1-1-2010

Molecular Mechanisms Of Pressure-Stimulated Cancer Cell Signaling

Christina Downey
Wayne State University

MOLECULAR MECHANISMS OF PRESSURE-STIMULATED CANCER CELL SIGNALING

by

CHRISTINA DOWNEY

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

In partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2010

MAJOR: CANCER BIOLOGY

Approved by:

_____

Advisor Date

_____

_____

_____

_____

ACKNOWLEDGEMENTS

I would like to thank Dr. Marc Basson for his ongoing mentorship and encouragement. His professional example, guidance and support have been key to my success as a graduate student. I would like to thank my committee members, Drs. Joseph Fontana, Adhip Majumdar and Ho-Sheng Lin for their thoughtful insights that have been an asset to the development and progression of my dissertation work. I also owe a great deal of gratitude to my fellow lab members (both past and present), Drs. Vijayalakshmi Thamilselvan, David Craig, Mary Walsh, Shouye Wang, Matt Sanders, Lakshmi Chaturvedi and Lisi Yuan, for their friendship and technical assistance through the years. Finally, I would like to thank my family and friends, especially my husband Mike, for all of their love, support and patience that they have given me through the difficult times that I've endured during my graduate training. Thank you.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 1

Introduction

Although more than 50% of colon cancer patients undergoing laparoscopic or open surgical procedures have viable colon tumor cells free in the peritoneum, only ~1% of these patients experience wound implantation [1,2,3,4,5]. In addition, only a fraction of patients with apparently resectable colon cancer will go on to experience metastasis at a distant site, approximately 33%. It is known that both wound implantation and metastasis require cell adhesion to the extracellular matrix and/or endothelium followed by rapid proliferation. However, this information suggests that the great majority of tumor cells never adhere [6]. For patients in whom errant tumor cells do adhere, the consequences may be radical surgery or possibly death. This unfortunate paradigm emphasizes the need to study the mechanisms underlying the cell adhesion and proliferation processes.

Previous studies have indicated that pressure stimulated adhesion is β1 integrin dependent [7]. Integrins are heterodimeric receptors embedded in the cell membrane that are composed of an α and a β subunit that are involved in cytoskeletal attachment to the extracellular matrix [8]. Integrins have no catalytic activity; however, their location within the membrane gives them the opportunity to participate in bi-directional signaling [9]. The ligands for integrins are components of the extracellular matrix, including laminin and collagen. Signaling initiated through extracellular ligand binding and/or ligand-induced integrin clustering is termed “outside-in” signaling, and may induce changes in cytoskeletal organization, protein phosphorylation and gene expression. Integrin affinity and avidity may both be modulated through the activation of “inside-out”

cytoplasmic signaling events that cause conformational changes in the extracellular ligand-binding sites (affinity) and cytoskeletal induced integrin clustering (avidity) [10]. Mediation of these properties provides an internal control over cell adhesiveness. Integrin-dependent cell adhesion has been shown to regulate colorectal carcinoma organ-specific metastasis through both changes in surface expression and alterations in integrin affinity [11,12].

Once a cell attaches to the extracellular matrix, integrin clustering leads to autophosphorylation and activation of focal adhesion kinase at tyrosine 397. This phosphorylation promotes the activation of intracellular signaling cascades. The phosphorylation of focal adhesion kinase at tyrosine 397 creates a high affinity binding site for the SH2 domain of Src family members [13]. Src is recruited and phosphorylated and leads to further phosphorylation of focal adhesion kinase at tyrosines 576 and 577 [14]. The C-terminal region of focal adhesion kinase contains proline rich sequences that give other molecules, such as $p130^{Cas}$, the ability to bind. The binding of these molecules lead to activation of Rac and Rho GTPases and influence cell migration and many other diverse cellular processes [15].

In 2000, Basson *et al.* reported that colon cancer cells adhere to collagen and endothelial cells upon exposure to 15mmHg increased pressure for 30 minutes. This result was confirmed in four colon cancer cell lines (SW620, Caco-2, HT-29, SW1116), as well as primary tumor cells from 20 colon cancer patients [7]. These results are clinically relevant, as patients are exposed to increases in pressure during laparoscopic insufflation, surgical manipulation and post operative edema [16]. Furthermore, additional evidence indicated that increasing

pressure to the same extent caused breast cancer cell adhesion to collagen [17]. These results are also clinically relevant, as studies have shown that breast tumors can experience intratumoral pressures exceeding 29mmHg [18,19].

In contrast to “outside in” signaling that occurs upon ligand binding to integrin receptors, “inside out” signaling causes internal activation of signaling cascades. “Inside out” signaling is β1 integrin dependent and can be influenced by various cations [7,20]. Cellular adhesion occurs without increased surface expression of β1 integrin, which suggests that receptor affinity modulation may be responsible for the pressure effect [21]. SW620 cells exposed to elevated pressure that are unable to adhere show activation of focal adhesion kinase at tyrosine 397, as well as Src activation. In addition, inhibiting focal adhesion kinase by specific siRNA or inhibiting Src using PP2, completely abrogated the pressure effect [21]. Further studies confirmed that an intact cytoskeleton is necessary for pressure stimulated adhesion. When the cytoskeleton is compromised by agents including colchicines, phalloidin and cytochalasin D, the pressure effect is abolished. Using these inhibitors, it was determined that Src phosphorylation is cytoskeleton independent, as Src phosphorylation continues to take place in the presence of cytoskeletal inhibitors. Focal adhesion kinase, on the other hand, does not become phosphorylated in the presence of these inhibitors [22]. Recent data also shows that PI3 kinase inhibitors (LY294002 and wortmannin), siRNA treatment to reduce Akt, and Akt inhibitors each also block pressure stimulated cell adhesion in SW620 cells as well as human primary tumor cells. Pressure was shown to activate PI3 kinase and phosphorylate Akt.

Pressure also stimulates the relocation of focal adhesion kinase to the cell membrane, except in the presence of Akt inhibitor IV [23]. While several molecules have been identified in the pressure stimulated adhesion pathway, the overall mechanism is still poorly understood. Paxillin may prove to be important in this pathway, as it is involved in focal adhesion complex assembly and linkage of the focal adhesion complex to integrins and the cytoskeleton.

Paxillin is a 68-kDa focal adhesion protein that has been implicated in diverse cellular events including cellular motility, cell spreading, embryogenesis, intracellular signaling pathways, and apoptosis [24]. Several types of cancer, including head and neck cancers and human osteosarcomas have shown increased paxillin expression relative to their normal counterparts [25]. Paxillin acts as a multi-domain adaptor protein that is found at the interface between the plasma membrane and the actin cytoskeleton. The carboxyl terminus of paxillin consists of four LIM domains arranged in tandem. The LIM3 domain is known to anchor paxillin to the plasma membrane, and though the exact mechanism is not entirely known, in vitro and in vivo co-precipitation studies have shown that paxillin co-precipitates with the cytoplasmic tail of β1 integrin [26]. The amino terminus of paxillin contains the SH2 and SH3 domains which may contain the binding site for Src, and lead to Src localization to focal adhesions. Focal adhesion kinase is known to phosphorylate paxillin at two main sites; tyrosine 31 and tyrosine 118 [27]. Upon this phosphorylation by focal adhesion kinase, two functional SH2 binding sites are generated for members of the Crk family of adaptor proteins to bind. This complex of paxillin and a Crk creates a docking site

for another focal adhesion protein; $p130^{Cas}$ [28]. This complex of paxillin, Crk and $p130^{Cas}$ has been shown to be necessary for integrin-mediated cell motility through activation of Rac and Rho family members [29,30].

In addition to increasing cancer cell adhesion, Walsh et al illustrated that 24 hours of increased pressure also induces colon cancer cell proliferation on fibronectin and collagen. This effect was observed beginning at six hours and continued for 24 hours. During this time, proliferation was increased 46% and the percentage of cells in S-phase was also elevated approximately 20% relative to cells that remained in ambient pressure conditions. In a manner similar to that observed when short term pressure stimulates adhesion, 24 hours of pressure increased phosphorylation of Src, MEK and p38. However, in contrast, Akt phosphorylation was not increased. Additional differences were observed when pharmacological inhibition of PI-3-kinase, actin depolymerization, Src, p38 and MEK did not abolish the effect of pressure on cellular proliferation. It was further determined that inhibition of protein kinase C was sufficient to abolish pressure stimulated proliferation [31].

Protein kinase C is a family of enzymes that serve many diverse functions and are known to stay activated for extended periods of time. Protein kinase C regulates cellular functions such as adhesion, migration, and immune cell regulation. Some protein kinase C isoforms can also regulate NF-kB activation as well as cellular proliferation [32,33].

NF-kB is a rapidly acting transcription factor that modulates gene transcription in cell-cycle regulation, apoptosis, and proliferation. NF-kB signaling

is complex, and can promote proliferation through increased expression of cell cycle regulators, or cell death through interactions with anti-apoptotic factors such as FLIP and TRAF1/2 [34]. NF-kB family members share a Rel homology domain in their N-terminus. However, RelA, RelB and c-Rel are the only members with transactivation domains. NF-kB family members form homodimers or heterodimers to participate in transactivation. RelA, the p65 subunit, regulates transcription directly, as it has a nuclear translocation sequence in addition to a direct DNA binding site. Traditional NF-kB signaling involves heterodimerization between RelA and p50. NF-kB can promote or inhibit apoptosis depending upon the stimulus [35].

Aberrant or constitutive NF-kB activation characterizes many human malignancies and indicates poor prognosis in colorectal and esophageal cancers [34,36,37]. Additionally, other studies have found that NF-kB immunoreactivity of correlates with poor prognosis in various cancers [38].

Based on these previous observations, we hypothesized that paxillin, protein kinase C and NF-kB may be necessary molecules in the cellular signaling that occurs as pressure stimulates adhesion or proliferation. We assessed the role of these molecules in various cancer cell lines and further evaluated the effect of pressure on distant metastasis using an in vivo murine model. These pathways may represent a target of opportunity to inhibit adhesion and metastasis, as well as slow the growth of unresectable tumors or tumors not candidates for conventional cytotoxics.

CHAPTER 2

Materials and Methods

Cell Culture. For initial characterization of CARP-1, estrogen receptor-negative, p53 negative MDA-MB-468 human breast cancer cells were used. These cells were cultured and maintained using Dulbecco's modified Eagle medium, Ham's F-12 medium and fetal bovine serum. All studies involving CARP-1 utilized these cells. SW620 and Caco-2 colon cancer cell lines, MCF-7 breast cancer and PC3 prostate cancer cell lines were obtained from the American Type Culture collection (Rockville, MD), and cultured according to the manufacture's recommendation. The luciferase-tagged CT-26 murine colon cancer cell line (Caliper Life Sciences, Hopkinton, MA) was cultured using 600 µg/mL geneticin. Primary human colon cancer cells were obtained from surgically resected specimens that were minced and collagenase digested into single cell suspensions. Viability of the isolated colonocytes was determined to be over 90% by trypan blue exclusion. The Wayne State University Human Investigation Committee approved the use of all human samples.

Mice. Balb/c mice were used for all in vivo tumor studies, as well as long term survival studies. All animal procedures were conducted with the approval of the Wayne State University Animal Investigation Committee and the John D. Dingell VA Medical Center Research and Development Committee.

Pressure Regulation. Pressure was regulated using an airtight Lucite box that was pre-warmed to 37℃ to prevent internal fluctuations. The box contained an inlet and outlet valve for gas application and a manometer

connection, respectively. Temperature was regulated to within ± 2ºC and pressure was maintained to within 1.5 mmHg. Cells were placed in the pressure box from 30 minutes to 72 hours depending on the study under investigation. Ambient pressure control plates were placed in identical incubation conditions for the same duration of time.

Pressure Measurements of Intraabdominal Malignant Tumors. Under IRB-approved protocol, we measured interstitial pressure during image-guided biopsy just outside of 13 human tumors using a Transpac IV Monitoring Kit pressure transducer (Hospira, Lake Forest, IL) attached to a coaxial needle which was then advanced into the tumor for a second measurement. Pressure was measured during routine biopsies of tumors subsequently confirmed to be malignant but before removing biopsied tissue.

Cell Adhesion Assay. SW620 cells in suspension were placed in collagen I (Sigma, St. Louis, MO) pre-coated 6 well plates for 30 minutes under ambient or 15 mmHg increased pressure conditions in a 37℃ incubator (10^5 cells/well). After 30 minutes the non-adherent cells were washed away with phosphate buffered saline solution and the adherent cells were fixed with formalin and stained with hematoxylin. Each well was divided into 20 random, individual, high-power fields and cells were counted in each field using an inverted microscope[7].

To determine adhesion of SW620 cells that were transfected with the paxillin plasmid phosphorylation mutants or p130cas rescue plasmid, 48 hours post transfection the cells were plated in collagen I precoated 6 well plates as

described above. After 30 minutes of ambient or 15mmHg increased pressure, the non-adherent cells were washed away and the adherent cells were permeablized and incubated overnight with antibody to myc or human influenza hemagglutinin (HA) depending upon the tag of the mutant. After 24 hours, the myc or HA antibody was removed, the cells were washed with phosphate buffered saline solution and Texas Red (Amersham Pharmacia Biotech, Piscataway, NJ) was added for 1 hour. The cells were then counted under a fluorescence microscope.

Proliferation Assay. 5,000 cells per well were seeded in collagen I precoated 96-well plates. Various inhibitors were added at the concentrations listed in Table 1. Cells were exposed to increased or ambient pressure for 24 hours. After 24 hours, MTT reagent was added and cells were incubated for 2-4 hours at 37℃. Finally, the cells were treated with a detergent reagent overnight. The plates were then read on a fluorescence plate reader at 570 nm.

Transfection. For studies involving CARP-1, MDA-MB-468 human breast cancer cells were transfected with vector plasmids pcDNA3, pcDNA3/Hygro, pcDNA3/Hygro CARP-1 antisense clone 1.6, or pcDNA3/CARP-1-myc-His clone 6.16. For stable transfections, this was followed by selection using 800 µg/mL of neomycin or 400 µg/mL of hygromycin. From this, multiple resistant stable sub-lines were generated and propagated.

Myc tagged wild type and Y31Y118 paxillin plasmid mutants were compliments of Dr. M. Cecilia Subauste. HA tagged wild type, as well as Y31, Y118, Y181 and Y31Y118Y181 paxillin plasmid mutants were generous gifts of

Dr. Ravi Salgia. All mutants had a Y⟶F mutation on their respective tyrosine residues. Using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), SW620 cells were transfected with 2 µg/ml of plasmids expressing myc tagged wild type or Y31Y118 mutant, or HA tagged wild type, Y31, Y118, Y181, or Y31Y118Y181 mutants.

Additionally, total Crk, p130cas, α-actinin and FAK levels were reduced using small interfering RNA *SMART*pools® produced by Dharmacon (Lafayette, CO), as well as oligofectamine and plus reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). NF-kB p65 protein levels were reduced using NF-kB specific small interfering siRNA (Cell Signaling), as well as lipofectamine 2000 (invitrogen) as recommended by the manufacture. Crk, p130cas and FAK expression was reduced using siRNA targeting the 5'-ACACTATTTGGACACTACA-3' segment of Crk mRNA, the 5'-GGTCGACAGTGGTGTGTAT-3' segment of p130cas mRNA and the 5'-AAGCAUGUGGCCUGCUAUGGA-3' segment of FAK mRNA. A Dharmacon si*CONTROL*® non-targeting siRNA #1 was used as a control. Scrambled siRNA sequences for paxillin, Crk and Cas were also used as a control. The scrambled sequences used were: paxillin 5'-TGGTGTCTTCCGTTTAGGG-3', Crk 5'-ATATAAGCAGCGTGATTCC-3' and Cas 5'-CAGTGATGGTATGTGCGGT-3'. All experiments were performed 48 hours post transfection.

Rac1 Activation Assay. Rac1 activity was assessed using a pulldown assay (Millipore, Billerica, MA). SW620 cells were grown to confluence and plated on dishes pre-coated with 1% heat inactivated BSA. They were then

exposed to ambient or 15 mmHg increased pressure as described above. Cells were collected by centrifugation and lysed using lysis buffer provided by the manufacturer. Protein concentrations were determined by the BCA method. Active Rac1 levels were determined by GST-PBD (glutathione *S*-transferase-conjugated p21 binding domain) of p21-activated kinase 1 pulldown assays. The cell lysates were incubated with PAK-PBD beads for 1 hour at 4 ℃ on a rotator, and the beads were pelleted by centrifugation at 5000 x *g* for 2 min at 4 ℃. The resulting pellet was then resuspended in Laemmli buffer resolved electrophoretically, transferred to nitrocellulose, and immunoblotted with monoclonal anti-Rac1 antibody (Pierce). Additionally, 20 µg of cell lysates were used for Western blotting for total Rac1.

Inhibitor Studies. The Src family kinase inhibitor PP2 (Calbiochem, Gibbstown, NJ) was dissolved in dimethyl sulfoxide (Me_2SO) and diluted in cell culture medium immediately prior to use. SW620 cells were treated with 65 nM of PP2 or an equivalent amount of Me_2SO (vehicle control) for 1 hour prior to adhesion or signaling studies. 30 nM of PP2 was added to cells for 24 hours during cellular proliferation studies and NF-kB activation studies. NSC23766 (Calbiochem, Gibbstown, NJ), an inhibitor of Rac1 activation was dissolved in sterile distilled water and diluted in cell culture medium immediately prior to use. SW620 cells were treated with 50 µM NSC23766 or cell culture medium (vehicle control) for 1 hour prior to adhesion or signaling studies. 30 µM NSC23766 was added to the cells for 24 hours during cellular proliferation studies and NF-kB activation studies.

To further determine molecular signaling involved in pressure stimulated proliferation and NF-kB activation, selected inhibitors were dissolved in sterile phosphate buffered saline solution, dimethyl sulfoxide or water according to the manufacture's protocol. Cells were treated with each inhibitor for 24 hours. Akt was inhibited using the 1 μM Akt inhibitor IV (EMD Chemicals, Gibbstown, NJ). NF-kB p50 nuclear localization was blocked with 12 μM SN50 (EMD Chemicals), in parallel with an SN50 inactive analog that does not affect NF-kB nuclear translocation. The NF-kB serine 276 inhibitory peptide (25 μM, Imgenex, San Diego, CA) acts as an NF-kB p65 nuclear decoy through phosphorylation at that site. Cells were treated with the inhibitory peptide of an inactive control. TCH-021, a novel imidazoline inhibitor, was a generous gift from our collaborator, Dr. Jetze Tepe. It was used (5 μM) to inhibit NF-kB gene transcription by modulating IkB degradation and subsequently inhibiting NF-kB DNA binding.

PKC was blocked globally using Calphostin C (100 nM). Inhibition of PKC-α and PKC-β was achieved using Go6976 (6 nM). PKC-β alone was inhibited using 3-(1-(3-Imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione (15 nM). Finally, the PKC-ε subunit was inhibited using a translocation inhibitory peptide (10 nM).

To evaluate the role of the IKK complex subunits on pressure-stimulated proliferation, the IKK-2 inhibitor [5-(p-fluorophenyl)-2-ureido]-thiophene-3-carboxamide (10 mM), IKK-3 inhibitor [5-(5,6-dimethoxybenzinidazol-1-yl)-3-(2-methanesulfonyl-benzyloxy)-thiophene-2-carbonitrile] (40 nM) and the IKK inhibitor N-(6-chloro-9H-β-carbolin-8-yl)-nicotinamide (90 nM), that blocks IkB

phosphorylation, were used per manufacturer's protocol, separately and in combination (EMD Chemicals). Table 1 lists all inhibitors used.

Flow Cytometric Analysis. To evaluate cellular proliferation, cells exposed to pressure for 24 hours were detached using trypsin/EDTA and fixed in 90% ice cold ethanol. The cells were then stained with propidium iodide (5 µg/mL PBS, pH 7.4, containing 200 µg/mL DNase free Rnase A and 0.1% Triton-X 100). Transition to S-phase was evaluated using a FACSCalibur flow cytometer (BD Bioscience) and FlowJo software (Tree Star). Assays were performed on cells following 24 hours of serum starvation to induce cellular quiescence.

To evaluate in vivo molecular signaling following cardiac puncture, isolated, CFSE labeled cells were fixed with 4% paraformaldehyde for 10 minutes and washed with a staining buffer that contained 0.2% bovine serum albumin and 0.02% sodium azide in phosphate buffered saline. Cells were then incubated with anti-FAK, anti-Akt, and anti-mouse CD29 (9EG7) for 30 minutes at 4ºC. Following antibody incubation, cells were washed twice with staining buffer and analyzed using the system and software stated above at the Wayne State University School of Medicine Flow Cytometry Core Facility.

TUNEL Staining. Cells were plated on collagen I pre-coated glass coverslips and exposed to increased or ambient pressure conditions for 24 hours. Proteinase K digestion was performed followed by peroxidase blocking. Coverslips were incubated with TdT reaction buffer and reaction mixture and detected using streptavadin-HRP and DAB. Hematoxylin was used as a counterstain and the slides were coversliped using a xylene based mounting

medium. Apoptotic cells were counted on each slide and control and pressure treated cells were compared (TUNEL staining kit, Roche Applied Science, Indianapolis, IN).

NF-kB Activity Assay. Cellular NF-kB activity was assayed using a luciferase-based NF-kB lentiviral reporter assay (Qiagen, Frederick, MD). 5,000 cells/well were plated in 96 well plates for 24 hours and lentiviral particles were introduced with SureENTRY transduction reagent (2 µg/mL, Qiagen) for 24 hours. The lentiviral suspension was replaced with normal medium, and cells were exposed to ambient or increased pressure for 24 hours. Luciferin was added using the bright-glow luciferase assay (Promega, Madison, WI) and luminescence quantitated by a FLUOstar Omega plate reader (BMG LabTech, Offenburg, Germany).

Nuclear Fractionation. Cytosolic and nuclear fractions wee obtained using the Qproteome nuclear subfractionation kit (Qiagen).

Immunoprecipitation and Western Blotting. Signaling studies were performed on suspended cells that were plated on bacteriological dishes precoated with 1% heat inactivated bovine serum albumin to prevent adhesion. To study cellular signaling following 24 hours of pressure treatment, cells were grown on collagen I pre-coated tissue culture dishes. The cells were exposed to ambient or increased pressure for the desired time point and collected by centrifugation. A lysis buffer containing 50mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1% deoxycholic acid, 0.1% SDS, 1mM EDTA, 1mM EGTA, 1mM PMSF, 1mM sodium vanadate, 50 mM NaF, 10mM sodium

pyrophosphate, 2 µg/mL aprotinin, and 2 µg/mL leupeptin was then used at 4℃. Total protein concentrations of the cell lysates were found using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). For co-immunoprecipitation studies, 400µg of protein for each sample was incubated with its appropriate antibody for 1 hour, after which time agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were added overnight. Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a Hybound nitrocellulose membrane (Amersham Pharmacia Biotech). Mouse monoclonal antibodies to total paxillin, p130cas, Crk, total Rac (BD Transduction lab, San Diego, CA), GAPDH (Biodesign International, Saco, MN), actin (Sigma Aldrich), HA (Covance, Berkley, CA), cyclin D1, phospho-IkB, and myc (Cell Signaling, Beverly, MA), as well as rabbit polyclonal antibodies for CARP-1, total IkB (Sigma Aldrich), Histone H1 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-paxillin (pY31 – Biosource, Camarillo, CA; pY118 – Cell Signaling), phospho-FAK (Biosource), NF-kB p65 (Millipore, Billerica, MA), alpha-tubulin, phospho-IKKα/β, NF-kB p50 and phospho-Rac (Cell Signaling) were used for immunoprecipitation studies and/or protein detection along with the appropriate horseradish peroxidase – conjungated secondary antibodies. Antibodies are provided in an alphabetical listing in Table 2. Proteins were visualized by ECL-Plus techniques (Amersham Pharmacia Biotech) and imaged and quantitated using a Kodak Phosphoimager (Perkin Elmer, Boston, MA). All blots were studied within the linear range of exposure.

NF-kB p50 and p65 Transcription Factor Activity. NF-kB p50 and p65 DNA binding activity was evaluated in nuclear lysates added to multi-well plates coated with the double stranded DNA consensus sequence by an ELISA based NF-kB p50 or p65 Transcription Factor Assay Kit (Cayman Chemical, Ann Arbor, MI).

In Vivo Splenic Injections. Luciferase tagged CT-26 murine colon cancer cells were exposed to ambient or increased pressure for 30 minutes. Following pressure exposure, cells were collected by centrifugation and washed with sterile phosphate buffered saline solution. Balb/c mice were anesthetized with 60 mg/kg of intraperitoneal sodium pentobarbital and a small incision was made just above the spleen. Using a 30 gauge needle, one million cells were slowly injected into the spleen. The cavity was aspirated and closed using a suture or small animal wound clip.

For long term survival studies following splenic injections, mice were euthanized when visible internal tumor burden reached a maximum or when the mice became scruffy, lethargic or unable to take in food or water, as determined by DLAR personnel.

In Vivo Tail Vein Injection and Cardiac Puncture. CT-26 murine colon cancer cells were labeled with CFSE for 30 minutes, collected by centrifugation and washed with sterile phosphate buffered saline solution. 1 million cells were injected into the tail vein of Balb/c mice. After 30 minutes, the mice were euthanized and cells were collected by injection of a 30 gauge needle into the left ventricle of the mouse. 1mL of heparin and 5 mL of red blood cell lysis buffer

were added to prevent blood clotting. The cellular suspension was centrifuged and the supernatant was discarded. The cellular pellet was used for further flow cytometric studies.

For long term survival studies following tail vein injections, mice were euthanized when visible internal tumor burden reached a maximum or when the mice became scruffy, lethargic or unable to take in food or water, as determined by DLAR personnel.

Bioluminescent Imaging of Metastasis. 10 days following splenic or tail vein injections, mice were re-anesthetized with 60 mg/kg of sodium pentobarbital and administered 150 mg/kg D-luciferin (BD Bioscience) intraperitoneally. After 10 minutes, mice were assessed for relative CT-26 luciferase bioluminescence using a Kodak IS4000MM small animal imaging device over a 10 minute time period. Exposure conditions that include time, aperture, stage position, binning, and time after D-luciferin injection, were all held constant for each measurement. Bioluminescence intensity images were pseudo-colored (pink least intense and red most intense). All images were quantified using MetaMorph software (Universal Imaging Corp). Average image intensity was quantified using a fixed region of interest and calculated values were expressed as relative light units per minute (RLU).

Active NF-kB, Cyclin D1 and IkB Immunohistochemistry in Human Tumors. Under IRB-approved protocol, archived colon, lung and head and neck malignant tumors were sectioned, deparaffinized, steamed at 95℃ with citrate antigen retrieval buffer (DAKO, Carpinteria, CA), rinsed with PBS and fixed with

3% hydrogen peroxide. Non-specific staining was prevented by adding horse serum (Vector Laboratories, Burlingame, CA). The slides were rinsed and primary antibody to active NF-kB (Invitrogen), cyclin D1, or IkB was added at room temperature. After PBS-washing, slides were incubated with the biotinylated secondary antibody, streptavidin-peroxidase and amino-ethyl carbazol chromogen (VectaStain Universal Rapid Kit, Vector). Staining intensity was monitored to prevent overstaining. Slides were hematoxylin-counterstained and coverslipped using Geltol (ThermoShandon, Fisher Scientific, Hanover Park, IL).

A reviewer blinded to the study assigned scores from 0 (no immunostaining) to 4 (maximal immunostaining intensity) to the three areas under review; tumor center, tumor periphery and adjacent non-malignant tissue. Areas were determined based on proximity to non-malignant tissue, cell morphology and density. All areas of the slide that were able to be evaluated were evaluated.

Discernable mitotic figures were also counted in each area of the tumor periphery and center. Mitotic figures were also counted separately in tumor areas defined subjectively by a blinded reviewer as highly immunoreactive for active NF-kB or less immunoreactive for active NF-kB. All areas of the slide were counted for these studies.

Statistical Analysis. SigmaStat (SPSS Inc., Chicago, IL) was utilized for statistical analysis. Results were expressed as mean ± standard error. Differences between two groups were analyzed using Student's t-test and

differences in immunostaining intensities were evaluated by chi-squared. Statistical significance was set at $p<0.05$.

Table 1. Inhibitors

Inhibitor	Target	Concentration (Adhesion, Proliferation)	Manufacture	Catalog Number
PP2	Src	65nM, 30nM	EMD Chemicals	529573
NSC23766	Rac1	50uM, 30uM	EMD Chemicals	553502
Akt Inh. IV	Akt	1uM	EMD Chemicals	124011
SN50	NF-kB	12uM	EMD Chemicals	481480
Serine 276 Inhibitory peptide	NF-kB	25uM	Imgenex	IMG-2001
TCH-021	NF-kB	5uM	Dr. Jetze Tepe	N/A
Calphostin C	PKC	100nM	EMD Chemicals	UCN-1028c
Go6976	PKC α/β	6nM	EMD Chemicals	365250
3-(1-(3-Imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione	PKC β	15nM	EMD Chemicals	539654
PKC-ε Inh. Peptide	PKC ε	10nM	EMD Chemicals	539522
IKK-2 Inh.	IKK	10mM	EMD Chemicals	401481
IKK-3 Inh.	IKK	40nM	EMD Chemicals	401488
IKK Phospho-Inhibitor	IKK	90nM	EMD Chemicals	401489

Table 2. Antibodies

Antibody	Source	Manufacture	Catalog Number
Actin	Mouse	Sigma Aldrich	A3853
CARP-1	Rabbit	Sigma Aldrich	HPA007856
Crk	Mouse	BD Biosciences	610035
Cyclin D1	Mouse	Cell Signaling	2922
FAK (phospho)	Rabbit	Biosource	700255
GAPDH	Mouse	Biodesign Intl.	H86045M
HA	Mouse	Covance	MMS-101R
Histone H1	Rabbit	Santa Cruz	SC-10806
IkB (phospho)	Mouse	Cell Signaling	9246
IkB (total)	Rabbit	Sigma Aldrich	I0505
IKK α/β (phospho)	Rabbit	Cell Signaling	2681
Myc	Mouse	Cell Signaling	2278
NF-kB (p50)	Rabbit	Cell Signaling	3035
NF-kB (p65)	Rabbit	Millipore	AB1604
p130cas	Mouse	BD Biosciences	610271
Paxillin (total)	Mouse	BD Biosciences	610568
Paxillin (phosphoY31)	Rabbit	Biosource	44720G
Paxillin (phosphoY118)	Rabbit	Cell Signaling	2541
Rac1 (total)	Mouse	BD Biosciences	610650

CHAPTER 3

CARP-1 Modulation of Pressure-Induced Cancer Cell Adhesion

Malignant tumor cells in cancer patients undergoing surgical resection may be susceptible to extracellular forces such as increased pressure and shear stress during tumor manipulation or during passage through the venous and lymphatic system during dissemination. Tumor cells shed into a body cavity may also be exposed to pressure, shear, and turbulence during laparoscopic insufflation or irrigation, whereas intraabdominal pressure increases substantially for 2 to 3 days after surgery because of bowel edema and third spacing [1,5]. Previous studies suggest that increases in extracellular pressure, similar in magnitude to those observed in vivo in some surgical patients, stimulate the adhesion of colon cancer cells by a focal adhesion kinase (FAK)-dependent mechanism, but the other mediators of this pathway are poorly understood [7].

Interstitial fluid pressure in patients with invasive ductal carcinoma is increased compared with normal breast parenchyma and other malignant and nonmalignant conditions [18,19] by as much as 29 mmHg. We hypothesized that the adhesion of breast cancer cells might therefore be stimulated by extracellular pressure in a manner similar to that observed in colon cancer cells.

CARP-1/CCAR1 is a novel protein that has been implicated in cell-cycle and apoptosis regulation [39,40]. CARP-1 is a perinuclear/cytoplasmic transducer of apoptosis signaling that binds with stratifin/14-3-3 sigma. Depletion of CARP-1 inhibits apoptosis caused by agents such as adriamycin. Conversely, overexpression of CARP-1 increases apoptosis and reduces expression of

various cell-cycle regulators including c-myc and cyclin B. CARP-1 is expressed in a variety of cell types including human breast and colon cancer cells. Because CARP-1 regulates diverse intracellular signaling pathways, we hypothesized that CARP-1 levels influence pressure-stimulated cancer cell adhesion by modulating mediators of the pathways of cell adhesion and motility such as FAK [39].

We tested these hypotheses by studying MDA-MB-468 breast cancer cells in which CARP-1 expression was down-regulated or enhanced by stable expression of antisense or full-length complementary DNA (cDNA), respectively. We performed adhesion assays under conditions of ambient or 15 mmHg increased extracellular pressure and assessed CARP-1 levels, CARP-1-dependent attenuation of FAK activation, and CARP-1 regulation of FAK by western blotting.

We previously performed various experiments involving different pressures and different time points and found that a 30-minute exposure to increased pressure was sufficient to both activate intracellular signals and enhance adhesion in colonic adenocarcinoma and other cancer cells [7]. We chose to replicate these conditions while evaluating CARP-1 in MDA-MB-468 cells, both for consistency with our previous signal transduction work and because breast cancer cells are likely to experience a range of increased pressures up to 30 mmHg; 15 mmHg pressure increases therefore seem relevant to breast cancer patho-physiology.

We first sought to determine whether adhesion of MDA-MB-468 breast cancer cells increases in response to pressure because pressure effects on

breast cancer cells had not previously been shown. Single cell suspensions of several subclones of MDA-MB-468 breast cancer cells were allowed to adhere to type-I collagen for 30 minutes under ambient, 15 mmHg, or 30 mmHg increased pressure conditions before washing away nonadherent cells. Because the sub-clones yielded similar results, data were pooled for analysis. MDA-MD-468 adhesion was increased by 25% ± 6% in response to 15 mmHg and 46% ± 9% in response to 30 mmHg increased pressure (Figure 1, n=3; p,0.05).

Because MDA-MB-468 breast cancer cells are known to express CARP-1 and because FAK activation has previously been shown to mediate the effects of pressure on cancer cell adhesion, we sought initially to determine whether manipulating CARP-1 levels alters basal FAK activation. Cells were either untreated (wild type) or treated with retroviruses expressing wild-type CARP-1 for 24 or 48 hours, followed by western blot analysis for expression of activated (p-FAK) and native FAK proteins. FAK-397 phosphorylation was dramatically inhibited by CARP-1 overexpression (Figure 2, n=3; $p<0.05$).

Because pressure-stimulated MDA-MB-468 breast cancer cells adhesion and CARP-1 appeared to modulate FAK signaling, we created stable cell lines that overexpress CARP-1 or display decreased CARP-1 expression to investigate whether CARP-1 modulation would alter the adhesive response to pressure. We have previously characterized MDA-MB-468 subclones, stably transfected with antisense to CARP-1, which express approximately 50% less CARP-1 protein compared with the parental line. We chose 2 such subclones

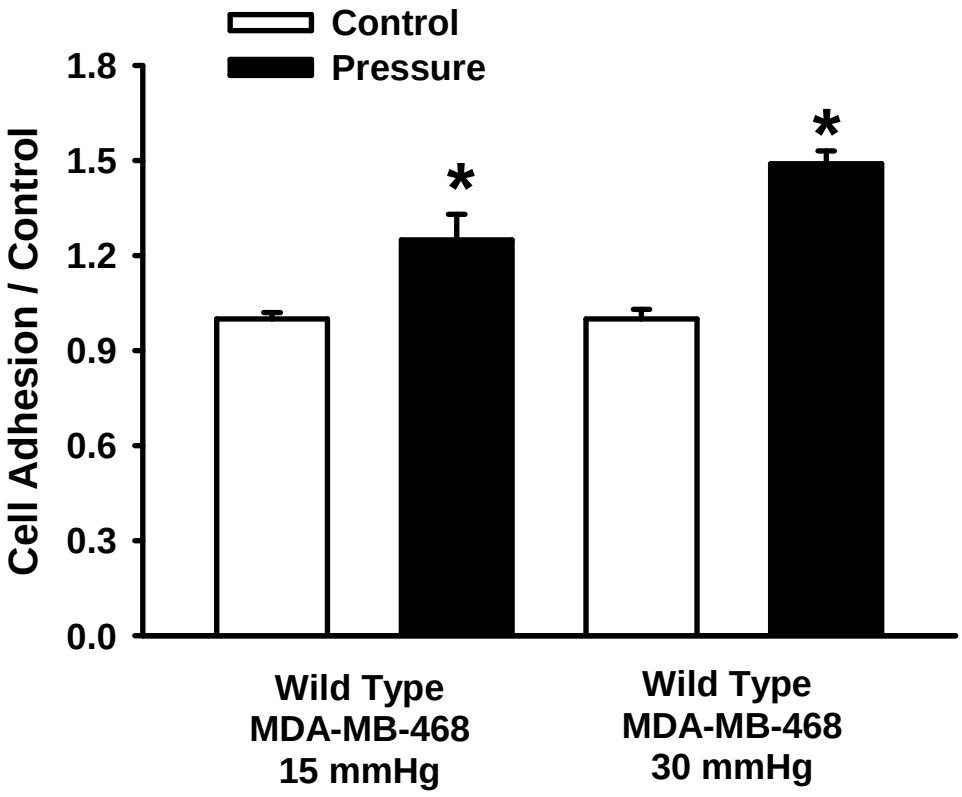


Figure 1. Effects of pressure on wild-type and vector-transfected cell adhesion. Adhesion to type I collagen under ambient pressure conditions (open bars) was compared with adhesion under conditions of 15 mmHg increased pressure and again under conditions of 30 mmHg increased pressure (shaded bars) for 30 minutes. At least 20 high-power fields were counted in each dish, and 3 separate dishes were studied in each experiment. Adhesion data for each cell line were normalized to their respective ambient pressure controls before pooling data from 2 separate experiments with similar results (*$P<0.05$).

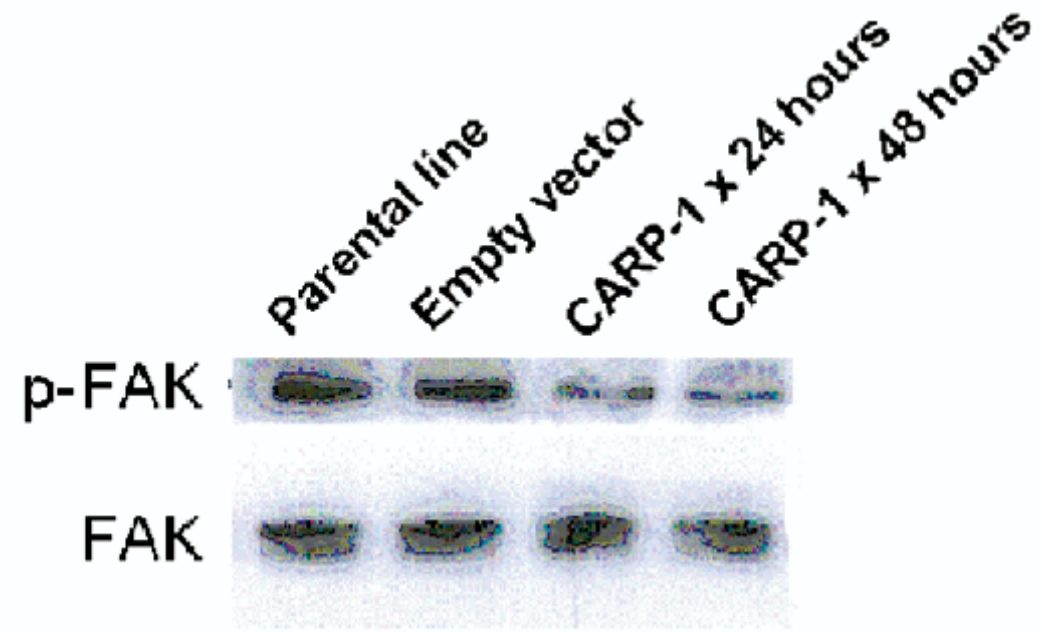


Figure 2. Overexpression of CARP-1 leads to inhibition of FAK activity. FAK-397 phosphorylation was compared in wild-type parental cells (lane 1), cells that were transduced with viruses encoding CARP-1 (lanes 3 and 4, for a period of 24 and 48 hours, respectively). Blots were stripped and reprobed for total FAK protein as a loading control. FAK-397 phosphorylation was dramatically inhibited by CARP-1 overexpression. Representative western blots are shown from 1 of 3 similar studies.

and compared their adhesive response to pressure with 2 antisense-transfected subclones with normal CARP-1 expression.

Despite successful reduction of CARP-1, both cell lines expressing reduced CARP-1 continued to display pressure stimulated adhesion (Figure 3, $n=16$; $p<0.05$). Pressure stimulated adhesion in the antisense-transfected cells that did not express reduced CARP-1 was similar to both the parental line and the CARP-1 reduced cells.

Because reducing CARP-1 did not appear to modulate pressure stimulated adhesion, we performed similar studies after stably overexpressing CARP-1 (Figure 4, $n=3$; $p<0.05$). Adhesive responses to increased extracellular pressures in CARP-1 overexpressing cells were also similar to those observed in matched subclones from the same transfections that did not express CARP-1 (Figure 5, $n=16$; $p<0.05$).

The regulation of cancer cell adhesion is an important phenomenon that may affect cancer metastasis in the perioperative period. Cell adhesion may be influenced by FAK signaling, and pressure stimulated adhesion is known to be mediated by FAK activation, but the mechanism by which this FAK signaling is activated by extracellular pressure is poorly understood [41]. Our data suggests that breast cancer cells display an increased adhesive response to extracellular pressure similar to that which we have previously described in colon cancer cells. Thus, the effect of extracellular pressure on cancer cell adhesion is not limited to colon cancers [41]. CARP-1 does modulate basal FAK signaling, but changes in

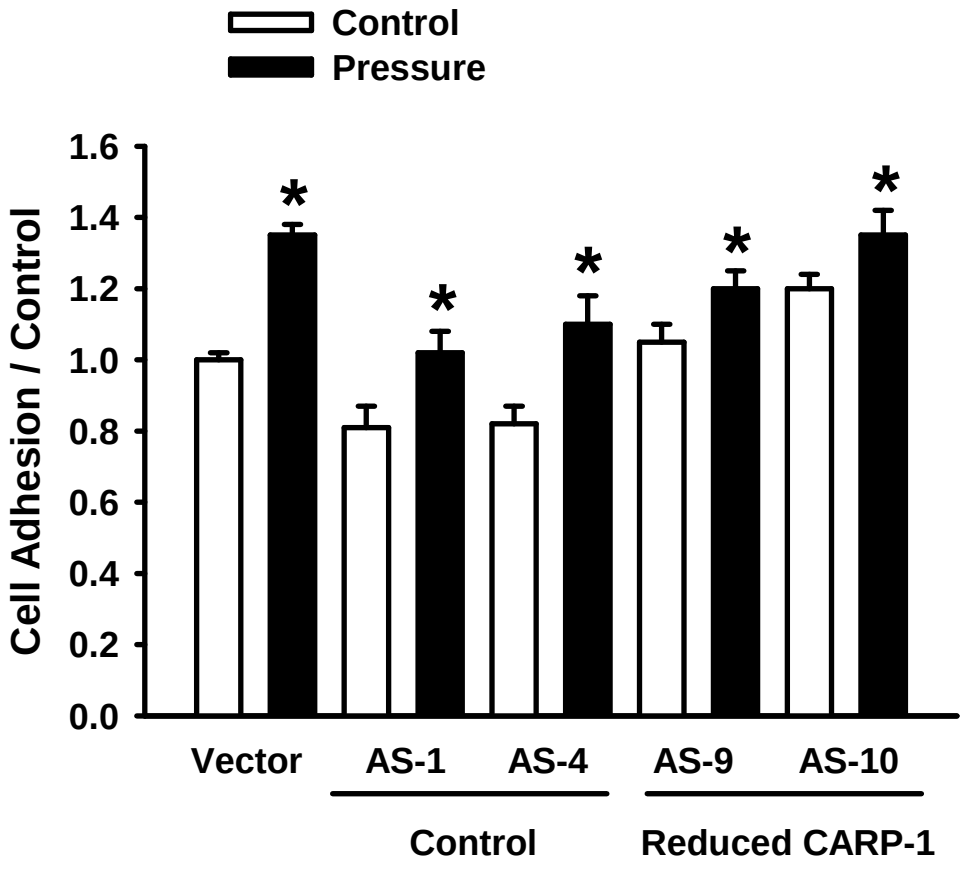


Figure 3. Reduction of CARP-1 does not alter pressure-stimulated adhesion. Cell adhesion under ambient pressure conditions (open bars), was compared with adhesion under 15 mmHg increased pressure conditions (shaded bars) for an empty vector, as well as 2 MD-MBA-468 subclones that did not display CARP-1 reduction (clone 1.6 subline 1 and clone 1.6 subline 4; AS-1 and AS-4, respectively) and 2 subclones that displayed approximately 50% CARP-1 reduction (clone 1.6 subline 9 and clone 1.6 subline 10; AS-9 and AS-10, respectively). Each subclone displayed increased adhesion in response to extracellular pressure, independently of modulation of CARP-1 expression. At least 20 high power fields were counted iin each dish, and 2 separate dishes were studied in each experiment. Adhesion data for each cell line were normalized to their respective ambient pressure controls before pooling data from at least 8 separate experiments with similar results (*P<0.05).

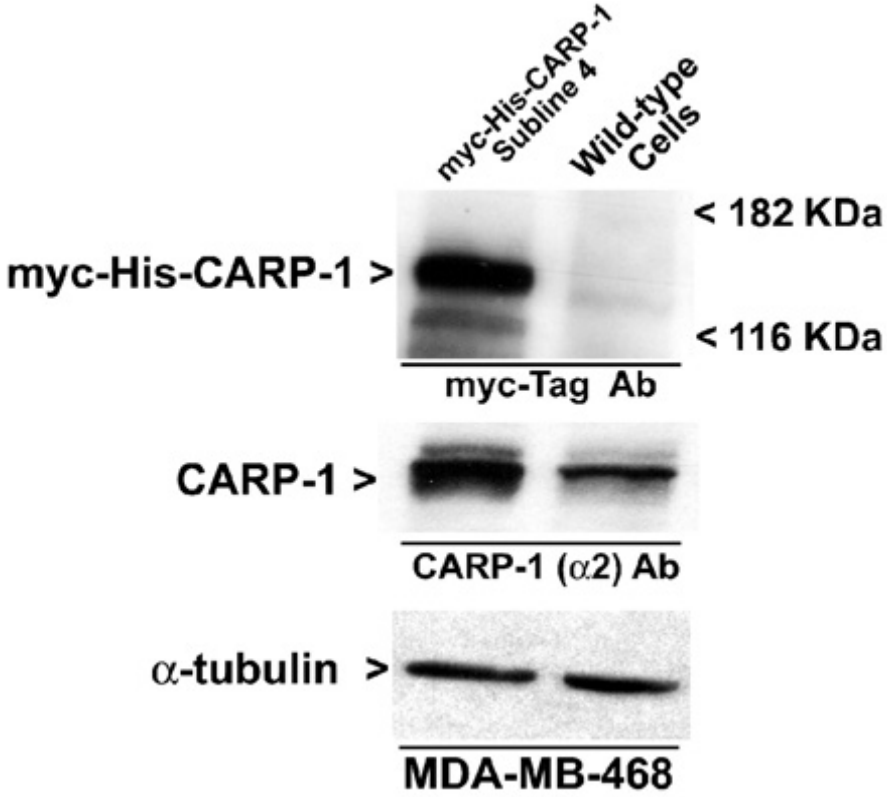


Figure 4. Overexpression of CARP-1. Stable overexpression of CARP-1 is shown in MDA-MB-468 CARP-1 subline 4 in lane 1 compared with the parental MDA-MB-468 line in lane 2. This figure depicts 1 of 3 similar western blots probed for the overexpressed myc-tagged transfectant with anti-myc antibody, for native CARP-1 itself with CARP-1 (α-2) antibody, and with α-tubulin as a protein loading control.

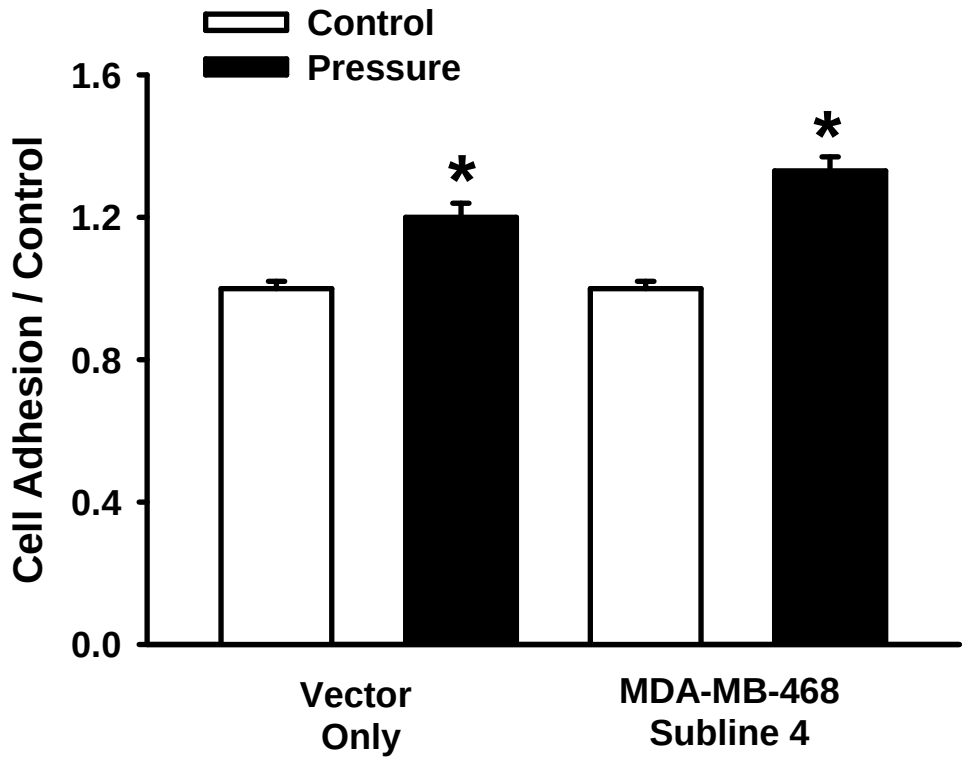


Figure 5. Overexpression of CARP-1 does not alter pressure-stimulated adhesion. Cell adhesion under ambient pressure conditions (open bars) was compared with adhesion under 15 mmHg increased pressure conditions (shaded bars) for the MDA-MB-468 CARP-1 subline 4, which overexpresses CARP-1 and for MDA-MB-468 cells transfected with the empty vector alone. Each subclone displayed increased adhesion in response to extracellular pressure, independently of modulation of CARP-1 expression. At least 20 high power fields were counted in each dish, and 3 separate dishes were studied in each experiment. Adhesion data for each cell line were normalized to their respective ambient pressure controls before pooling data from 2 separate experiments with similar results (*$P<0.05$).

CARP-1 expression does not affect the adhesive response to pressure, which suggests that CARP-1 is not involved in pressure stimulated adhesion.

Interstitial pressure is elevated in breast cancer patients undergoing needle biopsy [18]. In fact, increases in interstitial pressure seem to be a characteristic of rapidly growing breast cancers against their defining stroma. The activation of the adhesion of cancer cells by endogenous pressure within the tumor may affect the potential for metastasis both by increasing the incidence of local wound recurrence in the perioperative period, a complication requiring subsequent reoperation and mastectomy, or by increasing the metastatic potential of tumor cells as they pass through the lymphatics during dissemination even before surgical resection. Furthermore, when surgery is performed on breast cancer patients, even more pressure is exerted, which may cause a direct local effect specific to the perioperatiive period in which further pressure by surgical manipulation might cause shed tumor cells to adhere more efficiently. Thus, the pathway by which pressure stimulates adhesion may well be significant for breast cancer cells in vivo. We have previously shown that pressure stimulates colon cancer cell adhesion not only to purified matrix proteins but also to endothelial cell monolayers [41] and to surgical wounds in vivo [21].

Focal adhesion kinase (FAK) is a protein tyrosine kinase that is often overexpressed in cancer cells [41]. It is recruited to focal adhesions on the clustering of integrins through molecules such as talin or paxillin. FAK tyrosine 397 is of particular interest because it rapidly undergoes autophosphorylation on cell adhesion to extracellular matrix proteins. This leaves a docking site for the

Src homology 2 domains of the Src family protein kinases that bind and further activate FAK, leading to association with PI3 kinase and activation of the MAP kinase cascade [42,43,44]. Previous studies have shown that pressure stimulates cell adhesion via FAK autophosphorylation of tyrosine 397. Conversely, blocking FAK signaling by transfection of a dominant negative FAK construct or reducing FAK by specific siRNA each prevent pressure stimulated adhesion in colon cancer cells [45,46]. Similar FAK signaling pathways seem to mediate pressure effects in various cell types. Our study extends these observations to breast cancer cells. Although FAK activation has not been specifically shown to mediate pressure stimulated adhesion in breast cancer cells in these studies, FAK may be similarly involved. Our results indicate that although CARP-1 does inhibit basal FAK tyrosine 397 phosphorylation, CARP-1 overexpression does not appear to block the stimulation of FAK tyrosine 397 phosphorylation by extracellular pressure or at least one downstream consequence; increased adhesion. This does not exclude the possibility that CARP-1 effects on FAK activation could still be important to other aspects of FAK signaling.

We have previously studied the effects of pressure on colon cancer cell apoptosis and anoikis [31]. Thus, pressure mediated FAK signaling might have other effects, with which CARP-1 might conceivably also interact. Such other potential effects of pressure in these cells await further study. CARP-1 is involved in apoptosis signaling pathways through interactions with the cytoplasmic protein 14-3-3 [39]. Overexpression of CARP-1 inhibits cell growth regulatory proteins

such as c-myc. When CARP-1 was depleted, cell-cycle arrest and apoptosis signaling were altered [39,40]. Because CARP-1 modulates FAK signaling and FAK signaling is required for pressure stimulated adhesion, one would therefore similarly expect that modulation of CARP-1 would alter pressure stimulated adhesion. However, neither long term depletion nor overexpression of CARP-1 impeded pressure stimulated adhesion in breast cancer cells. Furthermore, no significant changes in CARP-1 levels were noted after short-term exposure to 15 mmHg increased pressure.

Our initial studies show that CARP-1 does modulate FAK activation in transient overexpression studies and raised the question of whether such a modulation might mediate pressure stimulated adhesion. The apparent dichotomy between the modulation of FAK in transient CARP-1 overexpression and the lack of effect of stable CARP-1 overexpression or underexpression might reflect adaptive processes to the chronic stable transfection. These might include compensatory modulation of other proteins that might interact with CARP-1, FAK or other proteins in the signal cascade. Indeed, database analyses recently revealed the presence of human deleted in breast cancer (DBC)-1 protein, which has significant homology to human CARP-1. Although CARP-1 and DBC-1 are encoded by separate genes located on chromosome 10 and 8, respectively, whether they possess overlapping or distinct functional domains and the extent to which DBC-1 may regulate signaling for pressure induced cell adhesion remain to be elucidated [39,40]. The characterization of such adaptations would be an interesting study in its own right, but our own results suffice to address our

initial hypothesis about CARP-1 because the stable transfection results strongly suggest that CARP-1 itself does not seem to modulate pressure stimulated adhesion. Alternatively, because CARP-1 modulates many other signal proteins besides FAK, the downstream effectors of CARP-1 may interact with the pressure pathway in other compensatory ways, co-modulating FAK signaling or reconstituting the pressure activated pathway downstream of FAK. Better understanding of how breast cancer cell pressure stimulated adhesion is reconstituted despite CARP-1 modulation therefore awaits delineation of the precise mechanism by which FAK itself modulates pressure stimulated adhesion in the future.

The results of this study do not exclude the possibility that CARP-1 may modulate other downstream consequences of FAK signaling. We studied the effects of inside out signaling in which intracellular signaling events triggered by increased extracellular pressure regulate integrin ligand binding function. However, FAK has been most frequently described as active in outside in signaling after cell adhesion, in which the occupancy of integrins by matrix ligands from outside the cell initiates signaling within the cell on ligand binding that then leads to downstream cellular consequences [41,46]. These results, therefore, do not exclude the possibility that CARP-1 modulation of FAK activation may influence outside-in signaling downstream of FAK, which is beyond the scope of our CARP-1/FAK study.

For the first time, we have shown that breast cancer cells do display pressure stimulated adhesion. This information may be important for

perioperative management to reduce local wound recurrence as well as for our ultimate understanding of the biology of these neoplasms. Because pressure also stimulates cancer cell proliferation [31], the proliferation of breast cancer cells within a constraining stroma may also be stimulated by extracellular pressure, causing a positive feedback cycle in which more rapidly proliferating tumors generate more pressure, which may cause increased proliferation. CARP-1 does inhibit FAK and could contribute to modulation of some of the signaling actions of this important kinase. Although the mechanisms by which breast cancer cells escape from CARP-1 modulation to continue to display pressure stimulated adhesion are unclear, our data suggests that CARP-1 modulation may not be an attractive target for interventions designed to interfere with this potentially deleterious pressure activated pathway.

CHAPTER 4

Role of Paxillin in Pressure-Induced Colon Cancer Cell Signaling

Paxillin is a 68 kDa focal adhesion associated protein that facilitates protein – protein interactions, focal adhesion assembly and functions in cell signaling downstream of integrins [47]. These events influence diverse cellular behaviors, including cell migration, proliferation, survival and apoptosis. In some cases, paxillin functions as a structural protein that links other proteins and facilitates formation of focal adhesions [26]. However, in other instances, kinases may phosphorylate paxillin directly and alter its binding affinity [24]. Paxillin localizes to focal adhesions and has been shown to interact directly with the α5 and α9 subunit of β1 – integrin [48,49,50] and provide a platform for tyrosine kinases including FAK and Src that can become activated in response to adhesion or growth factor stimulation [26,51,52,53]. Paxillin phosphorylation may also regulate the recruitment of downstream molecules such as Crk that can further lead to activation of $p130^{Cas}$ [29,47]. In addition, negative regulators of these pathways, including Csk and PTP-PEST bind directly to paxillin.

Previous studies from our laboratory have shown that paxillin is required for extracellular pressure to increase cancer cell adhesion, since paxillin reduction by siRNA blocks the effect and paxillin overexpression seems to reproduce it. Increased extracellular pressure also stimulates paxillin phosphorylation at tyrosines 31 and 118 [25,54]. However, it has not been clear whether paxillin acts simply as an adaptor protein or whether paxillin phosphorylation is necessary for the effects of extracellular pressure on cancer

cell adhesion. This is important because understanding how paxillin acts may ultimately facilitate the design of therapeutic interventions to reduce cancer cell adhesion and metastasis.

We therefore hypothesized that paxillin phosphorylation is important to the regulation of cancer cell adhesion, and sought to test this hypothesis using paxillin phosphorylation single mutants that are unable to be phosphorylated at tyrosine 31, 118 or 181, a Y31Y118 double mutant and a Y31Y118Y181 triple mutant. We transfected these constructs into human SW620 colon cancer cells and examined their effects on pressure stimulated cancer cell adhesion. We then determined the effects of increased pressure and transfection with non-phosphorylatable paxillin mutants on the potential paxillin associated molecules Crk, Cas and Rac1 to further delineate signaling events downstream of paxillin. Next, we examined the association of paxillin with α-actinin a molecules that is known to be involved in pressure – stimulated cancer cell adhesion [55], by reducing α-actinin levels and evaluating paxillin phosphorylation. Finally, we explored these signaling events in primary human colon cancer cells to confirm that our observations are not limited to a single cell line.

We have previously reported that increased extracellular pressure promotes paxillin phosphorylation in head and neck cancer cells [54]. However, we had yet to identify whether this phosphorylation is important to pressure - stimulated cancer cell adhesion. The cells transfected with single plasmid phosphorylation mutants all displayed increased adhesion in response to increased pressure. However, the cells transfected with the triple mutant did not

exhibit an increase in adhesion in response to pressure elevation (Figure 6A, n=5; $p<0.05$).

Tyrosine 31 and tyrosine 118 on paxillin are phosphorylated by FAK in response to various cellular events, including adhesion [52]. Since FAK is involved in pressure - stimulated cancer cell adhesion, we next sought to block these two tyrosine phosphorylation sites specifically. We transfected cells with a myc-tagged plasmid mutant that is unable to be phosphorylated at both tyrosines 31 and 118 and similarly evaluated basal and pressure stimulated adhesion. Transfection with a construct unable to be phosphorylated at these two tyrosine phosphorylation sites was sufficient to block pressure stimulated adhesion (Figure 6B, n=4; $p<0.05$).

In some other systems, paxillin phosphorylation at tyrosine 31 and tyrosine 118 creates a docking site for the adaptor protein Crk [27,52,56,57]. We therefore sought to determine whether Crk is also involved in pressure stimulated adhesion. Crk was reduced in SW620 cells approximately 74% by siRNA treatment and the cells were exposed in ambient or increased pressure (Figure 7A). When Crk was reduced, pressure did not stimulate cell adhesion (Figure 7B, n=4; $p<0.05$).

To evaluate the potential association between paxillin and Crk under pressure, we performed co-immunoprecipitation studies using SW620 cells exposed to ambient or increased pressure. Indeed, pressure increased the association of paxillin with Crk by 36 ± 8% compared to ambient pressure controls (Figure 7C, n=6; $p<0.05$). We therefore, next evaluated the association

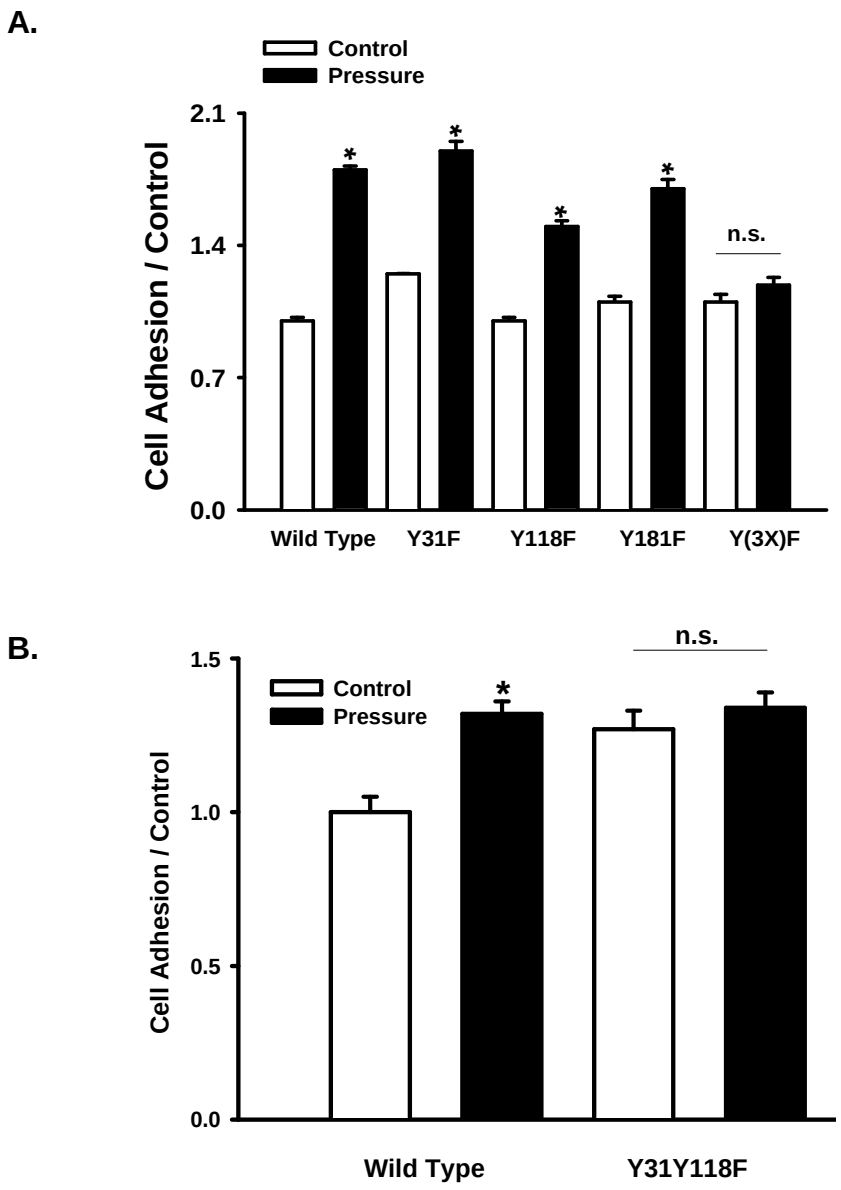


Figure 6. Effect of paxillin plasmid phosphorylation mutants on pressure-stimulated colon cancer cell adhesion. (A) 15mmHg increased extracellular pressure (shaded bars) stimulated SW620 cell adhesion to type I collagen in cells transfected with an HA-tagged Y31, Y118 and Y181 paxillin plasmid phosphorylation mutant, similar to that observed in wild type transfected cells. However, cells transfected with a Y31Y118Y181 plasmid phosphorylation mutant did not display increased adhesion in response to elevated pressure (n=5; $p<0.05$). (B) SW620 cells transfected with a Y31Y118 paxillin plasmid phosphorylation mutant did not display increased adhesion to type I collagen in response to elevated pressure (shaded bars) relative to ambient pressure controls (open bars) (n=4; $p<0.05$).

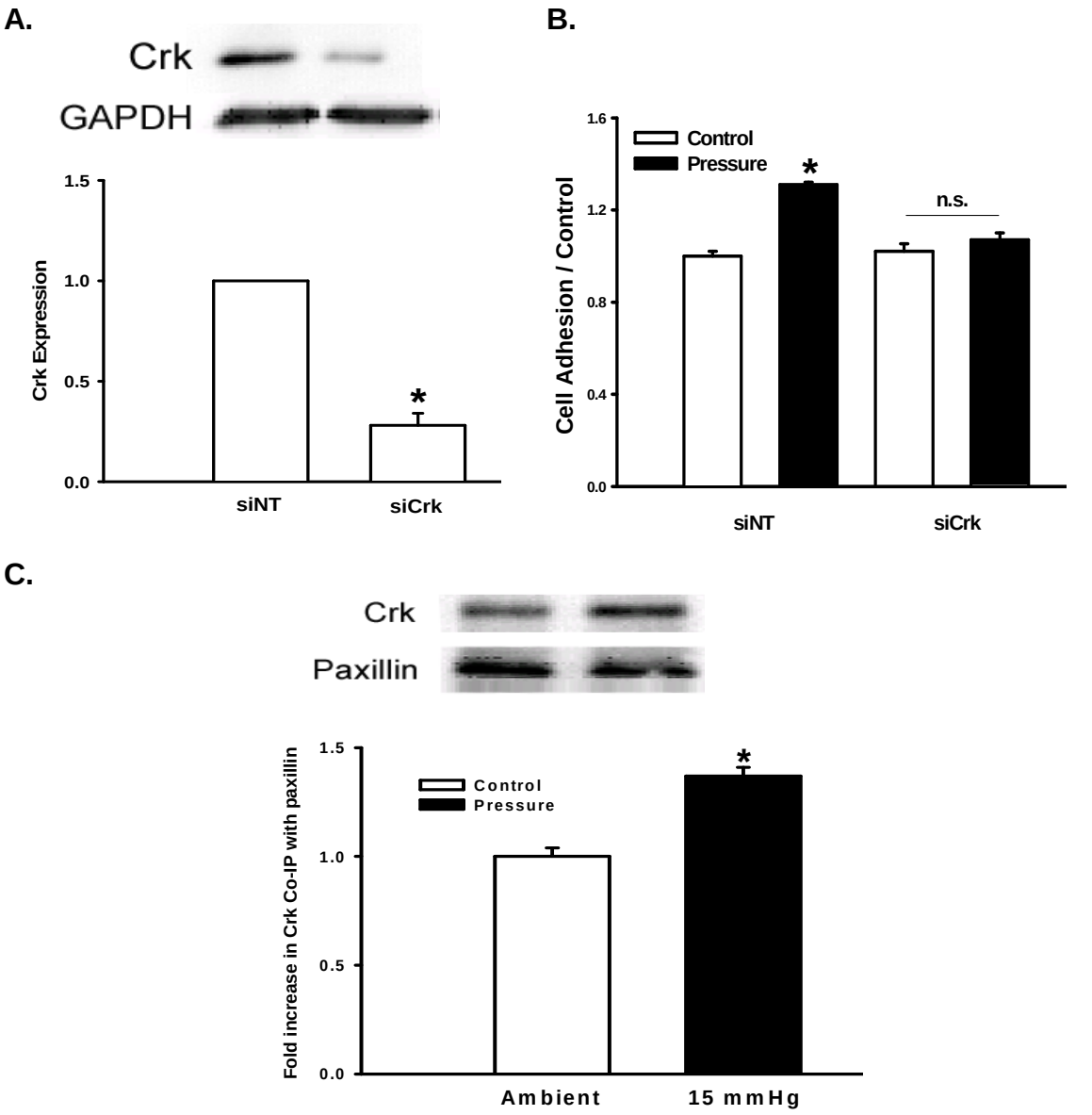


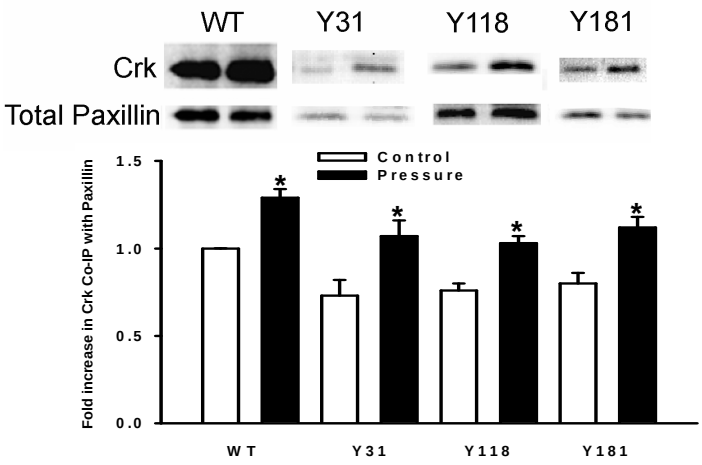
Figure 7. Crk is necessary for pressure stimulated colon cancer cell adhesion and Crk interacts with paxillin. (A) Typical reduction of Crk in SW620 cells transfected with Crk-specific siRNA. GAPDH was used as a loading control and Crk protein expression was normalized against non-targeting siRNA controls (n=4; $p<0.05$). (B) Cells transfected with a non-targeting siRNA control exhibited increased adhesion in response to 15 mmHg elevated pressure (shaded bars). In contrast, cells transfected with Crk-specific siRNA did not display increased adhesion in response to pressure (n=4; $p<0.05$). (C) Equal protein samples from SW620 cells that were exposed to ambient (open bar) or increased pressure (black bar) were immunoprecipitated for paxillin and probed for Crk by western blot. Samples were normalized against ambient controls (n=6; $p<0.05$). Paxillin-Crk association was increased in pressure-treated cells.

of Crk with paxillin in SW620 cells transfected with the paxillin single tyrosine mutants and the tyrosine double mutant by similar co-immunoprecipitation analysis. Paxillin-Crk interaction was increased under pressure by 34 ± 9%, 27 ± 4% and 32 ± 6% in the Y31, Y118 and Y181 transfected cells, respectively (Figure 8A, n=6; $p<0.05$). However, the association between paxillin and Crk was not significantly increased in the cells transfected with the Y31Y118 double mutant (Figure 8B, n=5; $p<0.05$).

To further validate these results, we assessed the interaction of paxillin and Crk under ambient and increased pressure conditions in primary human colon cancer cells isolated from 6 surgically resected specimens. Primary colon cancer cells yielded results similar to SW620 cells. Pressure increased the interaction of paxillin and Crk in these human cancer cells by 38 ± 4% (Figure 9, $p<0.05$).

Cas is a highly phosphorylated protein that may associate with Crk upon activation of Cas by Src [30,58,59]. We therefore investigated whether Cas is necessary for pressure - stimulated adhesion. We used siRNA to reduce Cas by approximately 62% in SW620 cells and exposed the cells to ambient or increased pressure (Figure 10A, n=3; $p<0.05$). Pressure - stimulated adhesion was abolished in these cells when siRNA was used to reduce Cas (Figure 10B, n=3; $p<0.05$). To determine if SW620 cells can be rescued from siRNA treatment, we transfected the cells with Cas specific siRNA in the presence of a Cas rescue plasmid or vector control. Cells transfected with Cas specific siRNA and Cas rescue plamid displayed increased adhesion in response to pressure,

A.



B.

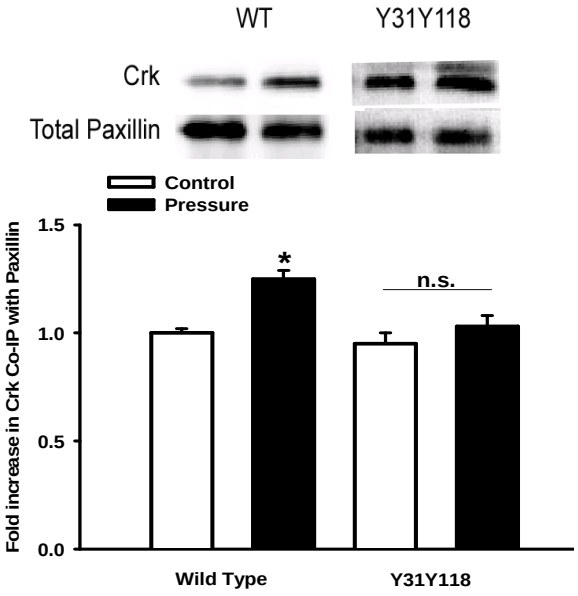


Figure 8. SW620 cells treated with paxillin point mutants display increased co-immunoprecipitation under pressure, double mutant treated cells do not. (A) Equal protein samples from SW620 cells that were transfected with a HA-tagged Y31, Y118 or Y181 paxillin plasmid phosphorylation mutants and exposed to ambient or increased pressure were utilized for co-immunoprecipitation studies. Crk co-immunoprecipitation with each mutant was significantly increased with elevated pressure (black bars) relative to ambient pressure controls (white bars) (n=6; $p<0.05$). (B) Co-immunoprecipitation studies were performed on equal protein samples from SW620 cells that had been transfected with a myc-tagged Y31Y118 paxillin plasmid phosphorylation mutant and exposed to ambient or 15 mmHg increased pressure. Crk co-immunoprecipitation with myc tagged paxillin was not increased in response to elevated pressure (n=5; $p<0.05$).

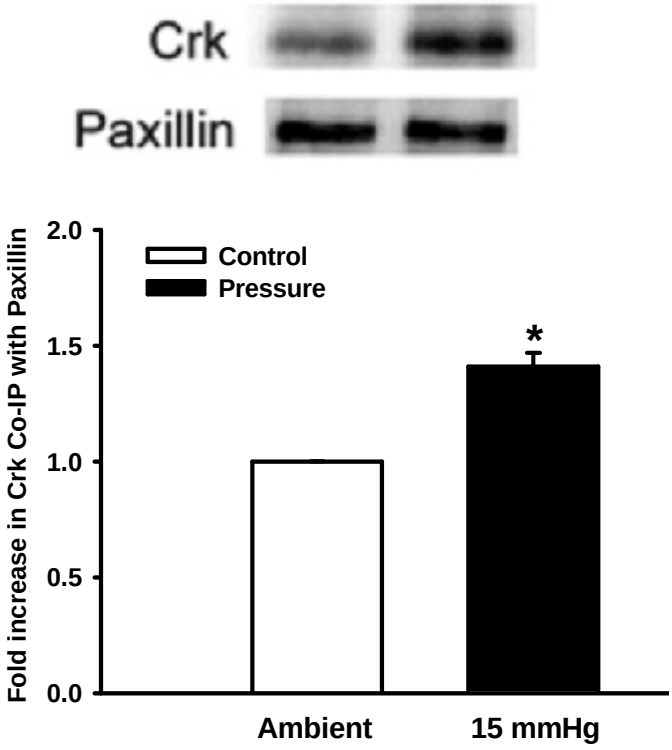
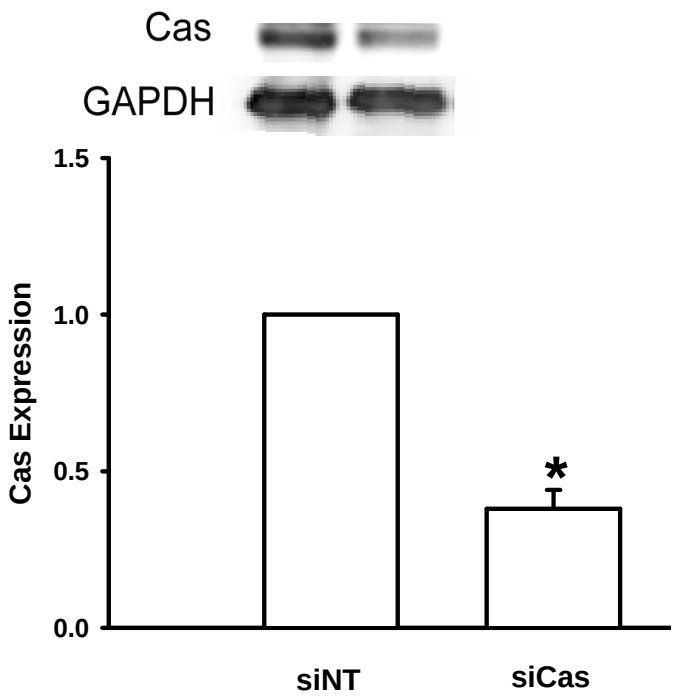


Figure 9. Pressure increases co-immunoprecipitation between paxillin and Crk in surgically resected human colon cancer cells. Primary human colon cancer cells isolated from surgically resected specimens were exposed to ambient or 15 mmHg increased pressure. Protein samples were used for co-immunoprecipitation studies and analyzed by western blot to evaluate the association of paxillin and Crk. Pressure increased paxillin-Crk association in primary colon cancer cells from six patients similarly to our observations in SW620 cells. ($p<0.05$).

A.



B.

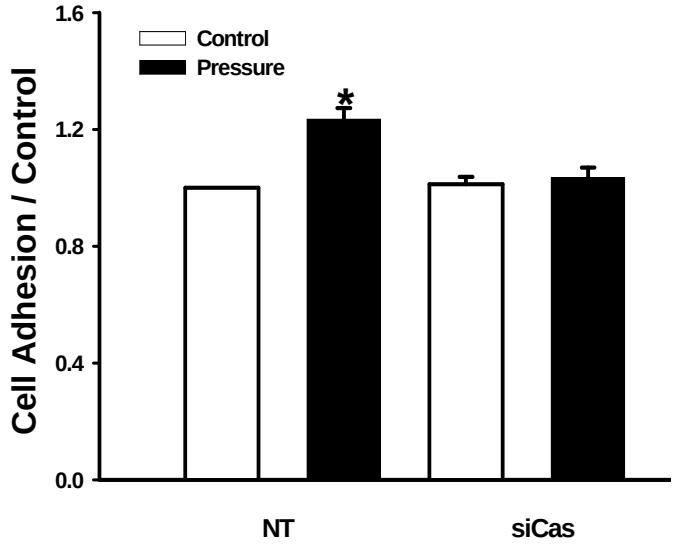


Figure 10. Cas is necessary for pressure-simulated adhesion. (A) Typical reduction of Cas in SW620 cells transfected with Cas siRNA. GAPDH was used as a loading control and Cas protein expression was normalized against non-targeting siRNA controls (n=3; $p<0.05$). (B) 15mmHg increased pressure (black bars) stimulated the adhesion of SW620 colon cancer cells that had been transfected with a non-target siRNA control. However, cells transfected with Cas-specific siRNA did not increase adhesion in response to increased pressure (n=3; $p<0.05$).

however, cells transfected with Cas siRNA and vector control did not increase adhesion in response to pressure (Figure 11, n=5; p<.05).

Since Crk and Cas may interact [60], we compared Crk-Cas interaction under conditions of ambient and elevated pressure using co-immunoprecipitation. Elevated pressure increases the association of Crk and Cas by 38 ± 7% (Figure 12, n=6, p<0.05).

We further evaluated whether Cas-paxillin association also changes in response to increased extracellular pressure. Cas did co-precipitate with paxillin under ambient pressure conditions, and the association increased 36 ± 5% under increased pressure conditions (Figure 13A, n=4; p<0.05).

We performed further studies to examine association between Cas and paxillin in cells transfected with the paxillin single and double plasmid tyrosine mutants. We found an increase of 30±9%, 24±5% and 32±6% between paxillin and Cas under increased pressure conditions in the cells transfected with the tyrosine 31, 118 and 181 plasmid mutants, respectively (data not shown; n=6; p<0.05). In contrast, the cells transfected with the double tyrosine mutant displayed no increase in association between paxillin and Cas after exposure to increased pressure (Figure 13B, n=5; p<0.05).

Activated Cas may phosphorylate and activate members of the small GTPase family, such as Rac1, that can in turn influence diverse cellular events including migration, motility and adhesion [61,62]. We therefore treated SW620 cells with NSC23766, an inhibitor of the small GTPase Rac1 and reduced Rac1 activation by approximately 88% (Figure 14A, n=5; p<0.05). Pressure –

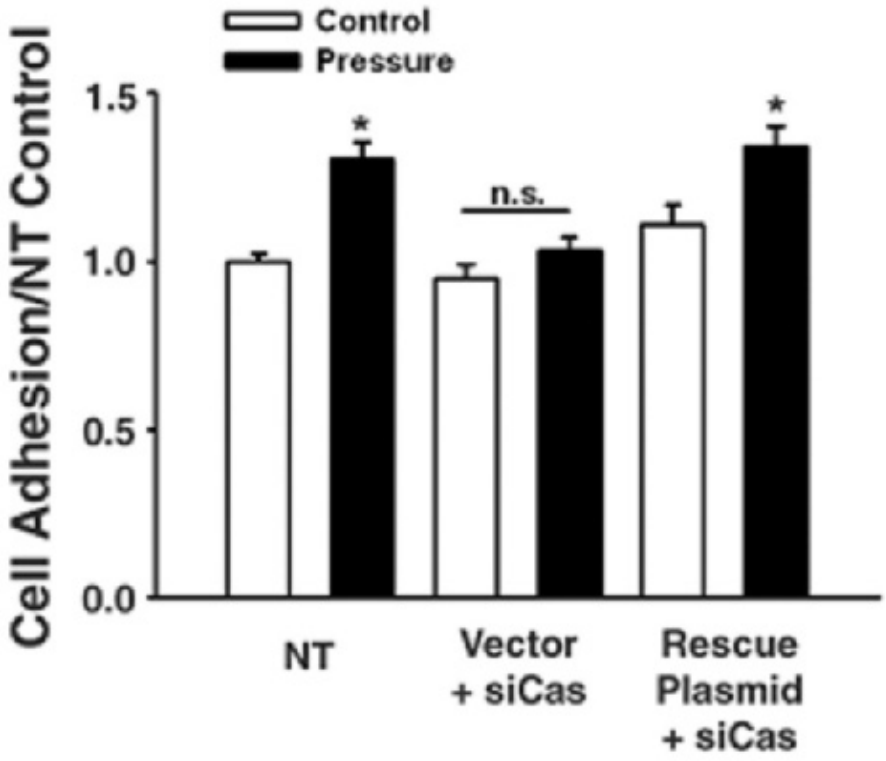


Figure 11. Treatment with Cas rescue plasmid recovers the effect of increased pressure on adhesion in the presence of Cas siRNA. Co-transfection of SW620 cells with Cas specific siRNA with a plasmid rescue construct containing a rat Cas construct resulted in an increased adhesion in response to pressure similar to non-targeting controls. Pressure had no effect in cells co-transfected with Cas-specific siRNA and the empty vector control (n=5; $p<0.05$).

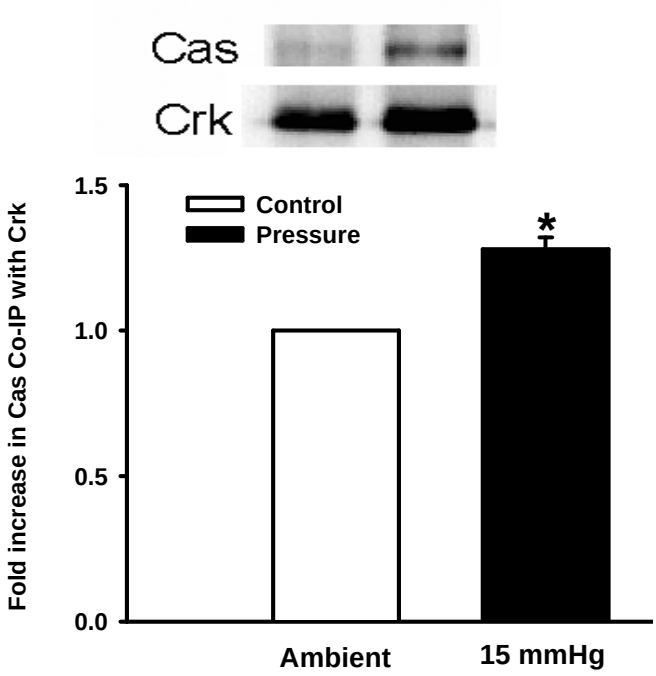
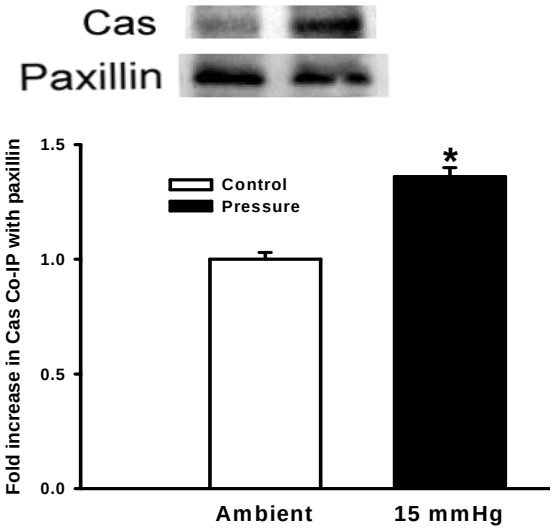


Figure 12. Cas and Crk co-immunoprecipitation increases under pressure. Co-immunoprecipitation studies were performed using equal protein samples from SW620 cells that had been exposed to ambient or increased pressure. The samples were immunoprecipitated for Crk, probed for Cas by western blot and normalized against total Crk. Pressure stimulated Crk-Cas association. (n=6; $p<.05$)

A.



B.

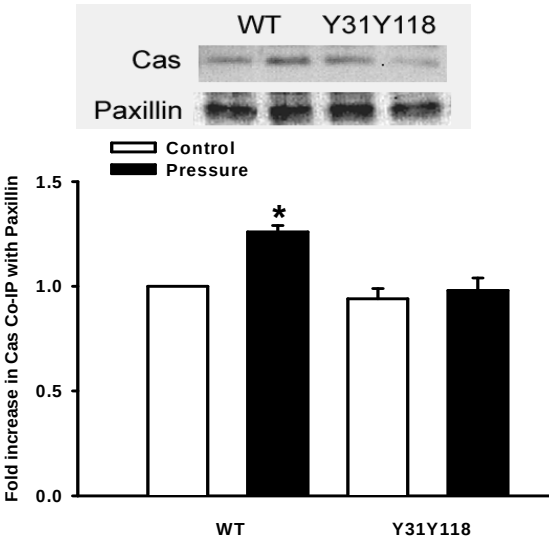


Figure 13. Co-immunoprecipitation of Cas and paxillin increases with pressure, however, effect is abolished following treatment with paxillin Y31Y118 mutant. (A) SW620 cells were exposed to ambient or increased pressure. Equal protein samples were subjected to co-immunoprecipitation. Paxillin was immunoprecipitated and Cas was probed for by western blot. Pressure stimulated paxillin-Cas association. (n=4, $p<0.05$). (B) Y31Y118 paxillin plasmid phosphorylation mutant was transfected into SW620 cells that were then exposed to ambient or increased pressure. Protein samples were immunoprecipitated for myc-tagged paxillin and cas protein levels were evaluated by western blot. Pressure did not stimulate paxillin-cas association in these cells. (n=5; $p<.05$).

stimulated adhesion was abolished in cells treated with this inhibitor (Figure 14B, n=5; $p<0.05$). Next, we sought to determine if Rac1 was activated in response to elevated pressure. We performed a Rac1 pull down assay and found that Rac1 was activation was increased by 28% in response to pressure (Figure 15A, n=6; $p<.05$).

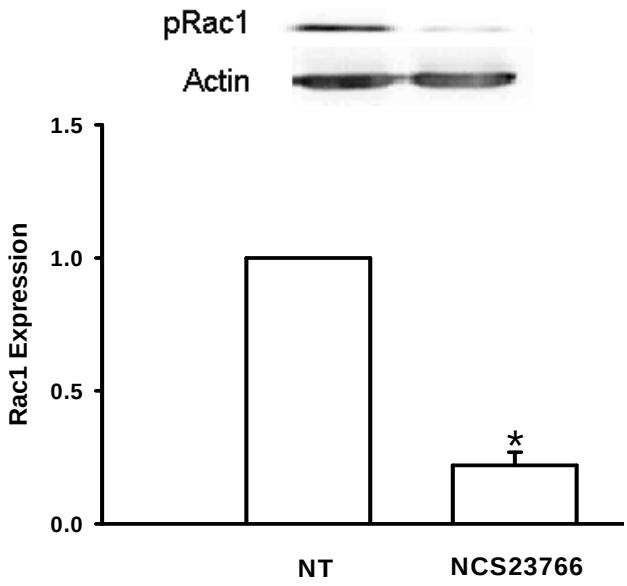
We therefore further evaluated the phosphorylation of Rac1 in response to increased extracellular pressure in SW620 cells transfected with either siRNA to reduce paxillin or a control non-targeted siRNA sequence. In the control cells, Rac1 phosphorylation was increased 38 ±4% under elevated pressure conditions (Figure 15B, n=7; $p<0.05$). However, the cells that were treated with siRNA to reduce paxillin showed no increase in phosphorylation in Rac1 in response to pressure.

We have previously shown that pressure – stimulated cancer cell adhesion requires FAK, Src and α-actinin. Therefore, we sought to evaluate the effect of reducing these molecules on paxillin phosphorylation.

We treated SW620 cells with siRNA to FAK and examined paxillin phosphorylation at Y31. The results indicated that paxillin Y31 phosphorylation is increased under elevated pressure conditions in cells transfected with a non-target siRNA control. In contrast, cells transfected with siFAK showed increased basal phosphorylation, but no additional increase in phosphorylation in response to elevated pressure (Figure 16, n=4; $p<.05$).

Next, we treated SW620 cells with PP2, a potent and specific inhibitor of Src family kinases. We found that cells treated with a DMSO control displayed

A.



B.

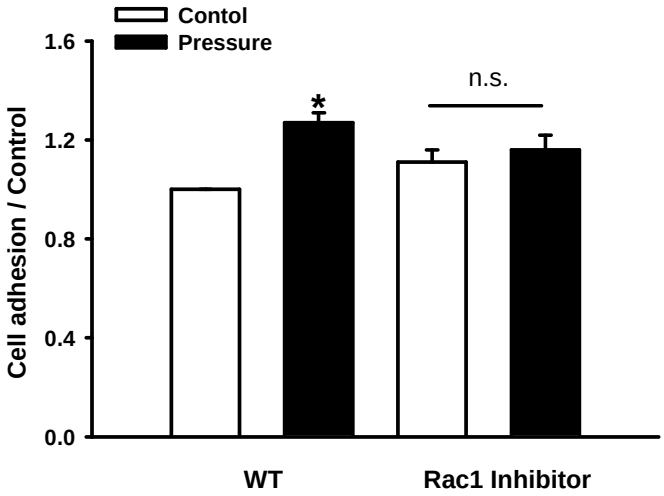
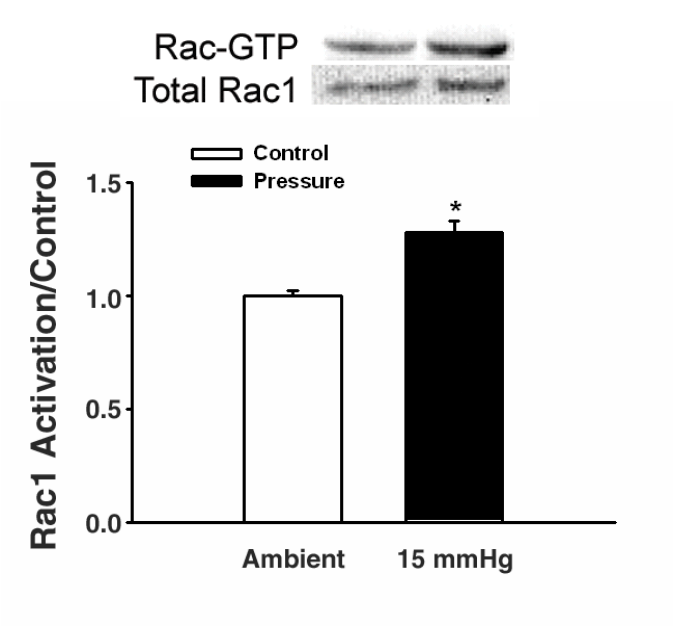


Figure 14. Rac1 is necessary for pressure-stimulated cancer cell adhesion. (A) Typical reduction in phosphorylated (active) Rac1 using NSC23766, an inhibitor of Rac1 activation. Phosphorylated Rac1 levels were normalized against actin (n=5; $p<.05$). (B) Wild type SW620 cells displayed increased adhesion in response to 15 mmHg increased pressure (shaded bars) relative to ambient controls. However, this effect was not seen in cells treated with NSC23766 (n=5; $p<.05$).

A.



B.

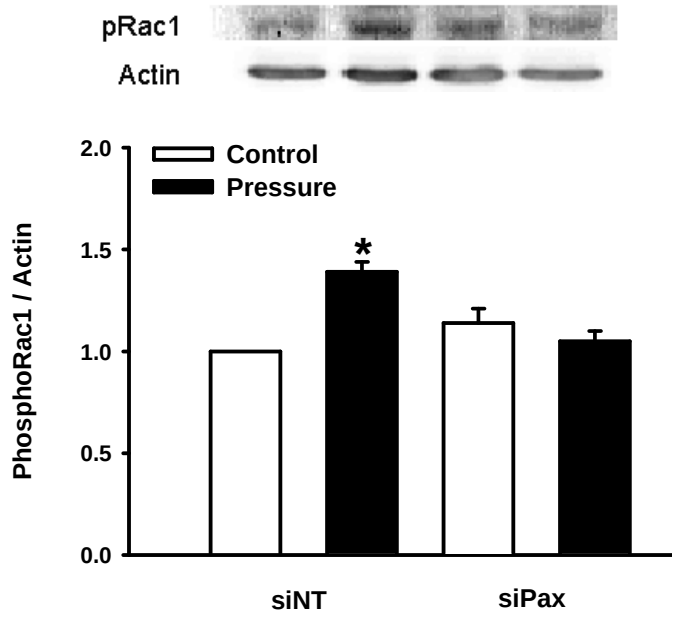


Figure 15. Paxillin affects Rac1 phosphorylation. (A) A Rac1 pull down assay was performed on SW620 cells that were exposed to ambient or increased pressure. Pressure increased the activation of Rac1 by 28% relative to ambient controls (n=6; p<.05). (B) SW620 cells transfected with a non-targeting siRNA display increased phosphorylation of Rac1 in response to elevated pressure (black bars) relative to ambient controls (white bars). Rac1 phosphorylation is not increased in response to elevated pressure in SW620 cells transfected with paxillin-specific siRNA (n=7; p<.05).

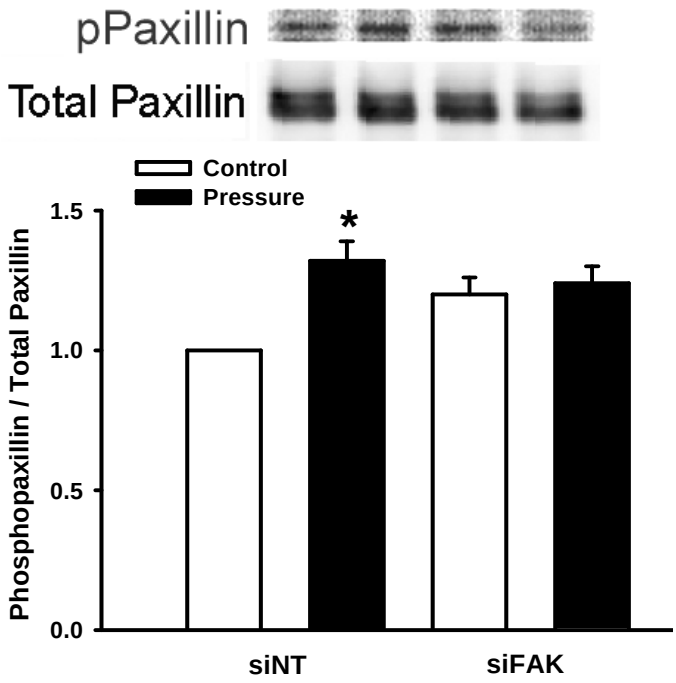


Figure 16. FAK influences paxillin phosphorylation. Phosphorylation of paxillin at tyrosine 31 increased under pressure in SW620 cells transfected with a non-targeting control. This effect was abrogated in cells transfected with FAK specific siRNA (n=4; $p<0.05$).

increased paxillin Y31 phosphorylation in response to pressure, however, the cells treated with PP2 had no increase in paxillin Y31 phosphorylation in response to pressure (Figure 17, n=5; $p<.05$). This result was consistent with similar observations in cells treated with Src specific siRNA that were probed for paxillin phosphorylation at Y31 (data not shown).

Finally, we sought to determine if silencing α-actinin with siRNA would affect paxillin Y31 phosphorylation. SW620 cells treated with a non-targeted siRNA control displayed increased paxillin Y31 phosphorylation. However, cells treated with siRNA to reduce α-actinin did not display increased phosphorylation in response to elevated pressure (Figure 18, n=6; $p<.05$).

Because non – targeting siRNA can potentially affect some proteins, we used scrambled siRNA sequences for several of the proteins examined to further confirm our results. Cells transfected with scrambled siRNA for paxillin, Crk and Cas all displayed increased adhesion in response to increased pressure similar to non – targeted controls (Figure 19, n=4; $p<.05$).

Tumor cell adhesion in response to elevated pressure is a physiologically relevant event, as metastasizing tumor cells are exposed to increased pressure during passage through the circulatory or lymphatic systems or during and after surgical procedures[63,64,65,66]. Elucidation of the intracellular signaling events that influence this adhesion may uncover useful targets to inhibit metastasis. Although previous preliminary studies had suggested that paxillin might be required for extracellular pressure to stimulate cancer cell adhesion [25,54], it was unclear whether paxillin was merely required for focal adhesion assembly

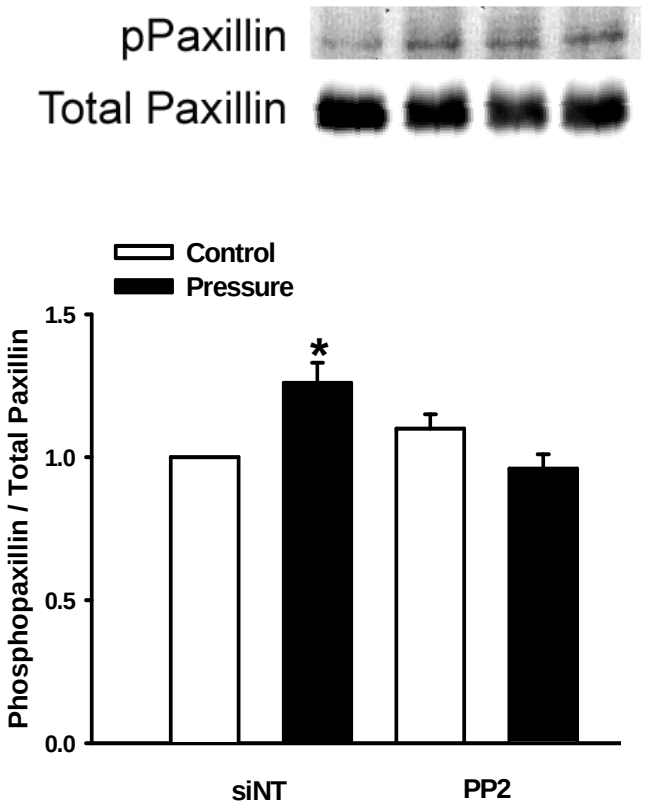


Figure 17. Src influences paxillin phosphorylation. Treatment of SW620 cells with PP2 blocked increased phosphorylation of paxillin at tyrosine 31 compared to non-targeting transfected control cells (n=5; $p<0.05$).

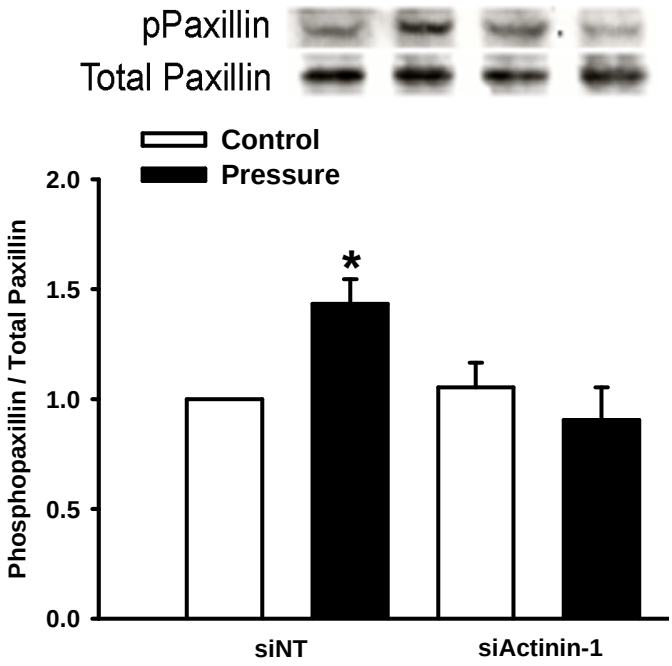


Figure 18. Alpha-actinin influences paxillin phosphorylation. Paxillin phosphorylation in response to pressure was higher in SW620 cells transfected with a control non-targeting siRNA but not in cells transfected with alpha-actinin-1 specific siRNA (n=6; $p<0.05$).

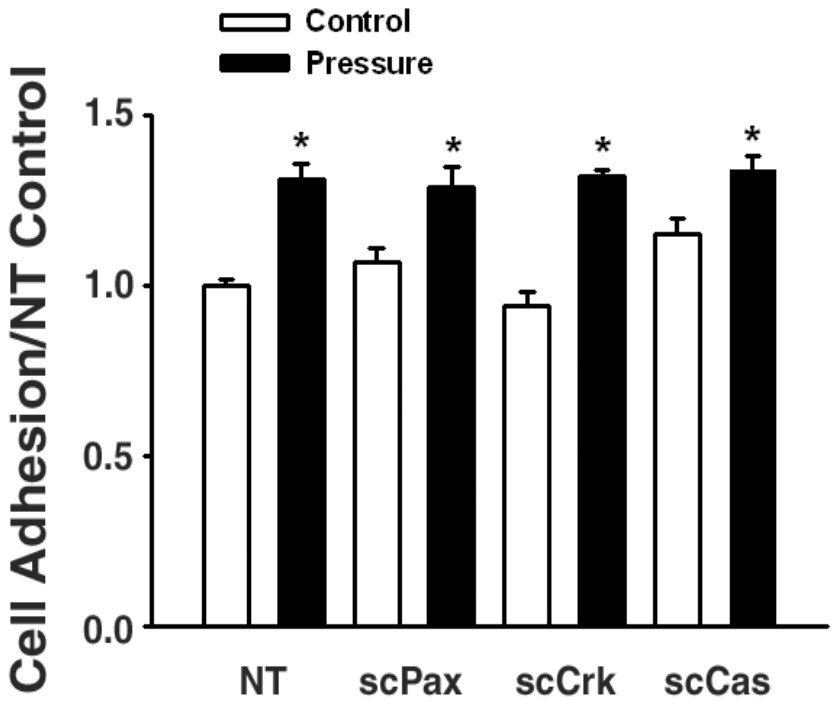


Figure 19. Scrambled siRNA does not inhibit pressure-stimulated colon cancer cell adhesion. Pressure elevations of 15 mmHg induced increases in adhesion in SW620 cells transfected with scrambled siRNA sequences for paxillin, Crk and Caswere similar to that in the non-targeting controls (n=5; $p<0.05$).

and linkage to the cytoskeleton or whether paxillin actually participated in a dynamic signal pathway stimulated by increased extracellular pressure. Our current data indicate that paxillin phosphorylation at tyrosines 31 and 118 together is necessary for pressure-induced adhesion. Furthermore, phosphorylation at these two sites creates a binding site for the adaptor protein Crk, leading to the recruitment of p130Cas and ultimately to the phosphorylation of Rac1. Each of these steps seems required for extracellular pressure to activate cancer cell adhesion.

Paxillin influences tumor biology in several ways. It participates in focal adhesion assembly and links the focal adhesion complex to integrins and to the cytoskeleton [26,67]. Paxillin expression is increased in metastatic head and neck cancer and human osteosarcomas as well as aggressive breast cancer cell lines [68,69,70].

In some instances, paxillin acts simply as a scaffolding protein that functions to provide docking sites for a variety of structural and signaling proteins. For example, in lymphoid cells, paxillin has been shown to bind directly to the α_4 integrin subunit [24]. Paxillin also provides a docking site for the protein tyrosine kinases FAK and Src, which can in turn participate in diverse signaling pathways [26]. A recent study by Geiger et al indicated that non-phosphorylated paxillin is essential for the formation of fibrillar adhesions, and leads to enhanced fibronectin fibrillogenesis in Porcine aortic endothelial cells [71]. In other situations, paxillin phosphorylation is necessary for downstream signaling and cellular functions. This necessity for phosphorylation is evident in a current study

by Vallés et al that showed blocking paxillin tyrosine phosphorylation at sites 31 and 118 in NBT-II rat bladder carcinoma cells decreased motility on collagen by approximately 50% [27]. In the current study we sought to determine the relevance of paxillin tyrosine phosphorylation to pressure-stimulated colon cancer cell adhesion.

Pressure appears to stimulate paxillin tyrosine phosphorylation at both tyrosine 31 and tyrosine 118. Using plasmid phosphorylation mutants, we found that tyrosine phosphorylation at sites 31 and 118 individually did not affect pressure stimulated cancer cell adhesion. However, when both sites were blocked simultaneously, the pressure effect was abolished. In contrast, point mutation of tyrosine 181 seemed irrelevant. These results are consistent with observations that paxillin tyrosine 31 and 118 phosphorylation is necessary for adhesion, migration and cell spreading in other systems [47,72,73]. Recent data has also implicated serine 273, as well as serine 188 and 190 phosphorylation as influencing cell adhesion [74,75]. Although this result was obtained in epithelial cells after adhesion had occurred, our present studies of paxillin tyrosine phosphorylation certainly do not preclude the possibility that this serine phosphorylation event may also occur in response to extracellular pressure.

Crk is a multifunctional adaptor protein that has been reported to bind to many different intracellular proteins, depending upon the cell type and stimulus studied. For instance, Crk can interact with phosphatidylinositol kinase (PI3 kinase), leading to the activation of the Akt signaling pathway [76], which also influences colon cancer cell adhesion in response to extracellular pressure [77].

Others have reported that Crk binds to the adaptor proteins DOCK180 and C3G in a variety of cell types, and that these downstream proteins then can then regulate cell migration or the mitogen activated protein kinase pathway, respectively [78,79]. The results presented here indicate that paxillin phosphorylation within colon cancer cell lines or primary human colon cancer cells in response to extracellular pressure facilitates paxillin-Crk binding, which is also required for the ultimate stimulation of adhesion. The potential role of Crk in modulating pressure stimulated cell adhesion has not previously been evaluated. Our studies were designed to delineate the intracellular signaling in suspended cells prior to adhesion that would then govern their subsequent adhesion; while most other studies have evaluated Crk signaling in cells after adhesion had occurred. Whether other proteins that interact with Crk may also participate in pressure – induced signaling in cells prior to adhesion awaits further study.

p130Cas is also an adaptor that promotes protein – protein interactions [30]. Cas interaction with other proteins modulates diverse cellular events [58,60,80,81]. Cas modulates cell motility, and integrin – mediated cell adhesion triggers Cas tyrosine phosphorylation that has been correlated with actin cytoskeleton reorganization and focal adhesion formation [58]. However, the ability of Cas to influence the binding affinity of suspended cells is less clear. Our observations of Cas paralleled our studies with Crk. Paxillin phosphorylation in response to pressure facilitates paxillin-Cas binding just as it does paxillin-Crk binding. Cas is required for the stimulation of adhesion, however, the pressure effect can be rescued from Cas siRNA treatment when a wild type Cas plasmid is

transfected in conjunction with Cas specific siRNA. Although Cas has been reported to associate with a wide variety of proteins, the present results suggest a model in which paxillin, Cas, and Crk form a trimeric complex in response to paxillin phosphorylation stimulated by pressure. This would be consistent with similar observations in collagen stimulated NBT-II cells [82] as well as in migrating FG-M carcinoma cells on vitronectin [60], although others have found that Cas binds to the Tec/Btk family member Bmx in Cos-7 cells in response to adhesion [83]. The differences between our observations and some of these other reports may reflect differences in the cell types and stimuli studied.

Rac1 is a known binding partner of Cas, although Rac1 also binds to and is activated by RhoG and aldosterone in other settings [84,85]. We therefore hypothesized that the paxillin-Crk-Cas interaction might relevantly phosphorylate and activate Rac1. Indeed, Rac1 is phosphorylated and activated in response to extracellular pressure. Furthermore, reduction of paxillin by targeted siRNA prevented increased Rac1 phosphorylation in response to elevated pressure. Whether paxillin interacts directly with Rac1 or simply facilitates Cas conformational changes that permit Cas phosphorylation of Rac1 awaits further study.

Several investigators have reported that Rac1 is important in actin polymerization, lamellipodial extension, and cell migration in various cell types, but that Rac1 is not critical for adhesion itself [86,87,88,89]. Indeed, Rac1 inhibition by NSC23766 produced little effect on basal cell adhesion in our studies. However, Rac1 inhibition completely prevented the stimulation of

adhesion by extracellular pressure. Force-activated adhesion may be regulated by a very different pathway than basal adhesion. We have previously reported that reduction of some other signals in this pathway, such as α-actinin; also have little effect on basal adhesion while nonetheless preventing the stimulation of adhesion by pressure [55].

If paxillin phosphorylation is a critical event in pressure-stimulated adhesion, what cellular events might trigger this phosphorylation in response to extracellular pressure increases? A recent report by Chien et al suggests that modifications of microtubule dynamics can increase paxillin phosphorylation, although a mechanism for this effect was not explored [90]. FAK and Src are focal adhesion proteins that are involved in intracellular signaling that regulates proliferation, apoptosis, migration, and adhesion in both normal and tumor cells [51]. FAK is phosphorylated at tyrosine 397 in response to various stimuli, including adhesion itself and extracellular pressure prior to adhesion [41,91]. This phosphorylation creates a binding site for Src where Src becomes activated and further phosphorylates FAK. The FAK-Src complex is thought to control focal adhesion turnover and target paxillin and Cas for phosphorylation [92]. Individually, FAK and Src have also been shown to be activated by various stimuli, including PDGF, insulin and growth hormone, where they can directly activate the MAP kinase pathway and promote cell migration [93,94,95]. Our previous studies have shown that pressure – stimulated cancer cell adhesion is dependent upon FAK and Src phosphorylation [41]. We therefore sought to determine if either FAK or Src are required to for the stimulation of paxillin

phosphorylation by elevated pressure. In our model, FAK reduction by targeted siRNA stimulated basal paxillin phosphorylation at ambient pressure but prevented further phosphorylation in response to pressure. The increase in ambient pressure phosphorylation is consistent with reports that FAK null fibroblasts display increased paxillin phosphorylation [96]. Although this increase in basal paxillin phosphorylation in FAK null cells has not been explained, it may reflect microtubular destabilization by FAK reduction, as has been reported in neuronal cells as well as in mouse fibroblasts [97,98]. However, the blockade of stimulation by pressure of paxillin phosphorylation in FAK-reduced cells suggests a role for FAK in this effect. Src inhibition also prevented the stimulation of paxillin phosphorylation by pressure, although without a notable effect on basal ambient pressure phosphorylation, again suggesting that paxillin is probably downstream of FAK and Src in the signal pathway by which suspended cancer cells respond to extracellular pressure. Previous studies have suggested that paxillin phosphorylation does increase in response to elevated pressure in cells treated with FAK-specific siRNA or PP2 [25]. However, we obtained a greater reduction in FAK levels (~70%) and treated with PP2 for a longer amount of time (4 hours) which could account for this difference.

α-Actinin is an actin crosslinking protein that interacts with multiple focal adhesion proteins and is able to bind to the cytoplasmic tail of β1 integrin. α-Actinin is also a FAK substrate and enhances the interaction between FAK and Src. Additionally, reduction of α-Actinin by siRNA blocks pressure – stimulated cancer cell adhesion [55]. In the current study, when α-Actinin was reduced by

siRNA treatment, paxillin phosphorylation at tyrosine 31 was inhibited. This would be consistent with a model in which FAK and Src interaction with α-Actinin is necessary for paxillin phosphorylation in response to pressure.

Finally, to further validate our results that used non – targeting control siRNA, we found that treatment with scrambled siRNA sequences for paxillin, Crk and Cas did not influence pressure – stimulated adhesion.

In summary, pressure stimulated colon cancer cell adhesion depends upon paxillin, and more specifically, upon paxillin phosphorylation at tyrosines 31 and 118. The phosphorylation of paxillin is dependent upon α-actinin and leads to association with Crk and Cas and downstream activation of Rac1. This pressure-activated pathway seems clinically relevant, as increased extracellular pressure stimulates colon cancer cell adhesion to endothelial cells *in vitro* and surgical wounds *in vivo*, and has been shown to influence tumor-free survival in a murine transplantable tumor model [22,41,99]. Although complete delineation of the pressure-sensitive signal pathway responsible for this effect awaits further study, paxillin and its binding partners seem critical to the adhesion process and may offer innovative therapeutic targets.

CHAPTER 5

NF-kB Dependent Pressure-Induced Proliferation

Extracellular pressure increases of 15mmHg above ambient for 1-30 minutes stimulate β1-integrin binding affinity in suspended cancer cells before adhesion through FAK, Src and Akt activation, and β1-integrin phosphorylation [100]. Prolonged pressures of similar magnitude stimulate proliferation in mesangial cells during glomerular hypertension, cardiac myocytes after abdominal aortic constriction, and in endothelial cells [101,102,103]. Preliminary studies from our laboratory demonstrated that 15mmHg increased pressure stimulates SW620 and HCT-116 colon cancer cell proliferation [31]. In contrast to the stimulation of cancer cell adhesion by extracellular pressure [100] or the stimulation of proliferation in Caco-2 cells by cyclic strain [104], the mitogenic effect of pressure appears independent of Src, but its underlying mechanism is largely unclear.

Physical forces may activate mitogenic signals varying with the cell and stimulus studied, including MAPK, Rho GTPases, and integrin activation. Two prominent candidates to mediate the mitogenic effects of pressure are Rac1 and NF-kB. Rac1 can be activated by forces such as deformation and pressure, and can modulate proliferation, adhesion, and migration [105,106]. NF-kB is a rapidly acting transcription factor that modulates gene transcription in cell-cycle regulation, apoptosis, and proliferation. NF-kB family members share a Rel homology domain in their N-terminus. However, RelA, RelB and c-Rel are the only members with transactivation domains. NF-kB family members form

homodimers or heterodimers to participate in transactivation. RelA, the p65 subunit, regulates transcription directly, as it has a nuclear translocation sequence in addition to a direct DNA binding site. Traditional NF-kB signaling involves heterodimerization between RelA and p50. NF-kB can promote or inhibit apoptosis depending upon the stimulus [35]. For example, NF-kB promotes apoptosis in colon cancer cells treated with aspirin [107]. In contrast, murine B cell proliferation is suppressed when NF-kB is blocked and constitutive NF-kB activation in gastric cancer cells promotes proliferation [36,108].

We hypothesized that pressure stimulates cancer cell proliferation via NF-kB activation. We measured intratumoral pressure directly in patients to confirm that pressure was increased within tumors, although we were not able to localize tumor center or periphery sufficiently to document the intratumoral pressure gradient observed by others [109]. We examined the proliferative response to increased extracellular pressure of 3 colon cancer cell lines, a breast cancer line, and a prostate cancer line. These studies were repeated after treatment with 3 NF-kB inhibitors or NF-kB-specific small interfering RNA (siRNA). We also evaluated pressure effects on NF-kB nuclear translocation, NF-kB transcription factor activity, and NF-kB-driven events that can stimulate proliferation, including IKK and IkB phosphorylation and cyclin D1 expression. We further examined NF-kB activity in pressure-treated cells treated with inhibitors of FAK, Akt and Protein Kinase C (PKC). Finally, we compared IkB phosphorylation, NF-kB, and cyclin D expression, and proliferation in the relatively lower pressure peripheries and the

relatively higher pressure centers of 28 large primary tumors to validate our in vitro findings.

We confirmed previous reports of increased extracellular pressure within human tumors [110] by measuring interstitial pressure just outside 13 human intraabdominal malignant tumors and then within them during image-guided biopsy. Pressures within the tumors were greater than pressures in adjacent tissue (Figure 20, $n=13, p<0.05$).

SW620, Caco-2 and CT-26 colon cancer, MCF-7 breast. and PC3 prostate cancer cells were exposed to 0-80mmHg increased extracellular pressure for 24 hours and proliferative activity was assessed by MTT assay. Each cell line displayed increased MTT fluorescent intensity across the range of increased pressures, with greatest responses at 40-60mmHg increased pressure (Figure 21, $n=13; p<0.05$). Parallel manual cell counting confirmed increased cell numbers after exposure to 40 mmHg increased pressure vs. ambient ($n=4; p<0.05$, not shown). The Rac1 inhibitor NSC23766 did not prevent increased MTT fluorescent intensity in pressure-treated cells (Figure 22, $n=4; p<0.05$), but the NF-kB inhibitor SN50, an NF-kB nuclear decoy, a novel imidazoline inhibitor, TCH-021, or NF-kB specific siRNA abolished the effect. Pressure remained mitogenic in cells treated with an inactive SN50 analog, an inactive NF-kB nuclear decoy, or a non-targeting control siRNA sequence (Figure 22, $n=6; p<0.05$). To contrast pressure stimulation of adhesion and proliferation, SW620 cells were treated with the NF-kB inhibitor SN50 and exposed to ambient or 40mmHg increased pressure during a 30 minute adhesion assay. Adhesion

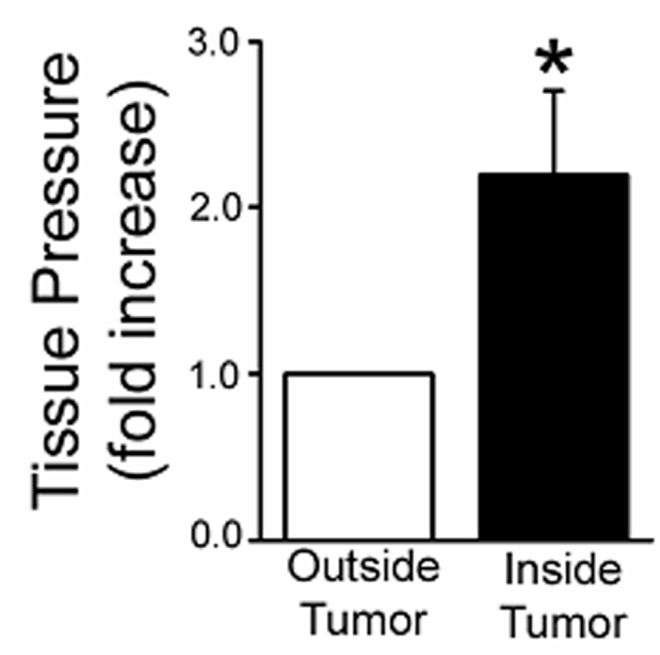


Figure 20. Extracellular pressure in human tumors. Tissue pressure was measured just outside and within 13 human intraabdominal malignant tumors. Pressure was increased inside the tumors relative to outside.

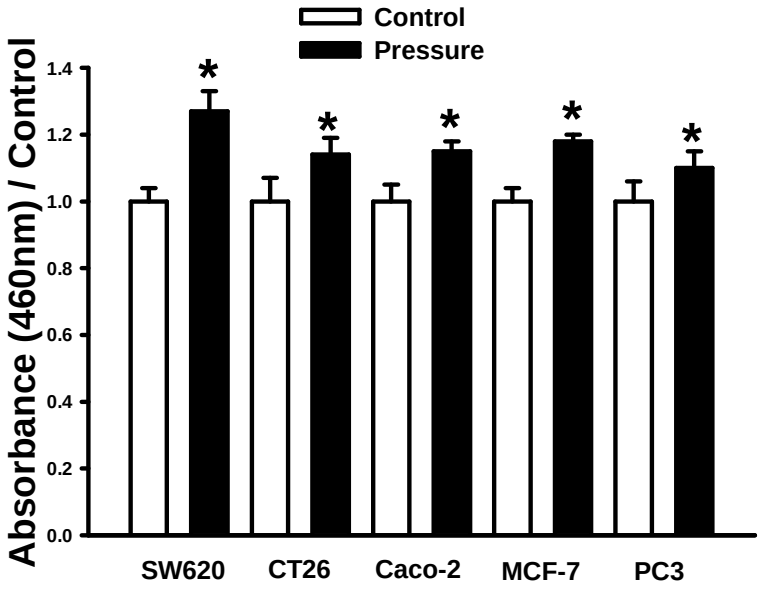


Figure 21. Extracellular pressure stimulates the proliferation of various cancer cell lines. 40mmHg increased pressure (shaded bars) stimulated proliferation in SW620, CT26 and Caco-2 colon cancer cells, MCF-7 breast cancer cells and PC3 prostate cancer cells.

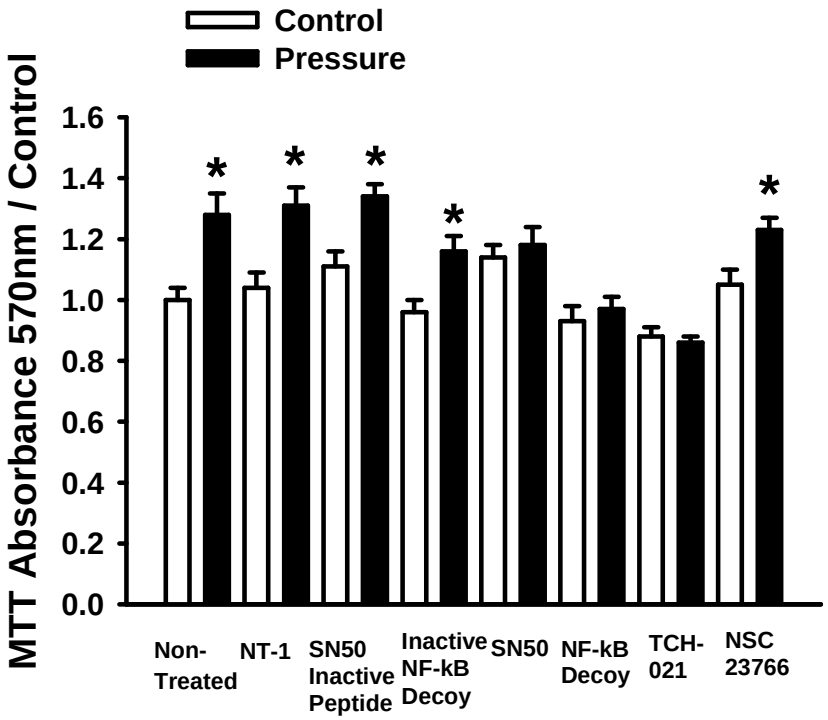


Figure 22. Increased proliferation in response to extracellular pressure is abolished in the presence of NF-kB inhibitors. Proliferation in response to 40mmHg increased pressure (shaded bars) is blocked in SW620 cells treated with SN50, a NF-kB nuclear decoy, TCH-021 and NF-kB specific siRNA, but not after treatment with the inactive SN50 analog, the inactive nuclear decoy or the Rac1 inhibitor NSC23766.

increased similarly in both control and NF-kB-inhibited cells (Figure 23, n=4;p<0.05). To further characterize the effect of increased pressure on cell cycle regulation, SW620 cells were treated with inhibitors, exposed to 24 hours of ambient or 40mmHg increased pressure, and cell cycle analysis was performed by flow cytometry. S-phase fractions increased 22±6% in untreated cells exposed to increased pressure (Figure 24, n=3;p<0.01). However, pressure did not increase S-phase fractions in inhibitor-treated cells (Figure 24, n=4;p<0.05). Finally, TUNEL staining demonstrated that pressure did not alter SW620 cell apoptosis (Figure 25, n=3;p<0.05).

To determine whether pressure activated NF-kB, we treated SW620 cells with NF-kB lentiviral reporter particles expressing firefly luciferase and incubated them under ambient or 40mmHg increased pressure for 24 hours. Pressure increased NF-kB activation 94±7% (Figure 26A, n=12;p<0.05). However, the NF-kB inhibitors SN50 and TCH 021, the NF-kB nuclear decoy, and siRNA to NF-kB each prevented pressure-associated NF-kB activation (Figure 26A, n=6;p<0.05). NF-kB activation is typically accompanied by nuclear translocation. We therefore evaluated NF-kB p50 and p65 levels in nuclear fractions derived from SW620 cells exposed to ambient or 40mmHg increased pressure for 24 hours. p50 and p65 nuclear translocation increased 58±6% and 67±8% respectively in pressure-treated cells compared to ambient pressure (Figure 26B, n=6;p<0.05).

NF-kB is sequestered within the cytosol by binding its inhibitor IkB. When IkB is phosphorylated by the kinase IKK, released NF-kB translocates to the nucleus [35,111]. IKK phosphorylation was increased 27±4% in pressure-treated

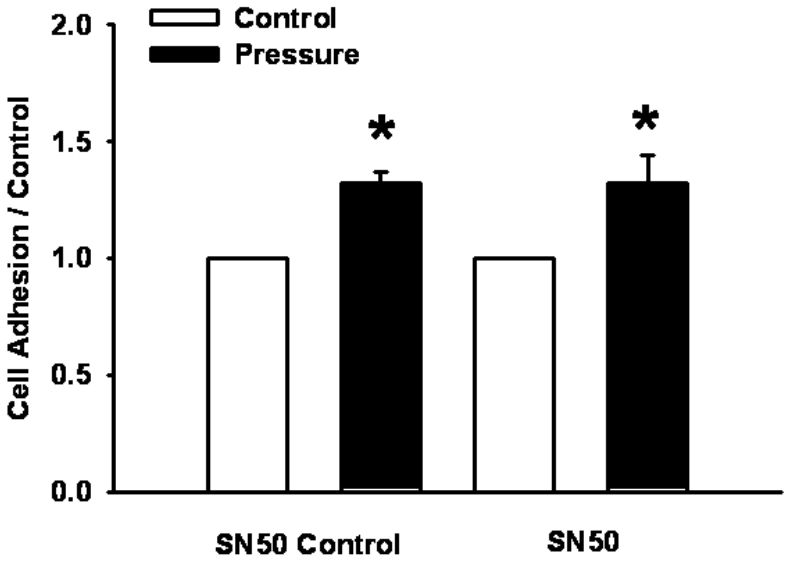


Figure 23. NF-kB inhibition has not effect on pressure stimulated cancer cell adhesion. 40mmHg increased pressure (shaded bars) increased SW620 cell adhesion on collagen in the presence of SN50 control and SN50 inhibitory peptide.

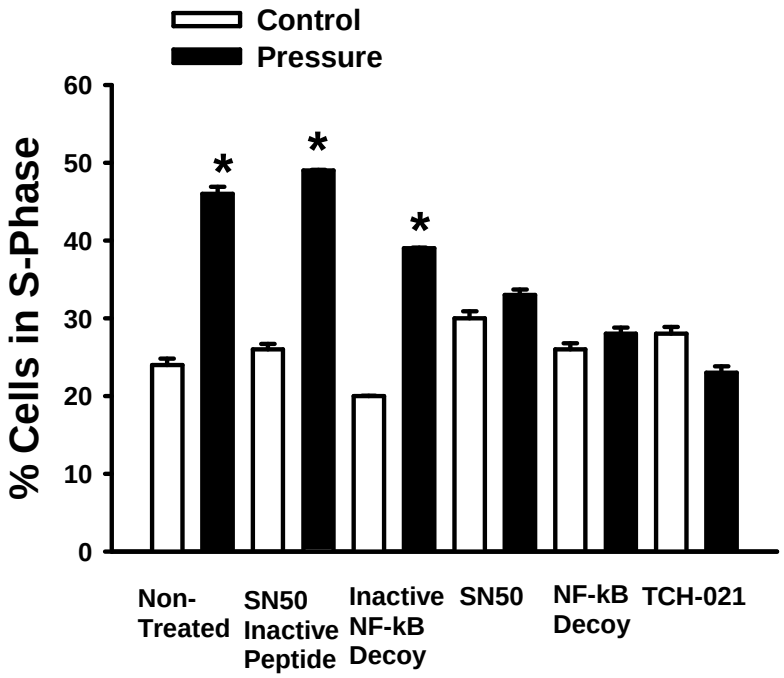


Figure 24. SW620 colon cancer cells increase S-phase fractions in response to elevated pressure. S-phase fractions increased in SW620 cells exposed to 40mmHg pressure (shaded bars) after no prior treatment or SN50 inactive peptide treatment. Cells treated with the NF-kB inhibitors SN50, a NF-kB nuclear decoy, or TCH-021 showed no increase.

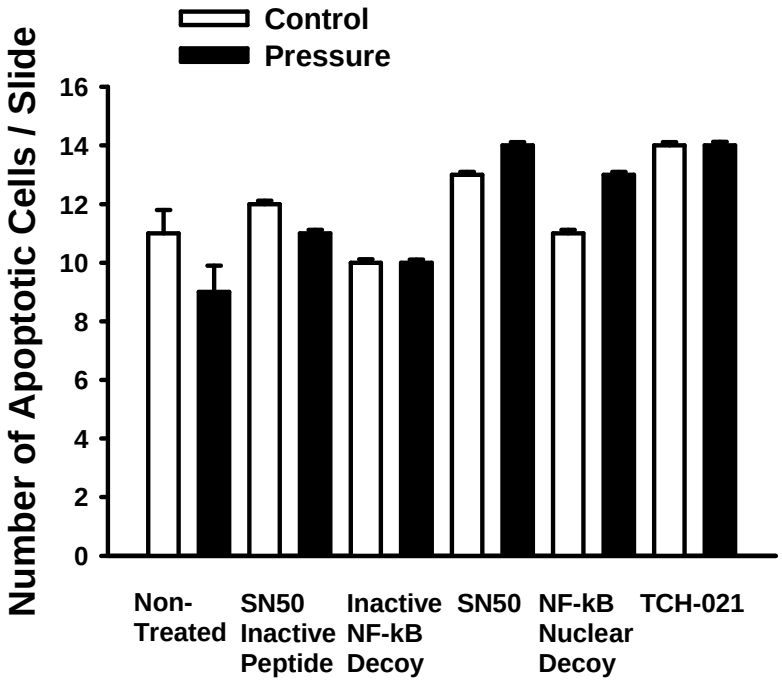
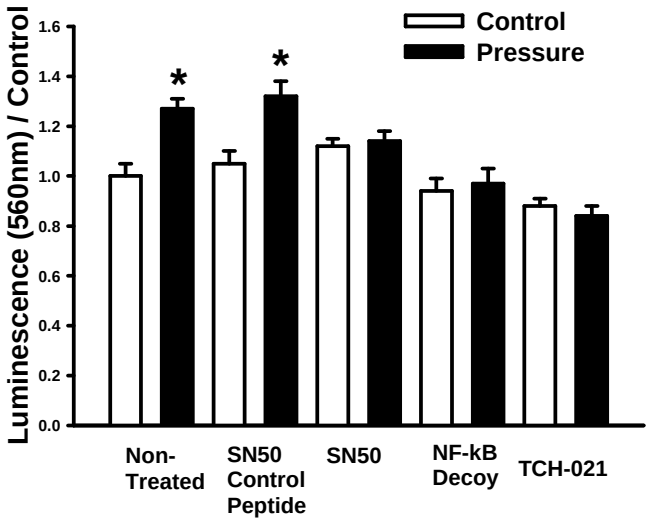


Figure 25. No changes in apoptosis are observed in pressure treated cells. TUNEL staining in SW620 cells after treatment with SN50, a NF-kB nuclear decoy or TCH-021 showed no changes in apoptosis with pressure.

A.



B.

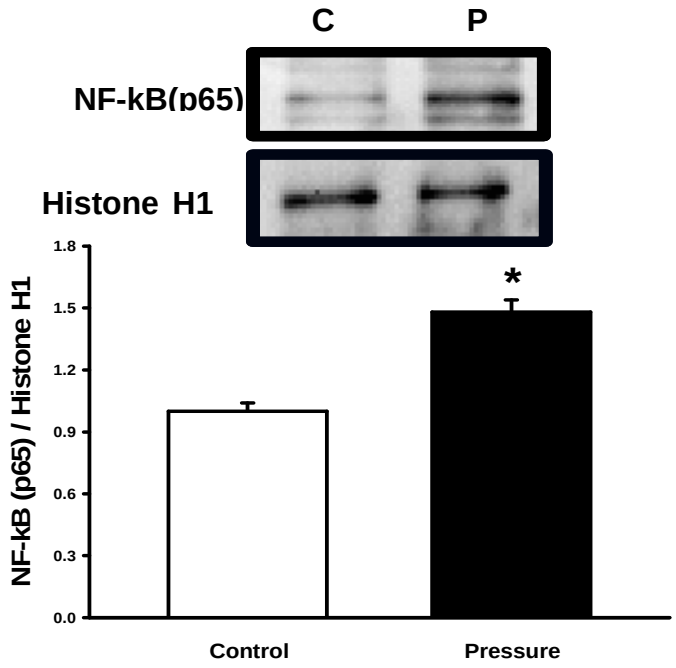


Figure 26. Effect of increased pressure on NF-kB activity and nuclear localization. (A) Pressure (shaded bars) increased SW620 NF-kB activity. This increase was abolished by SN50, a NF-kB nuclear decoy, or TCH-021. (B) NF-kB p65 and p50 increased in nuclear fractions from SW620 cells exposed to increased pressure, using histone H1 as a loading control.

cells relative to ambient control cells (n=8;p<0.05, not shown). We also observed a 61±8% increase in IkB phosphorylation in pressure-treated cells compared with cells at ambient pressure (Figure 27A, n=8;p<0.05). Elevated pressure also decreased NF-kB-IkB association 31±4% (Figure 27B, n=5;p<0.05). Cyclin D1 is influenced by NF-kB and regulates the G1-S phase transition [34]. We therefore compared cyclin D1 expression in SW620 cells at ambient or increased pressure for 24 hours. Pressure-treated cells expressed 26±5% more cyclin D1 than ambient pressure controls (Figure 28, n=6;p<0.05). This increase was abolished in cells pre-treated with the NF-kB inhibitors SN50 or TCH 021, with a nuclear decoy, or with siRNA to NF-kB (Figure 28, n=5;p<0.05). NF-kB family members form homo- or heterodimers prior to nuclear localization and transcription factor activation. Traditional NF-kB signaling involves the p50/p65 dimer. NF-kB p65 and p50 transcription factor activity increased 48±6% and 50±4% respectively in response to pressure (Figure 29, n=6;p<0.05).

NF-kB activation and subsequent signaling in response to some stimuli may be influenced by PKC. Go6976, which inhibits both PKC-α and PKC-β, and a pure PKC-β inhibitor abolished the effect of pressure on proliferation. However, elevated pressure increased proliferation in cells treated with a PKC-ε translocation inhibitor, similarly to untreated controls (Figure 30A, n=9;p<0.05). Inhibiting PKC-α/β in combination, or inhibiting PKC-β alone also abolished NF-kB activation in response to pressure (Figure 30B, n=9;p<0.05). FAK and Akt mediate the effects of extracellular pressure on integrin-mediated adhesion as well as the stimulation of proliferation by another physical force, cyclic

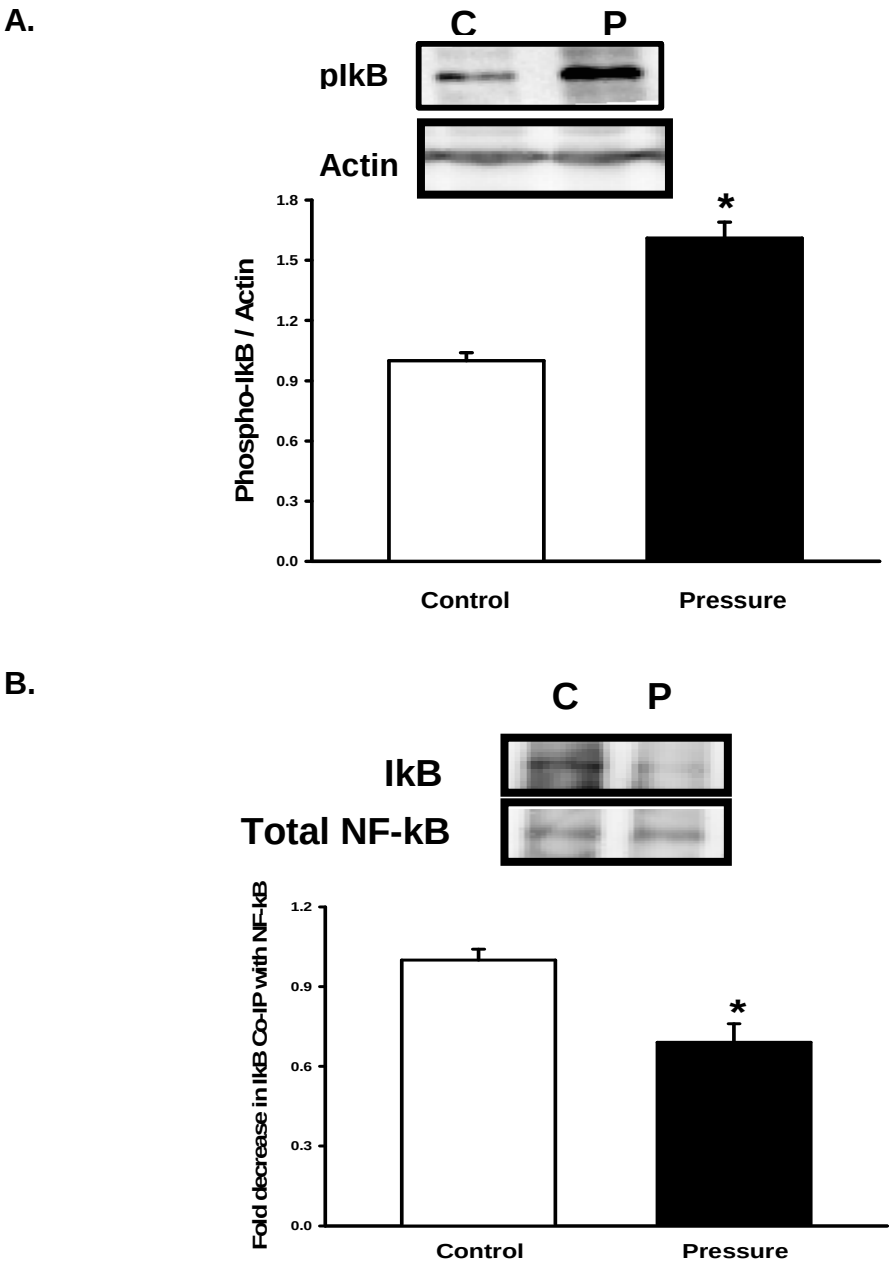


Figure 27. Effect of pressure on NF-kB signaling. (A) Pressure (shaded bars) increased SW620 IkB (serine273) phosphorylation vs. ambient pressure (open bars), using actin as a loading control. (B) Co-immunoprecipitation revealed reduced association between NF-kB(p65)/IkB under increased pressure (shaded bars). Total NF-kB was a loading control.

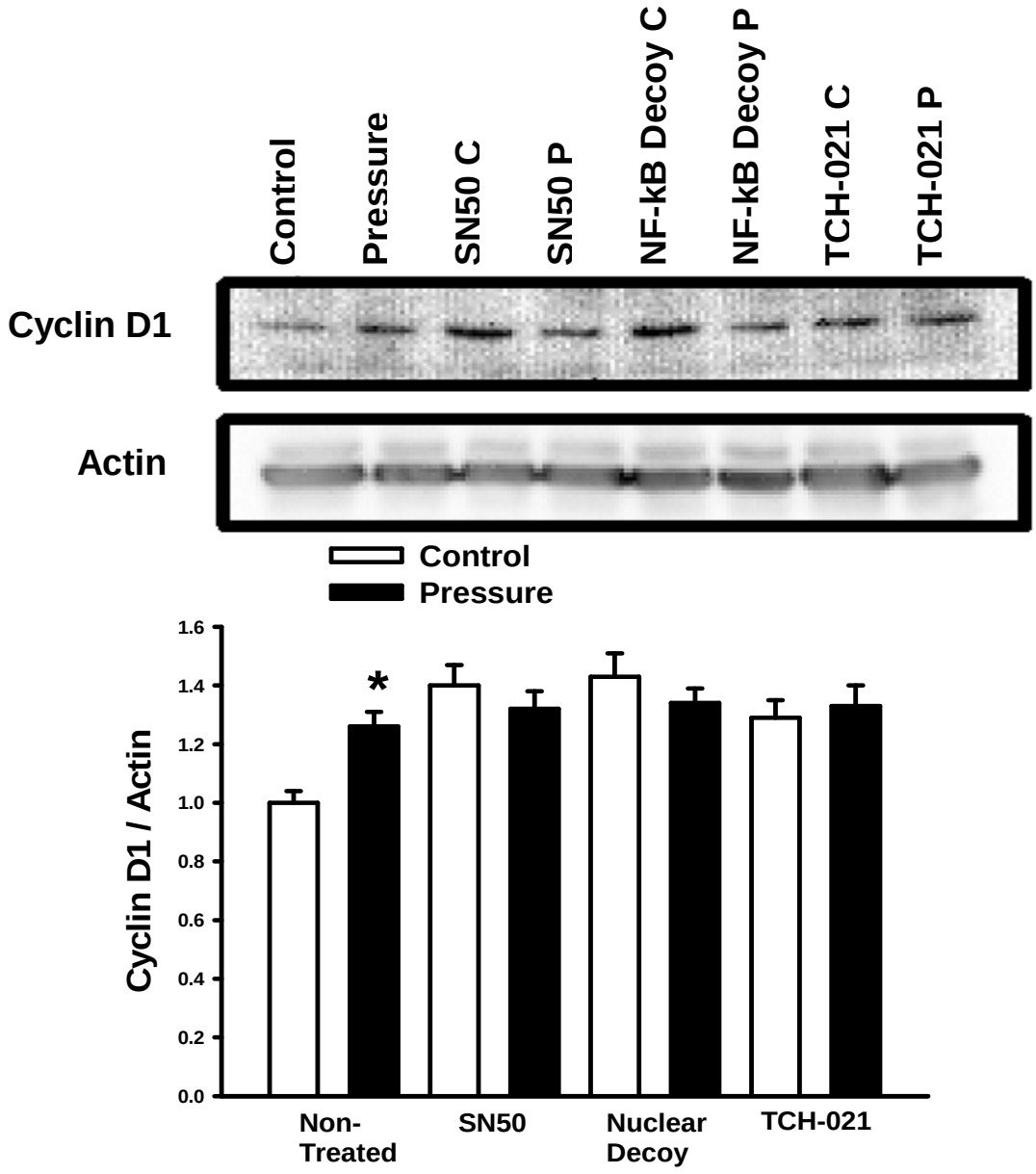


Figure 28. Cyclin D1 expression increases in response to pressure. SW620 cyclin D1 expression increased after exposure to increased pressure (shaded bars). This increase was abolished by SN50, a NF-kB nuclear decoy or TCH-021. Actin was a loading control.

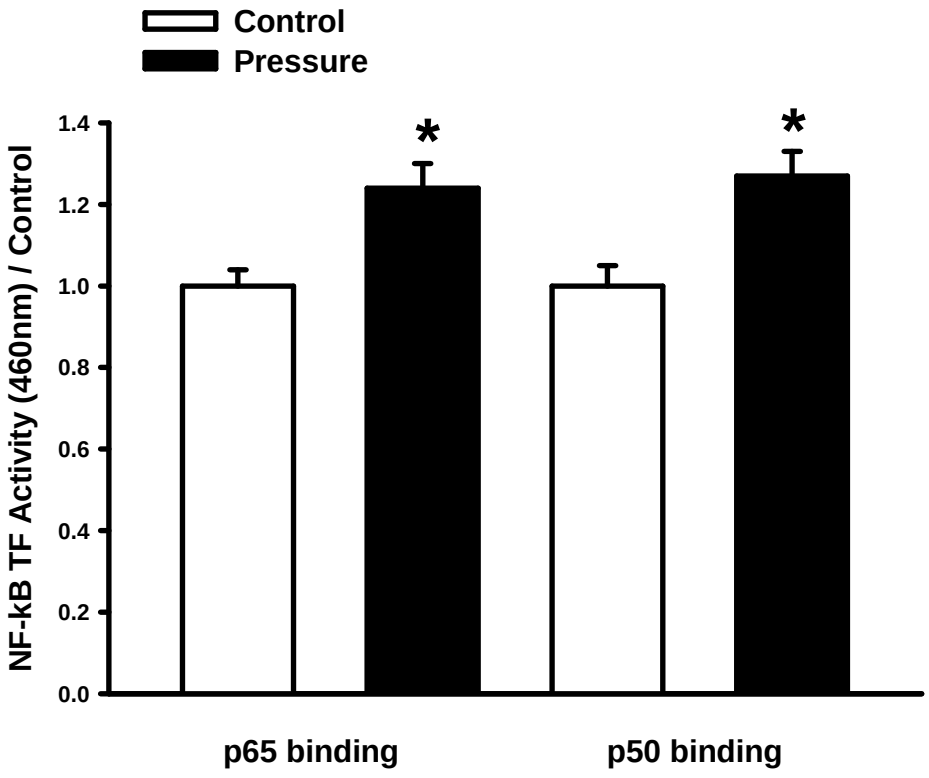
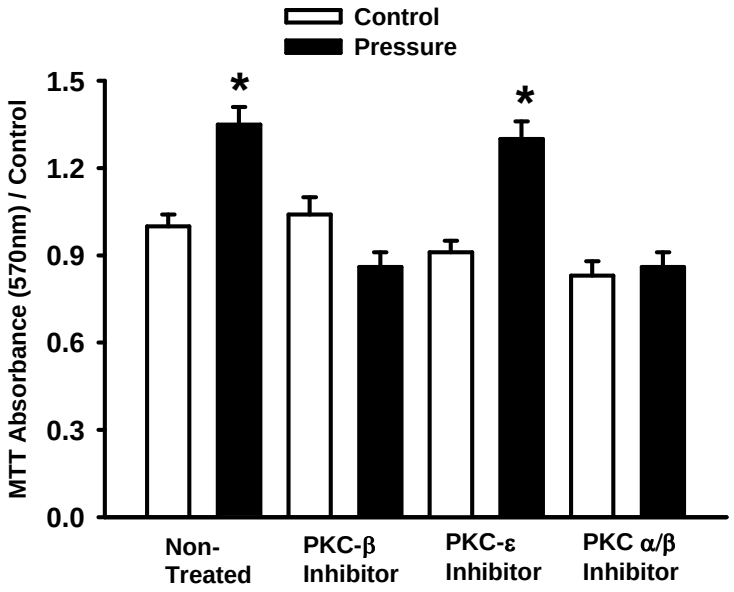


Figure 29. Extracellular pressure increases NF-kB p65 and p50 transcription factor activity. Pressure (shaded bars) increased NF-kB p50 and p65 transcription factor activity in SW620 nuclear fractions relative to ambient controls.

A.



B.

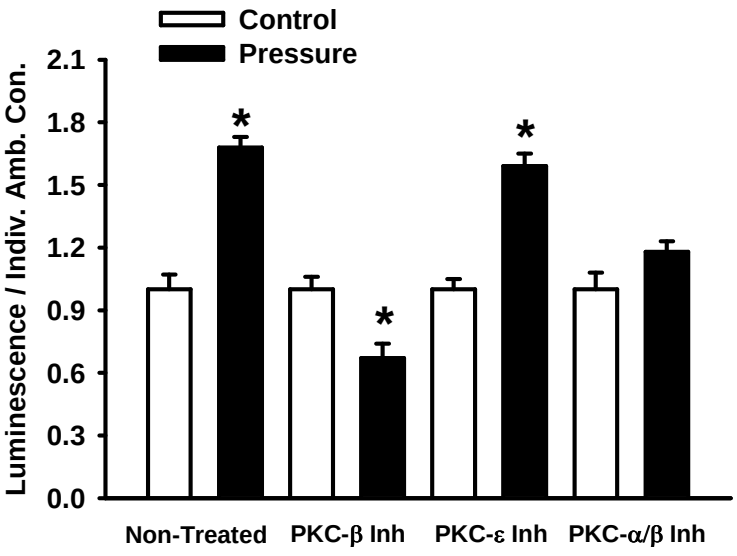


Figure 30. PKC α/β inhibition abolishes pressure-stimulated proliferation and NF-kB activation. (A) Inhibiting PKC-β or PKC α/β abolished pressure-stimulated proliferation. PKC-ε inhibition did not. (B) PKC-β and PKC α/β inhibition blocked the effect of increased pressure on NF-kB activation. PKC-ε did not.

deformation [100]. However, inhibition of FAK or Akt did not prevent the mitogenic effects of pressure on NF-kB activation, unlike global PKC inhibition using calphostin C (Figure 31, $n=9;p<0.05$). The IKK complex is required for IkB phosphorylation and subsequent NF-kB activation. To evaluate the effect of IKK subunits on pressure-stimulated NF-kB activation, we treated SW620 cells with inhibitors to IKK-2 or IKK-3, or an IKK inhibitor that blocks IkB phosphorylation. Individually, these inhibitors did not prevent pressure activation of NF-kB (Figure 32A, $n=12;p<0.05$). However, when IKK-2 and IKK-3, or IKK-2 and the IkB phosphorylation were blocked together, pressure activation of NF-kB was abolished. Pressure-induced NF-kB activation was also reduced when all three inhibitors were combined (Figure 32B, $n=12;p<0.05$).

Since extracellular interstitial pressures are higher in the center of many solid human tumors than at their peripheries [109,110], we compared mitotic rates at the periphery and center of 28 human colon, lung, and head and neck tumors, averaging 8±1.2cm diameter. A reviewer blinded to the hypothesis reported a 154±14% increase in mitotic figures/high power field in the central regions of the tumors relative to their peripheries (Figure 33, $n=15;p<0.05$). Parallel studies demonstrated very high immunoreactivity for active NF-kB, phosphoIkB, and cyclin D1 in the centers of these tumors which decreased towards their peripheries. Little or no staining was observed in adjacent non-malignant tissues from each specimen (Figures 34-36, $n=27;p<0.05$). Assessment of immunoreactivity on a scale from 0 (negative staining) to 4 (high staining), specimens immunostained for NF-kB were scored in 95 zones from

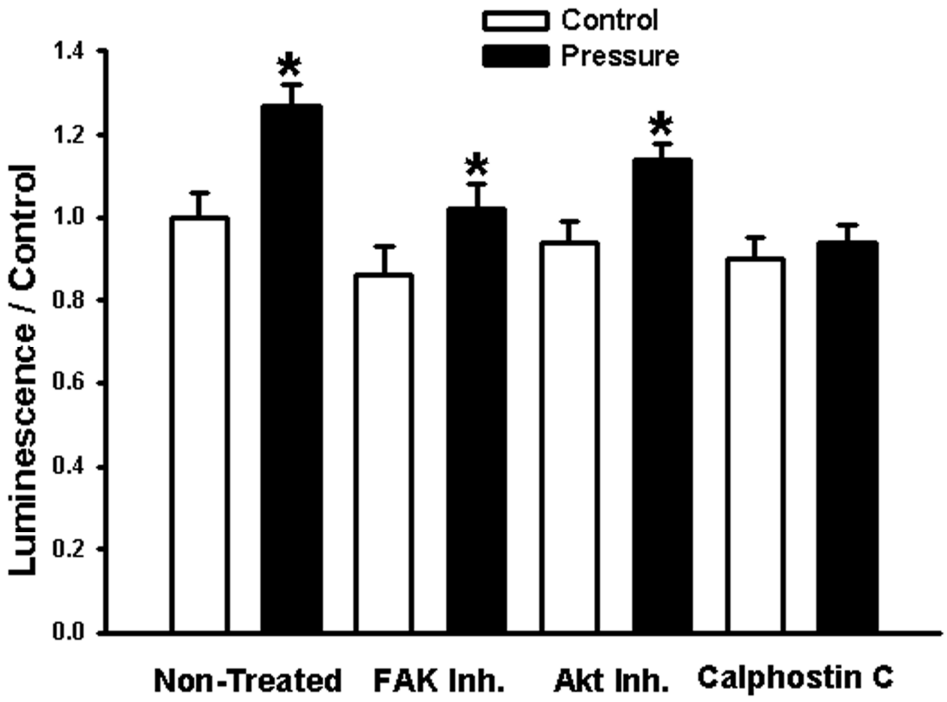
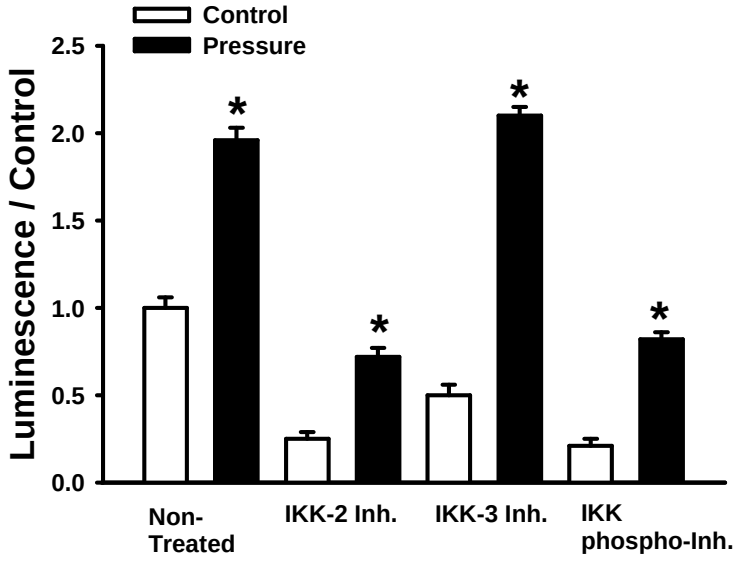


Figure 31. Effect of FAK, Akt and PKC inhibition on pressure-stimulated proliferation. Inhibiting FAK and Akt did not affect pressure-induced NF-kB activation, but PKC blockade abolished the effect.

A.



B.

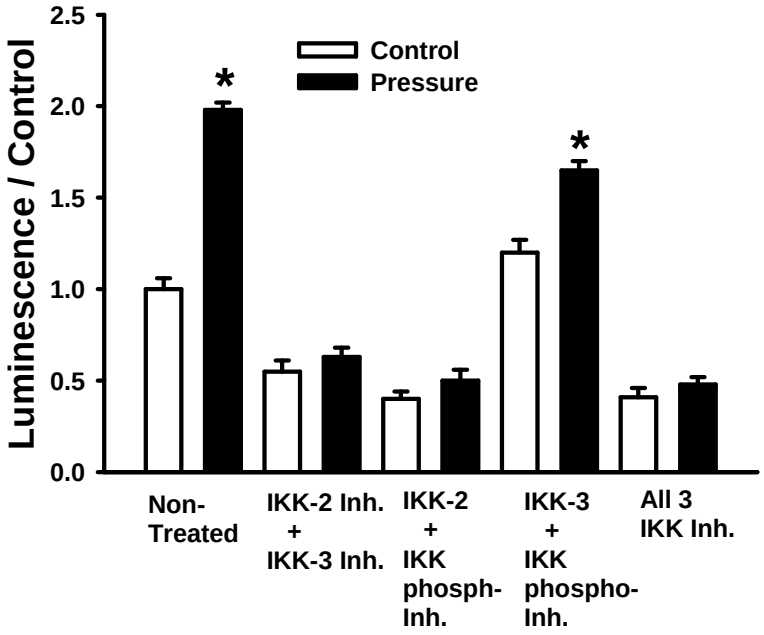


Figure 32. IKK inhibition affects pressure stimulated NF-kB activation in combination. (A) Inhibitors of IKK-2, IKK-3 and IKK phosphorylation of IkB did not reduce pressure-stimulated NF-kB activation when used individually. (B) Combination of IKK-2 and IKK inhibitor of IkB phosphorylation abolished pressure-stimulated NF-kB activation, as did IKK-2 and IKK-3, or all three inhibitors together.

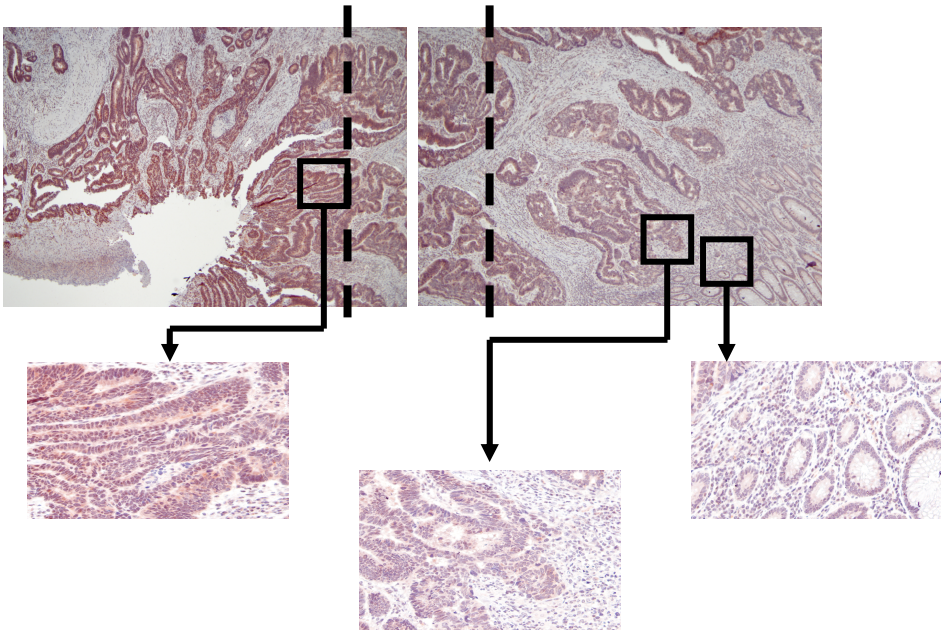


Figure 33. NF-kB immunostaining increases in the center of human tumors. Immunohistochemical staining of colorectal carcinomas, representative of staining observed in colorectal, head and neck and lung tumors. To obtain a full view of the sample, a panel of photographs were taken and placed together. Dashed lines represent zones of overlap within the image. Panel images were taken at 4X magnification and images within each zone at 20X. Staining represents active NF-kB.

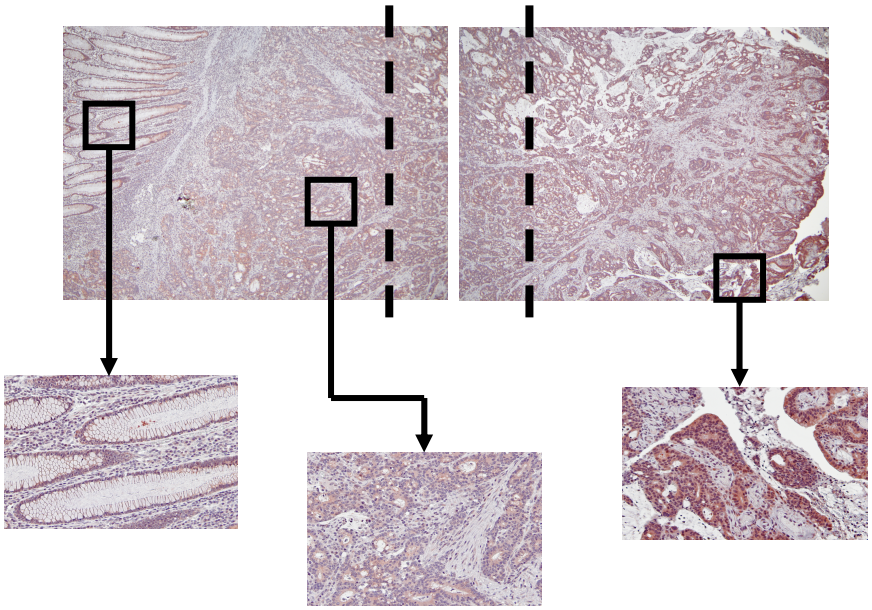


Figure 34. Phospho-IkB staining in the center of human tumors. Immunohistochemical staining of colorectal carcinomas, representative of staining observed in colorectal, head and neck and lung tumors. To obtain a full view of the sample, a panel of photographs were taken and placed together. Dashed lines represent zones of overlap within the image. Panel images were taken at 4X magnification and images within each zone at 20X. Staining represents phospho-IkB.

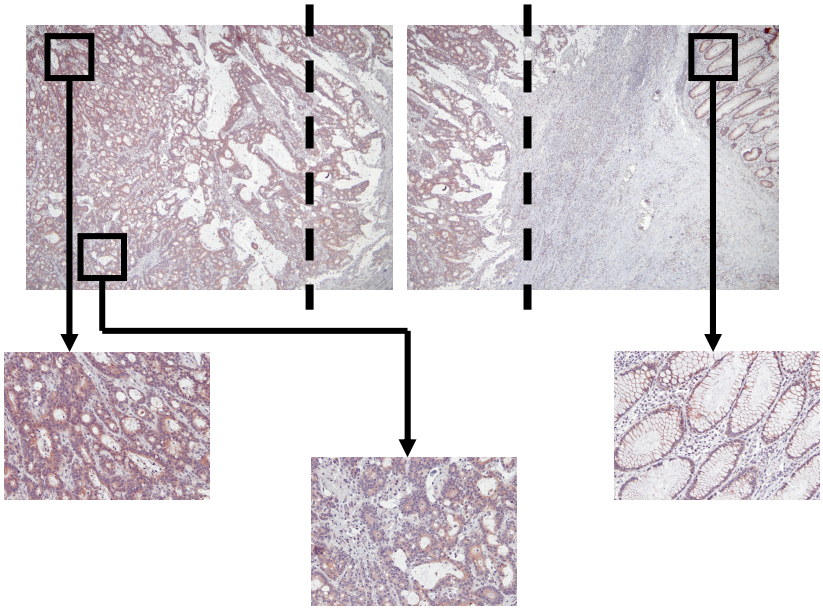


Figure 35. Cyclin D1 staining in the center of human tumors. Immunohistochemical staining of colorectal carcinomas, representative of staining observed in colorectal, head and neck and lung tumors. To obtain a full view of the sample, a panel of photographs were taken and placed together. Dashed lines represent zones of overlap within the image. Panel images were taken at 4X magnification and images within each zone at 20X. Staining represents cyclin D1.

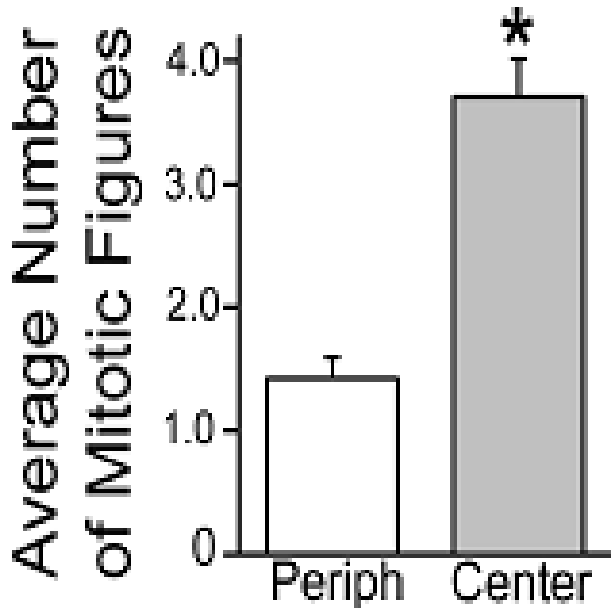


Figure 36. Mitotic index increases in the center of human tumors. Mitotic index increases in tumor centers vs. peripheries. Non-malignant tissue showed minimal staining and few mitotic figures.

adjacent histologically normal margins from 19 different tumor samples and averaged a score of 0.8. Blinded evaluation of areas at the periphery of the tumors in these same slides yielded a mean score of 2.2, while central tumor zones received a mean score of 3.6. Similar gradients occurred for phospho-IkB and cyclin D1 immunoreactivity (Table 3). Differences between the normal tissue, the peripheral zone, and the central tumor zone were each statistically significant ($p<0.01$). Mitotic figures were separately counted in 20 fields of highly NF-kB-immunoreactive areas within the tumors compared to less NF-kB-immunoreactive areas, confirming a 124±8% increase in mitotic figures within these more immunoreactive zones ($p<0.05$, not shown).

Tumor cells are exposed to increased extracellular pressure during passage through the circulatory and lymphatic systems [65,112]. Intraabdominal pressures can reach 15-30mmHg post-operatively as fluid is sequestered within the extracellular space of the viscera, causing swelling against the abdominal wall [113]. Although we and others have suggested that increased extracellular pressure can stimulate cancer cell proliferation, the mechanism has been unknown [31,114]. Cells respond to numerous mitogenic intracellular signals, including Src, FAK, MAPK, and Rac1. However, blocking Src, FAK or the MAPK does not prevent the mitogenic effects of pressure [31,115], and blocking Rac1 did not inhibit the mitogenic effects of pressure here. In contrast, NF-kB is a transcription factor that in some instances can be pro-apoptotic and in others mitogenic, depending on the stimulus and setting [34]. Our data suggests that increased extracellular pressure stimulates cancer cell proliferation through

Table 3. Immunohistochemical Scores

Antibody	Cases	Number of zones counted/average intensity			
		Non-Malignant	Periphery	Center	P-value
NF-kB	19	95/0.8	95/2.2	95/3.6	<0.01
pIkB	23	115/0.6	115/2.0	115/3.4	<0.001
Cyclin D1	21	105/0.9	105/2.4	105/3.6	<0.01

PKCα/β-dependent IKK and IkB phosphorylation, NF-kB activation and nuclear translocation, and increased cyclin D1 expression.

Mitogenic signaling in response to forces such as pressure may influence the proliferation of many cell types [17,31,116]. Less than 5mmHg of pressure stimulates endothelial proliferation, while 70mmHg pressure increases osteosarcoma proliferation and 160mmHg stimulates aortic myocyte proliferation [65,103,117]. In contrast, neuronal cell lines display apoptosis after 2 hours of 100mmHg increased pressure, and 40-80mmHg pressures increase human mesangial cell proliferation, while pressures of 100-180mmHg stimulate apoptosis in the same cells [118,119]. Although these results support an effect of pressure on proliferation in some cell types, how this occurs has not been elucidated. Several theories have been advanced. Some have hypothesized that pressure-exposed cells secrete a factor into the medium that influences proliferation, while others suggest that the disruption of the attachment between integrins and the cytoskeleton is responsible [114]. Additionally, non-malignant cells seem to respond to a lesser stimulus than their malignant counterparts, and malignant cells seem to have a threshold by which a proliferative response to pressure may turn apoptotic [103,117]. This study focused on pressures within the range typically experienced by tumor cells in vivo, and found that such pathophysiologically relevant pressure increases stimulate the MTT-based reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan, and increase S-phase fraction and cell numbers in colon, breast and prostate cancer cells. Apoptosis did not change. Taken together, these results

strongly suggest that pressure increases cell number by stimulating proliferation, rather than inhibiting apoptosis.

Previous studies suggest that pressure stimulates the adhesion of suspended cancer cells to matrix proteins, endothelial cell monolayers and surgical wounds in a manner that depends upon an intact cytoskeleton, as well as PI-3-kinase and Src signaling [100]. However, the pathway by which pressure stimulates proliferation seems different from the signaling mediating pressure-stimulated adhesion, because in the previous preliminary study the mitogenic effects of pressure were not prevented by inhibiting Src or PI-3-kinase, or the actin stablilization agent phalloidin, although these each prevent pressure stimulation of adhesion in suspended cells [31,100]. Our current study further confirms that pressure stimulates adhesion and proliferation by different pathways, and that pressure acts differently from repetitive deformation. Rac1 is required for pressure-stimulated adhesion [105] and the mitogenic effects of repetitive deformation in human Caco-2 colon cancer cells and non-malignant IEC-6 intestinal epithelial cells [120] but does not seem to contribute to the mitogenic effects of pressure. The differences between those previous observations and the present data likely reflect differences in the signaling of cancer cells not yet adherent to matrix proteins (thus lacking a background of integrin-driven intracellular signaling) and in the responses to constant pressure compared with cyclic deformation.

NF-kB signaling is complex, and can promote proliferation through increased expression of cell cycle regulators, or cell death through interactions

with anti-apoptotic factors such as FLIP and TRAF1/2 [34]. NF-kB is activated in response to deformation. Mechanical stretch activation of NF-kB in muscle cells increases TNF-alpha and IL-1B expression, while osteoblast-like MF-63 cells display NF-kB activation in response to low amplitude cyclic strain, but not low amplitude compressive strain. Interestingly, this effect was reversed at high amplitude cyclic and compressive strain [121,122]. High pressures can also activate NF-kB [123]. In our current study, pressure stimulated NF-kB nuclear translocation of the p50 and p65 subunits, as well as NF-kB activation. This occurred through phosphorylation of the IKK complex and subsequent IkB phosphorylation. NF-kB activation increased cyclin D1 expression and proliferation.

FAK and Akt are necessary for pressure-induced adhesion and the mitogenic effects of repetitive deformation [100], and also often mediate NF-kB activation [124,125]. However, inhibition of FAK or Akt did not prevent pressure activation of NF-kB here. Conversely, some PKC isoforms can also influence NF-kB activation and proliferation. Controversy exists over whether PKC-α or PKC-ε mediates NF-kB activation in T cells [126,127]. In colon cancer cells, PKC-τ has been reported to regulate NF-kB [128]. In our study, inhibiting PKC-β or PKC α/β, but not PKC-ε, prevented pressure activation of NF-kB. Intestinal epithelial proliferation in response to strain depends on PKC-α which, like PKC-β, requires calcium for activation [129]. In contrast, PKC-ε is representative of the calcium-independent family. The role of calcium in pressure-mediated proliferation awaits further study.

The IKK complex is responsible for IkB phosphorylation and subsequent NF-kB activation. TNF-α is required to activate this complex. The IKK-2 inhibitor we used is reported to function by inhibiting TNF-α production, while the IKK-3 inhibitor is an ATP-competitive inhibitor. The global IKK inhibitor is thought to block IkB phosphorylation directly. The roles of the IKK isoforms are diverse and vary with cell type. IKK-2 is necessary for myeloma cell proliferation, but IKK-2 deletion increases myocyte proliferation [130,131]. Although the individual inhibitors of IKK-2, IKK-3 and IKK phosphorylation of IkB did not prevent pressure activation of NF-kB, using these inhibitors in combination blocked this effect, indicating pathway redundancy but confirming IKK relevance.

Aberrant or constitutive NF-kB activation characterizes many human malignancies and indicates poor prognosis in colorectal and esophageal cancers [34,36,37]. We observed intense NF-kB, IkB, and cyclin D1 immunoreactivity in the high pressure center of the tumors which was reduced towards the lower pressure periphery and virtually absent in the adjacent normal tissue. These were all solid tumors without significant necrosis, which could have affected intratumoral pressures. NF-kB immunoreactivity of this magnitude correlates with poor prognosis [38]. Correlation does not prove causation, but our observations in human tumors suggest at least a potential causal link between pressure-mediated NF-kB activation and increased proliferation in tumors that is further supported by our in vitro mechanistic studies.

In conclusion, pathophysiologically relevant increases in pressure stimulate colon, breast and prostate cancer cell proliferation by a mechanism

involving activation of PKC and the IKK complex, and NF-kB p50 and p65 nuclear localization and activation, and increased cyclin D1 expression. This effect may explain, in part, the increased NF-kB, IkB and cyclin D1 immunoreactivity and increased proliferation that we observed within the higher pressure centers of human tumors. Tumor growth involves numerous signaling cascades. These results suggest that the increases in extracellular pressure generated by tumor growth and stiff tumor matrix may themselves stimulate proliferation within the tumor, generating a positive feedback loop. This pathway may represent a target of opportunity to slow the growth of unresectable tumors in patients and tumors not candidates for conventional cytotoxics.

CHAPTER 6

Pressure-Induced Metastatic Signaling In Vivo

We hypothesized that cancer cells are activated by the increased pressures and shear forces of the circulation, leading to increased adhesion and metastasis, and that blocking this activation can reduce metastasis.

It has been estimated that nearly 1 X 10^6 tumor cells per gram of tumor tissue are shed into the bloodstream each day [132]. In addition, viable tumor cells can be recovered from the peripheral blood of more than 40% of patients immediately following “curative” colon tumor resection surgery, indicating a poor prognosis [133,134]. Such circulating cancer cells encounter many host responses, including immune system interference, but some surmount these hurdles and progress to metastatic disease.

0.8-1.0% of patients may experience wound implantation following tumor resection surgery, while laboratory studies suggest that CT-26 murine colon cancer cells exposed to 30 minutes of 15mmHg increased pressure adhere to surgical wounds significantly greater than those cells left in ambient pressure conditions [135,136]. However, although local surgical site recurrence can be an important adverse event, most metastasis occurs via distant spread through the lymphatic, venous, and arterial circulation [137]. Such metastatic progression depends upon tumor cell adhesion at a distant site, but the factors that influence cancer cell adhesion are incompletely understood. Some studies suggest that adhesion at a distant site is a completely random process, while others have implicated the overexpression of adhesion molecules such as ICAM, E-Cadherin, or integrins [138,139]. Increased extracellular pressure promotes cancer cell adhesion in vitro by FAK and Src activation at the focal adhesion complex, Akt

activation, and ultimately increased β1 integrin binding affinity [100]. Shear stress similarly promotes adhesion in vitro [46]. These findings seem potentially clinically relevant, as cancer cells experience pressure and shear stresses as they circulate.

To test our hypothesis, CT-26 murine colon cancer cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and 1 million cells were injected into the tail vein of Balb/c mice. After 30 minutes, the cells were recovered by cardiac puncture and analyzed by flow cytometry. The flow cytometer gated only fluorescent CFSE labeled tumor cells that were further evaluated for FAK, Akt and 9EG7 phosphorylation or activation. Passage through the circulation increased phosphorylation of FAK at tyrosine 397 and Akt at serine 473, similar to that observed in vitro in response to increased extracellular pressure. Activation of the 9EG7 epitope, which specifically indicates activation of β1 heterodimers, was also increased in cells recovered from the circulation (Figure 37, $n=6;p<0.05$). Pretreatment with pharmacologic inhibitors to FAK or Akt prevented the increased phosphorylation of these molecules (not shown).

Pressures of the portal circulation are much lower (5-10mmHg) than those of the peripheral circulation (120-125mmHg) [140,141]. We first sought to determine whether pre-treating cells with pressure before injection into the portal circulation could increase adhesion and metastatic capability from within blood vessels. We exposed luciferase tagged CT-26 cells to ambient or 15 mmHg

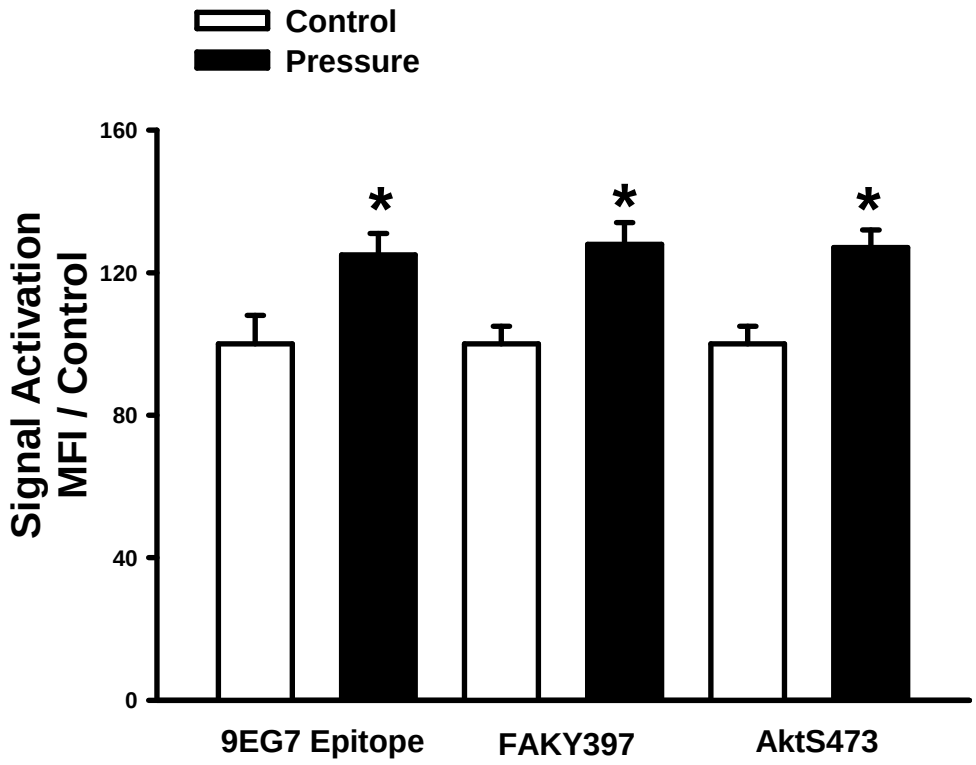


Figure 37. Pressures of the systemic circulation activate signaling in circulating cancer cells. Effect of circulatory pressures on β1 integrin heterodimer 9EG7 activation, FAK tyrosine 397 phosphorylation and Akt serine 473 phosphorylation. Data from individual experiments were normalized to their individual control. MFI, mean fluorescent intensity.

increased pressure and injected 750,000 cells into the spleen of Balb/c mice. Seven days later, we injected the mice with luciferin and imaged them using a

Kodak IS4000MM small animal imaging device. Exposure conditions were held constant for all measurements and all bioluminescence intensity of the images were pseudocolored (pink-low intensity; red-highest intensity). Quantitation using MetaMorph software indicated a 73±6% increase in luminescence in the spleen and liver of mice injected with pressure pre-treated cells relative to ambient controls (Figure 38, n=16; $p<0.05$). Splenic weight increased 62±9% and hepatic weight 41±11% in mice receiving pressure pre-treated cells (Figure 39, n=16;$p<0.05$), and long term survival was substantially decreased in mice injected with pressure pre-treated cells compared with mice injected with cells not first exposed to increased pressure (Figure 40, n=20; $p<0.05$). Prior treatment of the cells with inhibitors of FAK or Akt before pressure treatment and splenic injection resulted in reduced luminescence in mice receiving cells not exposed to increased pressure and blocked the increase in luminescence associated with injection with pressure-treated cells (Figure 41). Furthermore, long term survival increased substantially in mice receiving inhibitor-treated, with or without pressure pre-treatment (n=20 for each inhibitor; $p<0.05$, Figure 40). To distinguish alterations in adhesion from any effect of this single dose of each inhibitor on cellular proliferation, we performed MTT assays on cells treated with the FAK or Akt inhibitor. We observed an initial decrease in cell proliferation with each inhibitor, but a full recovery in proliferation by 24 hours compared with untreated cells (Figure 42, n=5;$p<0.05$). Since we imaged the mice seven days

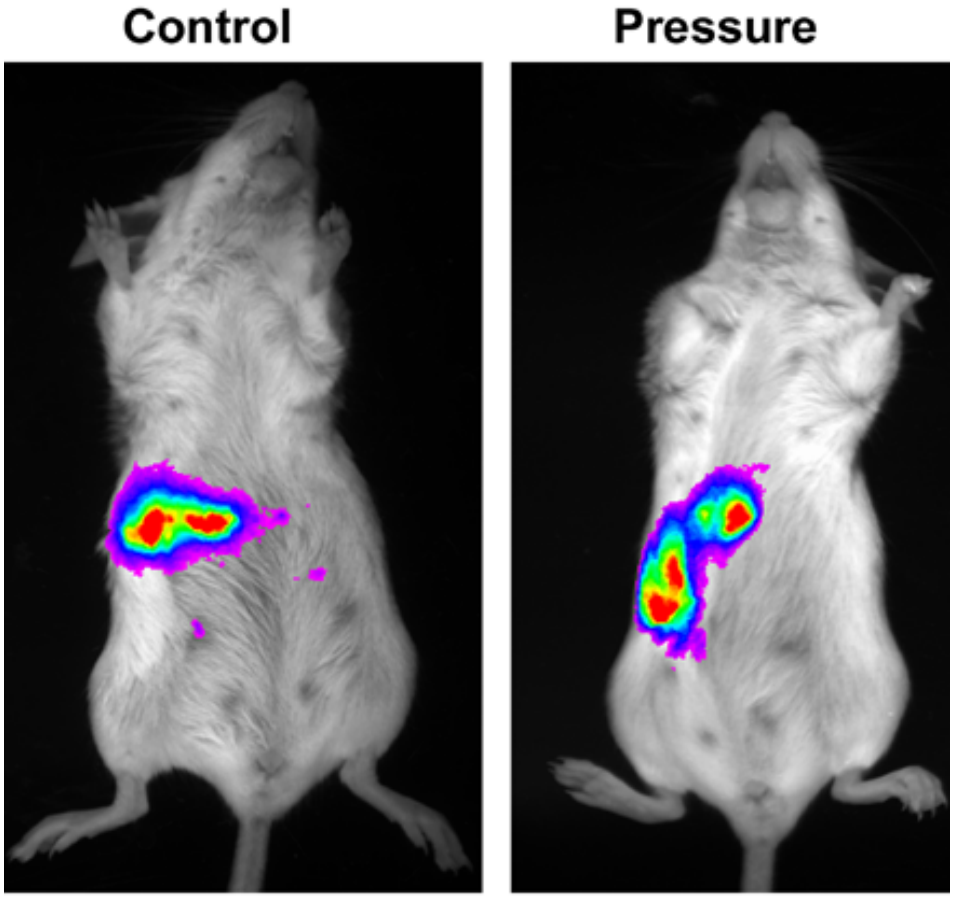


Figure 38. 15 mmHg increased pressure stimulates local metastasis via splenic injection. Splenic and hepatic tumor burden was visualized using luminescence following splenic injection with ambient or pressure pre-treated luciferase tagged CT-26 cells.

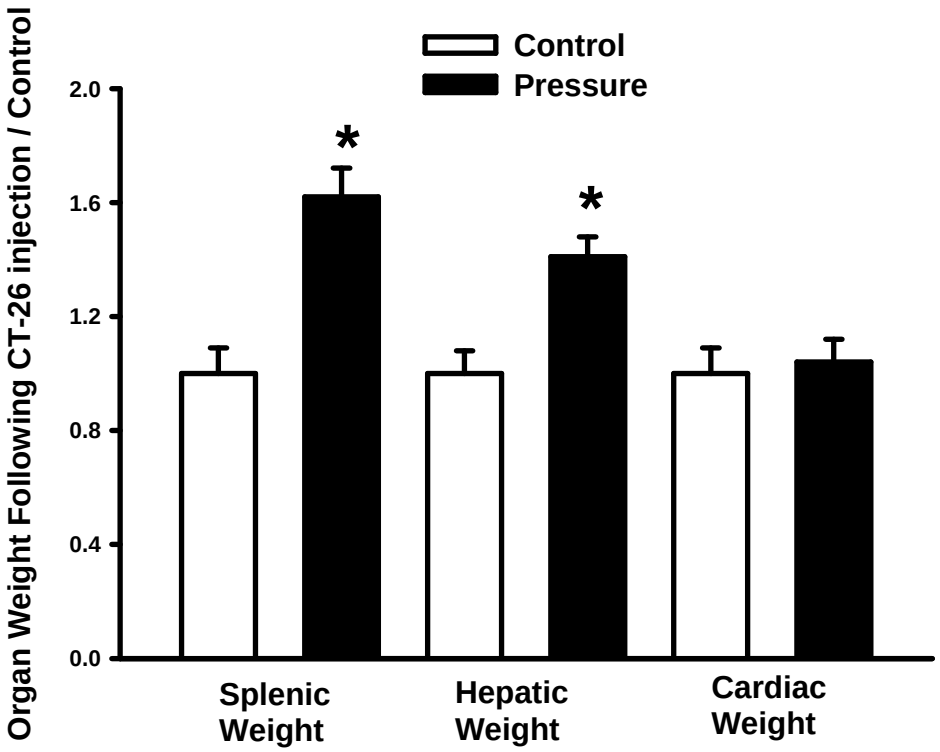


Figure 39. Splenic and hepatic weights following splenic injection. Splenic and hepatic weights were measured following splenic injection with ambient or pressure pre-treated CT-26 cells. Cardiac weight was used as a control.

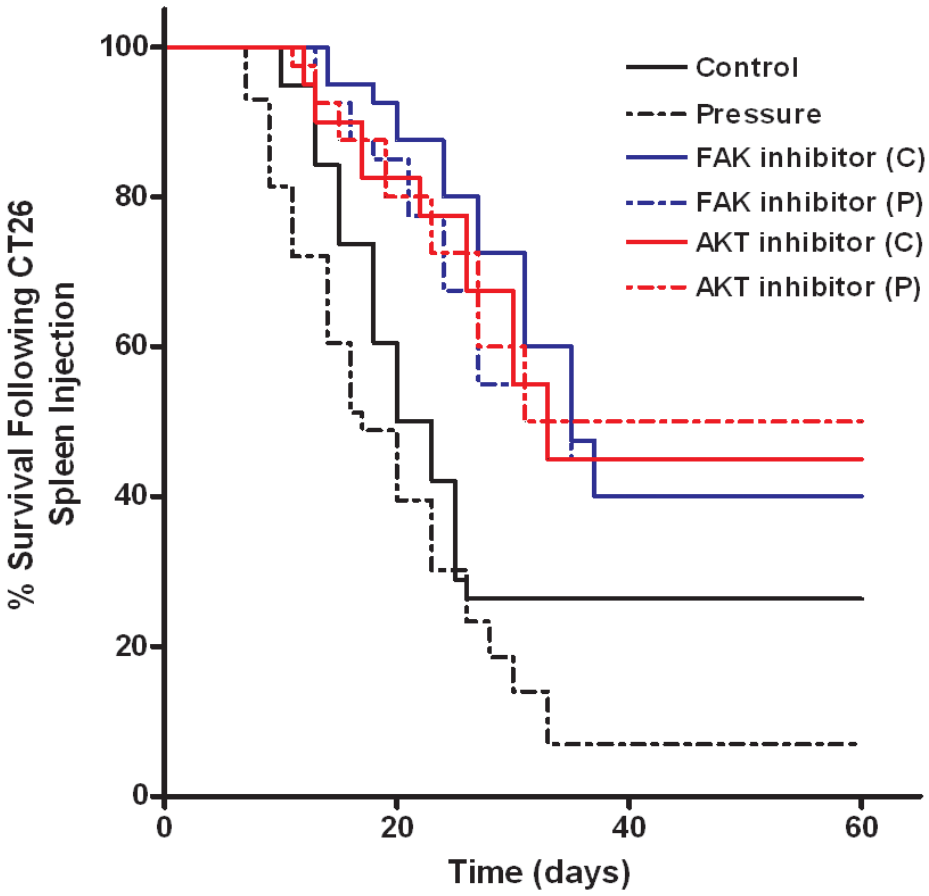


Figure 40. Long term survival following CT-26 splenic injection. Long term survival was measured in mice following splenic injection of CT-26 cells pre-treated with ambient or pressure conditions, or CT-26 cells pre-treated with FAK or Akt inhibitors prior to exposure to ambient or increased pressure conditions (* indicates $p<0.05$; ** indicates $p<0.01$).

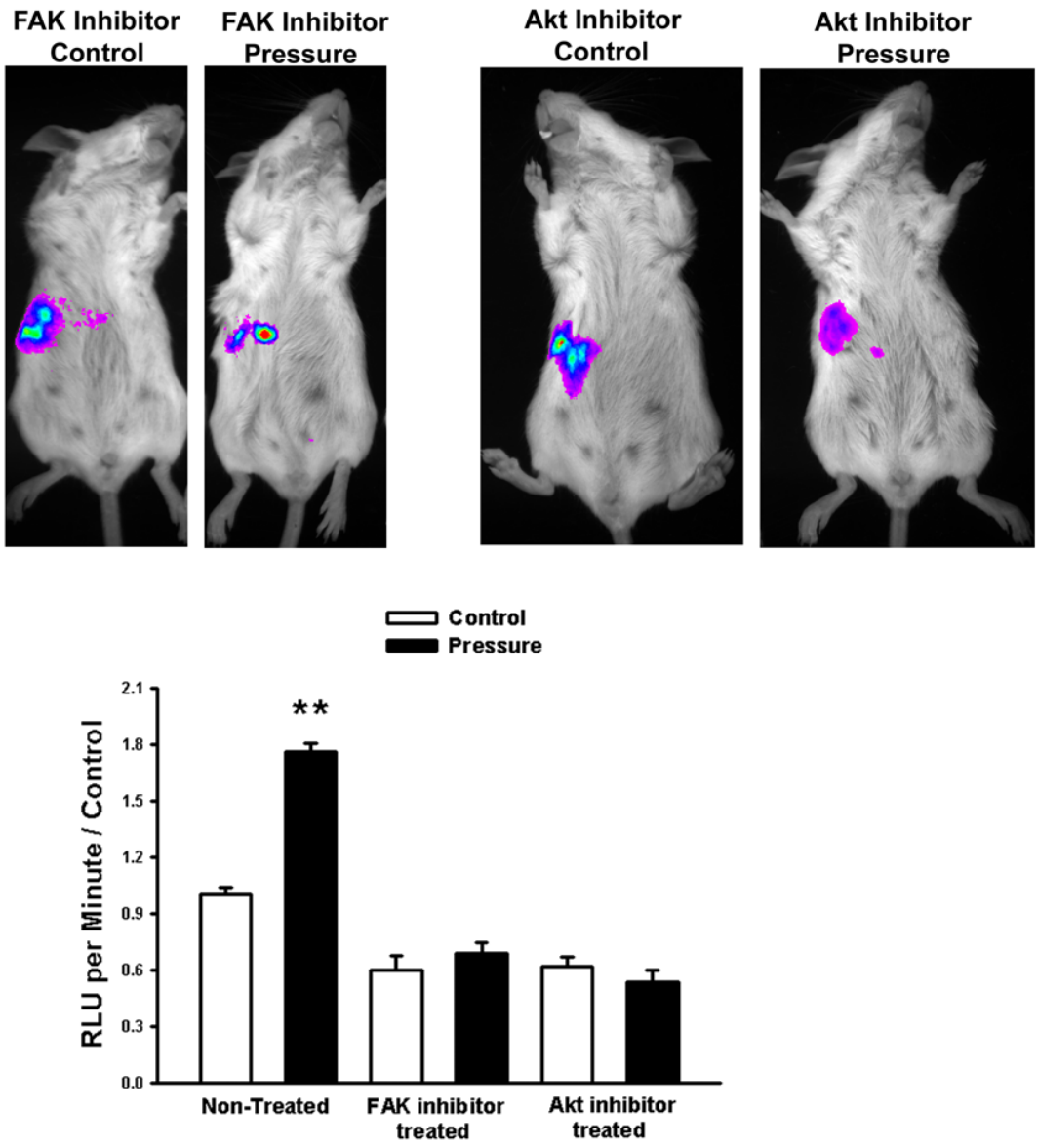


Figure 41. Splenic and hepatic burden following FAK or Akt inhibition. Splenic and hepatic tumor burden was visualized using luminescence following splenic injection of CT-26 cells pre-treated with FAK or Akt inhibitors prior to exposure to increased or ambient pressure.

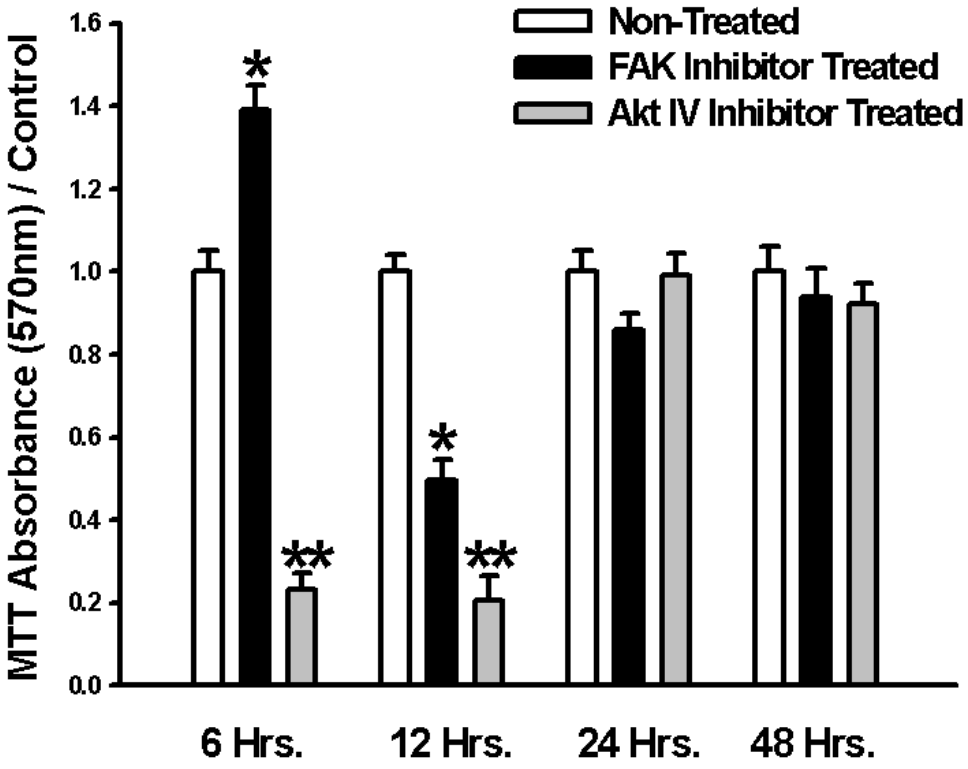


Figure 42. Cellular proliferation following treatment with FAK or Akt inhibitors. An MTT assay was performed on CT-26 cells following treatment with FAK or Akt inhibitors (* indicates $p<0.05$; ** indicates $p<0.01$).

after surgery, our findings are thus more likely to reflect modulation of initial tumor cell adhesion than interference with cellular proliferation.

To further confirm that cancer cells become activated during transit through the circulation, we exposed luciferase tagged CT-26 cells to ambient or 15 mmHg increased pressure for 30 minutes and injected 1 million into the tail vein of Balb/c mice. Seven days after surgery the mice were injected with luciferin and bioluminescent imaging was performed. In contrast to our results with portal injection, we observed no increase in lung metastasis in mice receiving pressure pre-treated cells relative to those receiving control cells preincubated at ambient (Figure 43, n=20) and we found no difference in long term survival (Figure 44, n=20), consistent with our observation (Figure 37) that the force-activated pathway was already activated by passage through the circulation. When we pre-treated the cells with FAK or Akt inhibitors to block this pathway, lung metastasis was reduced (Figure 45, n=12) and long term survival increased (figure 44, n=12).

Cancer metastasis is a complex process. Increased expression of cell surface receptors such as VEGF, loss of function of tumor suppressor genes such as p53 and over-expression of oncogenes all contribute [142]. Our results suggest that the extracellular forces experienced by cancer cells during circulation activate an adhesiogenic pathway that promotes adhesion and subsequent metastasis. Cells pretreated with increased pressure displayed increased adhesion and tumor burden locally, as illustrated by our splenic injection model. However, because pressure activation is already maximized as

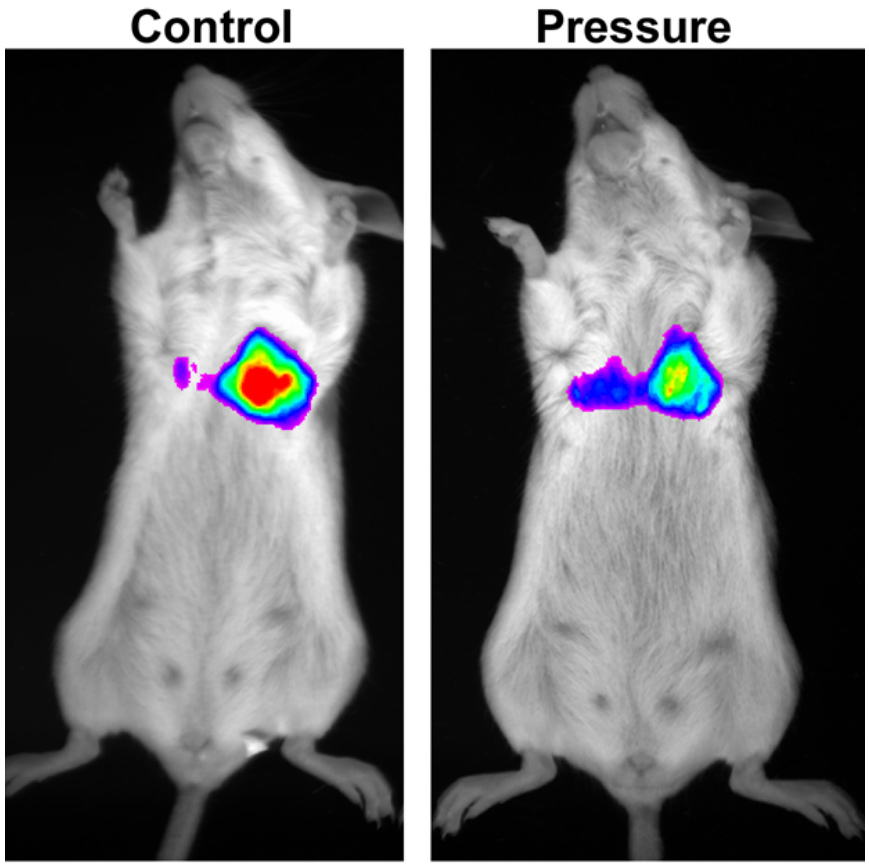


Figure 43. Metastasis of circulating tumor cells. Lung metastasis following tail vein injection of CT-26 cells exposed to 15 mmHg increased or ambient pressure.

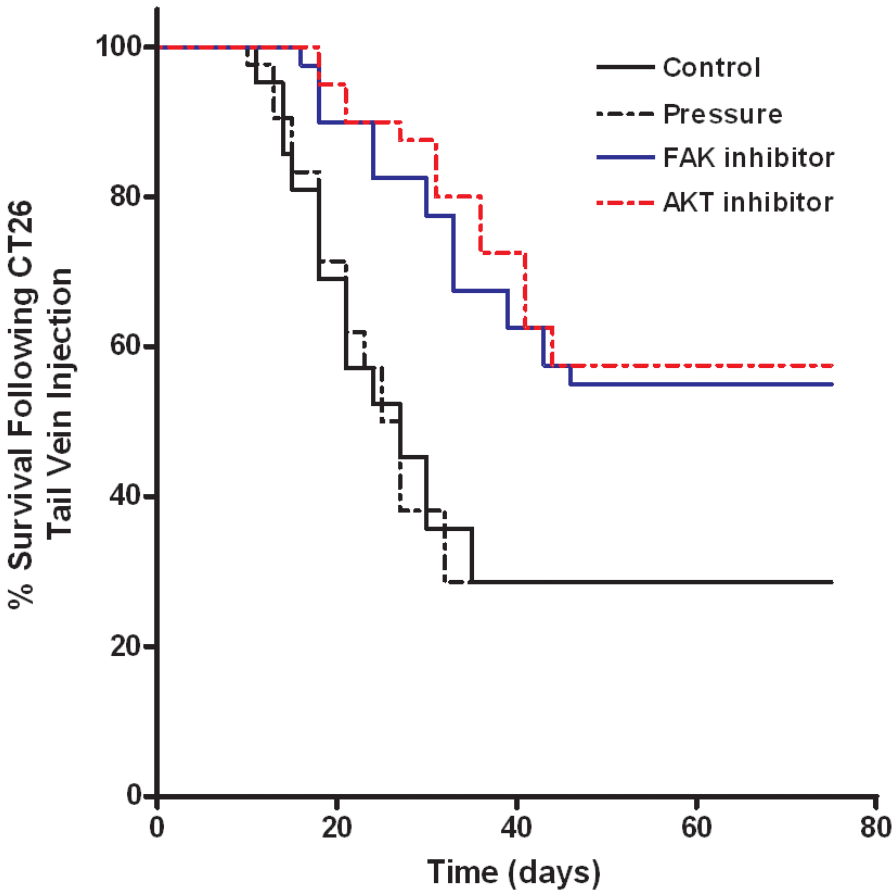


Figure 44. Long term survival following CT-26 tail vein injections. Long term survival of mice following tail vein injection of CT-26 cells pre-treated with 15mmHg increased or ambient pressure, or pre-treated with FAK or Akt inhibitors.

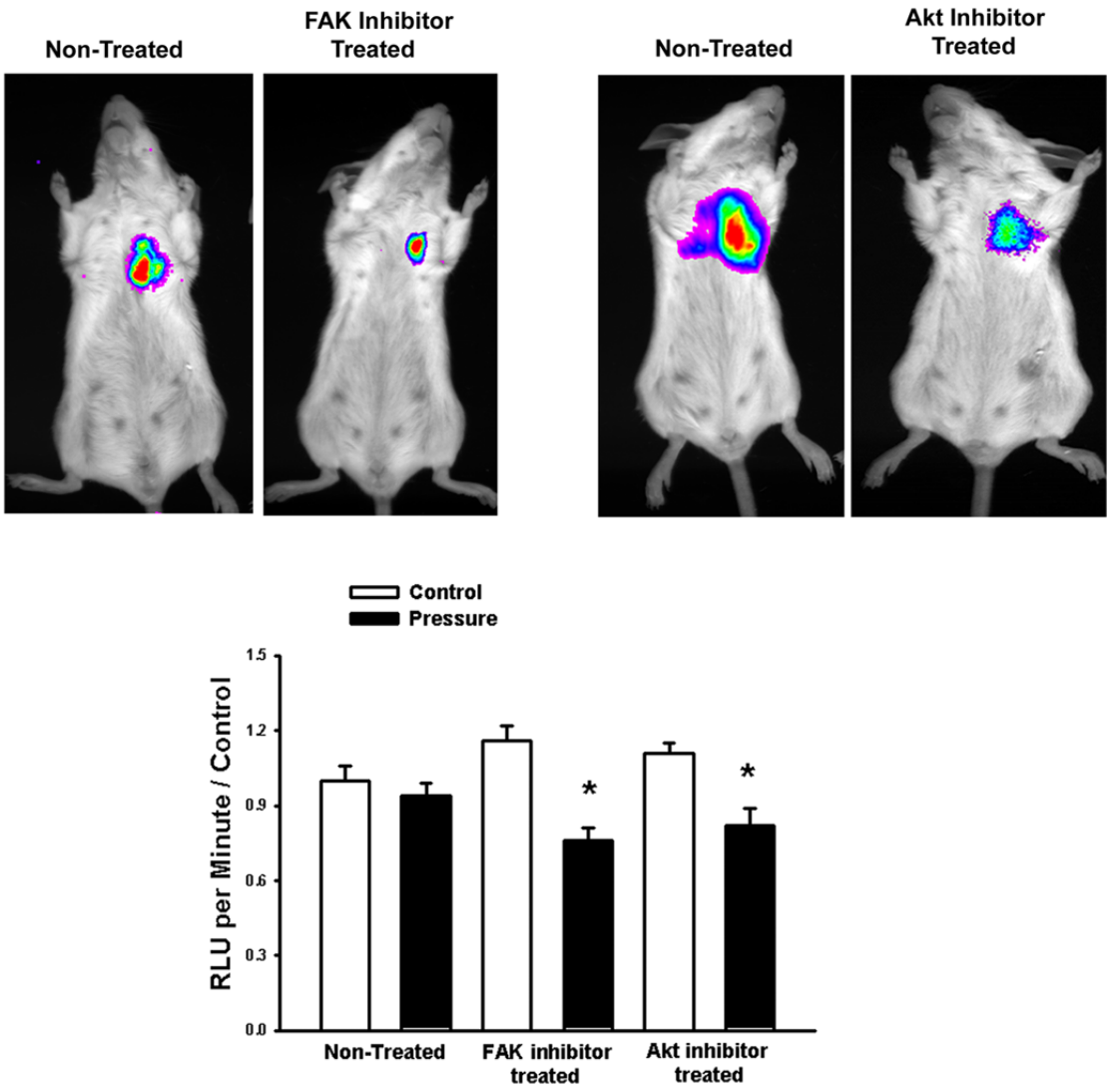


Figure 45. Lung metastasis following tail vein injection of inhibitor treated or untreated cells. Lung metastasis was reduced following treatment with FAK or Akt inhibitors.

cancer cells travel through the circulation, pretreatment with pressure did not further increase lung metastasis in our tail vein model.

Previous studies have confirmed the importance of FAK and Akt activation in pressure induced signaling in vitro, as well the necessity of a dynamically functional cytoskeleton [100]. FAK is a critical regulator in the signaling by which pressure stimulates adhesion, proliferation and migration of cancer cells. In vitro studies suggest that increased pressure results in FAK phosphorylation and activation and downstream signaling. Furthermore, FAK and Akt may participate in bi-directional signaling where FAK is necessary for Akt activation and Akt is necessary for FAK activation. Upon activation, FAK and Akt translocate to the cell membrane where FAK associates with β1 integrin. This association increases in response to elevated pressures leading to increased integrin affinity [100,136]. Although this novel mechanism has been previously elucidated in vitro, this is the first demonstration that physical forces promote metastasis in vivo, and that interruption of the force-activated signal pathway has biological consequences for metastasis.

In the present study, pretreatment of the cancer cells with a single dose of an inhibitor of FAK or Akt, critical kinases activated by physical forces, decreased metastasis and increased long term survival. FAK or Akt expression is increased in diverse tumors, including those of colon, breast, prostate and lung [143,144]. Increased FAK expression correlates with increased cancer cell migration, invasion, and metastasis [144,145]. Akt stimulates aerobic respiration in cancer cells [146]. Akt activation and localization correlates with tumor invasion,

oncogene expression and an overall poor prognosis in several cancers, including thyroid, lung and endometrial [147]. Early stage clinical trials of chronic therapy with FAK or Akt inhibitors have shown promise. TAE226 is a FAK inhibitor that inhibits cell proliferation and migration, induces apoptosis and inhibits cancer cell growth through inhibition of FAK tyrosine 397 phosphorylation. Another FAK inhibitor PF-562,271 inhibits FAK autophosphorylation and has lead to tumor regression or stable disease in ovarian cancer patients when taken daily [148]. Similarly, the Akt inhibitors KP372-1 and GSK690693 inhibit tumor cell growth and proliferation and induce apoptosis in a variety of cancers with daily treatment [149]. Although such trials imply a potential benefit of these new chemotherapeutic agents as part of a daily, long term regimen, our current results suggest that even a single perioperative dose of these inhibitors may interfere with perioperative tumor dissemination and metastasis when tumor cells are shed during surgery.

In conclusion, our results indicate that in vivo pressures modulate cancer cell adhesion and metastasis. Cancer cells traveling through the circulation experience phosphorylation of FAK and Akt leading to activation of signaling that induces adhesion. Increased adhesion at a distant site perpetuates the metastatic process and indicates a poor prognosis for patients. These findings may be particularly relevant, to patients with unresectable tumors or patients undergoing surgery in whom millions of cancer cells may be shed from a primary tumor prior to and during its removal.

CHAPTER 7

Discussion

Molecular signaling events that mediate cancer cell adhesion and proliferation are clinically relevant and necessary for our understanding of the metastatic process as well as inside out signaling. These results demonstrate that pressure activation of cancer cells influences tumor cell adhesion, and by a different mechanism, cellular proliferation. Furthermore, our results demonstrate the effect of pressure in vivo and potential targets to reduce metastatic events.

We have shown that pressure induces adhesion in colon and breast cancer cells. Colon cancer cell adhesion is dependent upon paxillin phosphorylation at tyrosines 31 and 118. Phosphorylation at these sites activates a signaling cascade inducing Rac1. Further evidence suggests paxillin signaling involves alpha-actinin, FAK and Src, and illustrates at least a partial working model by which physical forces may be translated into biochemical signals modulating integrin activation and cell adhesion.

In addition, our findings demonstrate the diverse pathway by which pressure induces cancer cell proliferation. Previous studies from our laboratory have shown that pressure stimulated adhesion is dependent upon Src, Akt and an intact cytoskeleton, however, pharmacological disruption of these molecules had no effect on pressure induced proliferation. Our current study elucidated a pathway by which pressure activates NF-kB, increasing transcription factor activity and proliferation. Furthermore, immunohistochemical staining confirmed

increased NF-kB staining intensities in the high pressure center of human tumors relative to the periphery.

Finally, we have shown the effect of pressure in vivo using a local and systemic metastasis model. Our findings confirm that cancer cell activation occurs as cells experience pressure changes while traveling through the circulation. Disruption of the signaling that mediates pressure stimulated adhesion was shown to reduce tumor burden and increase long term survival.

Taken together, these results indicate two diverse signaling mechanisms by which pressure mediates adhesion as well as proliferation. Elucidation of the final kinases regulating these mechanical signaling pathways, as well as redundancies within the pathways, may provide additional targets for therapeutic intervention, and thus merits further study. Although we have found that NF-kB is not necessary for pressure-induced adhesion, we have yet to determine its relevance in vivo. In addition, further investigation is needed to evaluate the role of calcium in this mechanical signaling study, as calcium dependent protein kinase C seems to be necessary for pressure-induced proliferation, but not calcium independent isoforms. Full elucidation of this pathway may provide molecular targets to inhibit metastasis in disseminated tumor cells following curative tumor resection surgery and potentially improve overall survival.

REFERENCES

1. Allardyce R, Morreau P, Bagshaw P (1996) Tumor cell distribution following laparoscopic colectomy in a porcine model. Dis Colon Rectum 39: S47-52.

2. Nduka CC, Darzi A (1997) Port-site metastasis in patients undergoing laparoscopy for gastrointestinal malignancy. Br J Surg 84: 583.

3. Reilly WT, Nelson H, Schroeder G, Wieand HS, Bolton J, et al. (1996) Wound recurrence following conventional treatment of colorectal cancer. A rare but perhaps underestimated problem. Dis Colon Rectum 39: 200-207.

4. Sugarbaker PH (1999) Successful management of microscopic residual disease in large bowel cancer. Cancer Chemother Pharmacol 43 Suppl: S15-25.

5. Umpleby HC, Fermor B, Symes MO, Williamson RC (1984) Viability of exfoliated colorectal carcinoma cells. Br J Surg 71: 659-663.

6. Haier J, Gallick GE, Nicolson GL (2002) Src protein kinase pp60c-src influences adhesion stabilization of HT-29 colon carcinoma cells to extracellular matrix components under dynamic conditions of laminar flow. J Exp Ther Oncol 2: 237-245.

7. Basson MD, Yu CF, Herden-Kirchoff O, Ellermeier M, Sanders MA, et al. (2000) Effects of increased ambient pressure on colon cancer cell adhesion. J Cell Biochem 78: 47-61.

8. Hynes RO (1992) Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69: 11-25.

9. Coppolino MG, Dedhar S (2000) Bi-directional signal transduction by integrin receptors. Int J Biochem Cell Biol 32: 171-188.

10. Hughes PE, Pfaff M (1998) Integrin affinity modulation. Trends Cell Biol 8: 359-364.

11. Haier J, Nicolson GL (2000) Tumor cell adhesion of human colon carcinoma cells with different metastatic properties to extracellular matrix under dynamic conditions of laminar flow. J Cancer Res Clin Oncol 126: 699-706.

12. Haier J, Nicolson GL (2001) The role of tumor cell adhesion as an important factor in formation of distant colorectal metastasis. Dis Colon Rectum 44: 876-884.

13. Schaller MD, Parsons JT (1994) Focal adhesion kinase and associated proteins. Curr Opin Cell Biol 6: 705-710.

14. Schaller MD (2001) Biochemical signals and biological responses elicited by the focal adhesion kinase. Biochim Biophys Acta 1540: 1-21.

15. Brakebusch C, Fassler R (2003) The integrin-actin connection, an eternal love affair. Embo J 22: 2324-2333.

16. Champault G, Almagro Ruiz M, Panchana G, Barrat C, Catheline JM (1997) [Port-site metastases. A prospective study of 131 cases]. J Chir (Paris) 134: 423-428.

17. Downey C, Alwan K, Thamilselvan V, Zhang L, Jiang Y, et al. (2006) Pressure stimulates breast cancer cell adhesion independently of cell cycle and apoptosis regulatory protein (CARP)-1 regulation of focal adhesion kinase. Am J Surg 192: 631-635.

18. Nathanson SD, Nelson L (1994) Interstitial fluid pressure in breast cancer, benign breast conditions, and breast parenchyma. Ann Surg Oncol 1: 333-338.

19. Rutz HP (1999) A biophysical basis of enhanced interstitial fluid pressure in tumors. Med Hypotheses 53: 526-529.

20. Thamilselvan V, Fomby M, Walsh M, Basson MD (2003) Divalent cations modulate human colon cancer cell adhesion. J Surg Res 110: 255-265.

21. van der Voort van Zyp J, Thamilselvan V, Walsh M, Polin L, Basson MD (2004) Extracellular pressure stimulates colon cancer cell adhesion in vitro and to surgical wounds by Src (sarcoma protein) activation. Am J Surg 188: 467-473.

22. van der Voort van Zyp J, Conway WC, Thamilselvan V, Polin L, Basson MD (2005) Divalent cations influence colon cancer cell adhesion in a murine transplantable tumor model. Am J Surg 190: 701-707.

23. Thamilselvan V, Craig DH, Basson MD (2007) FAK association with multiple signal proteins mediates pressure-induced colon cancer cell adhesion via a Src-dependent PI3K/Akt pathway. Faseb J.

24. Turner CE (2000) Paxillin interactions. J Cell Sci 113 Pt 23: 4139-4140.

25. van Zyp JV, Conway WC, Craig DH, van Zyp NV, Thamilselvan V, et al. (2006) Extracellular pressure stimulates tumor cell adhesion in vitro by paxillin activation. Cancer Biol Ther 5: 1169-1178.

26. Turner CE (2000) Paxillin and focal adhesion signalling. Nat Cell Biol 2: E231-236.

27. Petit V, Boyer B, Lentz D, Turner CE, Thiery JP, et al. (2000) Phosphorylation of tyrosine residues 31 and 118 on paxillin regulates cell migration through an association with CRK in NBT-II cells. J Cell Biol 148: 957-970.

28. Panetti TS (2002) Tyrosine phosphorylation of paxillin, FAK, and p130CAS: effects on cell spreading and migration. Front Biosci 7: d143-150.

29. Chodniewicz D, Klemke RL (2004) Regulation of integrin-mediated cellular responses through assembly of a CAS/Crk scaffold. Biochim Biophys Acta 1692: 63-76.

30. Defilippi P, Di Stefano P, Cabodi S (2006) p130Cas: a versatile scaffold in signaling networks. Trends Cell Biol 16: 257-263.

31. Walsh MF, Woo RK, Gomez R, Basson MD (2004) Extracellular pressure stimulates colon cancer cell proliferation via a mechanism requiring PKC and tyrosine kinase signals. Cell Prolif 37: 427-441.

32. Roffey J, Rosse C, Linch M, Hibbert A, McDonald NQ, et al. (2009) Protein kinase C intervention: the state of play. Curr Opin Cell Biol 21: 268-279.

33. Rosse C, Linch M, Kermorgant S, Cameron AJ, Boeckeler K, et al. (2010) PKC and the control of localized signal dynamics. Nat Rev Mol Cell Biol 11: 103-112.

34. Dolcet X, Llobet D, Pallares J, Matias-Guiu X (2005) NF-kB in development and progression of human cancer. Virchows Arch 446: 475-482.

35. Lee CH, Jeon YT, Kim SH, Song YS (2007) NF-kappaB as a potential molecular target for cancer therapy. Biofactors 29: 19-35.

36. Sasaki N, Morisaki T, Hashizume K, Yao T, Tsuneyoshi M, et al. (2001) Nuclear factor-kappaB p65 (RelA) transcription factor is constitutively activated in human gastric carcinoma tissue. Clin Cancer Res 7: 4136-4142.

37. Scartozzi M, Bearzi I, Pierantoni C, Mandolesi A, Loupakis F, et al. (2007) Nuclear factor-kB tumor expression predicts response and survival in irinotecan-refractory metastatic colorectal cancer treated with cetuximab-irinotecan therapy. J Clin Oncol 25: 3930-3935.

38. Ismail HA, Lessard L, Mes-Masson AM, Saad F (2004) Expression of NF-kappaB in prostate cancer lymph node metastases. Prostate 58: 308-313.

39. Rishi AK, Zhang L, Boyanapalli M, Wali A, Mohammad RM, et al. (2003) Identification and characterization of a cell cycle and apoptosis regulatory protein-1 as a novel mediator of apoptosis signaling by retinoid CD437. J Biol Chem 278: 33422-33435.

40. Rishi AK, Zhang L, Yu Y, Jiang Y, Nautiyal J, et al. (2006) Cell cycle- and apoptosis-regulatory protein-1 is involved in apoptosis signaling by epidermal growth factor receptor. J Biol Chem 281: 13188-13198.

41. Thamilselvan V, Basson MD (2004) Pressure activates colon cancer cell adhesion by inside-out focal adhesion complex and actin cytoskeletal signaling. Gastroenterology 126: 8-18.

42. Chang LC, Huang CH, Cheng CH, Chen BH, Chen HC (2005) Differential effect of the focal adhesion kinase Y397F mutant on v-Src-stimulated cell invasion and tumor growth. J Biomed Sci 12: 571-585.

43. Chen HC, Appeddu PA, Isoda H, Guan JL (1996) Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase. J Biol Chem 271: 26329-26334.

44. Li W, Duzgun A, Sumpio BE, Basson MD (2001) Integrin and FAK-mediated MAPK activation is required for cyclic strain mitogenic effects in Caco-2 cells. Am J Physiol Gastrointest Liver Physiol 280: G75-87.

45. Han O, Sumpio BE, Basson MD (1998) Mechanical strain rapidly redistributes tyrosine phosphorylated proteins in human intestinal Caco-2 cells. Biochem Biophys Res Commun 250: 668-673.

46. Thamilselvan V, Patel A, van der Voort van Zyp J, Basson MD (2004) Colon cancer cell adhesion in response to Src kinase activation and actin-cytoskeleton by non-laminar shear stress. J Cell Biochem 92: 361-371.

47. Turner CE (1998) Paxillin. Int J Biochem Cell Biol 30: 955-959.

48. Liu S, Slepak M, Ginsberg MH (2001) Binding of Paxillin to the alpha 9 Integrin Cytoplasmic Domain Inhibits Cell Spreading. J Biol Chem 276: 37086-37092.

49. Liu S, Thomas SM, Woodside DG, Rose DM, Kiosses WB, et al. (1999) Binding of paxillin to alpha4 integrins modifies integrin-dependent biological responses. Nature 402: 676-681.

50. Young BA, Taooka Y, Liu S, Askins KJ, Yokosaki Y, et al. (2001) The cytoplasmic domain of the integrin alpha9 subunit requires the adaptor protein paxillin to inhibit cell spreading but promotes cell migration in a paxillin-independent manner. Mol Biol Cell 12: 3214-3225.

51. Mitra SK, Schlaepfer DD (2006) Integrin-regulated FAK-Src signaling in normal and cancer cells. Curr Opin Cell Biol 18: 516-523.

52. Schaller MD, Schaefer EM (2001) Multiple stimuli induce tyrosine phosphorylation of the Crk-binding sites of paxillin. Biochem J 360: 57-66.

53. Schlaepfer DD, Hauck CR, Sieg DJ (1999) Signaling through focal adhesion kinase. Prog Biophys Mol Biol 71: 435-478.

54. Conway WC, Van der Voort van Zyp J, Thamilselvan V, Walsh MF, Crowe DL, et al. (2006) Paxillin modulates squamous cancer cell adhesion and is important in pressure-augmented adhesion. J Cell Biochem 98: 1507-1516.

55. Craig DH, Haimovich B, Basson MD (2007) {alpha}-Actinin-1 Phosphorylation Modulates Pressure-Induced Colon Cancer Cell Adhesion Through Regulation of Focal Adhesion Kinase-Src Interaction. Am J Physiol Cell Physiol.

56. Feller SM (2001) Crk family adaptors-signalling complex formation and biological roles. Oncogene 20: 6348-6371.

57. Schaller MD, Parsons JT (1995) pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for Crk. Mol Cell Biol 15: 2635-2645.

58. Vuori K, Hirai H, Aizawa S, Ruoslahti E (1996) Introduction of p130cas signaling complex formation upon integrin-mediated cell adhesion: a role for Src family kinases. Mol Cell Biol 16: 2606-2613.

59. Brabek J, Constancio SS, Siesser PF, Shin NY, Pozzi A, et al. (2005) Crk-associated substrate tyrosine phosphorylation sites are critical for invasion and metastasis of SRC-transformed cells. Mol Cancer Res 3: 307-315.

60. Klemke RL, Leng J, Molander R, Brooks PC, Vuori K, et al. (1998) CAS/Crk coupling serves as a "molecular switch" for induction of cell migration. J Cell Biol 140: 961-972.

61. Nomura N, Nomura M, Sugiyama K, Hamada J (2007) Src regulates phorbol 12-myristate 13-acetate-activated PKC-induced migration via Cas/Crk/Rac1 signaling pathway in glioblastoma cells. Int J Mol Med 20: 511-519.

62. Cho SY, Klemke RL (2002) Purification of pseudopodia from polarized cells reveals redistribution and activation of Rac through assembly of a CAS/Crk scaffold. J Cell Biol 156: 725-736.

63. Dregelid E, Svendsen E (1988) Endothelial cell injury in human saphenous veins after manipulation and tweezer grasping. J Cardiovasc Surg (Torino) 29: 464-469.

64. Moore-Olufemi SD, Xue H, Allen SJ, Moore FA, Stewart RH, et al. (2005) Effects of primary and secondary intra-abdominal hypertension on mesenteric lymph flow: implications for the abdominal compartment syndrome. Shock 23: 571-575.

65. Nathan SS, DiResta GR, Casas-Ganem JE, Hoang BH, Sowers R, et al. (2005) Elevated physiologic tumor pressure promotes proliferation and chemosensitivity in human osteosarcoma. Clin Cancer Res 11: 2389-2397.

66. Wu JS, Brasfield EB, Guo LW, Ruiz M, Connett JM, et al. (1997) Implantation of colon cancer at trocar sites is increased by low pressure pneumoperitoneum. Surgery 122: 1-7.

67. Brown MC, Turner CE (2004) Paxillin: adapting to change. Physiol Rev 84: 1315-1339.

68. Azuma K, Tanaka M, Uekita T, Inoue S, Yokota J, et al. (2005) Tyrosine phosphorylation of paxillin affects the metastatic potential of human osteosarcoma. Oncogene 24: 4754-4764.

69. Nagata M, Fujita H, Ida H, Hoshina H, Inoue T, et al. (2003) Identification of potential biomarkers of lymph node metastasis in oral squamous cell carcinoma by cDNA microarray analysis. Int J Cancer 106: 683-689.

70. Vadlamudi R, Adam L, Tseng B, Costa L, Kumar R (1999) Transcriptional up-regulation of paxillin expression by heregulin in human breast cancer cells. Cancer Res 59: 2843-2846.

71. Zaidel-Bar R, Milo R, Kam Z, Geiger B (2007) A paxillin tyrosine phosphorylation switch regulates the assembly and form of cell-matrix adhesions. J Cell Sci 120: 137-148.

72. Schaller MD (2004) FAK and paxillin: regulators of N-cadherin adhesion and inhibitors of cell migration? J Cell Biol 166: 157-159.

73. Vogel EM, Imperiali B (2007) Semisynthesis of unnatural amino acid mutants of paxillin: protein probes for cell migration studies. Protein Sci 16: 550-556.

74. Abou Zeid N, Valles AM, Boyer B (2006) Serine phosphorylation regulates paxillin turnover during cell migration. Cell Commun Signal 4: 8.

75. Nayal A, Webb DJ, Brown CM, Schaefer EM, Vicente-Manzanares M, et al. (2006) Paxillin phosphorylation at Ser273 localizes a GIT1-PIX-PAK complex and regulates adhesion and protrusion dynamics. J Cell Biol 173: 587-589.

76. Feller SM, Posern G, Voss J, Kardinal C, Sakkab D, et al. (1998) Physiological signals and oncogenesis mediated through Crk family adapter proteins. J Cell Physiol 177: 535-552.

77. Thamilselvan V, Craig DH, Basson MD (2007) FAK association with multiple signal proteins mediates pressure-induced colon cancer cell adhesion via a Src-dependent PI3K/Akt pathway. Faseb J 21: 1730-1741.

78. Okino K, Nagai H, Nakayama H, Doi D, Yoneyama K, et al. (2006) Inactivation of Crk SH3 domain-binding guanine nucleotide-releasing factor (C3G) in cervical squamous cell carcinoma. Int J Gynecol Cancer 16: 763-771.

79. Hasegawa H, Kiyokawa E, Tanaka S, Nagashima K, Gotoh N, et al. (1996) DOCK180, a major CRK-binding protein, alters cell morphology upon translocation to the cell membrane. Mol Cell Biol 16: 1770-1776.

80. Shin NY, Dise RS, Schneider-Mergener J, Ritchie MD, Kilkenny DM, et al. (2004) Subsets of the major tyrosine phosphorylation sites in Crk-associated substrate (CAS) are sufficient to promote cell migration. J Biol Chem 279: 38331-38337.

81. Wei L, Yang Y, Zhang X, Yu Q (2004) Cleavage of p130Cas in anoikis. J Cell Biochem 91: 325-335.

82. Valles AM, Beuvin M, Boyer B (2004) Activation of Rac1 by paxillin-Crk-DOCK180 signaling complex is antagonized by Rap1 in migrating NBT-II cells. J Biol Chem 279: 44490-44496.

83. Abassi YA, Rehn M, Ekman N, Alitalo K, Vuori K (2003) p130Cas Couples the tyrosine kinase Bmx/Etk with regulation of the actin cytoskeleton and cell migration. J Biol Chem 278: 35636-35643.

84. Katoh H, Hiramoto K, Negishi M (2006) Activation of Rac1 by RhoG regulates cell migration. J Cell Sci 119: 56-65.

85. Iwashima F, Yoshimoto T, Minami I, Sakurada M, Hirono Y, et al. (2007) Aldosterone induces superoxide generation via Rac1 activation in endothelial cells. Endocrinology.

86. Wennerberg K, Der CJ (2004) Rho-family GTPases: it's not only Rac and Rho (and I like it). J Cell Sci 117: 1301-1312.

87. Chrostek A, Wu X, Quondamatteo F, Hu R, Sanecka A, et al. (2006) Rac1 is crucial for hair follicle integrity but is not essential for maintenance of the epidermis. Mol Cell Biol 26: 6957-6970.

88. Gu Y, Filippi MD, Cancelas JA, Siefring JE, Williams EP, et al. (2003) Hematopoietic cell regulation by Rac1 and Rac2 guanosine triphosphatases. Science 302: 445-449.

89. Vidali L, Chen F, Cicchetti G, Ohta Y, Kwiatkowski DJ (2006) Rac1-null mouse embryonic fibroblasts are motile and respond to platelet-derived growth factor. Mol Biol Cell 17: 2377-2390.

90. Hu YL, Haga JH, Miao H, Wang Y, Li YS, et al. (2006) Roles of microfilaments and microtubules in paxillin dynamics. Biochem Biophys Res Commun 348: 1463-1471.

91. von Sengbusch A, Gassmann P, Fisch KM, Enns A, Nicolson GL, et al. (2005) Focal adhesion kinase regulates metastatic adhesion of carcinoma cells within liver sinusoids. Am J Pathol 166: 585-596.

92. Webb DJ, Donais K, Whitmore LA, Thomas SM, Turner CE, et al. (2004) FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat Cell Biol 6: 154-161.

93. Rosenfeldt HM, Hobson JP, Maceyka M, Olivera A, Nava VE, et al. (2001) EDG-1 links the PDGF receptor to Src and focal adhesion kinase activation leading to lamellipodia formation and cell migration. Faseb J 15: 2649-2659.

94. Tsakiridis T, Bergman A, Somwar R, Taha C, Aktories K, et al. (1998) Actin filaments facilitate insulin activation of the src and collagen homologous/mitogen-activated protein kinase pathway leading to DNA synthesis and c-fos expression. J Biol Chem 273: 28322-28331.

95. Zhu T, Goh EL, Lobie PE (1998) Growth hormone stimulates the tyrosine phosphorylation and association of p125 focal adhesion kinase (FAK) with JAK2. Fak is not required for stat-mediated transcription. J Biol Chem 273: 10682-10689.

96. Schlaepfer DD, Mitra SK, Ilic D (2004) Control of motile and invasive cell phenotypes by focal adhesion kinase. Biochim Biophys Acta 1692: 77-102.

97. Palazzo AF, Eng CH, Schlaepfer DD, Marcantonio EE, Gundersen GG (2004) Localized stabilization of microtubules by integrin- and FAK-facilitated Rho signaling. Science 303: 836-839.

98. Ezratty EJ, Partridge MA, Gundersen GG (2005) Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. Nat Cell Biol 7: 581-590.

99. Craig DH (In Press) SiRNA-mediated reduction of alpha-actinin-1 inhibits pressure-induced murine tumor cell wound implantation and enhances tumor-free survival. Neoplasia.

100. Basson MD (2008) An intracellular signal pathway that regulates cancer cell adhesion in response to extracellular forces. Cancer Res 68: 2-4.

101. Bevan RD (1976) An autoradiographic and pathological study of cellular proliferation in rabbit arteries correlated with an increase in arterial pressure. Blood Vessels 13: 100-128.

102. Kawata Y, Mizukami Y, Fujii Z, Sakumura T, Yoshida K, et al. (1998) Applied pressure enhances cell proliferation through mitogen-activated protein kinase activation in mesangial cells. J Biol Chem 273: 16905-16912.

103. Schwartz EA, Bizios R, Medow MS, Gerritsen ME (1999) Exposure of human vascular endothelial cells to sustained hydrostatic pressure stimulates proliferation. Involvement of the alphaV integrins. Circ Res 84: 315-322.

104. Zhang J, Li W, Sanders MA, Sumpio BE, Panja A, et al. (2003) Regulation of the intestinal epithelial response to cyclic strain by extracellular matrix proteins. FASEB J 17: 926-928.

105. Downey C, Craig DH, Basson MD (2008) Pressure activates colon cancer cell adhesion via paxillin phosphorylation, Crk, Cas, and Rac1. Cell Mol Life Sci 65: 1446-1457.

106. Gonzalez E, Kou R, Michel T (2006) Rac1 modulates sphingosine 1-phosphate-mediated activation of phosphoinositide 3-kinase/Akt signaling pathways in vascular endothelial cells. J Biol Chem 281: 3210-3216.

107. Stark LA, Reid K, Sansom OJ, Din FV, Guichard S, et al. (2007) Aspirin activates the NF-kappaB signalling pathway and induces apoptosis in intestinal neoplasia in two in vivo models of human colorectal cancer. Carcinogenesis 28: 968-976.

108. Wu M, Lee H, Bellas RE, Schauer SL, Arsura M, et al. (1996) Inhibition of NF-kappaB/Rel induces apoptosis of murine B cells. EMBO J 15: 4682-4690.

109. Boucher Y, Baxter LT, Jain RK (1990) Interstitial pressure gradients in tissue-isolated and subcutaneous tumors: implications for therapy. Cancer Res 50: 4478-4484.

110. Sarntinoranont M, Rooney F, Ferrari M (2003) Interstitial stress and fluid pressure within a growing tumor. Ann Biomed Eng 31: 327-335.

111. Yamamoto Y, Gaynor RB (2001) Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. J Clin Invest 107: 135-142.

112. Less JR, Posner MC, Boucher Y, Borochovitz D, Wolmark N, et al. (1992) Interstitial hypertension in human breast and colorectal tumors. Cancer Res 52: 6371-6374.

113. Wotton K, Redden M (2002) Third-space fluid shift in elderly patients undergoing gastrointestinal surgery: Part II: nursing assessment. Contemp Nurse 13: 50-60.

114. Diresta GR, Nathan SS, Manoso MW, Casas-Ganem J, Wyatt C, et al. (2005) Cell proliferation of cultured human cancer cells are affected by the elevated tumor pressures that exist in vivo. Ann Biomed Eng 33: 1270-1280.

115. Pirone DM, Liu WF, Ruiz SA, Gao L, Raghavan S, et al. (2006) An inhibitory role for FAK in regulating proliferation: a link between limited adhesion and RhoA-ROCK signaling. J Cell Biol 174: 277-288.

116. Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, et al. (2005) Tensional homeostasis and the malignant phenotype. Cancer Cell 8: 241-254.

117. Iizuka K, Morita N, Murakami T, Kawaguchi H (2004) Nipradilol inhibits atmospheric pressure-induced cell proliferation in human aortic smooth muscle cells. Pharmacol Res 49: 217-225.

118. Agar A, Yip SS, Hill MA, Coroneo MT (2000) Pressure related apoptosis in neuronal cell lines. J Neurosci Res 60: 495-503.

119. Hishikawa K, Oemar BS, Nakaki T (2001) Static pressure regulates connective tissue growth factor expression in human mesangial cells. J Biol Chem 276: 16797-16803.

120. Chaturvedi LS, Gayer CP, Marsh HM, Basson MD (2008) Repetitive deformation activates Src-independent FAK-dependent ERK motogenic signals in human Caco-2 intestinal epithelial cells. Am J Physiol Cell Physiol 294: C1350-1361.

121. Kumar A, Boriek AM (2003) Mechanical stress activates the nuclear factor-kappaB pathway in skeletal muscle fibers: a possible role in Duchenne muscular dystrophy. FASEB J 17: 386-396.

122. Liu J, Zou L, Zheng Y, Zhao Z, Li Y, et al. (2007) NF-kappaB responds to mechanical strains in osteoblast-like cells, and lighter strains create an NF-kappaB response more readily. Cell Biol Int 31: 1220-1224.

123. Lemarie CA, Esposito B, Tedgui A, Lehoux S (2003) Pressure-induced vascular activation of nuclear factor-kappaB: role in cell survival. Circ Res 93: 207-212.

124. Crosara-Alberto DP, Inoue RY, Costa CR (2009) FAK signalling mediates NF-kappaB activation by mechanical stress in cardiac myocytes. Clin Chim Acta 403: 81-86.

125. Shant J, Cheng K, Marasa BS, Wang JY, Raufman JP (2009) Akt-dependent NF-kappaB activation is required for bile acids to rescue colon cancer cells from stress-induced apoptosis. Exp Cell Res 315: 432-450.

126. Genot EM, Parker PJ, Cantrell DA (1995) Analysis of the role of protein kinase C-alpha, -epsilon, and -zeta in T cell activation. J Biol Chem 270: 9833-9839.

127. Silberman DM, Zorrilla-Zubilete M, Cremaschi GA, Genaro AM (2005) Protein kinase C-dependent NF-kappaB activation is altered in T cells by chronic stress. Cell Mol Life Sci 62: 1744-1754.

128. Lin X, O'Mahony A, Mu Y, Geleziunas R, Greene WC (2000) Protein kinase C-theta participates in NF-kappaB activation induced by CD3-CD28 costimulation through selective activation of IkappaB kinase beta. Mol Cell Biol 20: 2933-2940.

129. Han O, Li GD, Sumpio BE, Basson MD (1998) Strain induces Caco-2 intestinal epithelial proliferation and differentiation via PKC and tyrosine kinase signals. Am J Physiol 275: G534-541.

130. Bocker W, Docheva D, Prall WC, Egea V, Pappou E, et al. (2008) IKK-2 is required for TNF-alpha-induced invasion and proliferation of human mesenchymal stem cells. J Mol Med 86: 1183-1192.

131. Mourkioti F, Kratsios P, Luedde T, Song YH, Delafontaine P, et al. (2006) Targeted ablation of IKK2 improves skeletal muscle strength, maintains mass, and promotes regeneration. J Clin Invest 116: 2945-2954.

132. Bertazza L, Mocellin S, Nitti D (2008) Circulating tumor cells in solid cancer: tumor marker of clinical relevance? Curr Oncol Rep 10: 137-146.

133. Rahbari NN, Aigner M, Thorlund K, Mollberg N, Motschall E, et al. (2010) Meta-Analysis Shows That Detection of Circulating Tumor Cells Indicates Poor Prognosis in Patients With Colorectal Cancer. Gastroenterology.

134. Weitz J, Kienle P, Lacroix J, Willeke F, Benner A, et al. (1998) Dissemination of tumor cells in patients undergoing surgery for colorectal cancer. Clin Cancer Res 4: 343-348.

135. (2004) A comparison of laparoscopically assisted and open colectomy for colon cancer. N Engl J Med 350: 2050-2059.

136. Craig DH, Owen CR, Conway WC, Walsh MF, Downey C, et al. (2008) Colchicine inhibits pressure-induced tumor cell implantation within surgical wounds and enhances tumor-free survival in mice. J Clin Invest 118: 3170-3180.

137. Mehlen P, Puisieux A (2006) Metastasis: a question of life or death. Nat Rev Cancer 6: 449-458.

138. Aslakson CJ, Miller FR (1992) Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res 52: 1399-1405.

139. Meyer T, Hart IR (1998) Mechanisms of tumour metastasis. Eur J Cancer 34: 214-221.

140. Di Pasquale P, Cannizzaro S, Paterna S (2004) Does angiotensin-converting enzyme gene polymorphism affect blood pressure? Findings after 6 years of follow-up in healthy subjects. Eur J Heart Fail 6: 11-16.

141. Huang H, Deng M, Jin H, Dirsch O, Dahmen U (2010) Intraoperative vital and haemodynamic monitoring using an integrated multiple-channel monitor in rats. Lab Anim.

142. Alkim C, Savas B, Ensari A, Alkim H, Dagli U, et al. (2009) Expression of p53, VEGF, microvessel density, and cyclin-D1 in noncancerous tissue of inflammatory bowel disease. Dig Dis Sci 54: 1979-1984.

143. Neckers L (2002) Hsp90 inhibitors as novel cancer chemotherapeutic agents. Trends Mol Med 8: S55-61.

144. Toton E, Rybczynska M (2007) [The characteristics of focal adhesion kinase (FAK) and its role in carcinogenesis]. Postepy Hig Med Dosw (Online) 61: 303-309.

145. Parsons JT, Slack-Davis J, Tilghman R, Roberts WG (2008) Focal adhesion kinase: targeting adhesion signaling pathways for therapeutic intervention. Clin Cancer Res 14: 627-632.

146. Elstrom RL, Bauer DE, Buzzai M, Karnauskas R, Harris MH, et al. (2004) Akt stimulates aerobic glycolysis in cancer cells. Cancer Res 64: 3892-3899.

147. Tokunaga E, Oki E, Egashira A, Sadanaga N, Morita M, et al. (2008) Deregulation of the Akt pathway in human cancer. Curr Cancer Drug Targets 8: 27-36.

148. Hao HF, Naomoto Y, Bao XH, Watanabe N, Sakurama K, et al. (2009) Progress in researches about focal adhesion kinase in gastrointestinal tract. World J Gastroenterol 15: 5916-5923.

149. Kawauchi K, Ogasawara T, Yasuyama M, Otsuka K, Yamada O (2009) The PI3K/Akt pathway as a target in the treatment of hematologic malignancies. Anticancer Agents Med Chem 9: 550-559.

ABSTRACT

MOLECULAR MECHANISMS OF PRESSURE-STIMULATED CANCER CELL SIGNALING

by

CHRISTINA DOWNEY

August 2010

Advisor: Dr. Marc Basson, MD, PhD

Major: Cancer Biology

Degree: Doctor of Philosophy

Increased extracellular pressure stimulates cancer cell adhesion by a mechanism that is dependent upon β1-integrin activation, an intact cytoskeleton, and FAK and Src activation. By a different mechanism, increased extracellular pressure modulates cancer cell proliferation in a manner that is regulated by protein kinase C, but not Src or an intact cytoskeleton. Previous studies from our laboratory have shown that paxillin is a necessary mediator in the pathway by which pressure stimulates adhesion; however, it had not been determined whether paxillin acts simply as a scaffold, or participates in diverse signaling events. I therefore hypothesized that paxillin phosphorylation mediates pressure stimulated adhesion and further asked whether the independent pathway by which pressure induces proliferation is modulated by NF-kB. Finally, I postulated that increased pressures of the circulation activate cancer cells to increase adhesion, proliferation and overall tumor burden in vivo. My data illustrate that paxillin phosphorylation at tyrosines 31 and 118 is necessary for pressure stimulated adhesion, but tyrosine 81 phosphorylation is not. The phosphorylation

events at tyrosine 31 and 118 form a docking site for the adaptor molecule Crk, which has a binding site for the highly phosphorylated molecule Cas. Each member of the paxillin/Crk/Cas complex is necessary for pressure stimulated adhesion. Furthermore, this complex promotes the activation of the small GTPase, Rac1.

Next, I evaluated whether Rac1 was necessary for pressure induced proliferation, and found that it is not. However, it was found that this pathway is dependent upon NF-kB activation. Increased extracellular pressure increases NF-kB activation in colon, breast and prostate cancer cells. This increase is abolished in the presence of NF-kB inhibitors. Further studies showed that increased pressure activates protein kinase C α/β, increases IKK and IkB phosphorylation, cyclin D1 expression and increased S-phase fractions. Immunohistochemical staining for NF-kB, IkB and cyclin D1 increased in the high pressure center of human tumors and decreased toward the periphery.

Finally, I determined that increased pressures of the circulation activate cancer cells in vivo by phosphorylation of β1 integrin, FAK and Src. Pre-treatment with pressure prior to systemic injection did not increase metastasis. However, in a local metastasis model, increased tumor burden and decreased long term survival occurred when cells were pre-treated with pressure. These increases were abolished when cells were pre-treated with FAK or Akt inhibitors prior to injection.

In summary, my data suggests pressure activation of malignant cells promotes tumor development and impairs tumor free survival. Preoperative

inhibition of paxillin, NF-kB, or other interventions aimed at blocking pressure-induced integrin activation may abolish this effect.

AUTOBIOGRAPHICAL STATEMENT

PERSONAL

Born March 31, 1983, Detroit, Michigan

EDUCATION

2001 Roseville High School, Roseville, Michigan

2005 Wayne State University, Detroit, Michigan; B.S. in Biology

PUBLICATIONS

Downey C, Alwan K, Thamilselvan V, Zhang L, Jiang Y, Rishi AK, Basson MD. Pressure stimulates breast cancer cell adhesion independently of cell cycle and regulatory protein (CARP)-1 regulation of focal adhesion kinase. Am J Surg. 2006; 192(5): 631-5.

Craig DH, **Downey C**, Basson MD. siRNA mediated reduction of α-actinin-1 inhibits pressure-induced murine tumor cell wound implantation and enhances tumor-free survival. Neoplasia. 2008; 10(3): 217-222.

Downey C, Craig DH, Basson MD. Pressure activates colon cancer cell adhesion via paxillin phosphorylation, Crk, Cas, and Rac1. Cell Mol Life Sci. 2008; 65(9): 1446-57.

Craig DH, Owen CR, Conway WC, Walsh MF, **Downey C**, Basson MD. Colchicine inhibits pressure-induced tumor cell implantation within surgical wounds and enhances tumor-free survival in mice. J Clin Invest. 2008; 118(9): 3170-80.

Downey C, Craig DH, Hatfield JS, Tepe JJ, Basson MD. Increased extracellular pressure stimulates proliferation by activating NF-kB in cancer cells. (under review)

Downey C, Craig DH, Basson MD. Increased pressures activate signals in circulating cancer cells that promote metastasis in vivo. (under review)