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Physical forces enhance colon cancer cell adhesion through a mechanism involving Src kinase

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By
Ashish Ramesh Patel
2000

Dedicated to

My parents

Abstract

Colon cancer cells are subjected to physical forces during open or laparoscopic procedures due to insufflation pressure, manipulation of the tumor, and turbulence created by fluid jets. It has been shown that physical forces can activate intracellular signals that lead to increased adhesiveness and proliferation among variety of cells *in vitro*. We have studied the effects of pressure, shear stress, and turbulence on SW620 colon cancer cell adhesion using two paradigms--one studied effect of 15 mm Hg pressure, and the other studied combined effects of shear stress and turbulence. Pressure was applied to cells using a custom designed lucite box. Shear stress and turbulence were applied by stirring cells in suspension at 600 rpm for 10 minutes. SW620 cells plated on collagen I matrix at 15 mmHg increased pressure for 30 min showed a 30 % $\pm$ 4 % ($n = 15$, $p = 0.0009$) increase in adhesion by cell number compared to control cells plated at atmospheric pressure. When SW620 cells in suspension were pre-incubated at 15 mmHg increased pressure for 30 min and subsequently plated on collagen I matrix under ambient conditions, cell adhesion increased by 105 % $\pm$ 20 % ($n = 9$, $p = 0.0005$) compared to control cells pre-incubated at atmospheric pressure. Pressure induced enhancement of adhesion was inhibited by Src inhibitor PP1 at 1uM and 0.1 uM. In-vitro kinase assay for Src showed that Src activity was increased in cells exposed to pressure compared to control cells. Pre-treatment of cells with shear and turbulence combined by stirring at 600 rpm and subsequent plating on collagen I matrix enhanced cell adhesion by 85 % $\pm$ 22 % ($n = 9$, $p = 0.0047$) compared to non-stirred control cells. Shear induced enhancement was also inhibited by Src inhibitor PP1 at 0.1 uM. *In-vitro* kinase assay for Src confirmed that Src was activated in response to shear and turbulence.

We concluded that SW620 colon cancer cell adhesion *in-vitro* was stimulated by pressure, shear stress, and turbulence. This enhancement was directly mediated by Src kinase activity and could be blocked by Src family inhibitor PP1.

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Introduction

This thesis is focused on effects of physical forces, namely pressure, shear stress, and turbulence, on human SW620 colon cancer cell adhesion *in-vitro*. The project examines the effect of physical forces on colon cancer cell adhesion and the cellular mechanism behind this effect. The inspiration and background behind this project can be divided into three parts—1) The high rate of colon cancer in the United States 2) The relevance of understanding the effects of physical forces on colon cancer cells due to the motility of the tissue and potential problems during surgical intervention, and 3) The need to understand cellular signals activated by physical forces.

1) Colon Cancer in the United States

Colon cancer is the second largest cause of cancer death in the United States. Approximately 130,000 cases of colon cancer are detected every year, and 50,000 Americans die annually from this disease. The majority of the patients are adults above 50 years of age and more than 99% of the tumors are identified as adenocarcinomas (1). The prognosis of the patient with colon adenocarcinoma is highly dependent on the stage of the disease. Several staging systems have been used to classify colon cancer. The traditional staging system for colon adenocarcinoma is the Dukes staging system. In recent years, the Dukes system has been updated with the Astler-Coller modification. An international standard for classification has also been established through TNM classification, where T represents depth of tumor, N represents number of lymph nodes involved, and M represents presence or absence of metastasis. Lesions that are superficial to the muscularis layer and do not involve any nodes (T1N0M0) are Dukes stage A. Those that involve the bowel wall but do not involve nodes (T2N0M0) are

Dukes stage B. Stage B is further divided into B1 (T2N0M0) if the tumor is within the muscularis layer, and B2 (T3-4N0M0) if the tumor extends through the serosa. Tumors that involve nodes (T2-4N1M0) are staged as Dukes stage C which is further divided into C1 (T2N1M0) and C2 (T3-4N1M0). Finally, if metastases are present (T2-4NXM1), the patient is staged as Duke stage D (2).

The stage of the disease is the most important prognostic factor for survival for colon adenocarcinoma. The five-year survival for each Dukes stage is given in Table 1 (1). It is evident from Table 1 below that with currently available treatment modalities, patients with the best survival rates are those with tumors at earlier stages. This is the rationale behind screening programs, which try to detect colon adenocarcinomas at early stages. Early detection is the most successful factor in preventing mortality from colon cancer.

Prognosis and Staging of Colon Cancer		
Duke Stage	TNM Stage	5 year survival %
A	T1N0M0	>90
B1	T2N0M0	85
B2	T3-4N0M0	70-75
C1	T2N1M0	35-65
C2	T3-4N1M0	35-65
D	TXNXM1	<5

Table 1 Prognosis and Staging of Colon Cancer(1)

Treatment modalities for colon cancer include chemotherapy, radiation therapy, and surgical resection. Of these, surgical resection, which can be accomplished through open laparotomy, endoscopic excision, or transanal excision, is currently the only curative treatment. Chemotherapy is used for patients with advanced tumors that cannot be resected completely, or for patients with metastatic disease. The benefit of chemotherapy to survival is poor and there is no reported cure from chemotherapy alone for colon cancer at any stage of the disease. A more promising application of chemotherapy is its use as adjuvant treatment. Studies have shown survival benefit among patients receiving combination of 5FU and levamisole after resection of the tumor (3,4).

Radiation therapy is used as adjuvant therapy for patients with stage B2 or C rectal tumors. Rectal tumors recur locally at an unusually high incidence of 30-40% of patients. This high recurrence rate has been attributed to difficulty in resecting rectal tumors and the rich lymphatic drainage of the rectal region. Radiation has been shown to decrease the rate of local recurrence. Unfortunately, based on randomized trials, there has been no survival benefit with irradiation of rectal tumors.(5,6)

Surgical removal of the tumor is the primary treatment of choice for colon cancer. Even patients with metastatic disease generally undergo resection to prevent morbidity related to obstruction, as long as they are good surgical candidates. Resection of colon adenocarcinoma involves removal of the lesion with a minimum of 5 mm of the margin, removal of the regional draining lymph nodes, and removal of the associated blood vessels. Traditionally, resection has been performed through an open laparotomy. The

abdomen is opened through a midline incision, the tumor and the related tissue is removed with appropriate margins, the entire abdomen is examined, and the bowel run with hands to detect any gross malignant lesions.

A new alternative to laparotomy has been laparoscopic removal of colon tumors. Initially, laparoscopic colon resection (LCR) was used to treat non-oncologic diseases such as diverticulitis or inflammatory bowel disease (7). With advancements in this technology, however, surgeons began applying this technique to treat malignancies (8). Critics of laparoscopic colon resection raise concerns regarding increased manipulation of the tumor which may disperse more tumorigenic cells, a higher likelihood of inadequate lymphadenectomy, and an increased risk of recurrence at port sites (9-11). Benefits cited for laparoscopic colon resections, on the other hand, include shorter hospital stay (12), reduced pain, and quicker recovery (13).

2) Relevance of understanding the effects of physical forces on colon cancer cells

As stressed in the discussion above, complete tumor resection is critical in "curative" treatment of colon adenocarcinoma. Recurrence after "curative" surgery is a poor prognostic sign for the patient. The goal of a surgeon, therefore, is to undertake a procedure that minimizes the risk of recurrence. Although most of the contributing factors to recurrence are poorly understood, certain assumptions can be made from a surgical perspective. First, recurrence implies growth from the same clone of cells and not an emergence of a new malignancy. Second, total resection of the original tumor has to be possible. If gross tumor is left behind due to its unresectability or malignant nature,

it is expected to regrow. Thus, for recurrence to occur, cells from the original clone must have remained in the patient, and these cells must have had the capability to implant.

Several studies point to shedding of tumor cells during open laparotomy and laparoscopic tumor resections. Tumor cells have been recovered from surgical instruments (14-16), in the plume of smoke caused by cautery in laparoscopic procedures (17-20), during desufflation after a laparoscopic procedure (21), and from the peritoneal cavity in general (22-25). The fact that tumor cells can contaminate the abdomen during surgery is also evident from the observed implantation of tumors at open wounds and laparoscopic port sites. Recurrence rates at laparoscopic port sites have been reported between 0.4-21% (26-28). Tumor implantation at incision sites after open surgery are reported between 0.6-1.6% (29,30). If we make a conservative recurrence estimate of 1%, given the colon cancer incidence of 130,000 per year, 1300 people will have recurrence at wounds or laparoscopic ports every year. Yet, another route of tumor recurrence is through hematogenous shedding and traumatization of tumor during manipulation (31,32).

Once tumor cells are shed into the abdomen or the bloodstream, they must implant to establish a new clone. In a simplistic model, these tumor cells first have to adhere to an extracellular matrix, proliferate, interact with growth factors, escape immune surveillance, and stimulate angiogenesis (33,34). Each of these steps is actively under investigation.

The focus of this thesis has been on surgical factors that may affect tumor cell adhesion. In particular, we have investigated the effects of physical forces such as pressure, shear stress, and turbulence on colon cancer cells. This is relevant because

increased pressure is used during laparoscopic removal of tumors. If increased pressure is found to contribute to a detrimental outcome, options such as gasless laparoscopy can be applied. One study in an animal model has shown that gasless laparoscopy can decrease wound metastases from 10 out of 12 to 3 out of 12(35). Surgical manipulation also imposes forces such as shear stress and turbulence on tumor cells. Shear is a force exerted on a surface due to relative movement of fluid over the surface. Violent movement of fluid creates turbulence. Tumors are subjected to these forces during surgery by physical handling of the tumor, fluid jets and suction devices used to control the surgical field, as well as from the flow of gas into and out of the peritoneal cavity during the procedure. These forces may affect tumor recurrence. Previous experiments performed in our laboratory and by other investigators have shown that 15 mmHg pressure induces cell adhesion in colon cancer cell lines, including SW620 cell line. This response to pressure is blocked by the metabolic inhibitor sodium azide and therefore is energy dependent. Increase in cell adhesiveness is independent of the matrix used as demonstrated by plating on Matrigel, laminin, collagen I, or tissue culture plastic (36). If this is true for pressure, it may also be true of shear stress and turbulence. With an understanding of what effect such physical forces have on colon cancer cell adhesion, we may develop chemical inhibitors or modify procedural techniques that may allow better control of tumor implantation during colon cancer surgery.

3) Cellular signals activated by physical forces.

One may theorize that external physical forces such as pressure, shear stress, and turbulence initiate intracellular signals by activating cell surface proteins or by directly influencing the inner cytoskeleton of the cell. The cell surface proteins that anchor the cell to the extracellular matrix, such as integrins, are best suited for this signal transduction. This makes intuitive sense since for the cell to sense a mechanical force it needs a reference point. The extracellular matrix acts as a reference point (37). Integrins on the cell membrane provide an anchor for the cell by interacting with the extracellular matrix proteins such as collagen and fibronectin. The exact nature of the signals elicited by physical forces may vary depending on the nature of the stimulus and the cell type involved. Aside from activating signaling pathways through anchored proteins, physical forces are also known to transduce signals by mechanical linkages. In a concept known as tensegrity, physical forces such as pressure, shear stress, and strain can transduce signals through mechanical linkages between integrins, adhesion complex proteins, cytoskeletal filaments, and nuclear scaffolding(38-42).

Classically, the physical force acting on the cell is transduced through the integrin molecules towards their cytoplasmic domains. Certain integrins have a tyrosine kinase, named focal adhesion kinase (FAK), associated with their cytoplasmic domain. The term kinase refers to the ability of the protein to phosphorylate itself or another substrate. FAK autophosphorylates and interacts with and phosphorylates a series of other proteins such as paxillin, Shc, and Src kinases such as c-Src and Fyn. These events activate downstream pathways including the mitogen activated protein kinase (MAPK) pathway and ultimately influence gene regulation (43,44).

Of particular interest in this classical understanding of intracellular signal transduction is the activity of Src kinases. The Src family is one of eight known families of intracellular tyrosine kinases. Members of the Src family are Src, Fyn, Yes, Fgr, Lyn, Hck, Lck, Blk and Yrk. The determining characteristic of Src family kinases is their structure. From its N to C terminus, a Src family kinase has an anchoring domain (SH4), a poorly conserved but unique region of 40-70 aminoacid residues, a 50 aminoacid SH3 domain capable of binding to proline-rich sequences, a 100 aminoacid SH2 domain that binds to tyrosine phosphorylation sites, a 250 aminoacid catalytic domain, and C terminus domain with a specific conserved tyrosine residue. Src is the most widely studied member of the Src kinase family. The Src gene was the first defined proto-oncogene. The normal product of this gene is referred to as Src or c-Src. A mutated version is referred to as v-Src. Src has not only been shown to play a critical role in oncogenesis but is known to regulate the cellular response to extracellular stimuli (45).

Several extracellular agents and stimuli have been shown to activate Src family kinases. Growth factors, including platelet-derived growth factor (PDGF) activates Src (46). Increased intracellular calcium activates Src (47). Src family kinases are also activated by G-protein coupled receptors (48), cytokine receptors, and B cell antigen receptors (49). Furthermore, stresses such as oxidation and radiation have been shown to activate Src family kinases (50,51). There is no report, however, studying the effect of increased pressure, shear stress, or turbulence on Src activity, especially in colon cancer cells.

One may hypothesize that activation of Src is involved in colon cancer cell adhesion in response to physical forces such as pressure, shear stress, and turbulence. If

this is the case, inhibiting Src activation could decrease colon cancer cell adhesion in response to these forces. A recent discovery of a novel Src family inhibitor, PP1 (4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine), may allow us to test this hypothesis. PP1 inhibits a variety of proteins including JAK2, EGF-R kinase, ZAP-70, and Src family kinases at high concentrations. At low concentrations, it is selective for Src family kinases. The concentration of PP1 required to inhibit 50% activity of the Src family kinase (IC_{50}) Lck, is reported at 0.01 uM(52). Therefore, low concentrations of PP1 can be used to inhibit activity of Src family kinases. If inhibiting Src family kinases indeed affects adhesion, individual members of the family can be identified and studied. Thus by elucidating the role of Src in colon cancer cell adhesion, we can hope to devise strategies to inhibit cell adhesion, a critical step in tumor recurrence.

Hypothesis

Based on the literature available and previous experiments in our laboratory, we hypothesize that pressure, or a combination of shear stress and turbulence, can enhance colon cancer cell adhesion through a mechanism involving Src. An examination of this hypothesis was broken down into following questions:

1) Can pressure or a combination of shear stress and turbulence enhance colon cancer cell adhesion *in-vitro*?
2) Can PP1, an inhibitor of Src family kinases, inhibit cell adhesion?
3) Does pressure or a combination of shear stress and turbulence activate Src?

Methods and Materials

The experimental set-up chosen to study the effects of pressure on SW620 cells is demonstrated in figure 1a and 1b. Experimental set-up to study the effects of shear stress and turbulence is portrayed in figure 1c and 1d. Detailed recipes for media and solutions could be found in the Appendix on page 48.

A) SW620 Cell line

SW620 human colon cancer cell line was originally prepared from a lymph node metastasis from a 51-year-old Caucasian male (53). Since then it has been used as an *in-vitro* model for colon cancer cell biology by numerous laboratories (54-56). The cell line was maintained in 250 mL tissue culture flasks (Falcon 35-3111) in a 37 ^{o}C incubator (Fisher Scientific Model 5, 6%CO2).

B) Pre-coating plates

Adhesion assays were plated on six well plates (Falcon 35-3046) pre-coated with Collagen I (Sigma C-8897). Pre-coating solution was made by adding 500 uL of 1 mg/mL collagen I stock stored at 4 ^{o}C to 40 mL of ELISA buffer (recipe in appendix) also stored at 4 ^{o}C. Aliquots of 2 mL of pre-coating solution were transferred to each well of the 6 well plates. The plates were either stored at room temperature for one hour or wrapped in parafilm and stored at 4 ^{o}C overnight, prior to use in an assay.

C) Preparing SW620 Cells for Adhesion Experiments

The adhesion experiments were conducted using the established human colon cancer cell line SW620 maintained in a 37 ^{o}C incubator using 250 mL tissue culture flasks (Falcon 35-3111). For two experiments, two 75 % confluent flasks of SW620

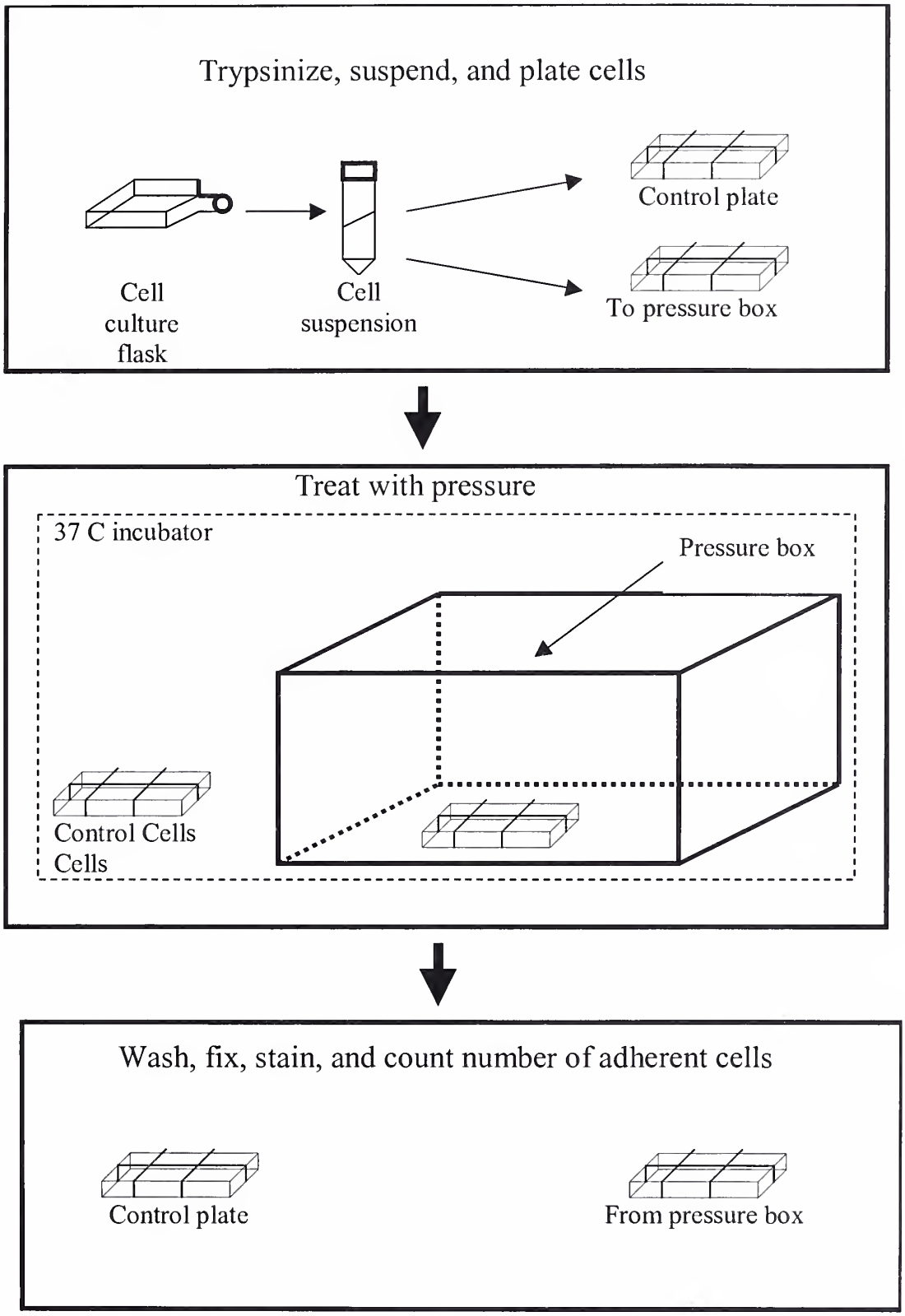

Figure 1a Pressure Experiment

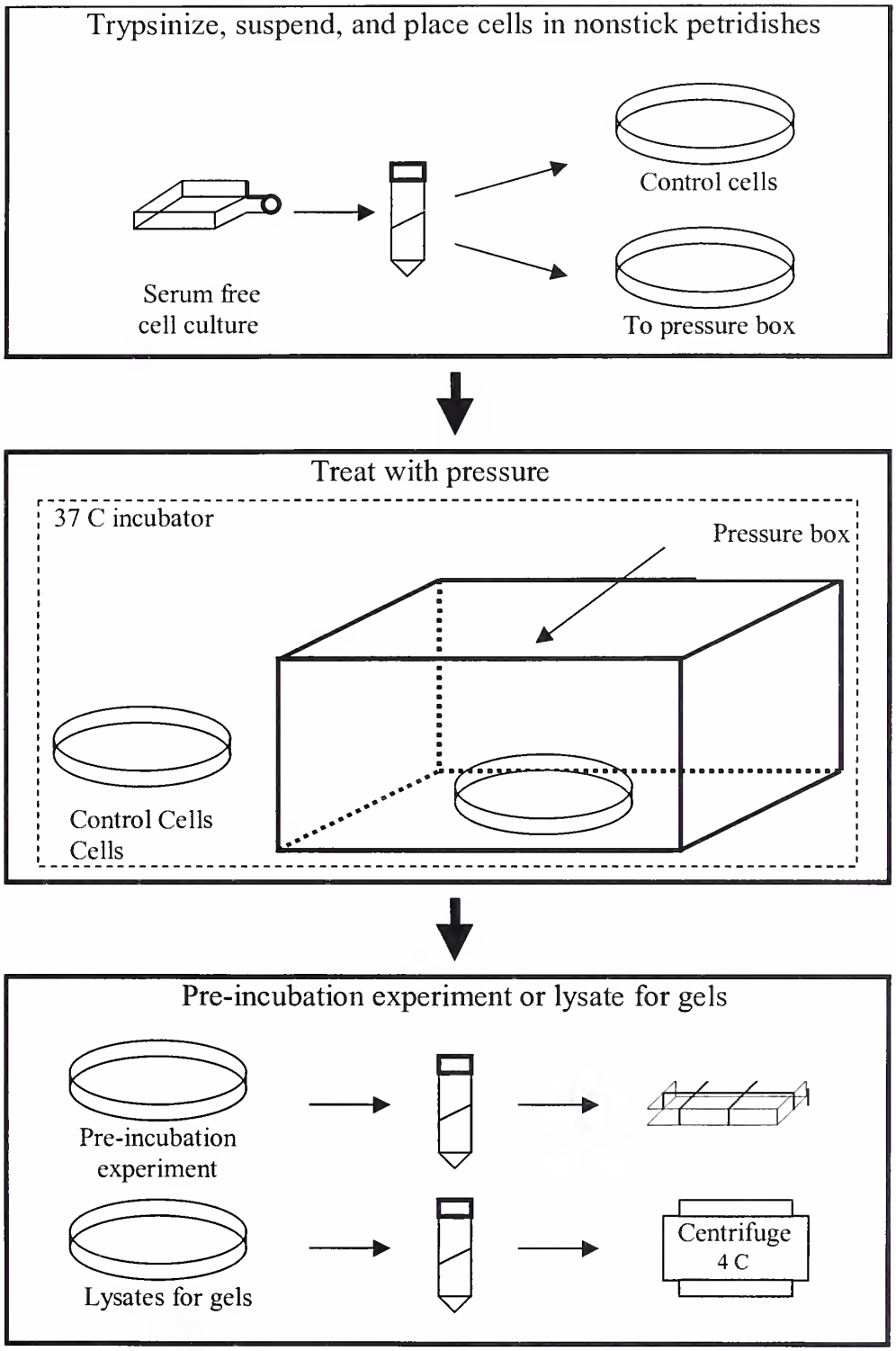

Figure 1b Lysates for Pressure Gels

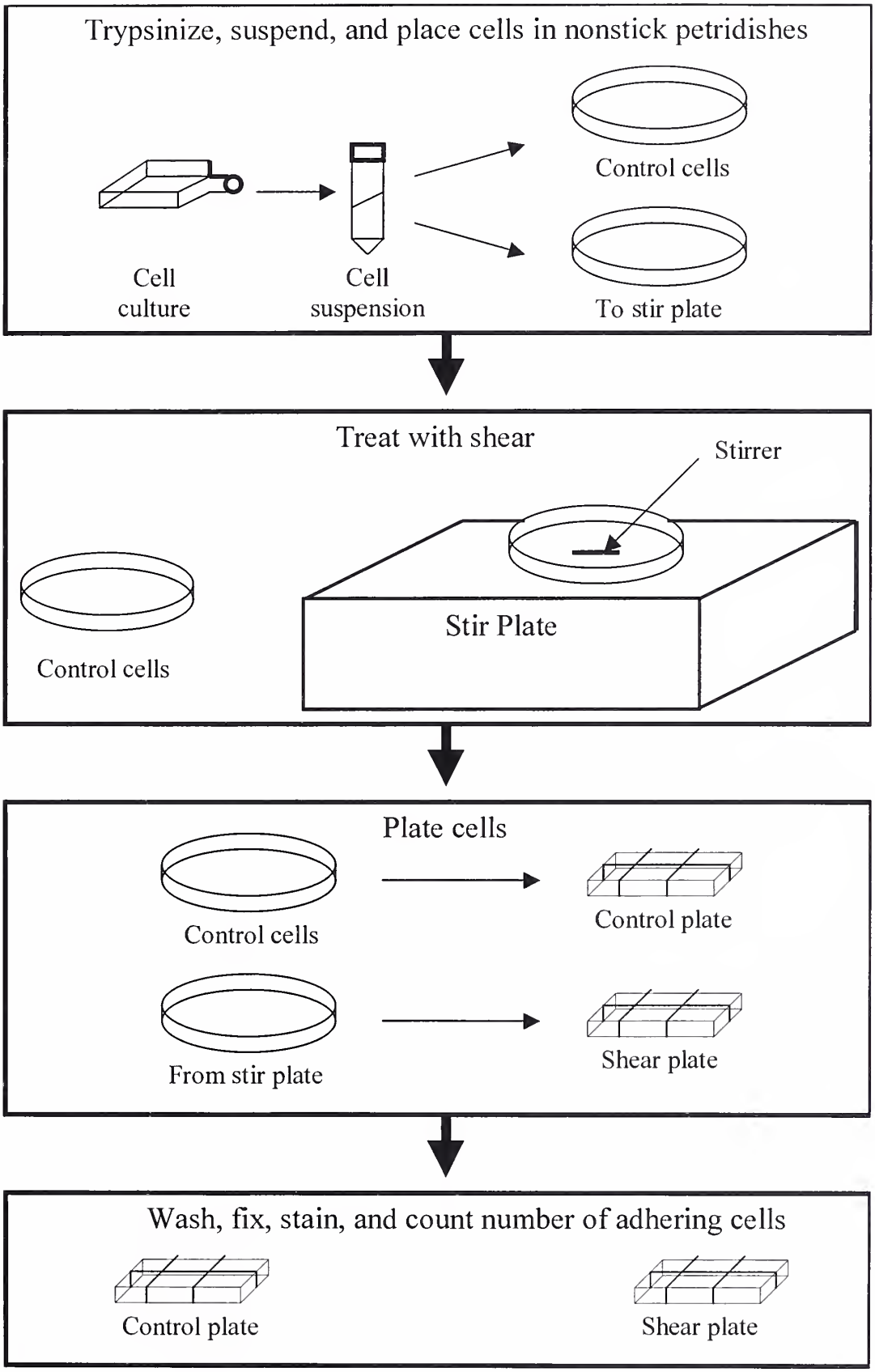

Figure 1c Shear Experiment

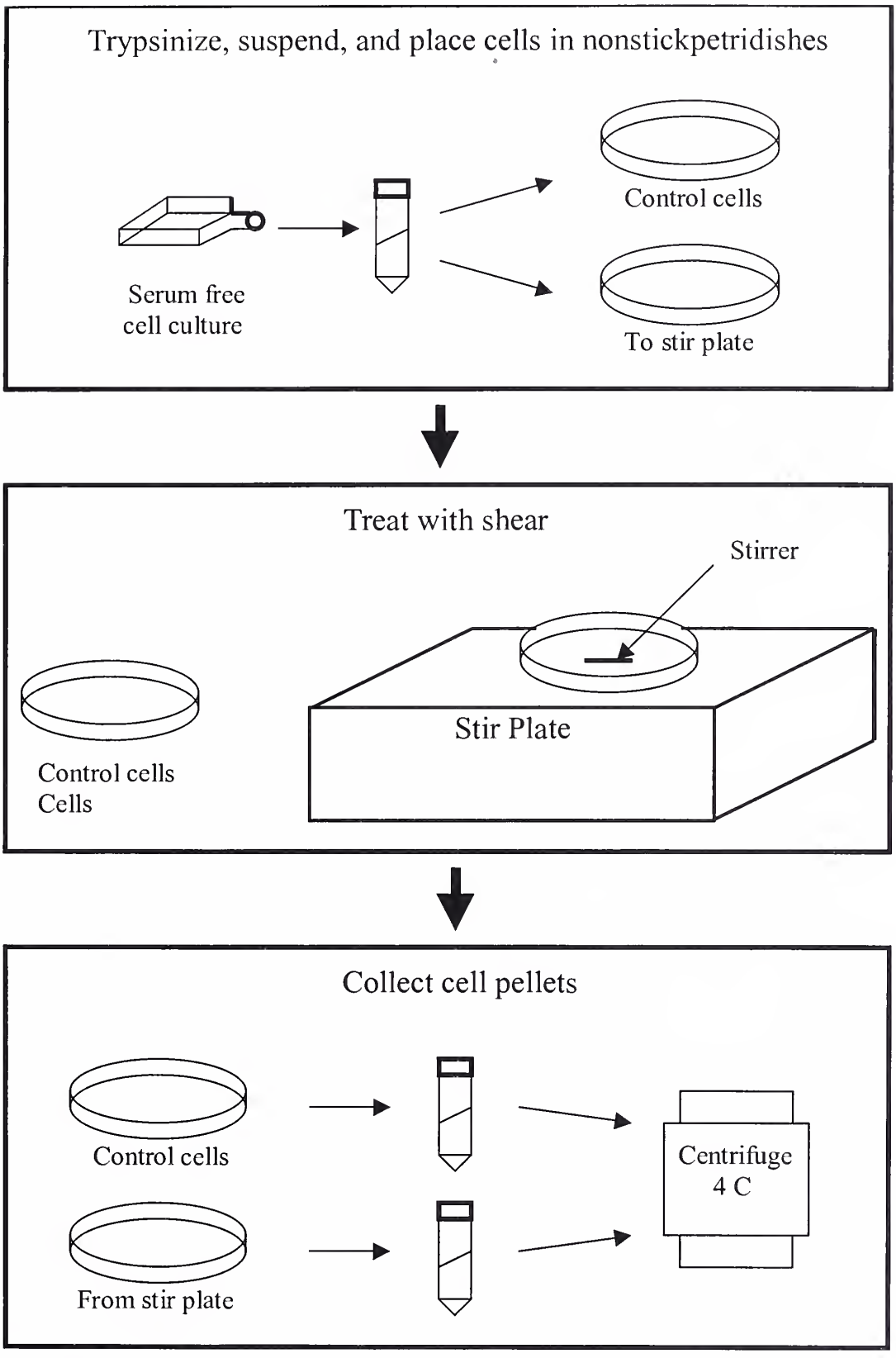

Figure 1d Lysates for Shear Gels

cells were washed gently with 10 mL of PBS each and trypsinized with 5 mL trypsin-EDTA each for 2 minutes at 37 °C (0.02% trypsin, Sigma T-8253, 0.27 mM EDTA, American Bioanalytical AB500). Two 15 mL tubes (Falcon 35-2096) were prepared each with 5 mL SW620 media (recipe in Appendix). At 2 minutes, cells were visually confirmed to be no longer adhering to the flask using an Olympus CK2 microscope at 100X. Cells that continued to adhere to the flask after a 2-minute digestion were suspended by gently tapping the side of the flask. Cells were transferred from each flask to respective 15 mL tubes containing 5 mL of SW620 media. This brought the total volume in each tube to 10 mL. Cells were mixed by inverting the tubes 2-3 times and then centrifuged for 5 minutes (Damon/IEC HNS2) at 1000 rpm. After centrifugation, the supernatant was removed from the cell pellets and 2 ml of fresh SW620 media was added to each of the two tubes. Pellets were resuspended gently using a 2 ml pipette. These stock cells were used to prepare a cell suspension for the experiments. The cell suspension was prepared by adding 100 ul of stock cells to 20 mL SW620 media in 50 mL tubes (Falcon 35-2074). A Coultercounter ZM set at sampling volume of 100 uL was used to determine the concentration of the cells in the suspension. The cell concentration was adjusted to the desired concentration of 160,000 cells/mL for each of the two experiments. If an inhibitor was to be used, it was added in the desired amount to the cell suspension. Appropriate DMSO vehicle control was prepared by adding DMSO (2.8 uL DMSO/10mL cell suspension) to a control cell suspension for experiments where PP1 was tested.

D) Pressure Adhesion Experiment

Two six well plates, one control and one experimental, were prepared for seeding by suctioning off the pre-coating solution and washing gently with 1.5 mL PBS at 37 °C per well. Each of the two adhesion experiments included three wells in the control plate and three wells in the experimental plate. Each well received 1.5 mL of cell suspension. Both control and experimental plates were gently swirled in a figure eight pattern to ensure adequate mixing of seeded wells. The experimental plate was placed in the pressure box pre-warmed to 37 °C in the incubator (Lab-line, 0% CO2). The pressure box was a custom made Lucite box with inlet and outlet valves, thumb screws and an O-ring for achieving an airtight seal. Previous experiments in the Basson lab have confirmed that the pressure box has no significant affect on variables such as internal air currents or the pH of cell suspension on cell adhesion(36). Controls were also performed by other investigators showing no enhancement of adhesion at 5 mmHg in the same pressure box(57). Since the box is pre-warmed to 37 °C in the incubator, there was no difference in temperature outside and inside the box. The control plate was placed outside the pressure box in the incubator. After the desired time, the plates were taken out of the incubator and brought to the hood. Cell suspension in each well was suctioned off, and each well was washed once gently with 1.5 mL PBS. Plates were fixed with 1.5 mL 10% formalin (Sigma HT50-1-128) per well and left overnight in 4 °C refrigerator. The next day the plates were washed once with 1.5 mL PBS per well and stained with 1.5 mL hematoxylin (Fisher Scientific SH30-500D) per well. After minimum of 6 hours, hematoxylin was removed and plates washed by submerging them in bucket of tap water;

plates were air dried. Twenty fields per well were counted randomly at 400X magnification using Olympus CK2 microscope. A consistent circular pattern was followed in choosing the area of the well for random counting to ensure that the entire well is represented in the count.

E) Shear Stress and Turbulence Experiment

Cell suspension for the shear stress and turbulence experiment was prepared exactly as above for the pressure experiment. If an inhibitor was to be used, it was added at the desired concentration to the cell suspension. Each shear and turbulence experiment had its own non-shear control plus a DMSO vehicle control if an inhibitor was used.

To conduct an experiment, a cell suspension was split into control and shear aliquots of 25 mL each into petri dishes (100 mm, Falcon 35-1001). Shear and turbulence were applied by stirring the experimental petri dish with a teflon coated stir bar (3.7 cm long X 1 cm diameter, 7.4g) at 600 rpm for 10 minutes at room temperature. A Sigma H3770 stirrer was used. Control dishes were placed next to the stir plate. After stirring for 10 minutes, cell suspensions from experimental dishes and control dishes were transferred to respective 50 mL Falcon tubes. Each dish was gently rinsed with its own cell suspension to insure that all cells were transferred. The cell suspensions were then plated, 3 wells per condition, into six well plates at 37 ^{o}C and ambient pressure. At 30 minutes, or the desired time, plates were fixed with 10% formalin and stained with hematoxylin following the procedure described in the pressure adhesion study above.

F) Preparing lysates for *In-vitro* Kinase Assay

Cell culture flasks were switched to serum free media twenty four hours prior to the experiment. Cells were trypsinized and resuspended in 2 mL of serum free media following the same procedure as described in the section on pressure adhesion assay. Trypsin inhibitor (0.4 mg/mL Sigma T-9003) was added to serum free media at the end of a 2 minute trypsinization to neutralize the trypsin. All of the 2 mL of stock was added to 20-25 mL SW620 media to prepare a concentrated cell suspension. The cell suspension was split into two 100 mm petri dishes, one acting as control and one as experimental. If the lysates were to be made for a pressure experiment, the control petri dish was placed outside the pressure box and the experimental petri dish was placed in the pressure box at 15 mmHg for the desired amount of time. If the lysates were to be made for shear and turbulence experiment, the control petri dish was placed next to the stir plate at room temperature and the experimental petri dish was stirred at 600 rpm for 10 minutes. At the end of the desired time, contents of petri dishes were transferred to respective 15 mL Falcon tubes chilled on ice. All subsequent work was performed on ice or at 4 ^{o}C. The 15 mL Falcon tubes were centrifuged for 5 min at 4 ^{o}C, media was suctioned off, and 10 mL of ice cold PBS was added. Tubes were centrifuged again for 5 min at 4 ^{o}C and the PBS was suctioned off. 300 uL of lysis buffer (recipe in appendix) was added to each Falcon tube, mixed, and allowed to sit on ice for 20 min. At 20 min, the resulting lysate was transferred to 1.5 mL eppendorf tubes chilled on ice. Lysates were centrifuged at 13,000 rpm for 10 min at 4 ^{o}C using Baxter Biofuge 13 centrifuge. The resulting supernatant was the final product, which was used for BCA colorimetric assay and *in-vitro* kinase assay.

G) *In-vitro* Kinase Assay

All steps unless otherwise specified were performed on ice.

Day 1

Total protein concentration of each sample was determined by the BCA colorimetric assay (Pierce Chemical, Rockford, IL). Albumin standard (bovine, Pierce Chemical, IL, No. 23210) was used to produce a standard curve and each sample was assayed in triplicates. A Whitaker Bioproducts plate reader was used at 562 nm wavelength. Given the total protein concentration of each sample, volume needed for 250 ug of protein was calculated for each sample. If 250 ug was not available, total protein requirement was reduced to as low as 200 ug. Thus all samples had equal amount of total protein. The calculated volume of each sample was transferred to 1.5 mL eppendorf tubes and the samples were brought to an equal with lysis buffer. Immunoprecipitation was carried out by adding 1 ug of anti-Src mouse monoclonal antibody (Anti-Src, clone GD11, Upstate Biotechnology, Lake Placid, NY) per 100 ug protein in each sample, samples were vortexed gently, and placed on a roller apparatus in 4 ^{o}C a room for 2 hours. Protein G sepharose 4 Fast Flow beads (Amersham Pharmacia Biotech AB, 17-0618-01) were prepared by transferring 40 uL/sample of ethanol bead suspension into 1.5 mL eppendorf tubes. Tubes were centrifuged, supernatants were discarded, and beads were washed with 500 uL of deionized water. This washing cycle was repeated one more time. Next, the beads were resuspended in lysis buffer using 10 uL per sample of lysis buffer. Pipette tips were clipped to allow adequate and accurate pipetting of beads. After the two hour incubation with primary antibody, 20 uL of bead

suspension was added to each sample. After brief vortexing, samples were placed back on a roller apparatus in a 4 °C room overnight (or at least 6 hours.)

Day 2

A 10 % polyacrylamide-SDS gel was prepared. Gel mix (4.0 mL H2O, 3.3 mL 30 % acrylamide mix, 2.5 mL 1.5M Tris (pH8.8), 0.1 mL 10% SDS) was degassed for 10 minutes using the house vacuum, then 100 uL 10% Ammonium persulfate and 4 uL Temed was added and the gel was poured into the minigel apparatus (Mighty Small Dual gel caster, Hoefer, SF). Gel was topped off with deionized water to prevent the entrapment of bubbles at top and to prevent the gel from drying out. After the end of one hour, a 5% polyacrylamide stacking gel (3.4 mL H2O, 0.83 mL 30% acrylamide mix, 0.63 mL 1.5M Tris (pH8.8), 0.05 mL 10% SDS) was prepared and degassed for 10 min. 50 uL 10% Ammonium persulfate and 5 uL Temed were added. Deionized water from the stacking gel was poured off. The template (comb) used for lanes was chosen based on the number and volume of samples to be run. Care was taken to ensure that the gel did not dry out.

Once the gel was ready, the samples were removed from the roller apparatus, and centrifuged at 4 °C at 5000 rpm for 5 min (Baxter Biofuge 13). The beads could be visualized at the bottom. Supernatant was discarded and the beads were washed with 200 uL of kinase buffer (25 mM HEPES pH 7.4, 20 mM McGill, 1 mM Sodium Vanadate, 2 mM DTT, 20 mM betaglycerophosphate, 10 ug/mL leupeptin, 100 mM PMSF) by adding the buffer, gently vortexing, centrifuging, and removing the supernatant. This wash cycle was repeated. Care was taken not to remove any beads while removing the supernatant. Radioactive kinase buffer (See Appendix A for recipe) was prepared. Radioactive ^{32}P

was added; each sample received 20 uL of radioactive kinase buffer. The beads were suspended in radioactive kinase buffer by gently tapping each sample at the bottom. The reaction was incubated at 30 ^{o}C in a water bath for 20 minutes.

All subsequent steps were performed at room temperature unless otherwise specified. Low range molecular weight marker was loaded onto the gel (Biorad 86581). After a 20-minute incubation, 6 uL of 5X loading buffer was added to each sample. Samples were denatured for 5 min in a boiling water bath. Samples then were centrifuged briefly to collect the beads. The supernatant was loaded onto the gel. Gel was run at 25 mA constant current using DC voltage. Once the bromophenol blue tracking die reached the bottom of gel (approximately in 2-3 hours), the power supply was shut off and the gel apparatus was disassembled, the stacking gel was removed, and a thin lower edge of the gel, which contained most of the free radioactivity, was removed. The gel was placed in a tray with deionized water and placed on a shaker. Deionized water was changed every 10 minutes (approximately 3-4 times) until most of the radioactivity was washed away. The gel was stored in deionized water on a shaker overnight.

Day 3

The next day, the deionized water was removed, and the gel stained with 50 mL of coomassie (Gelcode) stain for 30-60 minutes. Stain was removed and 100 mL of deionized water, along with a kimwipe was added to the gel tray. The kimwipe helps to remove the remaining traces of stain. After 60 minutes, the deionized water and kimwipe were removed and replaced with 50 mL 35% ethanol 2% glycerol solution. After another 60 minutes, gel was washed with deionized water for 3 minutes. The gel was sandwiched

with thick filter paper on one side and saran wrap on other and dried using a gel dryer for two hours. After two hours of drying, the gel was exposed to an X-ray film for development (Hyperfilm, MP, Amersham, England) at –70 ^{o}C. Exposure times were adjusted as needed to achieve a high quality image.

Data Analysis

An example calculation of results from an experiment is given in Appendix B. Experiment from one flask was plated over six wells—three for control cells and three for experimental cells. Thus, one flask gave n = 3. The number of adherent cells were counted at a 400 X magnification in twenty fields per well. These twenty measurements were added to get total cells counted in a given well. An average of three control wells gave average number of adherent control cells. Each of the three experimental wells was normalized to the average of the three control wells. Average of all normalized experimental wells gave the enhancement. Multiple experiments were grouped and statistical analysis performed with two tailed t-test. Acceptable p value for statistical significance was $p \leq 0.05$.

In-vitro kinase data was analyzed using densitometry. The autoradiogram was scanned using a Microtek Scanmaker IIxe scanner and Adobe Photoshop software at 360 dpi. The image was then transferred into Sigma scan software and the density and area of each band was calculated. Density was corrected by the subtraction of background density. The final result was the product of corrected density reading and area of the band. The final result of each experimental band was normalized to its control. Multiple experiments were grouped and statistical analysis performed with two tailed t-test. Acceptable p value for statistical significance was $p \leq 0.05$.

Results

Detailed numerical data for each figure is given in Appendix C for reference. All data presented was collected by the author.

Pressure

When SW620 cells were plated on a collagen I matrix for variable time points, cells plated at an increased pressure of 15 mmHg showed enhanced adhesion at 20, 30, 40, 60 and 120 minutes compared to their respective controls plated at ambient pressure. There was no enhanced adhesion seen at 10 minutes of plating. Enhancement at 20 minutes was 13 % $\pm$ 4 % ($n = 12$, $p = 0.017$). Adhesion was enhanced at 30 minutes by 30 % $\pm$ 4 % ($n = 15$, $p = 0.0009$). At 40 minutes, enhancement increased to 42 % $\pm$ 8 % ($n = 15$, $p = 0.0001$). Enhancement at 60 minutes was 12 % $\pm$ 4 % ($n = 15$, $p = 0.036$) and at 120 minutes was 9 % $\pm$ 3 % ($n = 15$, $p = 0.027$). Each experiment at each time point was normalized to its respective control. Variation among controls at each time point was 3 % at 10 minutes, 5 % at 20 minutes, 2 % at 30 minutes, 4 % at 40 minutes, 4 % at 60 minutes and 3 % at 120 minutes. The maximum enhancement of adhesion was at 40 minutes. When cell adhesion was compared among differing pressure conditions, and not to respective controls, adhesion at 30 minutes was statistically higher than adhesion at 10 minutes ($p = 0.002$), but not 20 minutes ($p = 0.069$). Adhesion at 40 minutes was statistically higher than adhesion at 10 and 20 minutes, but not at 30 minutes. Thus, although the enhancement was maximal at 40 minutes, basal adhesion rose over time.

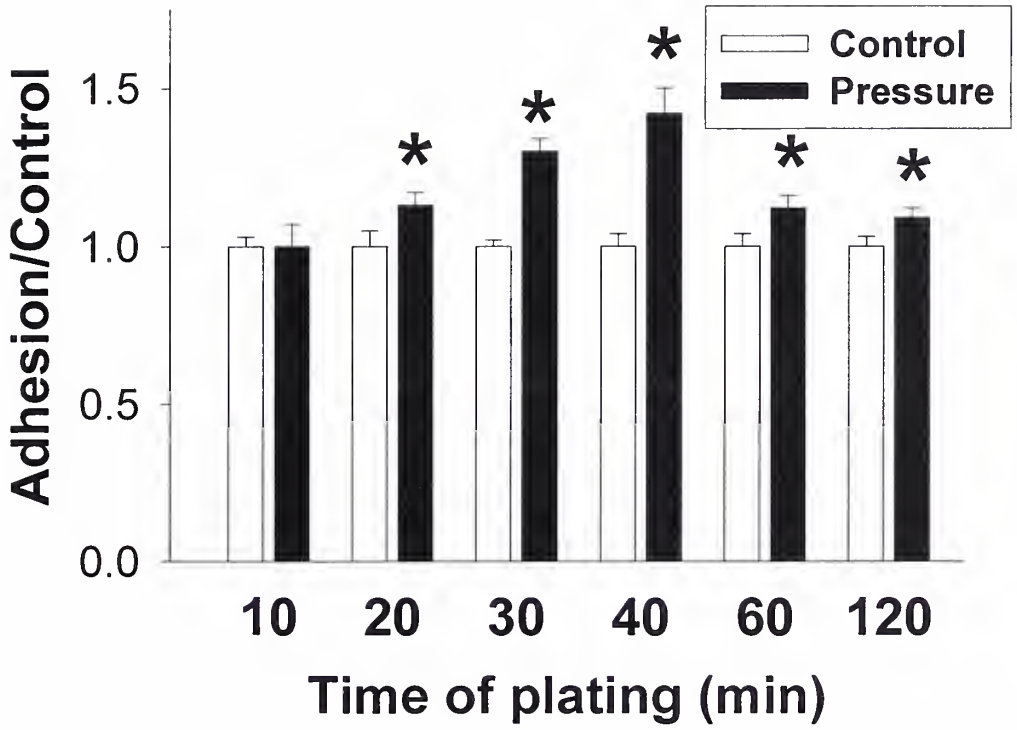

Figure 2 Pressure enhancement of cell adhesion vs. Time

SW620 Cells were plated on collagen I matrix under ambient pressure as a control (open bars) and under 15 mmHg increased pressure (black bars) for variable times. Cells under pressure showed enhanced adhesion compared to cells at ambient conditions at 20, 30, 40, 60 and 120 min ($n \geq 12$ for all points, * indicates a significant difference between control and pressure at that time point, $p \leq 0.04$).

This time-dependent enhanced adhesion is depicted in figure 3 which shows the percentage of cells seeded that adhered to collagen I matrix over time both under 15 mmHg increased pressure and at control ambient pressure conditions.

The effect of pressure on cell adhesion was not only seen when pressure was applied to cells as they adhere, but also when cells were plated after pre-incubation with pressure. Pre-incubation of cells in suspension in 100 mm petri dishes for 30 minutes at 15 mmHg, followed by plating on collagen I matrix for variable time points at ambient pressure showed enhanced adhesion among cells pre-incubated with 15 mmHg pressure compared to control cells pre-incubated at ambient pressure. This enhancement was seen for up to 60 minutes after pre-incubation as demonstrated in figure 4. Adhesion was enhanced 143 % $\pm$ 28 % ($n = 9$, $p = 0.0007$), 114 % $\pm$ 28 % ($n = 9$, $p = 0.003$), 105 % $\pm$ 20 % ($n = 9$, $p = 0.0005$), 110 % $\pm$ 29 % ($n = 9$, $p = 0.006$), and 33 % $\pm$ 6 % ($n = 6$, $p = 0.004$) at 10, 20, 30, 40, and 60 minutes, respectively. Each experiment at each time point was normalized to its respective control. The variation among controls at each time point was 6 % at 10 minutes, 6 % at 20 minutes, 3 % at 30 minutes, 3 % at 40 minutes, and 6 % at 60 minutes. Thus, the enhancement of adhesion among cells pre-incubated with 15 mmHg increased pressure continued over 60 minutes. Figure 5 shows the continued rise in adhesion of percentage of seeded cells.

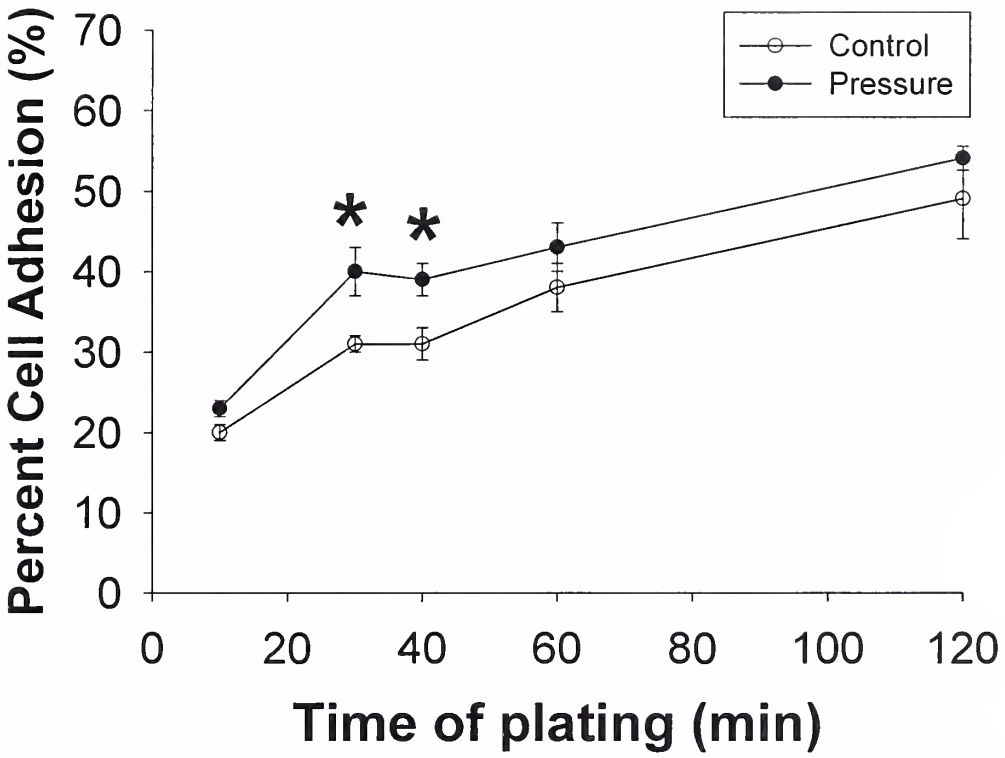

Figure 3 Percent of cells seeded-adhesion vs. Time

Percent of seeded SW620 cells adherent to collagen I matrix under ambient pressure as a control (open circles) and under 15 mmHg increased pressure (closed circles) approached 60% over time. Ratio of adhesion under 15 mmHg increased pressure vs. control is maximum at 30 minutes ($n \geq 12$ for all points, * indicates a significant difference between control and pressure at that time point, $p \leq 0.03$).

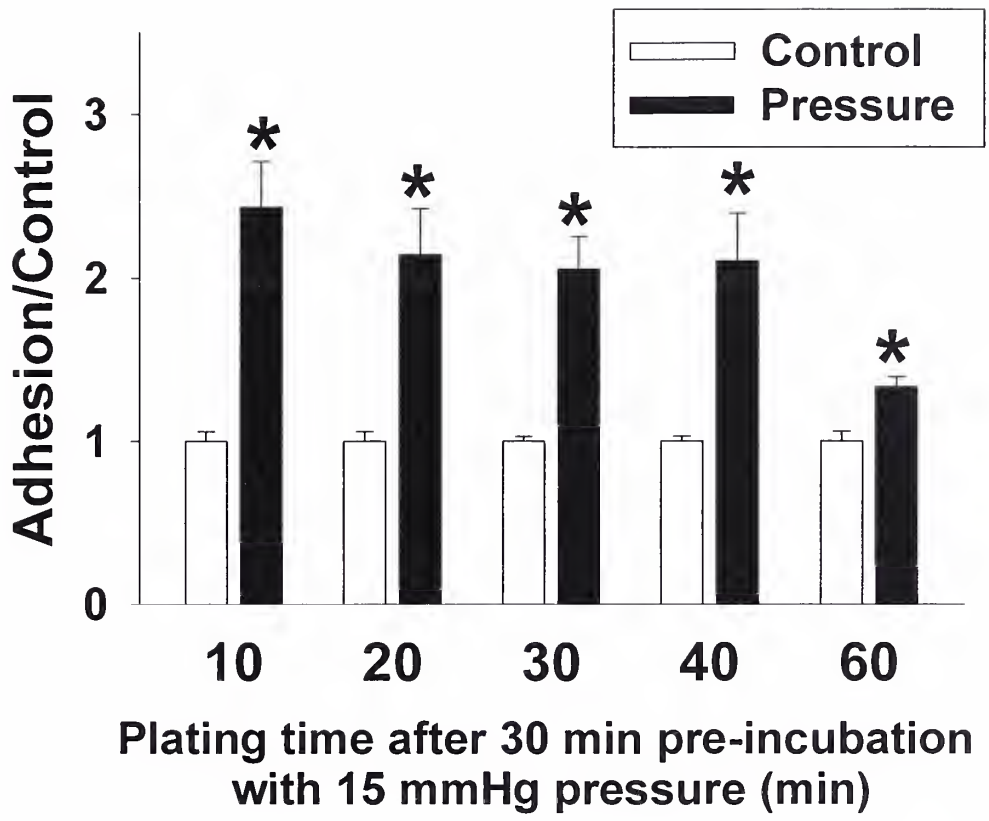

Figure 4 Pressure Pre-incubation, Relative Adhesion vs. Time

SW620 Cells are preincubated at ambient control conditions (open bars) or at 15 mmHg Pressure (black bars) for 30 minutes and subsequently plated at ambient pressure on a collagen I matrix for variable time points. Cells pre-incubated at 15 mmHg pressure showed enhanced adhesion compared to control cells pre-incubated at ambient condition for up to 60 minutes (n = 9 for all points, * indicates a significant difference between control and pressure at that time point, $p \leq 0.003$).

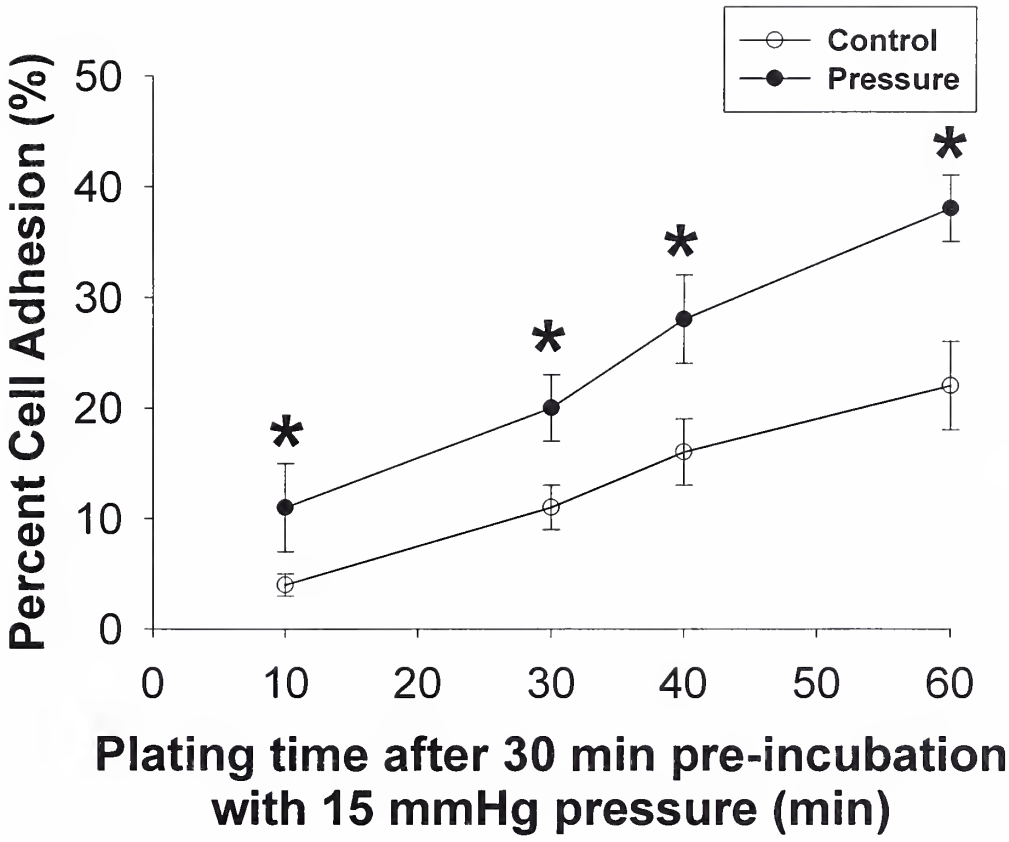

Figure 5 Percent of cell seeded-adhesion vs. Time

SW620 cells were pre-incubated at ambient pressure as a control (open circles) and at 15 mmHg increased pressure (closed circles) for 30 minutes and then plated on collagen I matrix at ambient pressure for variable times. The number of adherent cells continued to rise over time (n = 9 for all points, * indicates a significant difference between control and pressure at that time point, $p \leq 0.05$).

PP1, which is an inhibitor of the Src family of kinases, effectively inhibited pressure induced enhancement of adhesion. See figure 6. At 1 uM, cells with PP1 did not show any increase in adhesion (-4 % $\pm$ 2 %, n = 9, p = 0.3) when plated under 15 mmHg increased pressure compared to cells with PP1 plated at ambient pressure. Experimental variation among cells with PP1 at ambient pressure was 3 %. Cells with DMSO, acting as vehicle control, showed an enhanced adhesion of 30 % $\pm$ 5 % (n = 9, p = 0.0004) under 15 mmHg increased pressure compared to control cells with DMSO at ambient pressure. Experimental variation among cells with DMSO at ambient pressure was 2 %.

A similar effect was seen at PP1 concentration of 0.1 uM. Cells with 0.1 uM PP1 did not show any increase in adhesion (-3 % $\pm$ 4 %, n = 9, p = 0.5) when plated under 15 mmHg increased pressure compared to cells with PP1 plated at ambient pressure. Experimental variation among cells with 0.1 uM PP1 at ambient pressure was 4 %. Cells with DMSO, as vehicle control, showed an enhanced adhesion of 29 % $\pm$ 5 % (n = 9, p = 0.0006) under 15 mmHg increased pressure, compared to control cells with DMSO at ambient pressure. Experimental variation among cells with DMSO at ambient pressure was 3 %.

The inhibitory effect of PP1 was diminished somewhat at 0.01 uM. Cells treated with 0.01 uM PP1 showed increased adhesion of 7 % $\pm$ 2 % (n = 9, p=0.026) at 15 mmHg increased pressure compared to cells with 0.01uM PP1 at ambient pressure. DMSO vehicle control again showed a normal 23 % $\pm$ 3 % (n = 9, p=0.0001) enhancement.

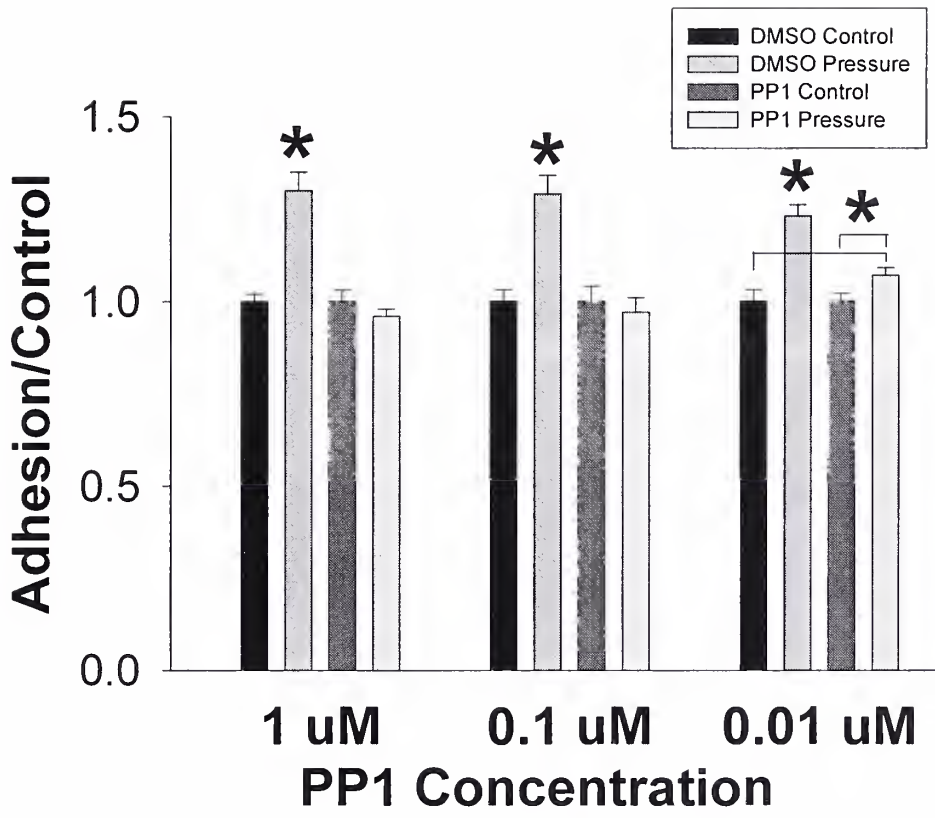

Figure 6 PP1 blocked pressure-stimulated cell adhesion

SW620 cells were treated with PP1dissolved in DMSO or DMSO alone before they are plated. Cells treated with DMSO showed pressure-induced enhancement of cell adhesion but cells treated with PP1 showed no effect of pressure at 1 uM and 0.1 uM PP1. At 0.01 uM, the inhibitory effect of PP1 was reduced (n = 9 for all points, * indicates a significant difference between control and pressure at that time point, $p \leq 0.02$).

When adhesion was compared between cells with PP1 and cells with DMSO vehicle control both at ambient conditions, there was no difference in adhesion. Thus, PP1 did not increase basal level of cell adhesion under ambient control conditions, but inhibited the pressure-induced enhancement of adhesion.

An *in-vitro* kinase assay for Src activity using enolase as a phosphorylating substrate showed increased signaling among cells pre-incubated with 15 mmHg increased pressure for 10 minutes compared to control cells at ambient pressure. A typical gel is shown in Figure 7a and the densitometry data are shown is Figure 7b. The average densitometry reading from three experiments normalized to respective controls was 1.4 ± 0.15 ($n = 8$, $p = 0.04$)

Shear and Turbulence

Shear and turbulence were applied to cells in suspension by stirring at 600 rpm in 100 mm petri dishes. The information from the manufacturer of the stir plate (Sigma, St. Louis, MO) showed a linear rise in revolutions per minute (rpm) in response to dial setting on the stirrer. Trypan blue studies showed cell viability after stirring for 10 minutes at 375 rpm to be 97 % and after 10 minutes at 750 rpm to be 94 % (see figure 8). Settings above 750 rpm led to excessive frothing and thus were avoided. A setting of 600 rpm was chosen as an appropriate setting for these experiments.

When cells were subjected to shear stress and turbulence for ten minutes and then plated on collagen I matrix for variable time points at ambient pressure, cells pre-treated with shear and turbulence showed an increased adhesion compared to untreated control cells. This enhancement was seen for up to 60 minutes after pre-treatment. This phenomenon is depicted in figure 9. Adhesion was enhanced after a 10 minute pre-

Figure 7a Pressure activated Src

In-vitro **kinase assay for Src showed increased Src activity in cells exposed to 15 mmHg increased pressure (P) compared to control cells (C) at ambient pressure both for 10 minutes (one of eight typical experiments).**

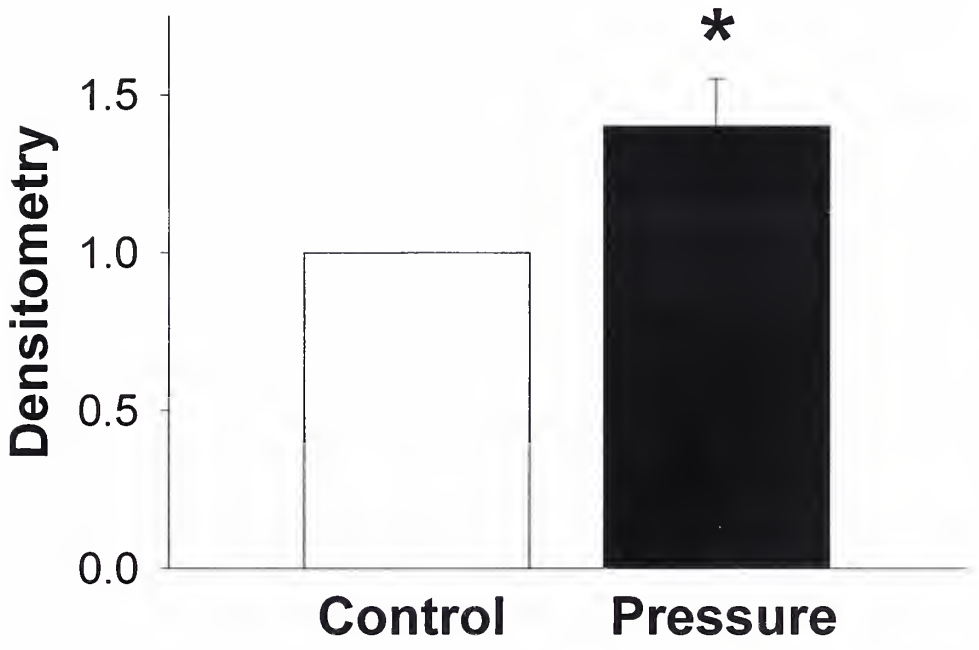

Figure 7b Control vs. Pressure; *in-vitro* Src kinase activity

Densitometric analysis of bands from the *in-vitro* kinase assay showed increased Src activity in cells exposed to 15 mmHg increased pressure compared to control cells at ambient pressure both for 10 minutes (n = 8, p = 0.04).

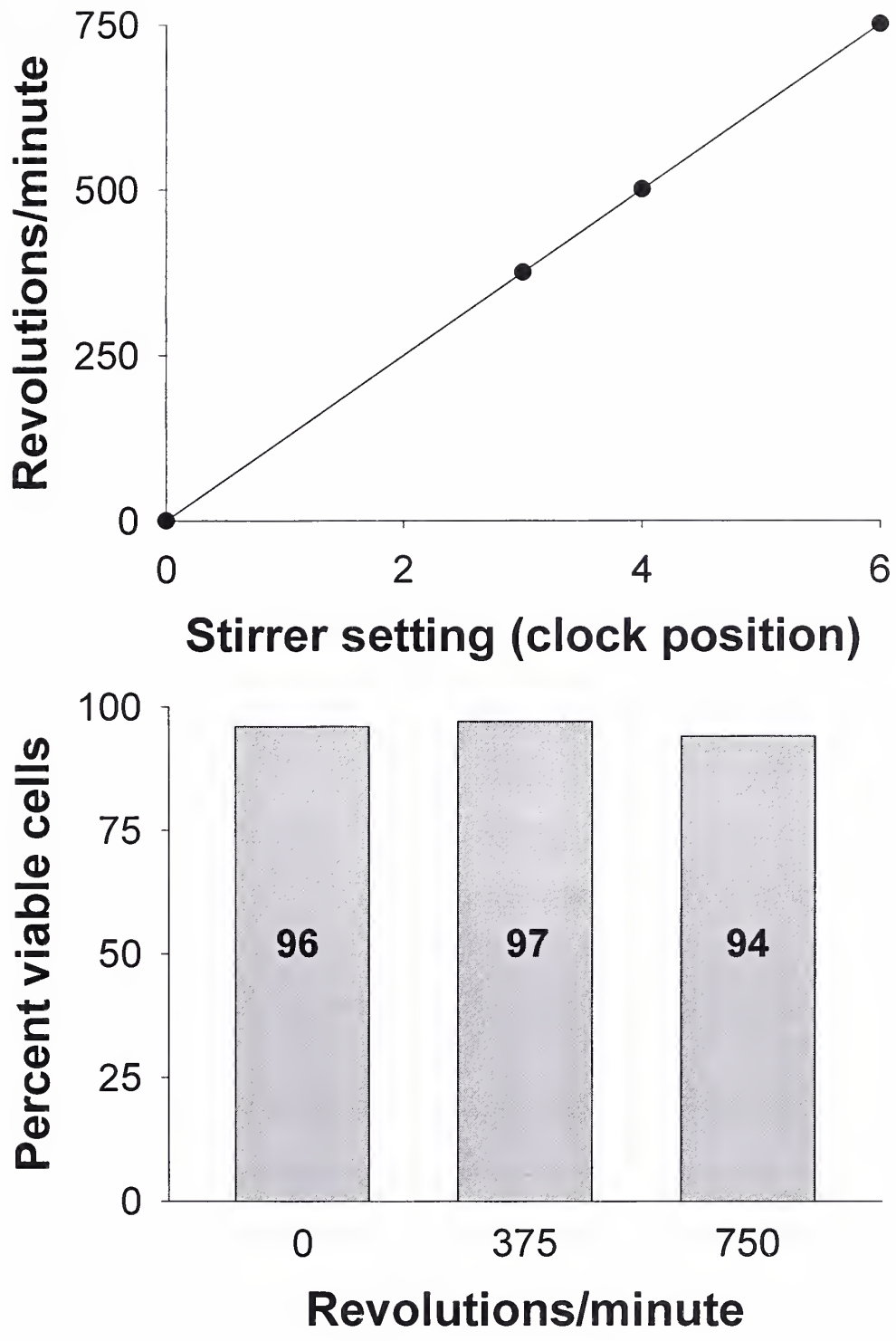

Figure 8 Cell viability with shear and turbulence

The figure upper shows the linear calibration of the Sigma stir plate H3770 dial setting and stir bar (3.7 cm x 1 cm dia, 7.4g) speed (rpm). The lower figure shows good cell viability when cells in suspension were stirred up to 750 rpm.

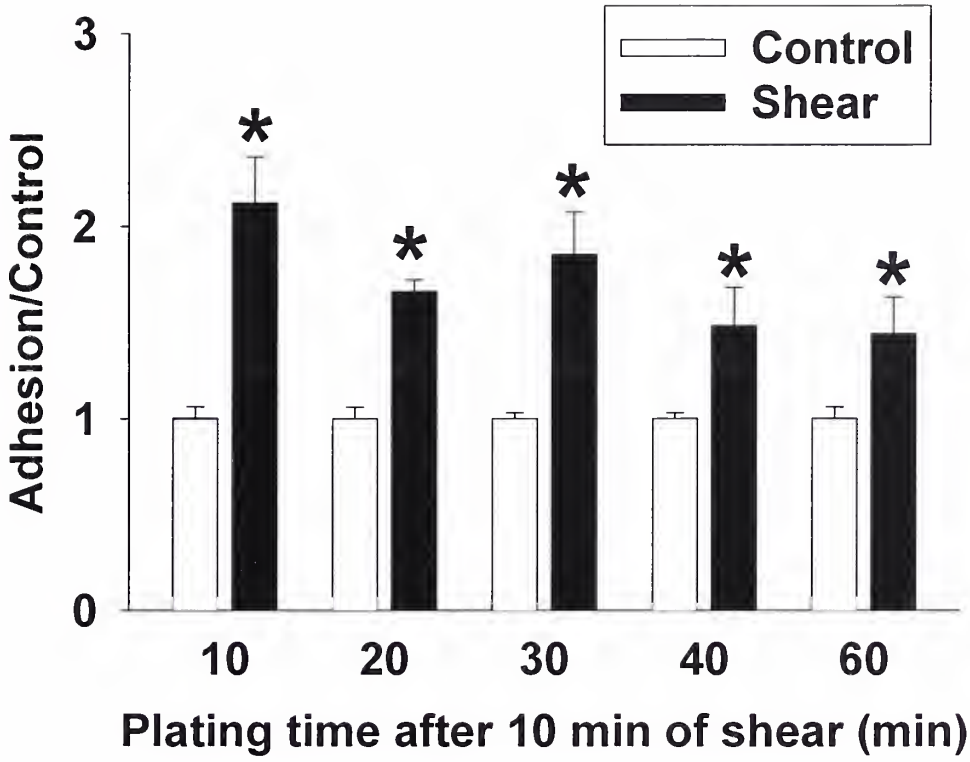

Figure 9 Shear and turbulence stimulated cell adhesion vs. Time

SW620 Cells were incubated at ambient control conditions (open bars) or pretreated with shear and turbulence (black bars) for 10 minutes and subsequently plated at ambient pressure on a collagen I matrix for variable time points. Cells pretreated with shear and turbulence showed enhanced adhesion compared to control cells incubated at ambient condition for up to 60 minutes (n = 9 for all points, * indicates a significant difference between control and pressure at that time point, $p \leq 0.05$).

treatment with shear and turbulence (112 % ± 24 %, n = 9, p = 0.0015). After a 20 minute pre-treatment adhesion was enhanced by 66 % ± 6 % (n = 9, p = 9.7E-7), while after 30 minutes of stirring at 600 rpm, the enhancement was 85 % ± 22 % (n = 9, p = 0.0047). Adhesion was enhancement after 40 and 60 minutes of pre-treatment 48 % ± 20 % (n = 9, p = 0.04) and 44 % ± 19 % (n = 6, p = 0.009) respectively. Each experiment at each time point was normalized to its respective control. Variations among controls were 6 % at 10 minutes, 6 % at 20 minutes, 3 % at 30 minutes, 3 % at 40 minutes, and 6 % at 60 minutes. Thus, the enhancement of adhesion among cells pre-treated with shear and turbulence continued for 60 minutes. The percentages of seeded cells that adhered after pre-treatment with shear and turbulence or control are shown in figure 10 as a function of time.

The effect of PP1 on shear and turbulence stimulated adhesion is shown in figure 11. SW620 cells pretreated with 0.1 uM PP1, stirred at 600 rpm for 10 minutes, then plated on collagen I matrix for 30 minutes, did not show an increase in adhesion (-1 % ± 3 %, n = 9, p = 0.65) compared to cells pretreated with 0.1 uM PP1 and left undisturbed as control. DMSO vehicle control showed a 34 % ± 4% (n = 9, p=0.0001) increase in adhesion in cells exposed to shear and turbulence compared to control cells. Experimental variation among cells with 0.1 uM PP1 in ambient controls was 4 % and among DMSO controls was 4 %. Under ambient control conditions, there was no difference in adhesion among cells with 0.1 uM PP1 or DMSO.

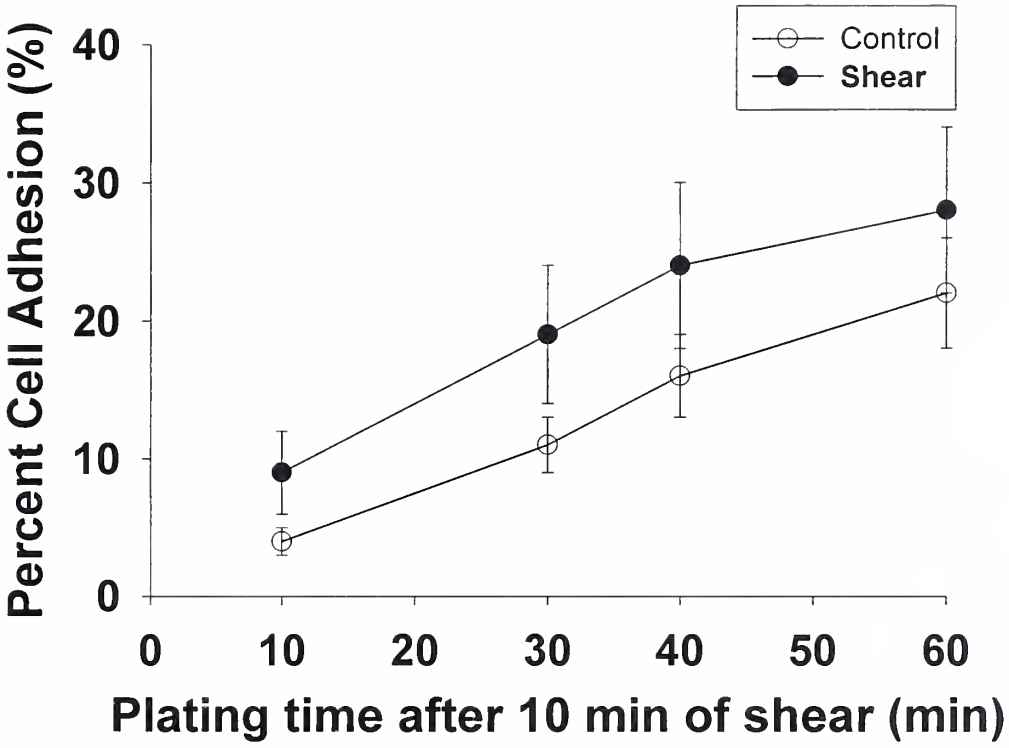

Figure 10 Percent of seeded cells that adhere vs. Time

SW620 cells were pre-treated with shear and turbulence for 10 minutes (closed circles) or left in cell suspension undisturbed as control for 10 minutes (open circles) and then plated on a collagen I matrix for variable times. The number of adherent cells continues to rise over time ($n = 9$, $p > 0.05$ for all points).

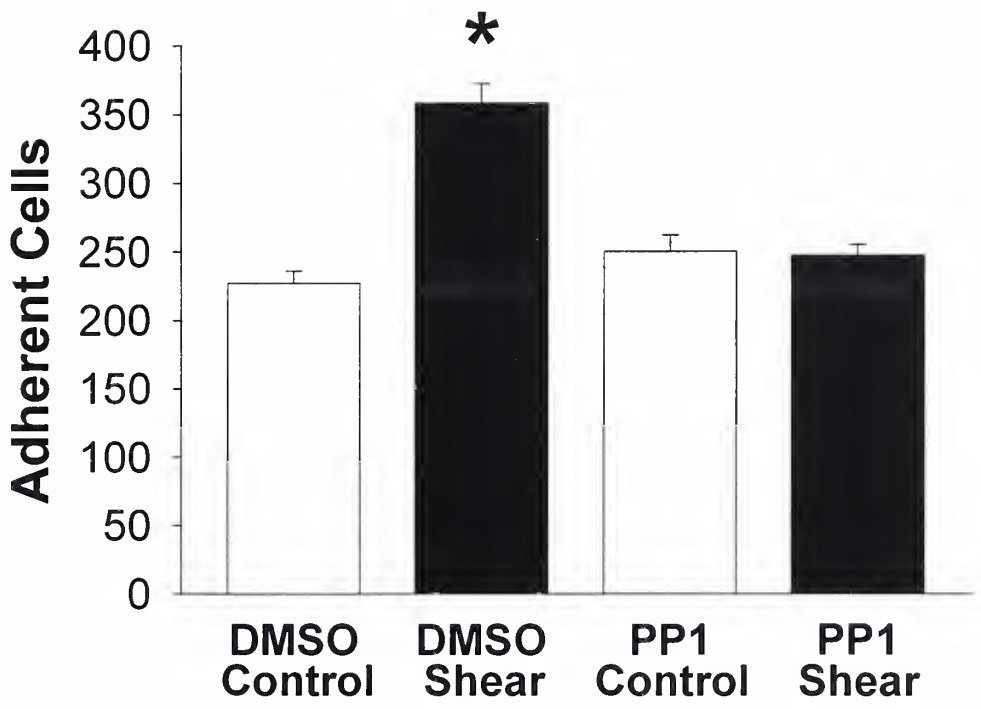

Figure 11 PP1 blocked shear and turbulence-stimulated cell adhesion

SW620 cells were treated with 0.1 μM PP1dissolved in DMSO or 0.03% DMSO alone before they were plated. Cells treated with DMSO showed shear and turbulence-induced enhancement of cell adhesion but cells with PP1 showed no effect of shear and turbulence (n = 9 for all points, * indicates a significant difference between control and pressure at that time point, $p \leq 0.003$).

The *in-vitro* kinase assay for Src activity that used enolase as a phosphorylation substrate showed increased Src signaling among cells pre-treated with shear and turbulence, compared to control cells. A typical gel is shown in Figure 12a and the densitometry data are shown is Figure 12b. The average densitometric reading of seven experiments normalized to respective controls was 1.5 ± 0.13 ($n = 7$, $p = 0.01$).

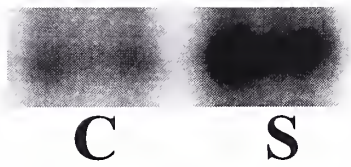

Figure 12a Shear and Turbulence activated Src

In vitro **kinase assay for Src showed increased Src activity in cells exposed to shear and turbulence (S) compared to control cells (C) (one of seven similar experiments).**

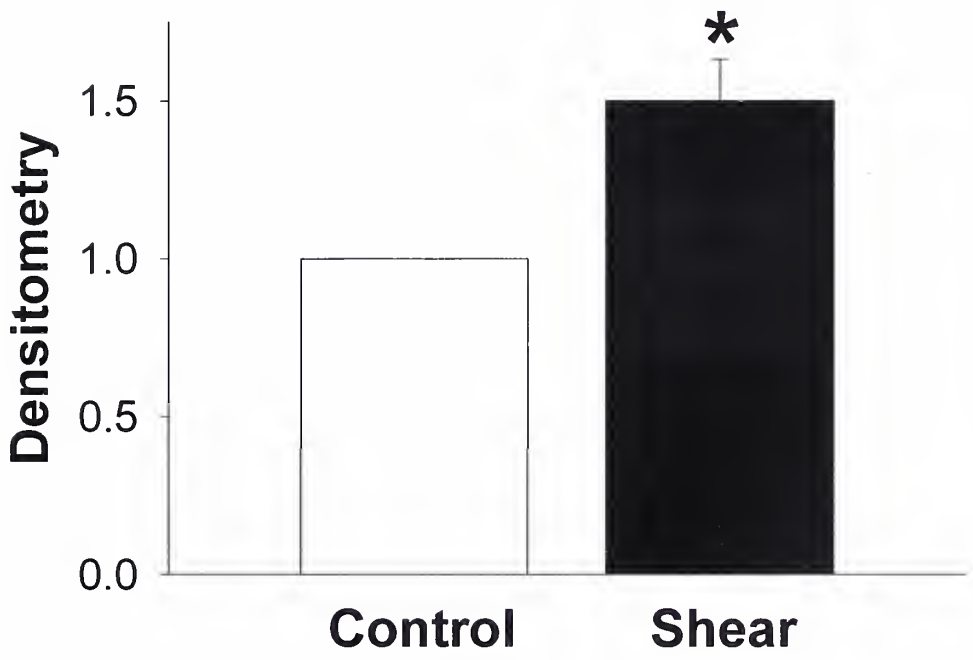

Figure 12b Control vs. Shear Src *in-vitro* kinase activity

Densitometric analysis of bands on *in-vitro* kinase assay showed increased Src activity in cells exposed to shear and turbulence for 10 minutes compared to control cells (n = 7, p = 0.01).

Discussion

There are three parts to the hypothesis proposed in this project as described in the hypothesis section. The results of the experiments can be discussed in the context of these three specific parts. First, we hypothesized that pressure, and a combination of shear stress and turbulence enhance colon cancer cell adhesion *in-vitro*. Of these three forces, pressure is the most defined and controlled paradigm in our experiments. We can not only control pressure as a variable in the Lucite box, but we can accurately measure it using a manometer. Shear and turbulence, on the other hand, are hypothesized as the two most crucial forces in the experimental model we used. Stirring cells in suspension may impose other forces on cells as well. It is evident that colon cancer cells are subjected to a variety of physical forces, including pressure, shear, and turbulence during open and laparoscopic resections. Together, the two paradigms of pressure and shear and turbulence combined suggest that colon cancer cell adhesion is enhanced in response to physical forces.

Our results suggest that both pressure, and shear and turbulence combined increase SW620 cell adhesion to collagen matrix. The results of the pressure study are consistent with previous observations by Basson et. al.(36) who demonstrated increased cell adhesion of colon cancer cell lines Caco-2, SW1116, HT-29 and SW620 in response to pressure in the range of 15 mmHg. Furthermore, pressure was found to stimulate adhesion in a matrix independent fashion, consistent with our observation that 15 mmHg pressure enhanced colon cancer cell adhesion on a collagen I matrix.

The first set of experiments, presented in figure 2, showed increased cell adhesion in response to 15 mmHg increased pressure over time. This result demonstrated that the

adhesion assay was working in the hands of the investigator. The enhancement of 30 % seen at 30 minutes was consistent with results published by Basson et. al(36). The time course demonstrated that the effect of pressure over control was maximized in the range of 30-40 minutes. Practically however, this effect may be in the range of 25-45 minutes. These data should be interpreted as showing a maximum difference in pressured vs. control adhesion in the 25-45 minute range, under the conditions used in this experiment. An alternative look at these data are shown in figure 3, where the percentage of seeded cells that adhere to the collagen I matrix showed that the number of adherent cells continued to rise beyond 40 minutes. As the number of adherent cells rose in the control condition, the adherent cells under pressure also increased, but not sufficiently to affect the ratio. Thus we may conclude from the time course that 30-40 minute exposure was optimal and should be used for future studies elucidating effects of pressure on cell adhesion. We chose a 30-minute adhesion as an ideal time.

One may argue that pressure induced adhesion is a physical phenomenon and may not involve a cellular response at a molecular level. Exposing cells to pressure while they are adhering to a matrix might result in a force vector acting on them pushing towards the matrix. However, pressure applied to the non-compressible fluid around the cells should also provide upward force from under the cells as well. This point is clarified by our next set of data (figure 4) which shows that simply pre-incubating cells with elevated pressure, while cells are in suspension, was sufficient to enhance adherence. This demonstration of increased adhesion of cells after removal of the pressure stimulus indicates that an exposure of cells in suspension to increased pressure effectively altered cellular physiology compared to cells in suspension at ambient conditions. Molecular signals

must be activated in cells exposed to pressure. An alternate way to perform future experiments may be to expose cells to pressure, allow them to remain at ambient conditions in suspension for a variable amount of time, and then plate them for 30 minutes. Although this was not done, the fact that the difference in adhesion among pressure and control was reduced at 60 minutes post pre-incubation compared to 10 minutes post pre-incubation (figure 4), suggests that the effect of pressure was transient.

The question is whether the effect is truly from pressure and not an artifact? Extensive work done by other members of our laboratory prior to this project suggests that the effect is indeed from 15 mmHg pressure(57). The numbers of cells seeded were counted to ensure equal plating. Trypan blue tests were used to confirm equal viability. Finally, the Lucite box used to apply pressure was calibrated to have no effect on adhesion secondary to pH changes, air currents, or temperature gradients. The most powerful control was a demonstration by other members of our laboratory, using the same Lucite box, that 5 mmHg increased pressure did not stimulate adhesion. Thus, if an effect was detected at 15 mmHg and not at 5 mmHg, it likely was a consequence of a change in pressure.

Observations in the shear and turbulence model paralleled observations in the pressure model. Shear and turbulence are among a variety of forces that act on colon cancer cells intraoperatively. We did not find literature that studied effects of shear and turbulence on colon cancer cells. However, others have investigated the effect of shear on endothelial cells. Ishida et. al.(37) propose that shear activates G-proteins which in turn activate downstream kinases. Src kinases play a role in long term changes in endothelial cell morphology and structure in response to shear. These changes are

responsible for controlling adhesion of monocytes and platelets, both of which are presumed to be critical players in the pathogenesis of atherosclerosis. Okuda, et. al, have shown activation of c-Src in response to shear (12 dynes/cm2)(58). This effect was inhibited by PP1 and obliterated by transfection of endothelial cells with kinase-inactive Src. In both of these studies, this model controlled the application of shear finitely, such as 12 dynes/cm2 by Okuda, et. al. Although, turbulence is a much more difficult phenomenon to model, it too can be standardized using tubular applications and calculating the Reynolds number. Reynolds number is a dimensionless quantity proportional to diameter of the vessel, velocity of fluid, and density of fluid. Reynolds number is inversely proportional to viscosity. If a system has as Reynolds number above 2500, the flow is likely to be turbulent(59). Studying turbulence using defined Reynolds number, however, requires tubular flow. Unfortunately, no model that concurrently studies shear and turbulence was suitable for our experimental needs. We needed a model to mimic the forces on colon cancer cells as realistically as possible. Thus we decided to stir the cells. The flow of solution due to stirring contributes to shear, while the fast stirring creates turbulence. Although this model does not allow quantification of each force, it was reproducible for both forces. Therefore, this model was chosen for the preliminary studies presented here. Furthermore, trypan blue studies (figure 8) confirmed the viability of cells subjected to shear and turbulence in our model.

Given our previous data that pre-incubation with pressure makes cells more adherent, these studies were extended to examine the effect of pre-treatment with shear and turbulence. These new data (figure 9) suggested that cells in suspension pretreated with shear and turbulence makes them more adherent, an effect sustained for at least 60

minutes. Unlike the pressure study, however, this effect apparently was more stable. Figure 10 shows that the percentage of adherent cells continues to rise at 60 minutes. The increased cell adhesion in response to shear and turbulence compared to control apparently results from a more prolonged effect secondary to shear than resulting from elevated pressure. To prove this, the time course needs to be extended beyond 60 minutes to determine the time required to achieve adherence comparable to control cultures. A reverse time course where cells are allowed to recover from shear and turbulence for varying time points before being plated for the adhesion assay may provide additional insight.

The second part of the hypothesis proposed that colon cancer cell adhesion in response to pressure, shear and turbulence was mediated at least in part by the Src family kinases. Our results clearly showed that PP1, a specific inhibitor of Src kinases, blocked the enhancement of adhesion both in response to pressure and shear and turbulence combined. PP1 blocks pressure induced cell adhesion at 1 uM and 0.1 uM as shown in figure 6. This inhibitory effect was partially lost at 0.01 uM, indicating a concentration dependent effect. This result is consistent with PP1 data presented by Hanke et. al.(52) who propose IC_{50} of 0.01 uM for PP1 action on Lck, a Src family kinase. The actions of C-Src and Lck likely are similar due to shared structural motifs that define Src family members. Thus at 0.01 uM, PP1 was only 50 % effective at inhibiting the effect of Src. At 0.1 and 1 uM, however, it effectively blocked Src as assessed by the *in-vitro* kinase assay. This may explain the inhibition of adhesion at 1 uM and 0.1 uM PP1 and points to role of Src in pressure induced colon cancer cell adhesion. Finally, it is important to note that PP1 does not increase cell adhesion at ambient conditions compared to the DMSO

vehicle control. Thus the inhibitory activity was directed against pressure induced stimulation.

Similar results were seen in the shear and turbulence model. Given the results in the pressure model, only 0.1 uM PP1 was investigated. Again, PP1 blocked shear and turbulence stimulated adhesion as demonstrated in figure 11. The correlation between pressure and shear results suggests a shared mechanism by which these stimuli affect cellular adhesion. Inhibition by PP1 points to the involvement of one or more members of the Src family kinases in this phenomenon. This is not surprising since Src family kinases are activated in response to a variety of stresses as discussed in the introduction

The third part of the hypothesis proposed that Src activity was activated by pressure, and shear stress and turbulence combined. Increased adhesion in response to pressure, and shear and turbulence combined was used to establish the phenomenology behind the effect of these physical forces. Inhibition of enhanced adhesion by PP1 provided a clue to the role of a Src family kinase in this effect. The *in-vitro* kinase assay provided a means to test for Src activity.

The *in-vitro* kinase assay was adapted from Hanke, et. al.(52) and Frangos, et. al(60). Hanke et. al., detected the Lck member of the Src family using enolase as substrate for phosphorylation. Frangos, et. al. applied this assay to Src using a anti-Src monoclonal antibody to concentrate the kinase prior to the assay. Our results are consistent with gel images from these two groups. The enolase band was clearly visible at 44 kDa. Gelcode stain was used to confirm protein loading. Cells exposed to 15 mmHg pressure for 10 minutes show 40 % + 15 % ($n = 8$, $p = 0.04$) increase in Src activity compared to control. Cells exposed to shear and turbulence for 10 min show 50

% + 13 % (n = 7, p = 0.01) increase in Src activity. This clearly indicated that Src was activated both by 15 mmHg pressure and shear and turbulence combined. The activation of Src was clearly visible on the autoradiograms. Since an equal amount of protein was loaded in each lane these data reliably assess Src kinase activity. The densitometric analysis and statistics are a semi-quantitative means to confirm these results. The absolute densitometric value may vary depending on the exposure time for the film. However, the relative effect on Src kinase activity for control and treated cultures was significant, with a p = 0.04 for pressure and p = 0.01 for shear and turbulence. The similarity between results using pressure and shear and turbulence combined is not surprising given the fact that both these paradigms responded similarly to PP1.

Finally, the next set of experiments should be an *in-vitro* kinase assay to directly demonstrate the effect inhibitory of PP1. This information will help to elucidate the mechanism of action of PP1 on adhesion. Finally, the question arises; how do the results from these *in-vitro* studies apply to effects *in-vivo*? This question cannot be answered accurately with the data in this thesis. The next step is to study the effect of these forces and inhibitors on tumors derived from surgical specimens. Future experiments may be needed in animal models to continue this work.

Conclusion

In conclusion we can state that pressure, and shear stress and turbulence enhance colon cancer cell adhesion *in-vitro*. This enhancement likely involves Src kinase activity since it can be blocked by the Src family kinase inhibitor PP1.

Appendix

A) Recipes for media and solutions

1) SW620 Media

50 mL Fetal Bovine Serum, (GibcoBRL Life Technologies, Grand Island, NY)
10 mL Penicillin-Streptomycin (GibcoBRL Life Technologies 15140-122)
10 mL Sodium pyruvate 100mM (GibcoBRL Life Technologies 11360-070)
10 mL Glutamine (Sigma, G9003, St Louis, MO)
10 mL HEPES buffer solution 1M (GibcoBRL Life Technologies 15630-080)
17.5 uL Transferrin from human serum (Boehringer Mannheim 652 202, Indianapolis, IN)
455 mL DMEM (GibcoBRL Life Technologies, Grand Island, NY)
455 mL RPMI (GibcoBRL Life Technologies, Grand Island, NY)
0.22 uM sterile filter (Millipore, Bedford, MA) and store at 4 C

2) Elisa Buffer

1L Deionized water
1.59g Sodium Carbonate, Anhydrous, Granular, Na2CO3 (Baker 3604-01)
2.93 g Sodium Bicarbonate, powder, NaHCO3 (J.T. Baker 3506-01)
Adjust pH to 9.4 using NaOH or HCL as needed
Sterile filter using 0.2 um millipore filter

3) PBS 1X (Can be made as 10X stock and then diluted to 1X when needed)

1 L Deionized water
8.76 g NaCl (American Bioanalytical, Natick, MA)
0.39 g Sodium phosphate, Monobasic, monohydrate, NaH2PO4 (Sigma S-9638)
1.0 g Sodium phosphate, Dibasic, anhydrous, Na2HPO4 (Sigma S-0876)
Sterile filter using 0.2 um millipore filter

4) Trypsin-Edta solution

800 mL PBS
200 mg Trypsin powder (Sigma T-8253)
100 mg EDTA powder (American Bioanalytical, AB500)
Bring volume up to 1 L using PBS
Sterile filter using 0.2 um millipore filter
Aliquot into 10 mL samples and freeze –20 C

5) Lysis Buffer for *In-vitro* kinase

10 mL buffer containing following
50 mM Tris
150 mM NaCl
10% Triton X 100 (Sigma, St Louis, MO)
0.25% Deoxycholic acid, sodium salt, C24H39O4Na (Sigma D-6750)
1 mM EDTA (American Bioanalytical, AB500)

plus add following prior to use
0.075 g Deoxycholic acid, sodium salt, C24H39O4Na (Sigma D-6750)
0.021 g Sodium Fluoride, NaF (Sigma S 6521)
10 ug Aprotinin (Sigma)
10 ug Leupeptin (Sigma)
10 ug Pepstatin (Sigma)
50 uL PMSF (200 mM)
50 uL Na3VO3 (200mM)

6) Kinase buffer

25 mM HEPES pH 7.4 (GibcoBRL Life Technologies)
20 mM MgCl2 (Baker, 2444-01, Phillipsburg, NJ)
20 mM DTT (American Bioanalytical, AB490, Natick, MA)
20 mM Betaglycerophosphate (Sigma)
100 uL PMSF
10 ug/cc Leupeptin (Sigma)
1 mM Sodium Vanadate

7) Radioactive buffer—add 20 uL of this cocktail to each sample before reaction

20 uL x number of samples = amount kinase buffer
2 uL x number of samples = amount of enolase (1 mg/ cc)
10 uCi x number of samples = 32P (ATP)
2 uL x number of samples = 1 mM ATP
2 uL x number of samples = 0.1 mM DTT

B) Example Data Analysis

Raw data for Exp 1 B) Example Data Analysis

Control

	Wells			
	1	2	3	
Count #				
1	2	3	22	
2	5	6	5	
3	13	19	27	
4	19	27	16	
5	13	50	33	
6	13	17	12	
7	12	8	0	
8	7	5	15	
9	24	16	10	
10	14	16	7	
11	35	26	10	
12	15	11	29	
13	24	20	24	
14	27	25	14	
15	20	17	18	
16	7	18	4	
17	13	9	10	
18	11	7	11	
19	10	13	19	
20	18	14	14	Average
Sum	302	327	300	310

Pressure

	Wells			
	1	2	3	
	12	40	10	
	10	23	15	
	24	22	20	
	22	22	18	
	37	48	15	
	26	28	27	
	18	16	16	
	16	30	41	
	18	20	20	
	21	12	20	
	28	36	18	
	18	26	15	
	14	21	24	
	12	10	46	
	10	17	19	
	15	37	16	
	20	23	11	
	22	31	19	
	17	20	18	
	19	20	11	Average
	379	502	399	427

Summary

Pressure	427
Control	310
Enhancement	425/310=1.38

Example data analysis page 2 of 3

Normalized data for Exp 1

Divide all numbers by average of cells in control experiment = 310

Control

	Wells 1	2	3		
1	0.006	0.010	0.071		
2	0.016	0.019	0.016		
3	0.042	0.061	0.087		
4	0.061	0.087	0.052		
5	0.042	0.161	0.107		
6	0.042	0.055	0.039		
7	0.039	0.026	0.000		
8	0.023	0.016	0.048		
9	0.078	0.052	0.032		
10	0.045	0.052	0.023		
11	0.113	0.084	0.032		
12	0.048	0.036	0.094		
13	0.078	0.065	0.078		
14	0.087	0.081	0.045		
15	0.065	0.055	0.058		
16	0.023	0.058	0.013		
17	0.042	0.029	0.032		
18	0.036	0.023	0.036		
19	0.032	0.042	0.061		
20	0.058	0.045	0.045	Average	Stdev
Sum	0.975	1.056	0.969	1.00	5%

Pressure

	Wells 1	2	3		
1	0.039	0.129	0.032		
2	0.032	0.074	0.048		
3	0.078	0.071	0.065		
4	0.071	0.071	0.058		
5	0.119	0.155	0.048		
6	0.084	0.090	0.087		
7	0.058	0.052	0.052		
8	0.052	0.097	0.132		
9	0.058	0.065	0.065		
10	0.068	0.039	0.065		
11	0.090	0.116	0.058		
12	0.058	0.084	0.048		
13	0.045	0.068	0.078		
14	0.039	0.032	0.149		
15	0.032	0.055	0.061		
16	0.048	0.119	0.052		
17	0.065	0.074	0.036		
18	0.071	0.100	0.061		
19	0.055	0.065	0.058		
20	0.061	0.065	0.036	Average	Stdev
Sum	1.224	1.621	1.288	1.38	21%

Normalized summary

Pressure	=427/310=1.38
Control	=310/310=1.00
Enhancem	=1.38/1.00=1.38

Example data analysis page 3 of 3

Multiple experiments normalized

Bold data=final data plotted in figures

		Control	Pressure
Exp 1	Well 1	0.98	1.22
	Well 2	1.06	1.62
	Well 3	0.97	1.29
Exp 2	Well 1	0.90	1.06
	Well 2	1.19	1.35
	Well 3	0.91	1.16
Exp 3	Well 1	1.01	1.17
	Well 2	0.93	1.24
	Well 3	1.06	1.32
Exp 4	Well 1	1.05	1.31
	Well 2	1.00	1.30
	Well 3	0.94	1.30
Exp 5	Well 1	1.16	1.33
	Well 2	0.83	1.44
	Well 3	1.08	1.31
Average		1.00	**1.30**
Stdev		10%	13%
SEM	**=stdev/sqrt(5)**	4%	**6%**

C) Figure Data

Figure 2 Pressure Enhancement of Cell Adhesion vs. Time				
	Value	Error (SEM)	n	p
Control 10 min	1.00	0.03	12	
Pressure 10 min	1.00	0.07	12	0.9768
Control 20 min	1.00	0.05	12	
Pressure 20 min	1.13	0.04	12	0.0166
Control 30 min	1.00	0.02	15	
Pressure 30 min	1.30	0.04	15	0.0009
Control 40 min	1.00	0.04	15	
Pressure 40 min	1.42	0.08	15	0.0001
Control 60 min	1.00	0.04	15	
Pressure 60 min	1.12	0.04	15	0.0359
Control 120 min	1.00	0.03	12	
Pressure 120 min	1.09	0.03	12	0.0268

Figure 3 Percent of cells seeded-adhesion vs. Time			
	Value %	Error %(SEM)	n
Control 10 min	20	1	12
Pressure 10 min	23	2	12
Control 30 min	31	1	15
Pressure 30 min	40	1	15
Control 40 min	31	2	15
Pressure 40 min	39	3	15
Control 60 min	38	3	15
Pressure 60 min	43	3	15
Control 120 min	49	5	12
Pressure 120 min	54	6	12

Figure 4 Pressure Pre-incubation Enhancement vs. Time				
	Value	Error (SEM)	n	p
Control 10 min	1.00	0.06	9	
Pressure 10 min	2.43	0.28	9	0.0007
Control 20 min	1.00	0.06	9	
Pressure 20 min	2.14	0.28	9	0.0031
Control 30 min	1.00	0.03	9	
Pressure 30 min	2.05	0.20	9	0.0005
Control 40 min	1.00	0.03	9	
Pressure 40 min	2.10	0.29	9	0.0057
Control 60 min	1.00	0.06	6	
Pressure 60 min	1.33	0.06	6	0.004

Figure 5 Percent of cells seeded-adhesion vs. Time			
	Value %	Error %(SEM)	n
Control 10 min	4	1	9
Pressure 10 min	11	4	9
Control 30 min	11	2	9
Pressure 30 min	20	3	9
Control 40 min	16	3	9
Pressure 40 min	28	4	9
Control 60 min	22	4	6
Pressure 60 min	38	3	6

Figure 6 PP1 blocks pressure stimulated cell adhesion					
		Value	Error (SEM)	n	p
1 μM	DMSO Control	1.00	0.02	9	
	DMSO Pressure	1.30	0.05	9	0.0004
	PP1 Control	1.00	0.03	9	
	PP1 Pressure	0.96	0.02	9	0.31
0.1 μM	DMSO Control	1.00	0.03	9	
	DMSO Pressure	1.29	0.05	9	0.0006
	PP1 Control	1.00	0.04	9	
	PP1 Pressure	0.97	0.04	9	0.54
0.01 μM	DMSO Control	1.00	0.03	9	
	DMSO Pressure	1.23	0.03	9	0.0001
	PP1 Control	1.00	0.02	9	
	PP1 Pressure	1.07	0.02	9	0.026

Figure 7b Control vs. Pressure Src *in-vitro* kinase activity				
	Value	Error (SEM)	n	p
Control	1		8	
Pressure	1.4	.15	8	0.04

Figure 8 Cell viability with shear and turbulence		
Stirrer Setting	Revolutions/minute	Percent cell viability
0	0	96
3	375	97
6	750	94

Figure 9 Shear and turbulence stimulated cell adhesion vs. Time				
	Value	Error (SEM)	n	p
Control 10 min	1.00	0.06	9	
Shear 10 min	2.12	0.24	9	0.0015
Control 20 min	1.00	0.06	9	
Shear 20 min	1.66	0.06	9	9.7E-7
Control 30 min	1.00	0.03	9	
Shear 30 min	1.85	0.22	9	0.0047
Control 40 min	1.00	0.03	9	
Shear 40 min	1.48	0.20	9	0.0437
Control 60 min	1.00	0.06	6	
Shear 60 min	1.44	0.19	6	0.049

Figure 10 Percent of cells seeded-adhesion vs. Time			
	Value %	Error %(SEM)	n
Control 10 min	4	1	9
Pressure 10 min	9	3	9
Control 30 min	11	2	9
Pressure 30 min	19	5	9
Control 40 min	16	3	9
Pressure 40 min	24	6	9
Control 60 min	22	4	6
Pressure 60 min	28	6	6

Figure 11 PP1 blocks shear and turbulence stimulated cell adhesion					
		Value	Error (SEM)	n	p
0.1 μM	DMSO Control	1.00	0.04	9	
	DMSO Pressure	1.34	0.04	9	0.0001
	PP1 Control	1.00	0.04	9	
	PP1 Pressure	0.99	0.03	9	0.65

Figure 12 b Control vs. Shear Src *in-vitro* kinase activity				
	Value	Error (SEM)	n	p
Control	1		7	
Shear	1.5	.13	7	0.01

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