according to the manufacturer's protocol. Cells were lysed 48 hours after transfection, as described below.

Cell Detachment Assays

Cells were grown to 80% confluence in standard tissue culture medium. Cells were then harvested by trypsinization (.05% trypsin-EDTA, Hyclone) or 2 mM EDTA (Sigma-Aldrich, St. Louis, MO). Trypsinized cells were subjected to neutralization with soybean trypsin inhibitor (Sigma-Aldrich). Cells were washed with Dulbecco's phosphate-buffered saline (DPBS) (Mediatech, Manassas, VA), pelleted by centrifugation at 2,000 rpm for 5 min, and resuspended in 10 ml cell culture media. Resuspended cells were placed in 15 ml conical centrifuge tubes and capped with vented lids. Cells were then placed in a rotator and incubated at 37 °C and 5% CO₂ for the indicated lengths of time. After incubation for the appropriate time, cells were either lysed in Nonidet P-40 (NP-40) buffer or re-plated on standard tissue culture dishes, or dishes pre-coated with poly-D-lysine, collagen I, fibronectin, or laminin for the indicated lengths of time prior to lysis. Pre-coated tissue culture dishes were obtained from BD Biosciences (BD Biosciences, San Jose, CA). Cell detachment assays were performed in the presence or absence of the Src family kinase inhibitor AP23846 (ARIAD pharmaceuticals, Cambridge, MA) [37], the broad-spectrum tyrosine phosphatase inhibitor sodium vanadate (Sigma-Aldrich), or the SHP-2 selective phosphatase inhibitor NSC-87877 (EMD Biosciences, San Diego, CA). For experiments in which inhibitors were used, AP23846, sodium vanadate, and NSC-87877 were added at the indicated concentrations (diluted from 10 mM stocks in DMSO for AP23846 and NSC-87877 or H₂O for sodium vanadate) for one hour (3 hours for NSC-87877) prior to lysis or detachment. Cells in suspension were maintained at the appropriate concentration of

inhibitor throughout the detachment period. For 0 μ M concentrations, DMSO or H₂O was added to resuspended cell cultures at a volume equivalent to that of the respective inhibitor.

Western Blot Analysis

Adherent cells were washed twice with ice-cold tris-buffered saline (TBS), followed by lysis for 10 min in NP-40 buffer (150 mM NaCl, 5 mM EDTA, 1% NP-40, pH 7.4), supplemented with one tablet complete mini-EDTA-free protease inhibitor cocktail (Roche Diagnostic, Mannheim, Germany), and sodium orthovanadate (1 mM, pH 7.4). Cells were scraped from plates, and cell lysates were clarified by centrifugation for 5 min at $13,000 \times g$ and 4 °C. Cells in suspension were centrifuged for 5 min at $800 \times g$ and 4 °C. Cells were washed with 10 ml cold TBS, re-pelleted, and resuspended in 450 μ l NP-40 lysis buffer. After 10 min, cells were drawn up and down 5 times through a 29-gauge needle. Cell lysates were transferred to a 1.5 ml centrifuge tube and clarified by centrifugation for 5 min at $13,000 \times g$ and 4 °C. Total protein concentration was determined via the Pierce Bicinchoninic Acid (BCA) protein assay (Thermo Fisher Scientific, Rockford, IL), followed by spectrophotometric analysis using the BioTek Elx-808 plate reader and KC4 software (BioTek Instruments, Winooski, VT). Thirty-five μ g of total cell protein were separated via 8% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA), and blocked for 30 min at room temperature with 5% non-fat milk/TBS-Tween (TBS-T) or overnight at 4 °C in 5% bovine serum albumin/TBS-T for anti-phosphotyrosine western blot analysis. Primary antibodies were diluted 1:1000 in blocking buffer and incubated overnight at 4 °C with gentle rocking. Horseradish peroxidase (HRP)-conjugated goat anti-mouse and sheep anti-rabbit secondary antibodies (Bio-Rad, Hercules, CA) were diluted 1:2000 in 5% milk/TBS-T and incubated for one hour at room temperature with gentle rocking. Proteins were visualized by incubation with Pierce SuperSignal Chemiluminescent Reagents (Thermo Fisher Scientific) and exposure to film (Kodak Biomax MR, Rochester, NY). Blots were scanned and quantitation was performed using the Kodak Molecular Imaging Software (v5.0.2) (Eastman Kodak, Rochester, NY). Statistical analysis was performed via Student's t test in Microsoft Excel (Microsoft Corporation, Redmond, WA).

Immunoprecipitations

Total cell protein (500 μ g) was normalized to a total volume of 650 μ l with NP-40 buffer and rotated with 6 μ l of anti-Src monoclonal antibody overnight at 4 °C. The next day, 50 μ l of a 1:1 slurry of protein G agarose (Millipore) in NP-40 buffer was added and incubated for an additional one hour with rotation at 4 °C. Bound proteins were pelleted by centrifugation, washed three times with NP-40 buffer, and eluted by boiling in 1 $\times$ Laemmli's sample buffer. Bound proteins were subjected to western blot analysis as described above.

Antibodies

The anti-Src monoclonal antibody (327) and the anti-p42/44 Erk MAPK polyclonal antibody were obtained from Calbiochem/Oncogene Research Products (EMD Biosciences). The anti-phospho-Src^{Y418} polyclonal, anti-phospho-Src^{Y529} polyclonal, anti-Src monoclonal (L4A1), anti-phospho-Akt^{S473} polyclonal, anti-Akt polyclonal, anti-phospho-Erk-1/2^{T202/Y204} polyclonal, anti-phospho-p38^{T180/Y182} polyclonal, anti-phospho-JNK^{T183/Y185} polyclonal, anti-JNK polyclonal, anti-phospho-p130 Cas^{Y165} polyclonal, anti-phospho-SHP-2^{Y542} polyclonal, and anti-SHP-2 polyclonal antibodies were obtained from Cell Signaling Technology (Cell Signaling Technology, Beverly, MA). The anti-phospho-FAK^{Y397} polyclonal antibody was obtained from Biosource (Invitrogen, Carlsbad, CA). The anti-FAK, anti-phospho-paxillin^{Y31}, anti-paxillin, and anti-p130 Cas polyclonal antibodies were obtained from Santa Cruz Biochemicals (Santa Cruz Biochemicals, Santa Cruz, CA). The anti-Yes monoclonal antibody was obtained from Wako Chemicals (Wako Chemicals,

Richmond, VA). The anti-total phosphotyrosine monoclonal antibody 4G10 was obtained from Millipore.

RESULTS

Src is Activated in Detached Pancreatic Cancer Cells

To gain initial insight into the regulation and signaling capacity of Src in pancreatic cancer cells detached from the extracellular substratum, we evaluated the activity of Src from pancreatic cancer cells in suspension, relative to adherent cells grown in standard tissue culture dishes. L3.6pl pancreatic cancer cells were lysed at 80% confluence or detached from the substratum by trypsinization. Detached cells were resuspended in tissue culture medium and rotated over a time course (0, 5, 10, 20, 60, 120 min) in a tissue culture incubator. Rotated cells were either lysed or re-plated into 100 mm tissue culture dishes. Re-plated cells were lysed after 30, 60, or 90 min of reattachment. Src activity was assessed via autophosphorylation at Tyr⁴¹⁸, a well-established indicator of Src kinase activity [38,39]. Total Src was immunoprecipitated from whole cell lysates, and Src^{Y418} phosphorylation was evaluated by immunoblot analysis. As observed in Figure 1, Src autophosphorylation from detached L3.6pl cells was over 10-fold higher than from adherent cells immediately after detachment (Figure 1A). Elevated Src autophosphorylation was observed throughout the two-hour time-course, although levels did gradually decrease (Figure 1A). Src autophosphorylation decreased to baseline within 30 min of re-plating L3.6pl cells onto tissue culture dishes and remained at baseline over a 90 min time-course (Figure 1A). No changes in Src levels were observed over the time course analyzed. These results indicate that Src is activated in a sustained fashion in detached pancreatic cancer cells. Elevated Src autophosphorylation in detached cells was determined not to be a trypsinization-specific phenomenon, as similar results were observed from L3.6pl cells detached via 2 mM EDTA (data not shown). Increased Src autophosphorylation was also not dependent on the state of cell confluence at the time of detachment, as Src autophosphorylation was elevated in cells that were detached at a range of different cell densities (data not shown).

To determine if the detachment-induced increase in Src autophosphorylation is a conserved response in pancreatic tumor cell lines, Src autophosphorylation was evaluated from a panel of pancreatic cancer cell lines (L3.6pl, PANC-1, Hs766T, and BxPC3). Whole cell lysates were prepared from attached, detached, and reattached cells as described above. Src autophosphorylation was again evaluated as a surrogate for kinase activity. As seen in Figure 1, Src autophosphorylation was elevated in detached cells, relative to adherent cells, for all pancreatic cancer cell lines tested (Figure 1B). These results suggest that elevated Src activity/autophosphorylation upon cell detachment occurs in pancreatic tumor cells with diverse genetic backgrounds.

To determine if detachment-specific activation of kinase activity/autophosphorylation was specific to Src, autophosphorylation of the closely related Src family kinase c-Yes (Yes) was also evaluated in attached, detached, and reattached pancreatic cancer cells. Yes was immunoprecipitated using a Yes-specific antibody, and western blot analysis was performed using the anti-phospho-Src^{Y418} antibody, as this residue is conserved across the Src family. Yes autophosphorylation was elevated approximately 10-fold in detached cells, relative to attached cells, and autophosphorylation levels again returned to baseline after reattachment (Figure 1C). These results suggest that the activities of Src and other Src family kinases are elevated in pancreatic cancer cells detached from the extracellular substratum, relative to adherent pancreatic cancer cells.

Phosphorylation of Src at Tyr⁴¹⁸ is Dependent on Src kinase Activity in Attached and Detached Cells

To verify that the elevated Src^{Y418} phosphorylation in detached cells was indicative of kinase activity, attached or detached (0 and 20 min) pancreatic cancer cells were incubated in the presence or absence of a small molecule ATP-competitive inhibitor of Src family kinase activity (AP23846) [37]. As observed in Figure 2A, incubation of pancreatic cancer cells with 1 μ M AP23846, previously established as the minimum concentration required to achieve complete Src inhibition in pancreatic cancer cells [37], completely abrogated Src^{Y418} phosphorylation in attached and detached cells. These results indicate that the elevated Src^{Y418} phosphorylation in detached cells is dependent on and indicative of Src activity.

Src Activation After Detachment is Independent of Specific Extracellular Matrix Proteins

As different extracellular matrices and their interactions with integrins and other adhesion proteins are known to alter cellular phenotype and biochemistry, the autophosphorylation of Src from pancreatic cancer cells was evaluated after re-plating cells on different extracellular substrata. L3.6pl pancreatic cancer cells were lysed at 80% confluence, detached for 20 min prior to lysis, or detached for 20 min and re-plated. Cells were re-plated on standard tissue culture plastic or pre-coated dishes (poly-D-lysine, collagen I, fibronectin, or laminin). As seen in Figure 2, Src autophosphorylation was elevated 4-fold in detached, relative to adherent cells, and returned to baseline levels after re-plating, regardless of the extracellular matrix on which the cells were plated (Figure 2B). These results indicate that the elevated Src autophosphorylation in detached pancreatic cancer cells is not due to loss of a specific integrin/extracellular matrix interaction.

Src Autophosphorylation is Regulated by Tyrosine Phosphatase Activity in Adherent Pancreatic Cancer Cells

Src kinase activity is regulated by opposing sites of tyrosine phosphorylation. Phosphorylation of the negative regulatory tyrosine residue, Tyr⁵²⁹, inhibits kinase activity by promoting acquisition of the closed conformation. In this conformational state, phosphorylated Tyr⁵²⁹ in the carboxy-terminus forms an intramolecular interaction with the SH2 domain, altering the conformation of the ATP binding pocket and ultimately limiting the accessibility of the kinase active site for ATP and substrates [39]. Conversely, phosphorylation of Tyr⁴¹⁸ helps stabilize the active conformation of the ATP binding pocket and is essential for full kinase activity [39]. It has previously been demonstrated that Src family kinases may be activated via protein tyrosine phosphatase-mediated dephosphorylation of the carboxy-terminal regulatory tyrosine residue [40,41], but which phosphatases are responsible under physiologic conditions remains unclear. To determine if detachment-mediated Src activity is dependent on dephosphorylation of Tyr⁵²⁹, Src was immunoprecipitated from attached, detached, and reattached L3.6pl pancreatic cancer cells, and western blot analysis was performed for anti-phospho-Src^{Y529}. Blots were stripped and re-probed for total Src. The ratio of phospho-Src^{Y529}/total Src was modestly decreased immediately after cell detachment, with a gradual increase towards baseline observed at later time points (Figure 3A). This small decrease in relative Tyr⁵²⁹ phosphorylation is likely not sufficient to account for the corresponding increase in Tyr⁴¹⁸ phosphorylation, suggesting that detachment-induced Src activation is not the result of Tyr⁵²⁹ dephosphorylation.

Doan and colleagues recently reported dephosphorylation of Src on Tyr⁴¹⁸ by the Src Homology 2 domain containing protein tyrosine phosphatase SHP-2 [42]. Thus, dephosphorylation of phospho-Tyr⁴¹⁸ may represent an alternate means of tyrosine phosphatase regulation of Src family kinase activity. To determine if Src^{Y418} phosphorylation is regulated by protein tyrosine phosphatases in pancreatic cancer cells,

L3.6pl cells were incubated in the presence or absence of 50 μ M sodium vanadate (a broad spectrum protein tyrosine phosphatase inhibitor) for one hour. Cells were then lysed, and Src autophosphorylation at Tyr⁴¹⁸ was evaluated by anti-phospho-Src^{Y418} western blot analysis. Blots were stripped and re-probed for phospho-Src^{Y529} and total Src. As seen in Figure 3, Src autophosphorylation was elevated nearly 5-fold in cells treated with sodium vanadate, relative to controls (Figure 3B). These results suggest that phosphorylation of Src at Tyr⁴¹⁸ is regulated by tyrosine phosphatase activity in attached pancreatic cancer cells. Phosphorylation of Src on Tyr⁵²⁹ was not affected by treatment with sodium vanadate (Figure 3B). Taken together, these results suggest that dephosphorylation of Tyr⁵²⁹ is not a major factor in regulation of Src activity in L3.6pl cells.

Src Autophosphorylation is Elevated in SHP-2 Knockdown Pancreatic Cancer Cells

As stated above, the Src Homology 2 domain containing protein tyrosine phosphatase SHP-2 has been demonstrated to be involved in regulation of Src family kinase activity and signaling in other systems [42–44]. To investigate a potential role for SHP-2 in regulation of Src^{Y418} phosphorylation, PANC-1 cells were transiently transfected with plasmids encoding SHP-2-targeted RNA interference (RNAi) sequences. As demonstrated in Figure 4A, Src autophosphorylation (relative to total Src) was elevated approximately 10-fold in SHP-2 knockdown cells, suggesting that SHP-2 regulates Src autophosphorylation in PANC-1 cells. To determine if these results were specific for SHP-2 inhibition and not generally attributable to tyrosine phosphatase inhibition, expression of protein tyrosine phosphatase 1B (PTP1B), which is widely expressed and has been reported to perform functions similar to SHP-2 [45], was targeted by RNAi in PANC-1 cells. RNAi-mediated knockdown of PTP1B did not result in elevated Src autophosphorylation (Figure 4B). As demonstrated in Figure 4B, PTP1B knockdown produced a modest decrease in Src autophosphorylation, suggesting that the increase in Src^{Y418} phosphorylation observed upon SHP-2 knockdown was specific for SHP-2 and not a general effect of protein tyrosine phosphatase functional inhibition. Elevated Src phosphorylation was also observed in PANC-1 cells in the presence of the SHP-2 selective small molecule inhibitor NSC-87877 [46] (data not shown). These data indicate that SHP-2 directly or indirectly regulates Src^{Y418} phosphorylation and may be an important regulator of Src activity in pancreatic cancer cells.

Inhibition of SHP-2 Activity Prevents the Detachment-Mediated Increase in Src Autophosphorylation

A dependence on SHP-2 activity for the reduced levels of Src autophosphorylation observed in attached pancreatic cancer cells, relative to detached cells, would suggest that inhibition of SHP-2 activity should increase Src autophosphorylation in adherent cells but have minimal effect in detached cells. To test this hypothesis, L3.6pl cells were again treated with NSC-87877. Adherent cells were treated with 0 or 50 μ M NSC-87877 for three hours, after which cells were lysed on tissue culture dishes or detached prior to lysis. The concentration of NSC-87877 used in these studies was previously determined as the concentration necessary to inhibit SHP-2 function in our model system and is in agreement with concentrations used by others [46,47]. As seen in Figure 5A, in the absence of treatment, Src^{Y418} phosphorylation is elevated in detached, relative to adherent cells. In contrast, in cells treated with NSC-87877, Src^{Y418} phosphorylation was equivalent in detached and adherent cells. These results suggest that Src activity is regulated by SHP-2 in adherent pancreatic cancer cells and that this regulation is lost in detached cells, promoting an increase in steady-state Tyr⁴¹⁸ phosphorylation.

Src and SHP-2 Co-Associate in Detached Pancreatic Cancer Cells

Two potential explanations for a loss of SHP-2 mediated Src regulation in detached pancreatic cancer cells include the loss of a SHP-2/Src signaling complex in detached cells

and loss of SHP-2 activity. To evaluate complex formation between SHP-2 and Src in pancreatic cancer cells, adherent or detached L3.6pl cells were lysed in NP-40 buffer, and cell lysates were immunoprecipitated with an anti-Src antibody. Co-immunoprecipitating proteins were resolved by SDS-PAGE, and western blot analysis was performed for SHP-2. Blots were stripped and reprobed for Src. Surprisingly, the amount of co-immunoprecipitating SHP-2, relative to immunoprecipitated Src, was increased in detached cells, relative to adherent cells (Figure 5B). Co-immunoprecipitation between Src and SHP-2 in adherent cells was only observed upon exposures of 10 min or longer. Similar results were obtained in PANC-1 cells (Figure 5B). These data indicate that the loss of SHP-2-dependent regulation of Src activity is not due to a loss of complex formation between the two proteins. We next evaluated SHP-2 tyrosine phosphorylation in attached and detached cells, as an indicator of phosphatase activity. Phosphorylation of SHP-2 on Tyr⁵⁴² in the carboxy-terminal tail of the protein has previously been demonstrated to correlate with elevated phosphatase activity [48]. Examination of SHP-2^{Y542} phosphorylation in detached versus attached pancreatic cancer cells revealed a rapid loss of phospho-Tyr⁵⁴² immunoreactivity after detachment (Figure 5B), supportive of a possible decrease in SHP-2 activity at a time when Src autophosphorylation is elevated.

Cell Survival and Stress Signaling Proteins Akt and JNK are Activated in Detached Pancreatic Cancer Cells in a Src Family Kinase Dependent Fashion

We hypothesized that activation of Src in detached cells facilitates growth and survival of pancreatic cancer cells in the absence of signals from the extracellular matrix. To determine which signaling pathways are activated by Src in detached pancreatic cancer cells, Src substrates and signaling partners known to regulate cell attachment, survival, and/or anoikis were evaluated from attached and detached cells. Adherent L3.6pl cells were lysed in NP-40 buffer, detached by trypsinization and maintained in suspension over a time course prior to lysis, or detached by trypsinization, maintained in suspension, and re-plated prior to lysis. Immunoblot analysis was performed using phospho-specific antibodies against focal adhesion kinase (FAK) (Tyr³⁹⁷) (Figure 6A), paxillin (Tyr³¹) (Figure 6B), and p130 Crk Associated Src substrate (CAS) (Tyr¹⁶⁵) (Figure 6C). Tyrosine phosphorylation of each of these proteins was decreased immediately in detached, relative to adherent cells (Figure 6A–C). A transient increase in tyrosine phosphorylation of FAK, paxillin, and p130 CAS was observed at 20 min after detachment; however, tyrosine phosphorylation of all three proteins was again decreased after 60 min in suspension (Figure 6A–C). Whole cell lysates were additionally analyzed by western blot analysis for activity of the Erk-1/2 mitogen activated protein kinase (MAPK), p38 MAPK, JNK, and phosphatidyl inositol 3' kinase (PI3K)/Akt pathways. These pathways were evaluated via phospho-specific western blot analysis (anti-phospho-Erk-1/2^{T202/Y204}, anti-phospho-p38^{T180/Y182}, anti-phospho-JNK^{T183/Y185}, and anti-phospho-Akt^{S473}). As observed in Figure 7, Erk-1/2 was activated in detached cells, with a peak of activity occurring within 5–10 min of detachment (Figure 7A). After 2 hrs in suspension, Erk-1/2 phosphorylation was undetectable. Erk-1/2 phosphorylation was again elevated in response to cellular reattachment (Figure 7A). Akt phosphorylation was reduced to undetectable levels immediately after detachment. Akt phosphorylation was detected from detached cells after 20 min and gradually increased throughout the 120 min in suspension (Figure 7B). Akt phosphorylation was further elevated after cellular reattachment (Figure 7B). JNK phosphorylation was undetectable in attached L3.6pl cells but was rapidly and transiently activated upon detachment (Figure 7C). After 20 min in suspension, JNK phosphorylation was no longer observed (Figure 7C). No p38 phosphorylation was observed in detached L3.6pl cells (data not shown). To determine the importance of Src family kinase activity for the activation of these signaling molecules, attached and detached cells were treated with or without the Src family kinase inhibitor AP23846 (1 μ M). Attached cells were treated for one hour prior to lysis. Detached cells were pretreated for one hour prior to

trypsinization and were maintained in the presence or absence of AP23846 throughout the time course of detachment. As observed in Figure 8, Erk-1/2 activity was at least partially Src-dependent at early and late time points after cell detachment (0 and 60 min time points); however peak Erk-1/2 activity (occurring at 20 min) was independent of Src family kinase activity, suggesting the importance of other factors in mediating Erk activation in detached pancreatic cancer cells (Figure 8A). Relative Akt phosphorylation was observed at peak levels 1–2 hours after pancreatic cancer cell detachment and was largely abrogated in the presence of Src family kinase inhibition (Figure 8B), indicating the importance of Src family kinase activity for activation of the Akt signaling network in detached pancreatic cancer cells. JNK activity was again elevated immediately after detachment of L3.6pl cells and was inhibited in the presence of AP23846 (Figure 8C). These data suggest that maximal detachment dependent Akt and JNK activation in pancreatic cancer cells is dependent on Src family kinase activity and indicative of a key role for Src and Src family kinases in the activation of canonical cell survival pathways in pancreatic cancer cells detached from the extracellular substratum.

DISCUSSION

In this study, we have demonstrated detachment-specific activation of Src in pancreatic cancer cells, as determined by autophosphorylation. This result was initially surprising, as activation of Src in response to cellular attachment to the extracellular matrix has been widely reported [49,50]. Attachment-dependent activation of Src family kinases has been reported to occur downstream of integrin clustering [49,50]. The mechanism of activation may vary, depending on the cellular system, and the particular extracellular matrix/integrin heterodimer involved. Proposed mechanisms of attachment-induced Src activation include direct interaction with integrin subunits and increased trans-autophosphorylation upon integrin clustering [50,51]; integrin-mediated FAK activation and subsequent Src/FAK complex formation [52]; dephosphorylation of Src^{Y529} by protein tyrosine phosphatases [41]; and/or inhibition of carboxy-terminal Src kinase (CSK)-mediated Src^{Y529} phosphorylation [44]. In recent years, however, reports of Src activation in detached cells have also begun to emerge.

Loza-Coll and co-workers demonstrated activation of Src in detached intestinal epithelial cells [53]. In their studies, Src activation in detached cells was transient, resulted in activation of the PI3K/Akt pathway, and produced a delay in the onset of anoikis [53]. Hofmann et al. recently made a similar observation regarding the importance of Src for anoikis resistance in primary human colonic epithelial cells [54]. Wei and colleagues demonstrated Src activation in detached lung adenocarcinoma cells [34]. In their studies, Src activity also served to protect the cells against anoikis. However, Src-mediated survival signaling was directed through the FAK-related kinase Pyk2 [34]. Src activity was characterized by elevated autophosphorylation, with no change in phosphorylation of the carboxy-terminal regulatory tyrosine [34], similar to our observations in the present studies. Src activity was reported to be dependent on the platelet-derived growth factor receptor (PDGFR) in these studies [34]. Desgrosellier et al. recently reported activation of Src in detached carcinoma cells dependent on association with the $\alpha\beta3$ integrin heterodimer [18]. In our studies, Src autophosphorylation at Tyr⁴¹⁸ appears to be regulated by protein tyrosine phosphatase activity.

Src family kinases harbor two major sites of tyrosine phosphorylation that regulate kinase activity [39]. In human Src, these residues are Tyr⁴¹⁸ and Tyr⁵²⁹. Phosphorylation of Src on Tyr⁵²⁹ downregulates kinase activity through formation of an intramolecular interaction with the SH2 domain that serves as the lynchpin of the closed conformation [39]. In this conformation, ATP and substrates are excluded from the kinase active site. Phosphorylation

of Tyr^{Y418} helps stabilize the active conformation of the kinase and promotes kinase activity [38,39]. Src family kinase activity has long been known to be activated by protein tyrosine phosphatases through dephosphorylation of the carboxy-terminal negative regulatory residue. Phosphatases that have been demonstrated to dephosphorylate this residue include CD45 and receptor protein tyrosine phosphatase (RPTP) alpha [40,41]. There is substantially less evidence to suggest that phosphatases may also negatively regulate Src family kinase activity through dephosphorylation of the autophosphorylation site. Kmiecik et al. demonstrated elevated Src autophosphorylation and kinase activity from vanadate-treated fibroblast cells overexpressing avian Src [55]. More recently, Doan and co-workers demonstrated dephosphorylation of Src Tyr⁴¹⁸ by SHP-2 in vascular smooth muscle cells stimulated with angiotensin II [42].

We provide evidence that SHP-2 is the tyrosine phosphatase responsible for downregulation of Src autophosphorylation in attached pancreatic cancer cells. Pre-treatment of L3.6pl cells with the SHP-2 inhibitor NSC-87877 or RNAi-mediated downregulation of SHP-2 expression elevated steady-state Src^{Y418} phosphorylation to levels equivalent to those observed from detached cells. Src^{Y418} phosphorylation from NSC-87877 treated cells was not further increased after detachment. These data are consistent with a model in which SHP-2 downregulates tyrosine phosphorylation of Src^{Y418} in adherent pancreatic cancer cells, preventing full activation of the kinase. We have previously demonstrated that tyrosine phosphorylation of SHP-2 in pancreatic cancer cells is largely Src dependent (unpublished data), and thus SHP-2 activation may represent a feedback mechanism to prevent prolonged periods of excess Src activity. In this model, SHP-2 tyrosine phosphorylation and activity are downregulated in detached cells, relieving the inhibition of Src^{Y418} phosphorylation and allowing full kinase activity. What remains unclear is why SHP-2 tyrosine phosphorylation is downregulated in detached cells, as this coincides with increased Src activity and SHP-2/Src co-immunoprecipitation. We believe that the answer lies in the differing nature of Src/SHP-2 interactions in attached and detached cells. Src and SHP-2 are likely brought together as part of a focal adhesion complex in attached cells, wherein SHP-2 may be phosphorylated and activated in a Src-dependent fashion. SHP-2/Src co-immunoprecipitation may be difficult to detect in adherent cells due to SHP-2 phosphatase activity. SHP-2 binding partners are often difficult to detect due to dephosphorylation of tyrosine residues that serve as SH2 domain ligands, hence the creation of SHP-2 substrate trapping mutants [56]. This may explain why SHP-2/Src co-immunoprecipitation is more easily detected in detached cells, when SHP-2 is dephosphorylated and inactivated. It remains unclear if the association between SHP-2 and Src is direct or indirect. We currently favor an indirect model of Src/SHP-2 co-association in detached cells, as interaction with one or more third party protein binding partners may inhibit the ability of SHP-2 to be properly presented for tyrosine phosphorylation by Src. The mechanism by which Src and SHP-2 co-associate in attached and detached pancreatic cancer cells is currently under investigation in our laboratory.

It has recently been demonstrated in other systems that Src activity is maintained in detached cells by sustained production of reactive oxygen species (ROS) [33]. Tyrosine phosphatases, including SHP-2, are sensitive to inactivation by ROS as a consequence of oxidation of a key cysteine residue in the catalytic domain (SHP-2^{C459}) [57,58]. ROS production in detached cells may inactivate SHP-2, leading to a subsequent increase in Src^{Y418} phosphorylation. The potential involvement of ROS in detachment-dependent regulation of Src and SHP-2 activity is an ongoing area of investigation in our laboratory.

Finally, in these studies, we have investigated the phosphorylation of canonical Src substrates in detached cells and the activity of signal transduction proteins known to regulate cell growth and survival. The Src substrates FAK, paxillin, and p130 CAS all demonstrated decreased tyrosine phosphorylation in detached cells, when Src activity is elevated. These

results initially appear incongruous; however we feel the likely explanation lies in the fact that, as with SHP-2, these proteins are normally brought together with Src at the focal adhesions after integrin engagement. When cells are detached, the focal contacts are disrupted, and focal adhesion proteins may no longer be optimally localized for Src-dependent tyrosine phosphorylation. We have noted an increase in tyrosine phosphorylation of a subset of protein bands in detached pancreatic cancer cells via anti-phosphotyrosine western blot analysis (data not shown). These bands appear to represent Src substrates, as Src family kinase inhibitors decrease their tyrosine phosphorylation in a dose-dependent fashion. We are currently in the process of identifying detachment-specific Src substrates, as they may be important for characterization of the role of Src in mediating pancreatic cancer cell anoikis resistance. Interestingly, tyrosine phosphorylation of FAK, paxillin, and p130 CAS was transiently elevated in detached pancreatic cancer cells after 20 min in suspension. This may reflect a transient reconstitution of integrin-mediated signaling events, as recently reported to occur in pancreatic cancer cells by Desgrosellier and co-workers [18]. It is thus possible, that a Src/FAK signaling axis in detached pancreatic cancer cells may facilitate the activation of downstream survival signals, such as those provided by the PI3K/Akt signaling pathway.

Src family kinase activity was required for full activation of Akt in detached pancreatic cancer cells. The role of the PI3K/Akt pathway in mediating cell survival is well established and, as mentioned above, has been demonstrated by others to be important for directing Src-dependent survival signals in detached cells [32,35,53]. In our experiments, Akt activation was detectable at low levels by 20 min post-detachment. The delay in Akt activation, relative to Src, indicates the likely importance of other factors in mediating activation of the PI3K/Akt signaling axis. FAK activation is also detected transiently 20 min post-detachment. It is thus possible that activation of the PI3K/Akt pathway is dependent on activation of a Src/FAK signaling complex in detached cells. The importance of FAK for Src-mediated Akt activation in detached cells will be examined in future studies.

The JNK pathway has been more commonly associated with cellular stress and the promotion of apoptosis. Some reports, however, have demonstrated a role for JNK in tumorigenesis and survival signaling, with discrepancies likely attributable to the nature and duration of the activating signal, as well as differences in cellular context [59,60]. Future studies will focus on how Src induces activation of the Akt and JNK pathways in detached pancreatic cancer cells, as well as the importance of the Src/Akt and Src/JNK axes for mediating resistance to anoikis.

In summary, in these studies, we have demonstrated elevated autophosphorylation of Src in pancreatic cancer cells detached from the extracellular substratum. The increase in Src autophosphorylation appears to be due to a loss of SHP-2 tyrosine-phosphatase-mediated regulation of steady-state Src^{Y418} phosphorylation. Activated Src in detached pancreatic cancer cells induces downstream activation of the Akt and JNK signaling proteins. We hypothesize that SHP-2 inactivation in detached cells allows for deregulation of Src activity and subsequent activation of the Akt and JNK signaling axes, promoting cell survival in the absence of attachment to the substratum. These results further emphasize the potential value of Src and Src family kinases as targets for pancreatic cancer therapy and suggest that inhibition of Src family kinase activity may short circuit multiple signaling pathways mediating resistance to anoikis.

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Abbreviations

5FU	5-fluorouracil
BCA	bicinchoninic Acid
BSA	bovine serum albumin
CAS	Crk associated Src substrate
CCK2R	cholecystokinin 2 receptor
CEACAM6	carcinoembryonic antigen cell adhesion molecule 6
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EmGFP	emerald green fluorescent protein
Erk	extracellular signal regulated kinase
FAK	focal adhesion kinase
HRP	horseradish peroxidase
IGFR	insulin like growth factor receptor
IL-8	interleukin 8
JNK	Jun N-terminal kinase
kDa	kilodalton
MAPK	mitogen activated protein kinase
miRNA	micro ribonucleic acid
NP-40	nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PDAC	pancreatic ductal adenocarcinoma
PI3K	phosphatidylinositol 3' kinase
PTP1B	protein tyrosine phosphatase 1B
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
RNAi	ribonucleic acid interference
SHP-2	Src homology 2 domain containing protein tyrosine phosphatase 2
TBS	tris buffered saline
Tyr	tyrosine

VEGF vascular endothelial growth factor
Y tyrosine

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Figure 1.

(A) Activation of Src in detached L3.6pl pancreatic cancer cells. Adherent, detached (indicated times), and reattached (indicated times) L3.6pl cells were lysed in NP-40 buffer. Total Src was immunoprecipitated, and Src activity was visualized via anti-phospho-Src^{Y418} western blot analysis. Blots were stripped and re-probed for total Src. The graph represents the ratio of phospho-Src^{Y418} staining to total Src, relative to attached cells (N=3). (B) Detachment-specific activation of Src in a panel of pancreatic cancer cell lines. Adherent, suspended (20 min), and reattached (60 min) L3.6pl, Hs766T, PANC-1, and BxPC3 cells were lysed in NP-40 buffer. Src was immunoprecipitated and anti-phospho-Src^{Y418} western blot analysis was performed as described in Materials and Methods. Blots were stripped and re-probed for total Src. (C) Activation of Yes in detached pancreatic cancer cells. Adherent, suspended (20 min), or reattached (60 min) L3.6pl cells were lysed in NP-40. Total Yes was immunoprecipitated, and Yes activity was visualized via anti-phospho-Src^{Y418} western blot analysis. Blots were stripped and re-probed for total Yes. The graph represents the ratio of phospho-Yes to total Yes, relative to attached cells (N=3).

Figure 2.

(A) Src^{Y418} phosphorylation in attached and detached pancreatic cancer cells is dependent on Src activity. PANC-1 cells were treated for one hour with 0 or 1 μ M AP23846. After one hour, cells were lysed in NP-40 buffer or detached via trypsinization and maintained in suspension for 0 or 20 min at 37 °C in the presence of 0 or 1 μ M AP23846 prior to lysis. Src was immunoprecipitated from whole cell lysates, and western blot analysis was performed for anti-phospho-Src^{Y418}. Blots were stripped and re-probed for total Src. (B) Activation of Src in detached L3.6pl cells, relative to cells adherent to extracellular matrix proteins. L3.6pl cells were released via trypsinization, rotated at 37 °C for 20 min, and re-plated on standard 100 mm tissue culture plastic (TCP) or dishes pre-coated with poly-D-lysine, collagen I, fibronectin, or laminin. Adherent, detached, and reattached cells were lysed in NP-40-buffer, and Src activity was evaluated as described in (A). All blots are representative of at least three experiments.

Figure 3.

(A) Phosphorylation of Src on Tyr⁵²⁹ in detached L3.6pl pancreatic cancer cells. Adherent, detached (indicated times), and reattached (indicated times) L3.6pl cells were lysed in NP-40 buffer. Total Src was immunoprecipitated, and anti-phospho-Src^{Y529} western blot analysis was performed. Blots were stripped and re-probed for total Src. The graph represents the ratio of phospho-Src^{Y529} to total Src, relative to attached cells (N=3). (B) Autophosphorylation of Src in the presence of the phosphatase inhibitor sodium vanadate. L3.6pl cells were treated with 0 or 50 μ M sodium vanadate for one hour. Cells were lysed, and total Src was immunoprecipitated. Anti-phospho-Src^{Y418} western blot analysis was performed. Blots were stripped and re-probed for phospho-Src^{Y529} and total Src. The graphs represent the ratio of phospho-Src^{Y418} or phospho-Src^{Y529}, as indicated, to total Src, relative to untreated cells (N=3).

Figure 4.

(A) Autophosphorylation of Src from pancreatic cancer cells with reduced SHP-2 expression. PANC-1 cells were transfected with plasmids encoding SHP-2 targeted interfering RNA sequences (SHP-2 RNAi 1 and SHP-2 RNAi 2) as described in Materials and Methods. After 48 hours, control, SHP-2 RNAi 1, and SHP-2 RNAi 2 cells were lysed, and lysates were prepared for western blot analysis. Blots were probed for phospho-Src^{Y418}, total Src, SHP-2, and actin. The graphs represent the ratios of phospho-Src^{Y418} to total Src and total SHP-2 to actin, respectively, relative to control cells (N=3). (B)

Autophosphorylation of Src from pancreatic cancer cells with reduced PTP1B expression. PANC-1 cells were transfected with PTP1B targeted interfering RNA sequences (PTP1B RNAi 1 and PTP1B RNAi 2) as described in Materials and Methods. After 48 hours, control, PTP1B RNAi 1, and PTP1B RNAi 2 cells were lysed, and lysates were prepared for western blot analysis. Blots were probed for phospho-Src^{Y418}, total Src, PTP1B, and actin. The graphs represent the ratios of phospho-Src^{Y418} to total Src and total PTP1B to actin, respectively, relative to control cells (N=3).

Figure 5. (A) SHP-2 inhibitor NSC-87877 abrogates detachment-mediated Src activation. Adherent L3.6pl cells were treated with 0 or 50 μ M NSC-87877 for three hours and either lysed or detached via trypsinization. Detached cells were incubated for 20 min in the presence or absence of 50 μ M NSC-87877. Src activity was evaluated via anti-phospho-Src^{Y418} western blot analysis. Blots were stripped and re-probed for total Src. The graph represents the ratio of phospho-Src^{Y418} to total Src, relative to untreated, attached cells (N=3). (B) Co-association of Src and SHP-2 in detached pancreatic cancer cells. Attached and detached (20 min) L3.6pl or PANC-1 cells were lysed in NP-40. Src was immunoprecipitated as described in Materials and Methods, and co-immunoprecipitating SHP-2 was detected by western blot analysis. Blots were stripped and re-probed for total Src. Whole cell lysates were probed for phospho-SHP-2^{Y542}, stripped, and re-probed for total SHP-2. The graphs represent the ratio of immunoprecipitated SHP-2 to Src, relative to attached cells (N=3).

Figure 6.

Tyrosine phosphorylation of Src substrates in detached L3.6pl pancreatic cancer cells. Adherent, detached (0, 20, and 60 min), and re-attached (60 min) L3.6pl cells were lysed, and tyrosine phosphorylation of FAK^{Y397} (A), paxillin^{Y31} (B), and p130 CAS^{Y165} (C) were evaluated by phospho-specific western blot analysis, as described in Materials and Methods. Blots were stripped and re-probed for total FAK (A), Paxillin (B), and p130 CAS (C), respectively. The graphs represent the ratio of phospho-FAK^{Y397} (A), phospho-paxillin^{Y31} (B), and phospho-p130 CAS^{Y165} (C) to total FAK, paxillin, and p130 CAS, respectively (N=3).

Figure 7.

Activation of Erk-1/2, Akt, and JNK in detached L3.6pl pancreatic cancer cells. Adherent, detached (indicated times), and reattached (indicated times) L3.6pl cells were lysed in NP-40 buffer. Cell lysates were resolved by 8% SDS-PAGE and western blot analysis was performed for phospho-Erk-1/2^{T202/Y204} (A), phospho-Akt^{S473} (B), or phospho-JNK^{T183/Y186} (C). Blots were stripped and re-probed for total Erk-1/2 (A), total Akt (B), or total JNK (C) respectively. The graphs represent the ratio of phospho-Erk-1/2^{T202/Y204} (A), phospho-Akt^{S473} (B), and phospho-JNK^{T183/Y186} (C) to total Erk-1/2, Akt, and JNK, respectively (N=3).

Figure 8. Effects of Src family kinase inhibition on detachment-dependent activation of Erk-1/2, Akt, and JNK. Attached and detached (indicated times) L3.6pl pancreatic cancer cells were treated with 0 or 1 μ M AP23846 as described in Materials and Methods. Cells were lysed in NP-40 buffer. Cell lysates were resolved by 8% SDS-PAGE and western blot analysis was performed for phospho-Erk-1/2^{T202/Y204} (A), phospho-Akt^{S473} (B), or phospho-JNK^{T183/Y186} (C). Blots were stripped and re-probed for total Erk-1/2 (A), total Akt (B), or total JNK (C) respectively. The graphs represent the ratio of phospho-Erk-1/2^{T202/Y204} (A), phospho-Akt^{S473} (B), and phospho-JNK^{T183/Y186} (C) to total Erk-1/2, Akt, and JNK, respectively (N=3).