DISRUPTION OF THE FAK-AKT1 INTERACTION: USING FAK-DERIVED INTERVENTIONS TO INHIBIT PRESSURE-STIMULATED CELL ADHESION

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ABSTRACT

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Pressure-stimulated cell adhesion contributes to the iatrogenic spread of cancer by increasing the likelihood of attachment of dislodged neoplastic cells. Central to the pressure-adhesion pathway is the interaction between focal adhesion kinase (FAK) and protein kinase B alpha, also known as Akt1. Akt1 binds and phosphorylates FAK at three serine sites (S517, 601, 695) to drive pressure-stimulated cell adhesion. Because both FAK and Akt1 are kinases, interventions targeting the interaction between the two without disturbing their kinase activity would seem worthwhile. Here I tested various peptidyl and small-molecule inhibitors of Akt1 derived from FAK and examined how they altered the FAK-Akt1 interaction.

The FAK-derived peptide inhibitors were developed by serially truncating FAK and testing the ability of these fragments to bind Akt1 using pull-down assays. I identified a 7-residue peptide that disrupted the FAK-Akt1 interaction. I*n vitro*, the 7-residue peptide was able to inhibit both pressure-stimulated FAK phosphorylation and the subsequent pressure-stimulated adhesion of colorectal adenocarcinoma cells to collagen matrices. *In vivo,* the same peptide prevented pressure-stimulated cell adhesion to murine surgical wounds and increase tumor-free survival times.

FAK-derived small-molecule inhibitor candidates were identified using computational screening. The 7-residue peptide was used as a query against a virtual library of 10,639,555 drug-like molecules. TanimotoCombo scoring, which considers volumetric and chemical similarity, was used to assess the overlays. The top results were tested for their ability to disrupt the FAK-Akt1 interaction. The small-molecule inhibitor designated as ZINC31501681 was able to prevent FAK pull-down of Akt1, as well as pressure-stimulated FAK phosphorylation and cell adhesion. These results render further studies of both ZINC31501681 and other leads generated by the virtual screen promising with the ultimate goal being the development of a therapeutic intervention for the iatrogenic spread of colorectal carcinoma.

To my wife and parents

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KEY TO ABBREVATIONS

FAK	Focal Adhesion Kinase
FAT	Focal Adhesion Targeting
FERM	Erythrocyte Band Four.1-Ezrin-Radixin-Moesin
FRNK	FAK-Related Non-Kinase
HA	Hemagglutinin
GFP	Green Fluorescent Protein
GST	Glutathione S-Transferase
PH	Pleckstrin Homology
PI3K	Phosphatidylinositol 3-Kinase
Ser	Serine
Tyr	Tyrosin

CHAPTER 1

Introduction

1.1 Cancer Metastasis

The study of cancer encompasses a large body of diseases that are often unique in their deviations from normalcy. Despite the varying pathologies, the “hallmarks of cancer,” as defined by Hanahan and Weinberg, distills the body of knowledge surrounding oncogenic progression into six fundamental components (Hanahan, Douglas, Weinberg 2000). The first five of these hallmarks focus on a singular issue: the growth potential of the cancer cell. Cancer cells promote their own growth, ignore signals that arrest growth, evade apoptosis, divide indefinitely, and stimulate angiogenesis to sustain their growth. These characteristics are reflected in the public perception of cancer as being uncontrolled and immortal. However, as critics have noted, these traits are shared with benign tumors, so it falls upon the sixth hallmark to truly underscore the erosive potential of the disease: the ability to invade surrounding tissue and spread to distant sites. Ultimately, it is this tendency that gives the disease its lethality. Benign tumors, and to some extent slow growing primary malignant tumors, may require little treatment or may be successfully treated with pharmacological and surgical interventions. Such a tumor is in a sense self-limiting or progresses slowly enough to allow the body to compensate for its presence. Conversely, upwards of 90% of solid tumor-associated deaths are attributed to cancer metastasis and the damage caused by the creation of secondary tumors (Gupta and Massagué 2006).

The “invasion-metastasis cascade” is a term given to the collective steps cancer cells undergo to create metastases. Initially, these cells detach from the primary tumor and migrate through the local extracellular matrix to invade the adjacent tissue. A

population of these invasive cells are able to enter nearby vascular or lymphatic vessels and gain access to other regions of the anatomy. Those that survive the journey may be deposited at secondary sites any distance from the primary tumor. Then the steps repeat in reverse as the cells exit the circulatory system and form metastatic foci. In murine models, only 0.01% of melonoma cells injected into the circulation completed this portion of the process (Fidler 1970). From the surviving population of cells, a small subset will proliferate in the new environment and create clinically significant metastases (Luzzi et al. 1998). While every step in the cascade poses an obstacle towards secondary tumor formation, the low collective probability of metastatic spread is overcome by the sheer number of inciting incidents. A one gram tumor can release one million cells into the circulation per day, thus reconciling the low rate of success with the high rate of mortality (Butler and Gullino 1975).

In normal pathology, the number of tumor cells entering the circulation limits the progression of the metastatic process. Cells capable of proliferating at distant locations may never get the opportunity if they are unable to detach from the primary tumor. Therefore, tumor cells indiscriminately introduced into the circulation could command a metastatic advantage over those forced to enter independently. Such artificial dissemination is seen in the cell shedding caused by surgical manipulation (Nishizaki et al. 1990). Surgical resection is the most common form of intervention among early stage tumors (Miller et al. 2016). However, the number of tumor cells in circulation increases dramatically during oncologic surgery, and they are readily recovered from the surgical site following these procedures (Allardyce, Morreau, and Bagshaw 1997; Yamaguchi et

al. 2000; Hayashi et al. 1999). Dislodging tumor cells in such a manner may lower the threshold for metastasis and contribute to surgical wound recurrence. It is difficult to determine the origins of the metastatic lesions found at or near a surgical site. Nevertheless, the presence of intraoperative circulating tumor cells is indeed associated with increased rates of tumor relapse (Weitz et al. 2000; Patel et al. 2002; Koch et al. 2005).

The complications caused by perioperative tumor dissemination are further compounded by the phenomenon of pressure-induced cell adhesion, which is especially pertinent in the context of laparoscopic-assisted resections (Thamilselvan and Basson 2004). In laparoscopic-assisted resections, the peritoneal cavity is often insufflated with carbon dioxide gas to a pressure of 15 mmHg to provide room to operate (all pressures referenced are presented as gauge pressures, which is the additional pressure in a system relative to the 760 mmHg of atmospheric pressure) (Dregelid and Svendsen 1988). 15 mmHg increased extracellular pressure stimulates cell adhesion in breast cancer, squamous cell carcinoma, and sarcoma cells (Downey et al. 2006; Conway et al. 2006; Perry, Wang, and Basson 2010). Such a modest increase in pressure also promotes the adhesion of colon cancer cells to collagen substrates, endothelial monolayers and murine surgical wounds (Basson et al. 2000; David H Craig, Haimovich, and Basson 2007). In murine models, the implantation of tumor cells, which had been exposed to 15 mmHg increased extracellular pressure, significantly reduced tumor-free survival relative to mice that received tumor cells that had been kept at ambient pressure (David H. Craig et al. 2008). In the clinical setting, 0.2% - 1% of

curative laparoscopic-assisted resections of colon cancer show verifiable signs of wound recurrence, and many more develop signs that are consistent with peritoneal spreading (Bonjer et al. 2007; Turnbull 1970).

1.2 Mechanotransduction

The ability of a physical force to affect cell behavior is neither unique to pressure nor limited to augmenting adhesion. Within an organism, the basic mechanical forces of compression and tension are generated by the anatomy as a part of normal cell functions. At the cellular level, single cells are constantly being compressed by adjacent cells or stretched through their intercellular attachments or connections to the extracellular matrix. Cells exposed to bulk fluid flow, such as those associated with the gastrointestinal, vascular or lymphatic systems, experience the additional force of shear stress. Cyclic strain is produced by the vessels themselves as they expand and contract with each heartbeat. Cyclic strain also drives the gut motility needed for digestion and is generated through the act of walking. A combination of hydrostatic and hydraulic pressures produces 5-10 mmHg in the portal vein and 90-120 mmHg in arteries. In tumors growing against constrictive stroma, average interstitial fluid pressures of 15-38 mmHg have been measured in breast carcinomas, metastatic melanomas, colorectal carcinomas, and head and neck carcinomas; this is within the context of an average interstitial fluid pressure of 2 mmHg in the surrounding normal tissue (Jain, Boucher, and Wolmark 1992; Madara et al. 1993; Leunig et al. 1992). These mechanical stimuli influence both physiological and pathological cellular behaviors. Shear stress induces cell adhesion in platelets and motility in corneal epithelial cells (Huynh et al. 2017;

Molladavoodi et al. 2017). Cyclic strain promotes epithelial wound closure and modulates directional alignment in osteoblasts (Zhang et al. 2006; Buckley et al. 1988). Extracellular pressure stimulates phagocytosis in macrophages and maturation in dendritic cells (Shiratsuchi and Basson 2004) ; Craig, Shiratsuchi, and Basson 2009). With respect to pathology, mechanical stretch has been shown to elicit hypertrophic responses in cardiac myocytes, pressure-induced wall stress contributes to the formation of atherosclerotic lesions, and increased extracellular pressure stimulates proliferation in colon, breast, and prostate cancer cells (Sadoshima and Izumo 1997; Thubrikar and Robicsek 1995; Basson et al. 2015). The connection between physical forces and cellular responses also extends beyond mammalian organisms. Change in the gravity vector induces cytoplasmic calcium flux in Arabidopsis seedlings to help reorient the plant (Tatsumi et al. 2014). Alternatively, microgravity cultivation doubles the concentration of the quorum-sensing molecule N-acylhomoserine lactone in Rhodospirillium rubrum, which is a candidate organism for food and oxygen production in long-term space travel (Mastroleo et al. 2013). Overall, the broadness of these observations suggests that the ability to sense and respond to physical stimuli may be as pervasive as the stimuli itself.

In order to detect these external physical forces, Ingber *et al.* proposed a concept called cellular tensegrity (Ingber, Wang, and Stamenović 2014). Named after the architectural term by R. Buckminster Fuller, tensegrity describes a structure that achieves its internal stability through the use of encompassing tensile forces (Motro 2003). While most structures are built by layering materials held together through

compressive forces, tensegrity stabilizes structures through a balance between compression-bearing struts and tensile cables. These self-contained sources of push and pull are arranged so that the compressive struts act as anchors for the tension producing cables, which in turn affix the struts to permanent positions in space relative to one another. Biologically, this creates a system that maximizes mechanical stability while using a minimal amount of materials. In Ingber's model of cellular tensegrity, microtubules play the part of the compression-bearing struts. They are formed through the polymerization of α- and β-tubulin dimers and become stiff, cylindrical structures. Filamentous actin makes up the tension-generating cables, which associate with myosin motor proteins to create stress fibers. This process generates tension within the stress fibers and pulls the cytoskeleton into a tensegritous structure that is always stressed but always stable. This constant state of isometric tension that the cytoarchitecture is in also allows the cell to immediately respond to mechanical stimuli. In such a prestressed structure, changes to any one component will be sensed across the system by all connected components in the manner of a collapsing house of cards. However, a house of cards can only transmit information from the bottom up, because it is held together by parallel compressive forces aligned by gravity, *i.e.*, removing the top card does not affect the bottom ones. On the other hand, a structure stabilized by tensegrity, such as a cell in suspension, is stressed in every direction and will collapse inward regardless of the component removed. The coupling of this architecture with kinases and signaling molecules creates the concept of mechanotransduction: the transformation of physical forces into biochemical signals (D. E. Ingber 2006).

Mechanotransduction operates in a manner similar to receptor-ligand or ion channel based signaling. However, by virtue of their intangible nature, physical forces are not constrained by membranes and thus can initiate signaling cascades from both within and without the cell. While receptor-ligand signaling begins at the surface receptors and progresses, pressure-induced adhesion begins within the cell and flows outwards: the increase in extracellular pressure changes the cytoarchitecture to initiate a cascade that ultimately modulates the affinity of integrins at the cell surface (Thamilselvan and Basson 2004). Within the cell, a dual, mechanosensitive signaling pathway involving the cytoskeleton, paxillin or Src and phosphatidylinositol 3-kinase (PI3K) converges at FAK and Akt1 as it progresses outwardly towards the cell surface to ultimately increase β1-integrin affinity and avidity (Basson 2008; Craig, Shiratsuchi, and Basson 2009; Craig et al. 2008). Specifically, this pathway phosphorylates threonines 788 and 789 within the cytoplasmic tail of the β1-integrin to induce conformational changes that expose its extracellular matrix binding domain (Craig et al. 2009). Consequently, pressure stimulation could effectively lower the ligand threshold required for integrin-mediated cell adhesion. Indeed, suspended colon cancer cells exhibit increased adhesiveness to collagen I and endothelial cells after exposure to 15 mmHg extracellular pressure for 30 minutes. Therefore, it is worth pursuing treatments that mitigate the metastatic advantages that tumor cells experience from pressurization.

1.3 Pressure-stimulated Adhesion and FAK and Akt1

The central roles FAK and Akt1 play in pressure-induced adhesion make them tempting pharmacologic targets. However, FAK and Akt1 influence a variety of cell

functions, which limits the utility of drugs that inhibit their catalytic activities. Classically, FAK activation is the result of surface integrin engagement, which places FAK near the top of several signaling cascades that transmit information from the external environment to actors within the cell (Parsons 2003). FAK associates with Cas and Src to promote the focal adhesion turnover required in cell migration, and it is also required in the cooperative signaling pathway that exists between integrins and growth factors, which controls cell cycle progression and proliferation through the MEK-ERK axis (Parsons 2003; Klinghoffer et al. 1999). Indeed, FAK signaling is required for cell survival in many adherent cells, and its absence triggers anoikis (Walsh et al. 2003). Akt also contributes to a range of cell processes such as cell proliferation and survival. Akt, classically, has a complex activation mechanism that involves PIP3-mediated membrane translocation and phosphorylation by both PDK1 and mTORC2. Upon activation, Akt loses its membrane restrictions and gains a high degree of intracellular mobility, which is directed in part by isoform preference (Toker and Marmiroli 2014). While Akt isoforms have a limited capability to compensate for one another, they also exhibit an assortment of unique functions (Toker and Marmiroli 2014). In murine knockout models, Akt1 has been proven to be responsible for overall growth, Akt2 required for proper insulin signaling, and the loss of Akt3 manifested in a decrease in brain size (Chen et al. 2001; Cho et al. 2001; Tschopp 2005). The non-specific inhibition of such wide reaching elements may produce unintended effects and limit their feasibility as drug targets.

Pressure-induced cell adhesion can be successfully deterred through pharmacological blockade with colchicine, which disrupts the signaling pathway by inhibiting actin polymerization; this form of intervention significantly increases tumor-free survival in animal models (Craig et al. 2008). But, as with FAK and Akt1, targeting common intracellular elements can cause non-specific toxicity, and here, the dose of colchicine required is not feasible for human use (Craig et al. 2008). Several pharmacological approaches exist to reduce cancer cell adhesion by targeting the expression or activity of the proteins involved. Some of these drugs perturb the integrin-cytoskeleton interface and weaken cell-matrix interactions by decreasing the expression of focal adhesions proteins such as talin (M. Y. Wu et al. 2015). Others interrupt receptor-ligand signaling, such as the pro-adhesion hepatocyte growth factor and c-MET, by targeting the ATP binding site on the receptor kinases (Moran-Jones, Brown, and Samimi 2015). However, the effectiveness of those treatments is limited due to unwanted side effects. Weakened cell-matrix interactions can affect all bound cells, ATP-competitive kinase inhibitors can block unintended targets, and global FAK or Akt1 inhibition can lead to difficulties striking a balance between efficacy and toxicity (Liu et al. 2008; Liu et al. 2007). These problems make clear the advantages of producing treatments that are more selective, which can be achieved by targeting the processes that enhance cancer cell adhesiveness instead of cell adhesion on the whole.

Brief pressurization of suspended cells facilitates association between FAK and Akt1 within the cells. After 30 minutes of exposure of colon cancer cells to 15 mmHg elevated extracellular pressure, FAK immunoprecipitated from colon cancer cell lysate

co-immunoprecipitates an increased amount of Akt1 when compared to unpressurized controls (Wang and Basson 2011). As a consequence of this association, the following sites are phosphorylated: Akt1 serine 473 (S473), FAK tyrosine 397 (Y397), and FAK serine 517/601/695 (S517/601/695); the first two are classical activation sites while the third event occurs across three novel serine phosphorylation sites on FAK. Akt1 is activated by S473 phosphorylation in a manner that is dependent on catalytically active FAK (S. Wang and Basson 2011; Thamilselvan, Craig, and Basson 2007). In a reciprocal fashion, FAK is activated by Y397 phosphorylation in a manner that is blocked by Akt inhibitor IV (S. Wang and Basson 2011). Interestingly enough, despite their mutual dependence, neither Akt1 nor FAK can directly phosphorylate the other at their respective activation sites. This is because Akt1 only phosphorylates serines and threonines while FAK only phosphorylates tyrosines. However, the third event bridges this gap and directly ties Akt1 to FAK. The three sites on FAK, S517/601/695, all contain consensus sequences for the catalytic domain of Akt1, and they are all phosphorylated in an Akt1-dependent manner upon pressurization. These phosphorylated serines are required for pressure-induced FAK Y397 phosphorylation, and all of the phosphorylation sites mentioned above are required for pressure-induced adhesion to occur; under ambient conditions, these processes still occur after loss of S517/601/695 (S. Wang and Basson 2011). Taken together, these data support a model of mechanotransduction that involves direct FAK-Akt1 binding; we may thus target this interaction to selectively inhibit pressure-induced adhesion.

1.4 The Structure and Function of FAK

The FAK molecule is composed of three domains, the N-terminal erythrocyte band four.1-ezrin-radixin-moesin (FERM) domain (residues 35-362), the central kinase domain (residues 416-676), and the C-terminal focal adhesion targeting (FAT) domain (residues 677-1025) (Parsons 2003). The FERM domain serves as an anchor between membrane and cytoskeletal components and plays a key role in directing FAK activity (Parsons 2003). The kinase domain is composed of N- and C-lobes with the active site situated in between the two. Additionally, it is noteworthy that the FAK kinase domain undergoes a very small conformational change as it transitions between active and inactive states; therefore, it is thought that FAK activity is controlled through active site occlusion (Lietha et al. 2007). The C-terminal FAT domain, as the name implies, targets FAK to focal adhesions and is required for integrin-mediated FAK signaling (Thomas et al. 1999). Of these three sections, data from our lab shows that FAK-Akt1 binding can occur in the absence of both the FAK kinase and C-terminal FAT domains with the highest Akt1 affinity dependent on the first 126 amino acids of the FERM domain (Basson, Zeng, and Wang 2017).

The FERM domain itself is composed of three lobes named F1 (residues 35-130), F2 (residues 131-255), and F3 (residues 256-362). (Fig. 1) The F2 lobe binds the C-lobe of the kinase domain folding the entire FERM domain over the kinase to inhibit FAK catalytic activity by means of active site denial (Lietha et al. 2007). In this manner, the F2 lobe regulates FAK activity and has indeed been implicated in FAK activation specifically following cell adhesion (Ceccarelli et al. 2006). The F3 lobe contains a site

that, while unavailable in the autoinhibited conformation of FAK, displays homology to a region of the talin FERM domain responsible for binding β integrins (Ceccarelli et al. 2006). On the other hand, the F1 lobe has been relatively silent in the FAK activation discussion. However, it is promising that our data implicates the F1 and not the better characterized F2 and F3 lobes, because this coincides with the “inside-out” dynamics of the pressure-adhesion pathway. Unlike the F2 lobe, we believe that the F1 plays a role in Akt1-dependent FAK activation specifically in suspended cells prior to adhesion. Likewise with the F3 lobe, which depends on FAK activation to bind β integrins, pressure itself induces FAK activation upstream of β1- integrin association (Thamilselvan, Craig, and Basson 2007). Therefore, a pathway in which Akt1 binds the F3 domain to facilitate β integrin interactions either before or while simultaneously activating FAK would be redundant.

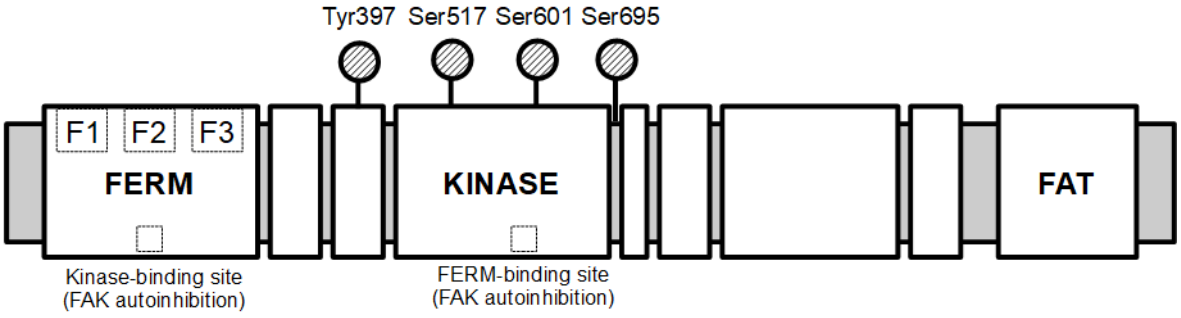


Figure 1. A model of the FAK molecule
This cartoon shows the FAK FERM, kinase, and FAT domains. The FERM domain is further divided into its F1, 2 and 3 lobes; the kinase-binding site on the FERM domain, and its counterpart in the kinase domain, are also shown. The four phosphorylation sites are Tyr397, Ser517/601/695. Tyr397 is associate with canonical FAK activation, and the three serine sites are implicated in pressure-stimulated FAK activation.

There exist structural characteristics within the F1 lobe that provides the potential to affect FAK activation, which is predicated on Y397 phosphorylation. The Y397 autophosphorylation site resides on a linker segment connecting the FERM F3 lobe to the N-lobe of the kinase domain, and this segment binds to a groove within the F1 domain (Lietha et al. 2007). Furthermore, distant changes in the F1 lobe, such as the mutation of lysine 38 which does not interact with the linker segment, can promote Y397 phosphorylation possibly through FERM-linker binding destabilization (Cohen and Guan 2005). Serial truncations of F1 lobe revealed a span of 33 amino acids, designated NT1-2-2 (aa. 94-126), to be both necessary and sufficient to pull down Akt1 from Caco2 cell lysate (Basson, Zeng, and Wang 2017). These findings led us to explore the secondary structure of NT1-2-2 to identify the residues responsible for interacting with Akt1. Using structural analysis and truncations of this fragment of human FAK as our starting point, we dissected the FAK-Akt1 interaction to define a short FAK-derived peptide that interrupts said interaction and demonstrated that this peptide inhibits cancer cell adhesion in vitro and in vivo and improves survival in a murine model of wound recurrence.

REFERENCES

REFERENCES

Allardyce, Randall A., Phil Morreau, and Philip F. Bagshaw. 1997. “Operative Factors Affecting Tumor Cell Distribution Following Laparoscopic Colectomy in a Porcine Model.” *Diseases of the Colon and Rectum*. https://doi.org/10.1007/BF02051202.

Arkin, Michelle R., Yinyan Tang, and James A. Wells. 2014. “Small-Molecule Inhibitors of Protein-Protein Interactions: Progressing toward the Reality.” *Chemistry and Biology*. https://doi.org/10.1016/j.chembiol.2014.09.001.

Arkin, Michelle R., and James A Wells. 2004. “Small-Molecule Inhibitors of Protein-Protein-Interactions: Progression Towards the Dream.” *Nature Reviews*. https://doi.org/10.1038/nrd1343.

Baell, Jonathan B., and Georgina A. Holloway. 2010. “New Substructure Filters for Removal of Pan Assay Interference Compounds (PAINS) from Screening Libraries and for Their Exclusion in Bioassays.” *Journal of Medicinal Chemistry*. https://doi.org/10.1021/jm901137j.

Baeyens, Nicolas. 2018. “Fluid Shear Stress Sensing in Vascular Homeostasis and Remodeling: Towards the Development of Innovative Pharmacological Approaches to Treat Vascular Dysfunction.” *Biochemical Pharmacology* 158 (October): 185–91. https://doi.org/10.1016/j.bcp.2018.10.023.

Basse, Marie Jeanne, Stéphane Betzi, Xavier Morelli, and Philippe Roche. 2016. “2P2Idb v2: Update of a Structural Database Dedicated to Orthosteric Modulation of Protein-Protein Interactions.” *Database*. https://doi.org/10.1093/database/baw007.

Basson, Marc D. 2008. “An Intracellular Signal Pathway That Regulates Cancer Cell Adhesion in Response to Extracellular Forces.” *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-07-2992.

Basson, Marc D., Cheng Fang Yu, Oliver Herden-Kirchoff, Marc Ellermeier, Matthew A. Sanders, Ronald C. Merrell, and Bauer E. Sumpio. 2000. “Effects of Increased Ambient Pressure on Colon Cancer Cell Adhesion.” *Journal of Cellular Biochemistry*. https://doi.org/10.1093/brain/114.3.1297.

Basson, Marc D., B. Zeng, and S. Wang. 2017. “The C-Terminal Region of the Focal Adhesion Kinase F1 Domain Binds Akt1 and Inhibits Pressure-Induced Cell Adhesion.” *Journal of Physiology and Pharmacology*.

Basson, Marc D., Bixi Zeng, Christina Downey, Madhu P. Sirivelu, and Jetze J. Tepe. 2015. “Increased Extracellular Pressure Stimulates Tumor Proliferation by a

Mechanosensitive Calcium Channel and PKC-β.” *Molecular Oncology*. https://doi.org/10.1016/j.molonc.2014.10.008.

Bonjer, H. Jacob, Wim C J Hop, Heidi Nelson, Daniel J. Sargent, Antonio M. Lacy, Antoni Castells, Pierre J. Guillou, et al. 2007. “Laparoscopically Assisted vs Open Colectomy for Colon Cancer: A Meta-Analysis.” *Archives of Surgery*. https://doi.org/10.1001/archsurg.142.3.298.

Buckley, M J, A J Banes, L G Levin, B E Sumpio, M Sato, R Jordan, J Gilbert, G W Link, and R Tran Son Tay. 1988. “Osteoblasts Increase Their Rate of Division and Align in Response to Cyclic, Mechanical Tension in Vitro.” *Bone & Mineral*.

Butler, Thomas P., and Pietro M. Gullino. 1975. “Quantitation of Cell Shedding Into Efferent Blood of Mammary Adenocarcinoma.” *Cancer Research*.

Ceccarelli, Derek F.J., Kyu Song Hyun, Florence Poy, Michael D. Schaller, and Michael J. Eck. 2006. “Crystal Structure of the FERM Domain of Focal Adhesion Kinase.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M509188200.

Chaturvedi, Lakshmi S., H. Michael Marsh, Xun Shang, Yi Zheng, and Marc D. Basson. 2007. “Repetitive Deformation Activates Focal Adhesion Kinase and ERK Mitogenic Signals in Human Caco-2 Intestinal Epithelial Cells through Src and Rac1.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M605817200.

Chaturvedi, Lakshmi S, Harold M Marsh, and Marc D Basson. 2007. “Src and Focal Adhesion Kinase Mediate Mechanical Strain-Induced Proliferation and ERK1/2 Phosphorylation in Human H441 Pulmonary Epithelial Cells.” *American Journal of Physiology-Cell Physiology*. https://doi.org/10.1152/ajpcell.00529.2006.

Chen, William S., Pei Zhang Xu, Kathrin Gottlob, Mei Ling Chen, Karen Sokol, Tanya Shiyanova, Igor Roninson, et al. 2001. “Growth Retardation and Increased Apoptosis in Mice with Homozygous Disruption of the Akt1 Gene.” *Genes and Development*. https://doi.org/10.1101/gad.913901.

Cheng, Zhaokang, Laura A. DiMichele, Mauricio Rojas, Cyrus Vaziri, Christopher P. Mack, and Joan M. Taylor. 2014. “Focal Adhesion Kinase Antagonizes Doxorubicin Cardiotoxicity via P21Cip1.” *Journal of Molecular and Cellular Cardiology*. https://doi.org/10.1016/j.yjmcc.2013.12.002.

Cho, H., J. Mu, J. K. Kim, J. L. Thorvaldsen, Q. Chu, E. B. Crenshaw, K. H. Kaestner, M. S. Bartolomei, G. I. Shulman, and M. J. Birnbaum. 2001. “Insulin Resistance and a Diabetes Mellitus-like Syndrome in Mice Lacking the Protein Kinase Akt2 (PKBβ).” *Science*. https://doi.org/10.1126/science.292.5522.1728.

Clackson, Tim, and James A. Wells. 1995. “A Hot Spot of Binding Energy in a Hormone-Receptor Interface.” *Science*. https://doi.org/10.1126/science.7529940.

Cohen, Lee Ann, and Jun Lin Guan. 2005. “Residues within the First Subdomain of the FERM-like Domain in Focal Adhesion Kinase Are Important in Its Regulation.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M412021200.

Collawn, James F., Martin Stangel, Leslie A. Kuhn, Victor Esekogwu, Shuqian Jing, Ian S. Trowbridge, and John A. Tainer. 1990. “Transferrin Receptor Internalization Sequence YXRF Implicates a Tight Turn as the Structural Recognition Motif for Endocytosis.” *Cell*. https://doi.org/10.1136/jnnp.s1-12.48.320.

Conway, William C., Jochem Van Der Voort Van Zyp, Vijayalakshmi Thamilselvan, Mary F. Walsh, David L. Crowe, and Marc D. Basson. 2006. “Paxillin Modulates Squamous Cancer Cell Adhesion and Is Important in Pressure-Augmented Adhesion.” *Journal of Cellular Biochemistry*. https://doi.org/10.1002/jcb.20819.

Craig, D H, C P Gayer, K L Schaubert, Y Wei, J Li, Y Laouar, and M D Basson. 2009. “Increased Extracellular Pressure Enhances Cancer Cell Integrin-Binding Affinity through Phosphorylation of Beta1-Integrin at Threonine 788/789.” *American Journal of Physiology. Cell Physiology*. https://doi.org/10.1152/ajpcell.00355.2008.

Craig, David H., Cheri R. Owen, William C. Conway, Mary F. Walsh, Christina Downey, and Marc D. Basson. 2008. “Colchicine Inhibits Pressure-Induced Tumor Cell Implantation within Surgical Wounds and Enhances Tumor-Free Survival in Mice.” *Journal of Clinical Investigation*. https://doi.org/10.1172/JCI34279.

Craig, David H., Hiroe Shiratsuchi, and Marc D. Basson. 2009. “Increased Extracellular Pressure Provides a Novel Adjuvant Stimulus for Enhancement of Conventional Dendritic Cell Maturation Strategies.” *Biochemical and Biophysical Research Communications*. https://doi.org/10.1016/j.bbrc.2009.07.010.

Craig, David H, Christina Downey, and Marc D Basson. 2008. “SiRNA-Mediated Reduction of α-Actinin-1 Inhibits Pressure-Induced Murine Tumor Cell Wound Implantation and Enhances Tumor-Free Survival.” *Neoplasia*. https://doi.org/10.1593/neo.07945.

Craig, David H, Beatrice Haimovich, and Marc D Basson. 2007. “␣-Actinin-1 Phosphorylation Modulates Pressure-Induced Colon Cancer Cell Adhesion through Regulation of Focal Adhesion Kinase-Src Interaction.” *Am J Physiol Cell Physiol*. https://doi.org/10.1152/ajpcell.00118.2007.

Craig, Lisa, Paul C. Sanschagrin, Annett Rozek, Steve Lackie, Leslie A. Kuhn, and Jamie K. Scott. 1998. “The Role of Structure in Antibody Cross-Reactivity between Peptides and Folded Proteins.” *Journal of Molecular Biology*. https://doi.org/10.1006/jmbi.1998.1907.

Datta, Sandeep Robert, Henryk Dudek, Tao Xu, Shane Masters, Fu Haian, Yukiko Gotoh, and Michael E. Greenberg. 1997. “Akt Phosphorylation of BAD Couples

Survival Signals to the Cell- Intrinsic Death Machinery." *Cell*. https://doi.org/10.1016/S0092-8674(00)80405-5.

DeLano, Warren L. 2002. "PyMOL: An Open-Source Molecular Graphics Tool." *CCP4 Newsletter On Protein Crystallography*. https://doi.org/10.1038/s41598-017-03842-2.

Dorlo, Thomas P.C., Manica Balasegaram, Jos H. Beijnen, and Peter J. de vries. 2012. "Miltefosine: A Review of Its Pharmacology and Therapeutic Efficacy in the Treatment of Leishmaniasis." *Journal of Antimicrobial Chemotherapy*. https://doi.org/10.1093/jac/dks275.

Downey, Christina, Kamal Alwan, Vijayalakshmi Thamilselvan, Liyue Zhang, Yan Jiang, Arun K. Rishi, and Marc D. Basson. 2006. "Pressure Stimulates Breast Cancer Cell Adhesion Independently of Cell Cycle and Apoptosis Regulatory Protein (CARP)-1 Regulation of Focal Adhesion Kinase." *American Journal of Surgery*. https://doi.org/10.1016/j.amjsurg.2006.08.006.

Dregelid, E., and E. Svendsen. 1988. "Endothelial Cell Injury in Human Saphenous Veins after Manipulation and Tweezer Grasping." *Journal of Cardiovascular Surgery*.

Fidler, Isaiah J. 1970. "Metastasis: Quantitative Analysis of Distribution and Fate of Tumor Emboli Labeled with 125l-5-Lodo-2'-Deoxyuridine." *Journal of the National Cancer Institute*. https://doi.org/10.1093/jnci/45.4.773.

Fosgerau, Keld, and Torsten Hoffmann. 2015. "Peptide Therapeutics: Current Status and Future Directions." *Drug Discovery Today*. https://doi.org/10.1016/j.drudis.2014.10.003.

Fuller, Jonathan C., Nicholas J. Burgoyne, and Richard M. Jackson. 2009. "Predicting Druggable Binding Sites at the Protein-Protein Interface." *Drug Discovery Today*. https://doi.org/10.1016/j.drudis.2008.10.009.

Gayer, Christopher P., Lakshmi S. Chaturvedi, Shouye Wang, David H. Craig, Thomas Flanigan, and Marc D. Basson. 2009. "Strain-Induced Proliferation Requires the Phosphatidylinositol 3-Kinase/AKT/Glycogen Synthase Kinase Pathway." *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M804576200.

Giantonio, Bruce J., Christine Derry, Cecilia McAleer, Joseph J. McPhillips, and Peter J. O'Dwyer. 2004. "Phase I and Pharmacokinetic Study of the Cytotoxic Ether Lipid Ilmofosine Administered by Weekly Two-Hour Infusion in Patients with Advanced Solid Tumors." *Clinical Cancer Research*. https://doi.org/10.1158/1078-0432.CCR-0837-02.

Gupta, Gaorav P., and Joan Massagué. 2006. “Cancer Metastasis: Building a Framework.” *Cell*. https://doi.org/10.1016/j.cell.2006.11.001.

Han, Okhee, Guang Di Li, and Marc D Basson. 1998. “Strain Induces Caco-2 Intestinal Epithelial Proliferation and Differentiation via PKC and Tyrosine Kinase Signals.” *American Journal of Physiology*.

Hanahan, Douglas, Weinberg, Robert. 2000. “Hallmarks of Cancer.” *Cell*. https://doi.org/10.1007/s00262-010-0968-0.

Hartman, Z. R., M. D. Schaller, and Y. M. Agazie. 2013. “The Tyrosine Phosphatase SHP2 Regulates Focal Adhesion Kinase to Promote EGF-Induced Lamellipodia Persistence and Cell Migration.” *Molecular Cancer Research*. https://doi.org/10.1158/1541-7786.MCR-12-0578.

Hawkins, Paul C.D., and Anthony Nicholls. 2012. “Conformer Generation with OMEGA: Learning from the Data Set and the Analysis of Failures.” *Journal of Chemical Information and Modeling*. https://doi.org/10.1021/ci300314k.

Hawkins, Paul C.D., A. Geoffrey Skillman, and Anthony Nicholls. 2007. “Comparison of Shape-Matching and Docking as Virtual Screening Tools.” *Journal of Medicinal Chemistry*. https://doi.org/10.1021/jm0603365.

Hayashi, Naoko, Hiroshi Egami, Mikio Kai, Yuji Kurusu, Sadamu Takano, and Michio Ogawa. 1999. “No-Touch Isolation Technique Reduces Intraoperative Shedding of Tumor Cells into the Portal Vein during Resection of Colorectal Cancer.” *Surgery*. https://doi.org/10.1016/S0039-6060(99)70003-6.

Huynh, Khon C., Marianna Gyenes, Thi Hiep Nguyen, Toi Van Vo, and Volker R. Stoldt. 2017. “Impact of Shear Stress on Src and Focal Adhesion Kinase Phosphorylation in Fibrinogen-Adherent Platelets.” *Blood Coagulation and Fibrinolysis*. https://doi.org/10.1097/MBC.0000000000000593.

Hwang, Howook, Thom Vreven, Joël Janin, and Zhiping Weng. 2010. “Protein-Protein Docking Benchmark Version 4.0.” *Proteins: Structure, Function and Bioinformatics*. https://doi.org/10.1002/prot.22830.

Ingber, D. E. 2006. “Cellular Mechanotransduction: Putting All the Pieces Together Again.” *The FASEB Journal*. https://doi.org/10.1096/fj.05-5424rev.

Ingber, Donald E., Ning Wang, and Dimitrije Stamenović. 2014. “Tensegrity, Cellular Biophysics, and the Mechanics of Living Systems.” *Reports on Progress in Physics*. https://doi.org/10.1088/0034-4885/77/4/046603.

Irwin, John J., and Brian K. Shoichet. 2005. “ZINC - A Free Database of Commercially Available Compounds for Virtual Screening.” *Journal of Chemical Information and Modeling*. https://doi.org/10.1021/ci049714+.

Jain, Rakesh K., Yves Boucher, and Norman Wolmark. 1992. “Interstitial Hypertension in Human Breast and Colorectal Tumors.” *Cancer Research*.

Jakalian, Araz, David B. Jack, and Christopher I. Bayly. 2002. “Fast, Efficient Generation of High-Quality Atomic Charges. AM1-BCC Model: II. Parameterization and Validation.” *Journal of Computational Chemistry*. https://doi.org/10.1002/jcc.10128.

Johnson, David K., and John Karanicolas. 2013. “Druggable Protein Interaction Sites Are More Predisposed to Surface Pocket Formation than the Rest of the Protein Surface.” *PLoS Computational Biology*. https://doi.org/10.1371/journal.pcbi.1002951.

Kao, Y. C., J. R. Jheng, H. J. Pan, W. Y. Liao, C. H. Lee, and P. L. Kuo. 2017. “Elevated Hydrostatic Pressure Enhances the Motility and Enlarges the Size of the Lung Cancer Cells through Aquaporin Upregulation Mediated by Caveolin-1 and ERK1/2 Signaling.” *Oncogene*. https://doi.org/10.1038/onc.2016.255.

Kaspar, Allan A., and Janice M. Reichert. 2013. “Future Directions for Peptide Therapeutics Development.” *Drug Discovery Today*. https://doi.org/10.1016/j.drudis.2013.05.011.

Kleinschmidt, Elizabeth G., and David D. Schlaepfer. 2017. “Focal Adhesion Kinase Signaling in Unexpected Places.” *Current Opinion in Cell Biology*. https://doi.org/10.1016/j.ceb.2017.01.003.

Klinghoffer, Richard A., Christoph Sachsenmaier, Jonathan A. Cooper, and Philippe Soriano. 1999. “Src Family Kinases Are Required for Integrin but Not PDGFR Signal Transduction.” *EMBO Journal*. https://doi.org/10.1093/emboj/18.9.2459.

Ko, Hyo Rim, Il Sun Kwon, Inwoo Hwang, Eun Ju Jin, Joo Ho Shin, Angela M. Brennan-Minnella, Raymond Swanson, Sung Woo Cho, Kyung Hoon Lee, and Jee Yin Ahn. 2016. “Akt1-Inhibitor of DNA Binding2 Is Essential for Growth Cone Formation and Axon Growth and Promotes Central Nervous System Axon Regeneration.” *ELife*. https://doi.org/10.7554/eLife.20799.

Koch, Moritz, Peter Kienle, Ulf Hinz, Dalibor Antolovic, Jan Schmidt, Christian Herfarth, Magnus Von Knebel Doeberitz, and Jürgen Weitz. 2005. “Detection of Hematogenous Tumor Cell Dissemination Predicts Tumor Relapse in Patients Undergoing Surgical Resection of Colorectal Liver Metastases.” *Annals of Surgery*. https://doi.org/10.1097/01.sla.0000151795.15068.27.

Krieger, Elmar, Tom Darden, Sander B. Nabuurs, Alexei Finkelstein, and Gert Vriend. 2004. “Making Optimal Use of Empirical Energy Functions: Force-Field Parameterization in Crystal Space.” *Proteins: Structure, Function and Genetics*. https://doi.org/10.1002/prot.20251.

Labbé, Céline M., Guillaume Laconde, Mélaine A. Kuenemann, Bruno O. Villoutreix, and Olivier Sperandio. 2013. “IPPI-DB: A Manually Curated and Interactive Database of Small Non-Peptide Inhibitors of Protein-Protein Interactions.” *Drug Discovery Today*. https://doi.org/10.1016/j.drudis.2013.05.003.

Lamore, Sarah D., Ernst Ahlberg, Scott Boyer, Michelle L. Lamb, Maria P. Hortigon-Vinagre, Victor Rodriguez, Godfrey L. Smith, et al. 2017. “Deconvoluting Kinase Inhibitor Induced Cardiotoxicity.” *Toxicological Sciences*. https://doi.org/10.1093/toxsci/kfx082.

Le Devedec, SE.,Geverts B, de Bont H, Yan K, Verbeek FJ, Houtsmuller AB, and van der Water B. 2018. “The Residence Time of Focal Adhesion Kinase (FAK) and Paxillin at Focal Adhesions in Renal Epithelial Cells is Determined by Adhesion Size, Strength, and Life Cycle Status.” *J Cell Sci*.

Leunig, Michael, Rakesh K. Jain, Alwin E. Goetz, Konrad Messmer, and Ernst Kastenbauer. 1992. “Interstitial Hypertension in Head and Neck Tumors in Patients: Correlation with Tumor Size.” *Cancer Research*.

Li, Wei, Aydin Duzgun, Bauer E. Sumpio, and Marc D. Basson. 2001. “Integrin and FAK-Mediated MAPK Activation Is Required for Cyclic Strain Mitogenic Effects in Caco-2 Cells.” *Am J Physiol Gastrointest Liver Physiol*.

Li, Weiqun, Jiachang Zhang, Lawrence Flechner, Teresa Hyun, Alan Yam, Thomas F. Franke, and Jacalyn H. Pierce. 1999. “Protein Kinase C-α Overexpression Stimulates Akt Activity and Suppresses Apoptosis Induced by Interleukin 3 Withdrawal.” *Oncogene*. https://doi.org/10.1038/sj.onc.1203065.

Lietha, Daniel, Xinming Cai, Derek F.J. Ceccarelli, Yiqun Li, Michael D. Schaller, and Michael J. Eck. 2007. “Structural Basis for the Autoinhibition of Focal Adhesion Kinase.” *Cell*. https://doi.org/10.1016/j.cell.2007.05.041.

Lipinski, Christopher A., Franco Lombardo, Beryl W. Dominy, and Paul J. Feeney. 2012. “Experimental and Computational Approaches to Estimate Solubility and Permeability in Drug Discovery and Development Settings.” *Advanced Drug Delivery Reviews*. https://doi.org/10.1016/j.addr.2012.09.019.

Liu, T.-J., T. LaFortune, T. Honda, O. Ohmori, S. Hatakeyama, T. Meyer, D. Jackson, J. de Groot, and W.K. A. Yung. 2007. “Inhibition of Both Focal Adhesion Kinase and Insulin-like Growth Factor-I Receptor Kinase Suppresses Glioma Proliferation in

Vitro and in Vivo.” *Molecular Cancer Therapeutics*. https://doi.org/10.1158/1535-7163.MCT-06-0476.

Liu, Weiguo, David A. Bloom, William G. Cance, Elena V. Kurenova, Vita M. Golubovskaya, and Steven N. Hochwald. 2008. “FAK and IGF-IR Interact to Provide Survival Signals in Human Pancreatic Adenocarcinoma Cells.” *Carcinogenesis*. https://doi.org/10.1093/carcin/bgn026.

London, Nir, Barak Raveh, and Ora Schueler-Furman. 2013. “Druggable Protein-Protein Interactions - from Hot Spots to Hot Segments.” *Current Opinion in Chemical Biology*. https://doi.org/10.1016/j.cbpa.2013.10.011.

Luzzi, Keith J., Ian C. MacDonald, Eric E. Schmidt, Nancy Kerkvliet, Vincent L. Morris, Ann F. Chambers, and Alan C. Groom. 1998. “Multistep Nature of Metastatic Inefficiency: Dormancy of Solitary Cells after Successful Extravasation and Limited Survival of Early Micrometastases.” *American Journal of Pathology*. https://doi.org/10.1016/S0002-9440(10)65628-3.

Madara, Karen, John E. Janik, John W. Smith, and Dan L. Longo. 1993. “Interstitial Pressure of Subcutaneous Nodules in Melanoma and Lymphoma Patients: Changes during Treatment1.” *Cancer Research*.

Mastroleo, Felice, Rob van Houdt, Steve Atkinson, Max Mergeay, Larissa Hendrickx, Ruddy Wattiez, and Natalie Leys. 2013. “Modelled Microgravity Cultivation Modulates N-Acylhomoserine Lactone Production in Rhodospirillum Rubrum S1H Independently of Cell Density.” *Microbiology (United Kingdom)*. https://doi.org/10.1099/mic.0.066415-0.

McGaughey, Georgia B., Robert P. Sheridan, Christopher I. Bayly, J. Chris Culberson, Constantine Kreatsoulas, Stacey Lindsley, Vladimir Maiorov, Jean Francois Truchon, and Wendy D. Cornell. 2007. “Comparison of Topological, Shape, and Docking Methods in Virtual Screening.” *Journal of Chemical Information and Modeling*. https://doi.org/10.1021/ci700052x.

Miller, Kimberly D., Rebecca L. Siegel, Chun Chieh Lin, Angela B. Mariotto, Joan L. Kramer, Julia H. Rowland, Kevin D. Stein, Rick Alteri, and Ahmedin Jemal. 2016. “Cancer Treatment and Survivorship Statistics, 2016.” *CA: A Cancer Journal for Clinicians*. https://doi.org/10.3322/caac.21349.

Molladavoodi, Sara, Matthew Robichaud, David Wulff, and Maud Gorbet. 2017. “Corneal Epithelial Cells Exposed to Shear Stress Show Altered Cytoskeleton and Migratory Behaviour.” *PLoS ONE*. https://doi.org/10.1371/journal.pone.0178981.

Moran-Jones, Kim, Laura M. Brown, and Goli Samimi. 2015. “INC280, an Orally Available Small Molecule Inhibitor of c-MET, Reduces Migration and Adhesion in Ovarian Cancer Cell Models.” *Scientific Reports*. https://doi.org/10.1038/srep11749.

Motro, René. 2003. *Tensegrity: Structural Systems for the Future*. *Tensegrity: Structural Systems for the Future*. https://doi.org/10.1016/B978-1-903996-37-9.X5028-8.

Nishizaki, Takashi, Takashi Matsumata, Takashi Kanematsu, Chikao Yasunaga, and Keizo Sugimachi. 1990. “Surgical Manipulation of VX2 Carcinoma in the Rabbit Liver Evokes Enhancement of Metastasis.” *Journal of Surgical Research*. https://doi.org/10.1016/0022-4804(90)90116-J.

Oki, Yasuhiro, Michelle Fanale, Jorge Romaguera, Luis Fayad, Nathan Fowler, Amanda Copeland, Felipe Samaniego, et al. 2015. “Phase II Study of an AKT Inhibitor MK2206 in Patients with Relapsed or Refractory Lymphoma.” *British Journal of Haematology*. https://doi.org/10.1111/bjh.13603.

Oltersdorf, Tilman, Steven W. Elmore, Alexander R. Shoemaker, Robert C. Armstrong, David J. Augeri, Barbara A. Belli, Milan Bruncko, et al. 2005. “An Inhibitor of Bcl-2 Family Proteins Induces Regression of Solid Tumours.” *Nature*. https://doi.org/10.1038/nature03579.

Pachioni, Juliana de Almeida, Juliana Gallottini Magalhães, Elys Juliane Cardoso Lima, Luciana de Moura Bueno, Joyce Ferreira Barbosa, Matheus Malta de Sá, and Carlota de Oliveira Rangel-Yagui. 2013. “Alkylphospholipids - A Promising Class of Chemotherapeutic Agents with a Broad Pharmacological Spectrum.” *Journal of Pharmacy and Pharmaceutical Sciences*. https://doi.org/10.18433/J3CW23.

Parisi, Cristian, Vikesh V. Chandaria, and Niamh C. Nowlan. 2018. “Blocking Mechanosensitive Ion Channels Eliminates the Effects of Applied Mechanical Loading on Chick Joint Morphogenesis.” *Philosophical Transactions of the Royal Society B: Biological Sciences*. https://doi.org/10.1098/rstb.2017.0317.

Parsons, J. T. 2003. “Focal Adhesion Kinase: The First Ten Years.” *Journal of Cell Science*. https://doi.org/10.1242/jcs.00373.

Patel, Hitesh, Nadia Le Marer, Richard Q. Wharton, Zulfiqar A.J. Khan, Ruth Araia, Clare Glover, Michael M. Henry, and Timothy G. Allen-Mersh. 2002. “Clearance of Circulating Tumor Cells after Excision of Primary Colorectal Cancer.” *Annals of Surgery*. https://doi.org/10.1097/00000658-200202000-00010.

Perry, Brandon C., Shouye Wang, and Marc D. Basson. 2010. “Extracellular Pressure Stimulates Adhesion of Sarcoma Cells via Activation of Focal Adhesion Kinase and Akt.” *American Journal of Surgery*. https://doi.org/10.1016/j.amjsurg.2010.07.013.

Petsalaki, Evangelia, and Robert B. Russell. 2008. “Peptide-Mediated Interactions in Biological Systems: New Discoveries and Applications.” *Current Opinion in Biotechnology*. https://doi.org/10.1016/j.copbio.2008.06.004.

Prevelige, Peter, and Gerald D. Fasman. 1989. “Chou-Fasman Prediction of the Secondary Structure of Proteins.” In *Prediction of Protein Structure and the Principles of Protein Conformation*. https://doi.org/10.1007/978-1-4613-1571-1_9.

Raj, Monika, Brooke N. Bullock, and Paramjit S. Arora. 2013. “Plucking the High Hanging Fruit: A Systematic Approach for Targeting Protein-Protein Interactions.” *Bioorganic and Medicinal Chemistry*. https://doi.org/10.1016/j.bmc.2012.11.023.

Raschka, S, S K More, D Devadoss, B Zeng, L A Kuhn, and M D Basson. 2018. “Identification of Potential Small-Molecule Protein-Protein Inhibitors of Cancer Metastasis by 3D Epitope-Based Computational Screening.” *Journal of Physiology and Pharmacology : An Official Journal of the Polish Physiological Society* 69 (2). https://doi.org/10.26402/jpp.2018.2.11.

Sadoshima, Junichi, and Seigo Izumo. 1997. “THE CELLULAR AND MOLECULAR RESPONSE OF CARDIAC MYOCYTES TO MECHANICAL STRESS.” *Annual Review of Physiology*. https://doi.org/10.1146/annurev.physiol.59.1.551.

Shapovalov, Maxim V., and Roland L. Dunbrack. 2011. “A Smoothed Backbone-Dependent Rotamer Library for Proteins Derived from Adaptive Kernel Density Estimates and Regressions.” *Structure*. https://doi.org/10.1016/j.str.2011.03.019.

Shiratsuchi, Hiroe, and Marc D. Basson. 2007. “Akt2, but Not Akt1 or Akt3 Mediates Pressure-Stimulated Serum-Opsonized Latex Bead Phagocytosis through Activating MTOR and P70 S6 Kinase.” *Journal of Cellular Biochemistry*. https://doi.org/10.1002/jcb.21295.

Shiratsuchi, Hiroe, and Marc D Basson. 2004. “Extracellular Pressure Stimulates Macrophage Phagocytosis by Inhibiting a Pathway Involving FAK and ERK.” *American Journal of Physiology. Cell Physiology*. https://doi.org/10.1152/ajpcell.00553.2003.

Souers, Andrew J., Joel D. Leverson, Erwin R. Boghaert, Scott L. Ackler, Nathaniel D. Catron, Jun Chen, Brian D. Dayton, et al. 2013. “ABT-199, a Potent and Selective BCL-2 Inhibitor, Achieves Antitumor Activity While Sparing Platelets.” *Nature Medicine*. https://doi.org/10.1038/nm.3048.

Tamura, Masahito, Jianguo Gu, Erik H J Danen, Takahisa Takino, Shingo Miyamoto, and Kenneth M. Yamada. 1999. “PTEN Interactions with Focal Adhesion Kinase and Suppression of the Extracellular Matrix-Dependent Phosphatidylinositol 3-Kinase/Akt Cell Survival Pathway.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.274.29.20693.

Tatsumi, Hitoshi, Masatsugu Toyota, Takuya Furuichi, and Masahiro Sokabe. 2014. “Calcium Mobilizations in Response to Changes in the Gravity Vector in

Arabidopsis Seedlings Possible Cellular Mechanisms." *Plant Signaling and Behavior*. https://doi.org/10.4161/psb.29099.

Thamilselvan, Vijayalakshmi, and Marc D. Basson. 2004. "Pressure Activates Colon Cancer Cell Adhesion by Inside-Out Focal Adhesion Complex and Actin Cytoskeletal Signaling." *Gastroenterology*. https://doi.org/10.1053/j.gastro.2003.10.078.

Thamilselvan, Vijayalakshmi, David H Craig, and Marc D Basson. 2007. "FAK Association with Multiple Signal Proteins Mediates Pressure-Induced Colon Cancer Cell Adhesion via a Src-Dependent PI3K/Akt Pathway." *The FASEB Journal : Research Communication*. https://doi.org/10.1096/fj.06-6545com.

Thomas, J W, M A Cooley, J M Broome, R Salgia, J D Griffin, C R Lombardo, and M D Schaller. 1999. "The Role of Focal Adhesion Kinase Binding in the Regulation of Tyrosine Phosphorylation of Paxillin." *The Journal of Biological Chemistry*. https://doi.org/10.1074/JBC.274.51.36684.

Thubrikar, Mano J., and Francis Robicsek. 1995. "Pressure-Induced Arterial Wall Stress and Atherosclerosis." *The Annals of Thoracic Surgery*. https://doi.org/10.1016/0003-4975(94)01037-D.

Toker, Alex, and Sandra Marmiroli. 2014. "Signaling Specificity in the Akt Pathway in Biology and Disease." *Advances in Biological Regulation*. https://doi.org/10.1016/j.jbior.2014.04.001.

Tschopp, O. 2005. "Essential Role of Protein Kinase B (PKB /Akt3) in Postnatal Brain Development but Not in Glucose Homeostasis." *Development*. https://doi.org/10.1242/dev.01864.

Turnbull, R. B. 1970. "Cancer of the Colon. The Five- and Ten-Year Survival Rates Following Resection Utilizing the Isolation Technique." *Annals of the Royal College of Surgeons of England*. https://doi.org/10.5833/jjgs.9.539.

Vassilev, Lyubomir T., Binh T. Vu, Bradford Graves, Daisy Carvajal, Frank Podlaski, Zoran Filipovic, Norman Kong, et al. 2004. "In Vivo Activation of the P53 Pathway by Small-Molecule Antagonists of MDM2." *Science*. https://doi.org/10.1126/science.1092472.

Walsh, Mary F., Vijayalakshmi Thamilselvan, Rainer Grotelueschen, Lulu Farhana, and Marc D. Basson. 2003. "Absence of Adhesion Triggers Differential FAK and SAPKp38 Signals in SW620 Human Colon Cancer Cells That May Inhibit Adhesiveness and Lead to Cell Death." *Cellular Physiology and Biochemistry*. https://doi.org/10.1159/000071864.

Wang, S., and M. D. Basson. 2011. “Akt Directly Regulates Focal Adhesion Kinase through Association and Serine Phosphorylation: Implication for Pressure-Induced Colon Cancer Metastasis.” *AJP: Cell Physiology*. https://doi.org/10.1152/ajpcell.00377.2010.

Wang, Shouye, and Marc D Basson. 2011. “Protein Kinase B/AKT And Focal Adhesion Kinase: Two Close Signaling Partners in Cancer.” *Anti-Cancer Agents in Medicinal Chemistry*. https://doi.org/10.2174/187152011797927661.

Weitz, Jürgen, Moritz Koch, Peter Kienle, Andrea Schrödel, Frank Willeke, Axel Benner, Thomas Lehnert, Christian Herfarth, and Magnus Von Knebel Doeberitz. 2000. “Detection of Hematogenic Tumor Cell Dissemination in Patients Undergoing Resection of Liver Metastases of Colorectal Cancer.” *Annals of Surgery*. https://doi.org/10.1097/00000658-200007000-00010.

Wells, James A., and Christopher L. McClendon. 2007. “Reaching for High-Hanging Fruit in Drug Discovery at Protein-Protein Interfaces.” *Nature*. https://doi.org/10.1038/nature06526.

Wen, Li, Tanveer A. Javed, Dean Yimlamai, Amitava Mukherjee, Xiangwei Xiao, and Sohail Z. Husain. 2018. “Transient High Pressure in Pancreatic Ducts Promotes Inflammation and Alters Tight Junctions via Calcineurin Signaling in Mice.” *Gastroenterology*. https://doi.org/10.1053/j.gastro.2018.06.036.

Wolfenson, Haguy, Bo Yang, and Michael P Sheetz. 2018. “Steps in Mechanotransduction Pathways That Control Cell Morphology.” *Annual Review of Physiology* 81 (1): annurev-physiol-021317-121245. https://doi.org/10.1146/annurev-physiol-021317-121245.

Wu, Meng Yao, Rong Rui Liang, Kai Chen, Meng Shen, Ya Li Tian, Dao Ming Li, Wei Ming Duan, et al. 2015. “FH535 Inhibited Metastasis and Growth of Pancreatic Cancer Cells.” *OncoTargets and Therapy*. https://doi.org/10.2147/OTT.S82718.

Wu, Rong, Xiao Meng Liu, Jian Guang Sun, Hong Chen, Jun Ma, Meng Dong, Shengyi Peng, et al. 2017. “DJ-1 Maintains Energy and Glucose Homeostasis by Regulating the Function of Brown Adipose Tissue.” *Cell Discovery*. https://doi.org/10.1038/celldisc.2016.54.

Yamaguchi, Kazuya, Yukihiro Takagi, Shinichirou Aoki, Manabu Futamura, and Shigetoyo Saji. 2000. “Significant Detection of Circulating Cancer Cells in the Blood by Reverse Transcriptase-Polymerase Chain Reaction during Colorectal Cancer Resection.” *Annals of Surgery*. https://doi.org/10.1097/00000658-200007000-00009.

Zahiri, Javad, Joseph Bozorgmehr, and Ali Masoudi-Nejad. 2013. “Computational Prediction of Protein–Protein Interaction Networks: Algorithms and Resources.” *Current Genomics*. https://doi.org/10.2174/1389202911314060004.

Zanotelli, Matthew R., and Cynthia A. Reinhart-King. 2018. “Mechanical Forces in Tumor Angiogenesis.” In *Advances in Experimental Medicine and Biology*, 1092:91–112. https://doi.org/10.1007/978-3-319-95294-9_6.

Zeng, Bixi, Dinesh Devadoss, Shouye Wang, Emilie E. Vomhof-DeKrey, Leslie A. Kuhn, and Marc D. Basson. 2017. “Inhibition of Pressure-Activated Cancer Cell Adhesion by FAK-Derived Peptides.” *Oncotarget* 8 (58): 98051–67. https://doi.org/10.18632/oncotarget.20556.

Zhang, Jianhu, Cheri R. Owen, Matthew A. Sanders, Jerrold R. Turner, and Marc D. Basson. 2006. “The Motogenic Effects of Cyclic Mechanical Strain on Intestinal Epithelial Monolayer Wound Closure Are Matrix Dependent.” *Gastroenterology*. https://doi.org/10.1053/j.gastro.2006.08.007.

CHAPTER 2

Binding domains of FAK and Akt1

The following papers are the work of Dr. Shouye Wang and myself. The papers were authored by me and the experiments were performed by Dr. Wang. They are included here to provide a more detailed background behind how we came to discover the regions of FAK and Akt1 implicated in their binding. The findings in this chapter have been previously published:

Marc D. Basson, **Bixi Zeng,** and Shouye Wang (2015). Akt1 binds focal adhesion kinase via the Akt1 kinase domain independently of the pleckstrin homology domain. **J Physiol Pharmacol**, 66(5):701-9.

Marc D. Basson, **Bixi Zeng,** and Shouye Wang (2017). The C-terminal region of the FAK F1 domain binds Akt1 and inhibits pressure-induced cell adhesion. **J Physiol Pharmacol,** 68(3):375-383.

2.1 Akt1 binds FAK via the Akt1 Kinase Domain Independently of the Pleckstrin Homology Domain

Marc D. Basson, **Bixi Zeng,** and Shouye Wang (2015). Akt1 binds focal adhesion kinase via the Akt1 kinase domain independently of the pleckstrin homology domain. **J Physiol Pharmacol,** 66(5):701-9.

2.1a Introduction

Cancer metastasis requires adhesion to a new substrate by cancer cells that have traveled from the primary tumor to the metastatic site. Circulating tumor cells increase dramatically during oncologic surgery while tumor cells are also easily recovered from the peritoneal cavity after colon cancer resection (Weitz et al. 2000; Choy et al. 1996; Uchikura et al. 2002; Hayashi et al. 1999; Yamaguchi et al. 2000; Summy et al. 2003; Allardyce et al. 1996). It is not possible to quantitate the impact of surgical intervention upon tumor dissemination, since new metastases cannot be distinguished from pre-existing metastases that were simply too small to detect. However, laparoscopic surgery is associated with a 1.14% increase in wound recurrence, which is the appearance of tumor growth along surgical wounds, when compared to laparotomy (Walker et al. 2012). Additionally, murine models show that tumor dissemination remains a concern for the oncologic surgeon, and designing a drug to block tumor cell adhesion would seem a worthwhile goal. Forces such as pressure and shear are present in the circulation due to hemodynamic forces and in the surgical environment due to laparoscopic insufflation pressures, tumor manipulation, and irrigation forces. Modest (15 mmHg) increases in extracellular pressure activate a signal cascade within cancer cells that ultimately results in phosphorylation of the cytoplasmic tail of the beta 1 integrin subunit, propagating a conformational change that

opens the extracellular matrix binding domain of beta 1 integrin heterodimers and increases cancer cell adhesiveness (Basson 2008; Craig et al. 2009). Other physical forces, such as shear force have similar effects (Thamilselvan et al. 2008). This pathway can be targeted either pharmacologically or by more specific molecular techniques to block this increase in adhesion and substantially increase tumor free survival in animal models (Craig et al. 2008; Craig, Downey, and Basson 2008). However, blocking common intracellular signals leads also to non-specific toxicity, as these signals are likely to regulate other important aspects of the organism's biology. Indeed, the dose of colchicine used in previous pharmacologic blockade studies of pressure stimulated cancer adhesion is too high for human therapy (Craig, Downey, and Basson 2008). Blocking a less common element of the signal pathway that regulates cancer cell adhesiveness would seem more likely to be tolerated by a patient.

Promising candidates for pharmacologic targeting are the non-receptor tyrosine kinase focal adhesion kinase (FAK) and the serine/threonine kinase Akt. Both FAK and Akt play important roles in normal cell physiology, and both kinases are activated and/or overexpressed in a variety of cancers (Yoon et al. 2015; Clark et al. 2014). Classically, FAK activation is the result of surface integrin engagement, which places FAK near the top of several signaling cascades that work to transmit information from the external environment to actors within the cell (Parsons et al. 2003). FAK associates with Cas and Src to promote the focal adhesion turnover required in cell migration, and it is also required in the cooperative signaling pathway that exists between integrins and growth factors, which controls cell cycle progression and proliferation through the MEK-ERK

axis (Parsons et al. 2003; Klinghoffer et al. 1999). Indeed, FAK signaling is required for cell survival in many adherent cells, and its absence triggers anoikis (Walsh et al. 2003). However, FAK also participates in a range of physical force-mediated signaling events. For instance, repetitive deformation of adherent intestinal epithelial monolayers flexes the cytoskeleton and modulates cell proliferation, motility, and differentiation via FAK in a manner that varies with matrix substratum and integrin-binding (Han et al. 1998; Zhang et al. 2006; Li et al. 2001; Zhang, Li, and Sanders 2003). Conversely, increases in extracellular pressure can stimulate cancer cell adhesiveness by increasing FAK activation before the cell's integrins have engaged with the matrix (Craig et al. 2007). Interestingly, similarly increased extracellular pressure stimulates phagocytic cells to increase their phagocytic ability via a decrease in FAK activation (Shiratsuchi and Basson 2004; Shiratsuchi and Basson 2005).

Akt also contributes to a range of cell processes such as cell proliferation and survival. Akt classically has a complex activation scheme that involves PIP3-mediated membrane translocation and phosphorylation by both PDK1 and mTORC2. Upon activation, Akt loses its membrane restrictions and enjoys a high degree of intracellular mobility, which is directed in part by isoform preference (Toker et al. 2014). While Akt isoforms have a limited capability to compensate for one another, they also exhibit an assortment of unique functions (Toker et al. 2014) In murine knockout models, Akt1 proved to be responsible for overall growth, Akt2 was required for proper insulin signaling, and the loss of Akt3 manifested in a decrease in brain size (Chen et al. 2001; Cho et al. 2001; Tschopp et al. 2005). In the context of cancer, unregulated FAK and Akt

activity have been repeatedly implicated in the machinations of tumors. However, as previously mentioned, the non-specific inhibition of such wide-reaching cellular elements may prove to be inextricably tied to unintended toxicities that limit their feasibility as drug targets.

While it may not be prudent to base treatments off of global kinase blockade, a viable option could be the inhibition of unique interactions exhibited by these kinases. FAK and Akt have been tied to numerous processes that are initiated by physical forces. Pressure-mediated macrophage phagocytosis progresses through a mechanism that inhibits FAK but activated Akt2 (Shiratsuchi and Basson 2004; Shiratsuchi and Basson 2007). In Caco-2 colon cancer cells, both cyclic strain-mediated migration and proliferation require the activation of FAK and Akt (Gayer et al. 2009; Gayer, Chaturvedi, Wang, and Craig 2009). Similarly, pressure-mediated Caco-2 adhesion also requires FAK and Akt activation (Wang and Basson 2011). We have previously delineated a novel interaction between Focal Adhesion Kinase (FAK) and Akt1 that is required for pressure to stimulate cancer cell adhesion. While FAK is normally considered to act at the focal adhesion complex, there are much larger pools of FAK within the cytosol, and there is a constant equilibrium between the two (Le Devedec et al. 2018). The signals activated by increases in extracellular pressure in cancer cells in suspension cause Akt-1 to bind to FAK in the cytosol and phosphorylate FAK at three previously uncharacterized serines (Wang and Basson 2011). This interaction is required for subsequent FAK tyrosine 397 autophosphorylation, activation, and translocation to the focal adhesion complex where it further influences integrin binding strength (Wang and

Basson 2011). Although it is not known how this specific FAK-Akt1 interaction responds to variations in the magnitude and duration of extracellular pressure, at the cellular level, increases in adhesion have been observed over a range of pressures (10-30 mmHg) and can be elicited equally well by exposures of 1 minute as by those lasting 30 minutes (Craig et al. 2009; Basson et al. 2000). The increase in adhesiveness engendered by increased pressure persists for at least 30 minutes after the higher pressure has been returned to baseline (Craig et sl 2009).

Direct interaction between Akt-1 and FAK has not been identified outside of pressure-stimulated cell adhesion suggesting that blocking the Akt-1-FAK interaction might be less toxic than blocking all FAK activity. Indeed, even with regard to physical force effects, the mitogenic effects of pressure in adherent cancer cells are completely independent of the PI-3-Kinase-Akt axis while the effects of repetitive deformation on Caco-2 colon cancer cell proliferation require Akt-2, not Akt-1 (Gayer, Chaturvedi, Wang, Alston 2009; Walsh et al. 2004). Previous studies using chimeras made by splicing different domains of Akt-1 and Akt-2 have suggested that the specificity of this interaction for FAK activation might rest in the PH-domain and hinge region of Akt-1 (Wang and Basson 2008). We therefore now sought to further characterize the site on Akt which binds to FAK as a preliminary to attempting to design a drug to block this interaction. Serial truncations of Akt-1 were constructed and tested for their ability to bind to FAK.

2.1b Materials and Methods

Materials

Caco-2 colon cancer cells were cultured according to American Type Culture Collection (ATCC, Rockville, MD) recommendations. We obtained Lipofectamine 2000 and other transfection supplies from Invitrogen (Carlsbad, CA), Glutathione Sepharose 4B beads from GE Life Sciences (Pittsburg, PA), Akt1 and GST antibodies from Cell Signaling Technology (Beverly, MA), anti-hemagglutinin (HA, clone 12CA5) monoclonal antibodies from Roche Applied Science (Indianapolis, IN). pCMV-HA vector was obtained from Clontech (Mountain View, CA). pcDNA3 myr HA Akt1 was provided by Dr. Paula Herman (Dana Farber Cancer Institute) through Addgene (Cambridge, MA). pcDNA3 HA-FAK was generated as previously described (Wang and Basson 2011). pGEX GST-Akt1 and its truncations were a generous gift from Dr. Chi Bun Chan (Emory University School of Medicine). All primers were purchased from Integrated DNA Technologies (Coralville, IA). QIAquick Gel Extraction, QIAprep spin Miniprep, QIAquick PCR purification and QIAfilter Plasmid Maxi kits were purchased from Qiagen (Valencia, CA).

Generation of Constructs

Mammalian expression vectors pCMV-HA-Akt1 and its truncations were constructed via PCR by introducing 5'*Eco*R I and 3'*Kpn* I cut sites into a pcDNA3 myr HA Akt1 template. Products were then subcloned into the *Eco*R I/*Kpn* I double digested pCMV-HA cassette to get pCMV-HA-Akt. A similar protocol was used to generate pGEX-4T-1 GST FAK-NT and pGEX-4T-1 GST FAK-NT1 from pcDNA3 HA-FAK.

Transfections

Caco-2 cells were plated on p100 dishes at 30–35% confluence one day prior to transfection. Briefly, the constructed plasmids or empty plasmid were transfected into Caco-2 cells at final concentrations of 2 μg/ml plasmid and 5 μg/ml Lipofectamine 2000. Five hours after transfection, the medium was replaced with 15 ml pre-warmed Caco-2 media without antibiotics. Forty eight hours after DNA transfection, the cells were lysed for pull-down assays.

Glutathione S-transferase Pull-down

Glutathione Sepharose 4B beads were blocked with 1% bovine serum albumin for 1hr at room temperature and washed before being conjugated with recombinant GST-tagged proteins under similar conditions. Conjugated beads were incubated with lysate from transfected cells overnight at 4°C. The beads were washed to remove the unbound proteins. Bound proteins were eluted by addition of loading buffer with sodium dodecyl sulfate (SDS) and heating at 95°C for 5 min in preparation for western analysis.

Western Blotting

Western blots were performed as previously described [38]. Eluate from the pull-downs were resolved by SDS-polyacrylamide gel electrophoresis and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were blotted with specific antibodies directed against either their wild-type structures or recombinant tags with the appropriate secondary antibody coupled to horseradish peroxidase. Bands were detected with enhanced chemiluminescence

(Amersham) and analyzed with a Kodak Image Station 440CF (Perkin Elmer, Boston, MA).

Akt1 Structural Analysis

Structures were obtained from the Protein Data Bank (http://www.rcsb.org): 3CQW (active Akt1 with ATP-competitive inhibitor bound kinase) and 3O96 (cytoplasmic PH domain of Akt1) (Lippa et al. 2008; Wu et al. 2010). Structures were analyzed using Pymol from DeLano Scientific (San Carlos, CA).

Statistical analysis

Results were compared by Student's unpaired t-test and considered statistically significant when $p < 0.05$. All experiments were done independently at least three times unless indicated otherwise. All data are expressed as mean ± SEM.

2.1c Results

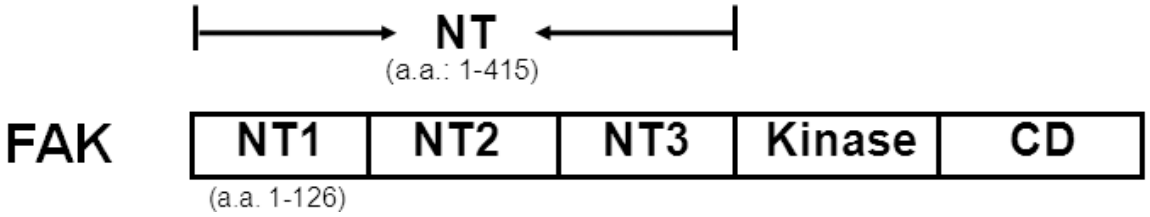
The FAK (NT1) Region is Sufficient to Bind Akt1

The wild-type full-length FAK molecule was truncated at its band 4.1, ezrin, radixin, and moesin (FERM) domain to discern its necessity for Akt1 binding. These truncations created two shortened FAK molecules. NT (residues 1-415) consisted of only the FERM domain, and NT1 (residues 1-126) contained the F1 subdomain of the FERM domain (Figure 2.1A). Each of these truncations was able to bind Akt1 (Figure 2.1B. One of four representative blots).

Akt1 Binds FAK (NT1) Independently of the Pleckstrin Homology Domain and Hinge Region

Akt1 truncations were generated to determine the role of the pleckstrin homology domain (residues 1-106) and the hinge region (residues 107-147) in binding FAK. These initial serial truncations began with the (N)-terminal half of the PH domain, extended

A



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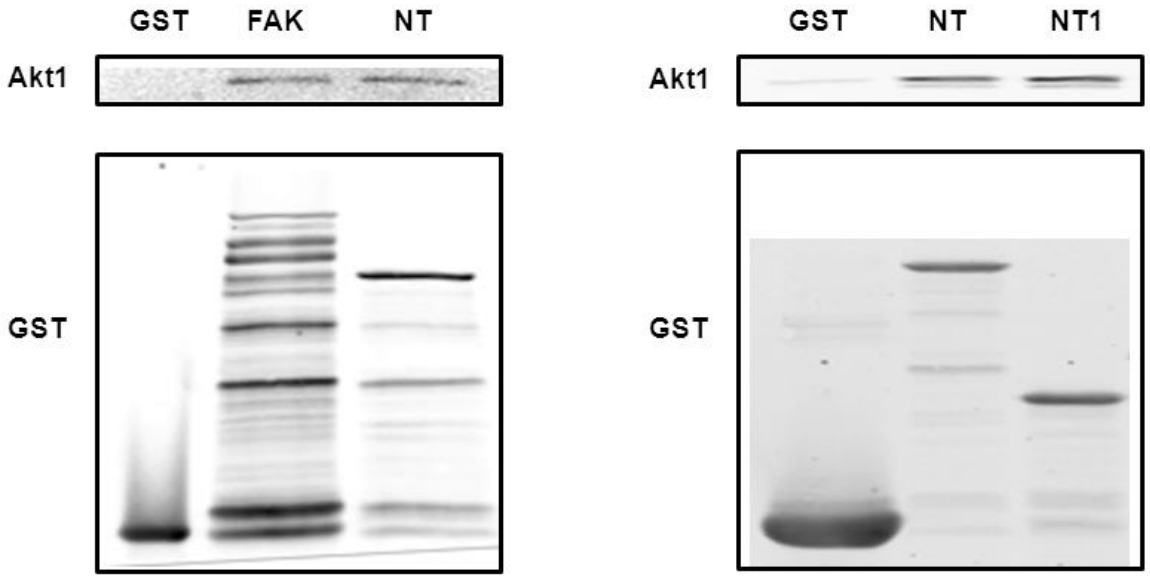


Figure 2.1. The FAK (NT1) region binds Akt1
Recombinant GST-FAK truncations NT (residues 1-415) and NT1 (residues 1-126) (shown in A) were conjugated to Glutathione Sepharose 4B beads and used to pull down recombinant HA-Akt1. All four truncations were able to pull down Akt1 (B, N=4 similarly). In each lane, the GST blot shows the presence of the FAK truncations (top band), the detached GST tags (bottom band), and protein degradation products (middle bands).

to include the (N)-terminal half of the hinge region, and ended with the complete deletion of both domains (Figure 2.2A). The endpoints of these truncations were chosen to either disrupt the natural folding of the PH and hinge domains (HA-Akt1Δ1-66, HA-Akt1Δ1-125, respectively) or to remove them outright (HA-Akt1Δ1-147). GST-FAK (NT1), which is a FAK truncation consisting of the first 126 amino acids from its (N)-terminal, was used as bait to bind the Akt1 truncations. Both the most conservative (HA-Akt1Δ1-147) and least conservative (HA-Akt1Δ1-66) Akt1 truncations were pulled down well by the GST-FAK (NT1) coated beads (Figure 2.2B, N=2). However, the intermediate truncation (HA-Akt1Δ1-125) was not detectably bound to the GST-FAK (NT1) coated beads (Figure 2.2B, N=2).

A

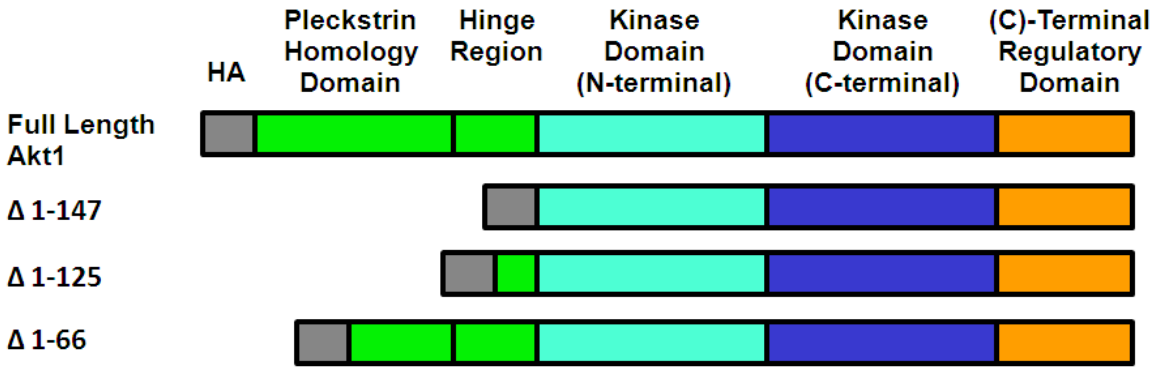
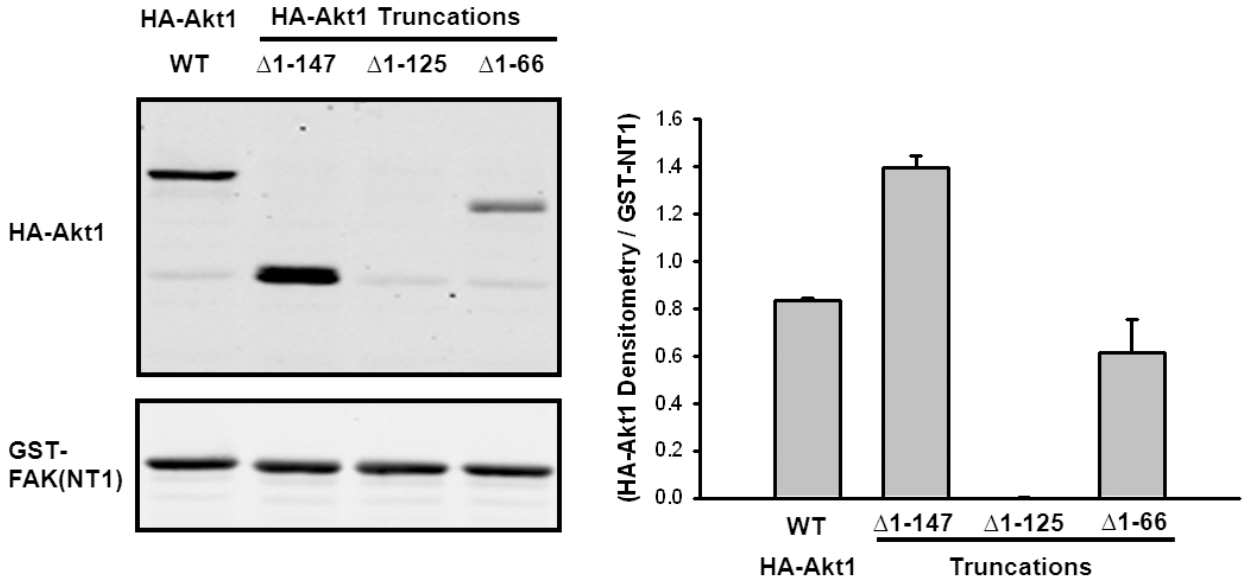


Figure 2.2. Akt1 / FAK (NT1) binding does not require the pleckstrin homology domain and hinge region
(A) A delineation of the constructs generated from the original Akt-1 structure. The Akt1 pleckstrin homology domain spans residues 1-106 and the hinge region residues 107-147. Akt1 truncations were generated lacking both the entire PH domain and hinge region (Δ1-147); all of the PH domain and the (N)-terminal half of the hinge region (Δ1-125); and only the (N)-terminal half of the PH domain (Δ1-66). (B) Glutathione Sepharose 4B beads were coated with GST-FAK(NT1) to pull down HA-Akt1 truncations

Figure 2.2 (cont'd)

obtained from lysate of transfect Caco-2 cells. Appreciable amounts of HA-Akt1, HA-Akt1Δ1-147, and HA-Akt1Δ1-66 were all pulled down by the GST-NT1 coated beads. However, HA-Akt1Δ1-125 was not detectable (N=2 similarly).

B



The Akt1 Kinase Domain is Required to Bind FAK

Further Akt1 truncations were generated to determine the role of the kinase domain (residues 149-408) and the (C)-terminal regulatory domain (residues 409-480) in binding FAK. Construct 1 consisted of only the (C)-terminal regulatory domain. Constructs 2 and 3 consisted of the (C) and (N)-terminal halves of the Akt1 molecule, respectively. Construct 4 consisted of only the kinase domain (Figure 2.3A). All constructs were fused to GST at the (N)-terminus and were used to pull down endogenous wild type FAK from Caco-2 cell lysate. Construct 3 demonstrated an affinity for FAK that was significantly greater than that of the wild-type full-length Akt1 (Figure 2.3B, N=4; $p<0.05$). This is consistent with our data showing the full-length PH and

hinge domains to permit Akt1/FAK binding. Construct 2 and construct 4 each displayed an ability to bind FAK similar to that of full-length Akt1 (Figure 2.3B, N=4). Construct 1, however, which consisted of only the (C)-terminal regulatory domain, demonstrated a significantly lower affinity for FAK (Figure 2.3B, N=6; $p<0.05$).

A

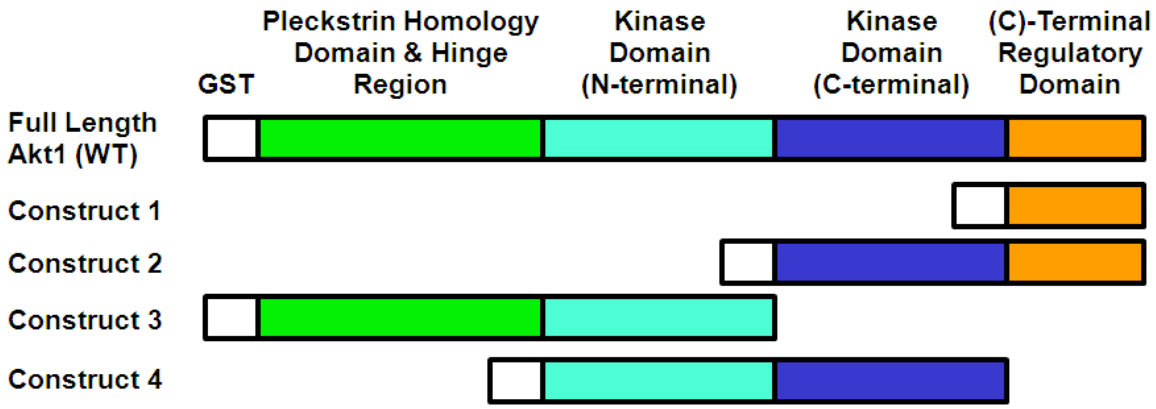
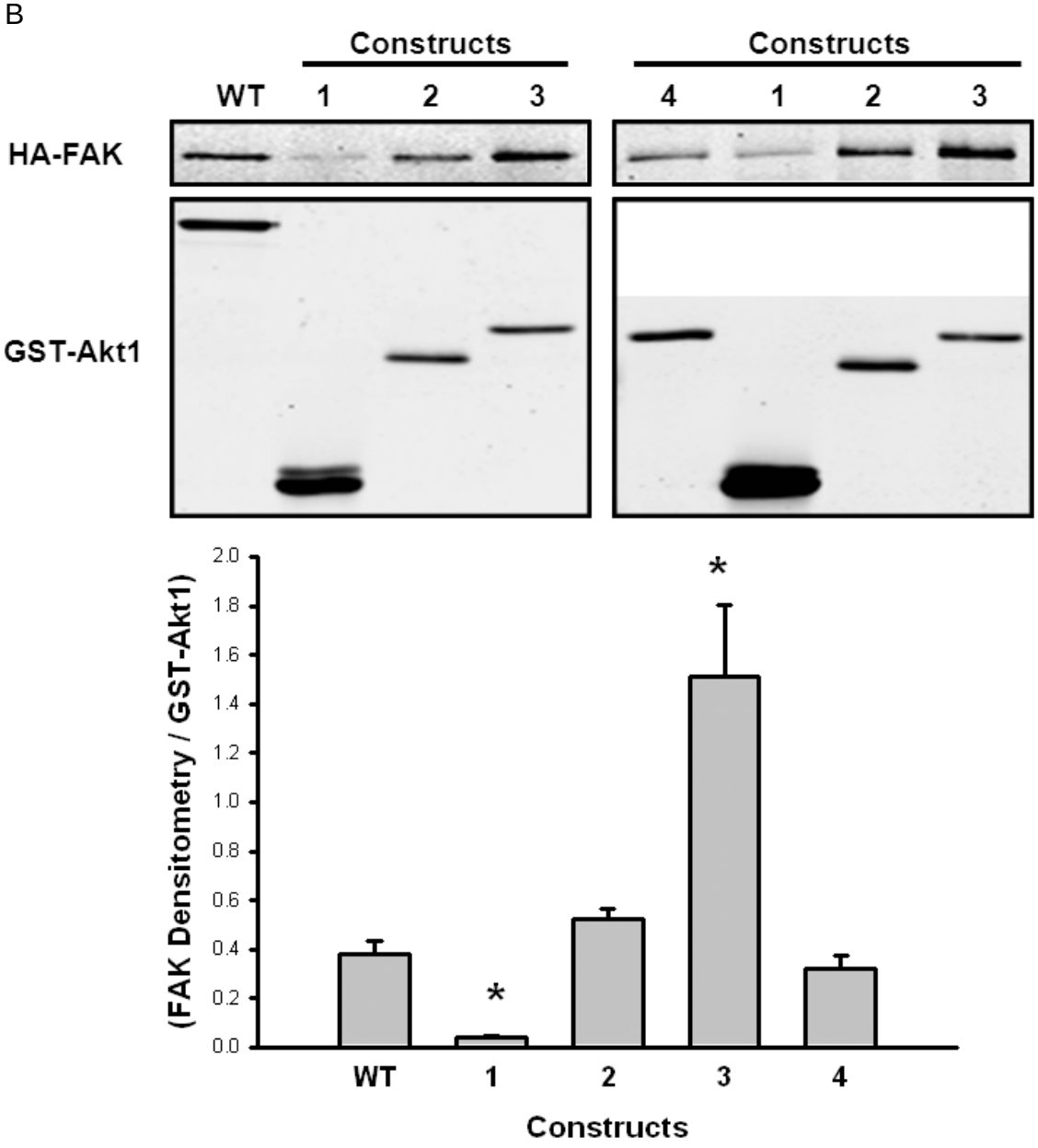


Figure 2.3. Akt1 / FAK binding requires the Akt1 kinase domain
(A) The Akt1 pleckstrin homology domain and hinge region span residues 1-147; the kinase domain spans residues 149-408; and the (C)-terminal regulatory domain spans residues 409-480. GST-fused constructs were generated with truncations targeting these domains in their entirety. Construct 1 is a deletion of the PH/hinge domain and the kinase domain. Construct 2 is a deletion of the PH/hinge domain and the (N)-terminal half of the kinase domain. Construct 3 is a deletion of the (C)-terminal half of the kinase domain and the (C)-terminal regulatory domain. Construct 4 is a deletion of the PH/hinge domain and the (C)-terminal regulatory domain. (B) Akt1 constructs were conjugated to Glutathione Sepharose 4B beads and incubated with lysate from transfected Caco-2 cells; and subsequent westerns were blotted for HA-FAK. Akt1 constructs 2 and 4 showed affinities for HA-FAK similar to that of wild-type full-length Akt1 (N=4) while construct 3 pulled down significantly more FAK than the wild-type Akt1 (N=4; $p<0.05$). Compared to the wild-type Akt1, construct 1 demonstrated significantly lower affinity for FAK (N=6; $p<0.05$).

Figure 2.3 (cont'd)

B



2.1d Discussion

We have previously delineated an intricate intracellular signal pathway activated by extracellular forces such as pressure and shear stress up-regulate integrin binding

affinity and metastatic potential in cancer cells (Basson 2008; Craig et al. 2009; Thamilselvan et al. 2004). While many of the elements of this pathway are common signaling elements, one novel aspect of the pathway is the binding of Akt1 to FAK and subsequent serine phosphorylation of FAK by Akt1, which seems required for pressure-stimulated FAK activation in this setting. This unusual interaction seems a promising target for manipulation to inhibit metastasis since FAK activation in response to other stimuli has not been found to require Akt-binding. In this manuscript, we used serial truncations to show that the interaction between Akt1 and FAK is independent of the Akt1 pleckstrin-homology (PH) domain and hinge region. Domain-directed truncations further demonstrate that the Akt1 kinase domain alone is sufficient to pull down FAK. Taken together with our previous studies of Akt1/Akt2 chimeras, these results would be consistent with a model in which the Akt isoforms to bind FAK through their homologous catalytic domain with an affinity which is modulated by their varying PH, hinge, and (C)-terminal regulatory domains.

We have previously described a relationship between Akt1 and FAK wherein pressure-induced membrane translocation of Akt1 and its activation by Ser473 phosphorylation is indirectly dependent upon FAK (FAK itself is a tyrosine kinase and so could not be directly responsible for Akt1 Ser473 phosphorylation (Wang and Basson 2008). This translocation of Akt1 and its phosphorylation at S473 occur in response to a 15 mmHg increase in extracellular pressure and requires the presence of both activatable FAK and the specific PH and hinge regions of the Akt1 isoform (Wang and Basson 2008). While the PH domains of the Akt isoforms share a similar functionality of

binding phospholipids, their unique identities contribute to isoform-specific functions ranging from the promotion of cell growth and migration to the inhibition of apoptosis in response to shear stress (Brazil et al. 2001; Heron et al. 2006; Zhou et al. 2006; Dimmeler et al. 1998). The membrane translocation and activation responses of Akt1 to pressure can be transferred to Akt2-like chimeric molecules that contain the Akt1 PH and hinge regions (Wang and Basson 2008).

In contrast to what might have been expected from those previous studies, we show here that FAK binds to Akt1 even when the Akt1 PH and hinge regions are deleted. Omitting either only the (N)-terminal half of the PH domain or the entire PH and hinge regions did not affect the ability of Akt1 to bind FAK fragments (GST-FAK (NT1)). Both the truncation missing the (N)-terminal half of the PH domain (HA-Akt1Δ1-66) and the truncation with completely deleted PH and hinge regions (HA-Akt1Δ1-147) bound FAK strongly. However, the partial hinge region truncation HA-Akt1Δ1-125 was unable to bind FAK (NT1). Akt1 activity can be strongly suppressed by allosteric inhibitors acting through PH domain conformational changes. Crystal structures show these inhibitors locking the PH domain into the kinase domain to prevent both Akt1 activation and membrane translocation, and highlight the inhibitory potential of a disrupted PH domain (Wu et al. 2010). Thus, one model consistent with these results would postulate that FAK binds to Akt outside the PH and hinge regions but that the PH domain can interfere with access by FAK to the kinase domain depending upon the conformation of the PH domain and hinge region. This would explain why the PH and hinge region confer specificity on Akt1 to permit its interaction with FAK even though they are not

directly involved in FAK binding. The deviation from the wild-type structure engendered by the inactivating HA-Akt1Δ1-125 deletion could then disrupt intermolecular interactions more effectively than the full length Akt1 PH and hinge region.

In reciprocal binding experiments using FAK as bait, the FAK (NT1) fragment proved to be sufficient to pull down Akt1. The FERM domain is responsible for FAK's ability to bind a variety of proteins and seemed a suitable candidate for mediating FAK-Akt1 interaction (Frame et al. 2010). The FERM domain consists of three subdomains F1, F2 and F3. The F2 and F3 subdomain resemble acyl-CoA binding proteins and PH domains, respectively (Pearson et al. 2000). No region of Akt1 proposes to be a good binding target for an acyl-CoA binding protein, and while PH-PH interactions do exist, they are highly specific as the PH domains from even related isoforms of Akt fail to bind (Datta et al. 1995). The F1 subdomain, however, displays structural similarities to ubiquitin (Pearson et al. 2000). Not only is this ubiquitin-like fold found in several unrelated proteins, which makes it a good contender in the context of protein-protein interactions, Akt1 also plays a role in several ubiquitylation pathways (Lin et al. 2002; Yang et al. 2010).

Indeed, our second series of Akt1 truncations instead demonstrated the Akt1 kinase domain alone to be sufficient to bind wild-type FAK. Domain-directed truncations of Akt1 consistently showed strong FAK binding to be dependent on the presence of some portion of the kinase domain. Truncations lacking either the (N) or (C)-terminal half of the Akt1 kinase domain (constructs 2, 3) were able to pull down wild type FAK

equally, if not more strongly, than the full Akt1 kinase domain by itself (construct 4). The only truncation in this series that lost FAK-binding affinity was construct 1, which consisted of only the (C)-terminal regulatory domain. Taken along with the PH and hinge domain truncation data, these results point to the kinase domain as the key region needed to allow for FAK binding, and suggests that there may be at least two separate sites in the Akt1 kinase domain, one in the C terminal segment and one in the N terminal segment, that each interact with and bind FAK. A model of the Akt1 surface structure shows the area where the PH domain and (N) and (C)-terminal halves of the kinase domain meet (Figure 2.4). In light of the ability of either half of the kinase domain to bind FAK, and our previous data showing the PH domain to confer FAK specificity amongst Akt isoforms, this region of Akt1 presents a promising target for disrupting FAK-specific binding.

There are several global FAK inhibitors undergoing trials, but they share the distinct disadvantage of targeting FAK catalytic activity indiscriminately. In doing so they either also inhibit other kinases with some structural similarity or have profound downstream consequences because of global FAK inhibition. Inhibitors such as TAE226 also inhibit IGFR-1, MAPK, and Akt activity while PF-228 inhibits only motility and not cell growth or viability (Liu et al. 2007; Slack-Davis et al. 2007). The promising FAK inhibitor Y15 decreased FAK Y397 autophosphorylation, cancer cell viability, and colony formation, and in mice xenograft in vivo models, intraperitoneal Y15 (30mg/kg) blocked tumor growth of colon, pancreatic, and breast cancer (Heffler et al. 2013; Hochwald et al. 2009; Golubovskaya et al. 2008). However, toxicity studies conducted in mice

showed intraperitoneal Y15 at 45 mg/kg to be lethal with mortality associated with peritonitis (Golubovskaya et al. 2014). In addition to exhibiting a narrow range of safe effectiveness, intraperitoneal Y15 at 30 mg/kg only reaches plasma concentrations of 110 nM whereas in vitro inhibition of cell viability of colon, pancreatic, and breast cancer

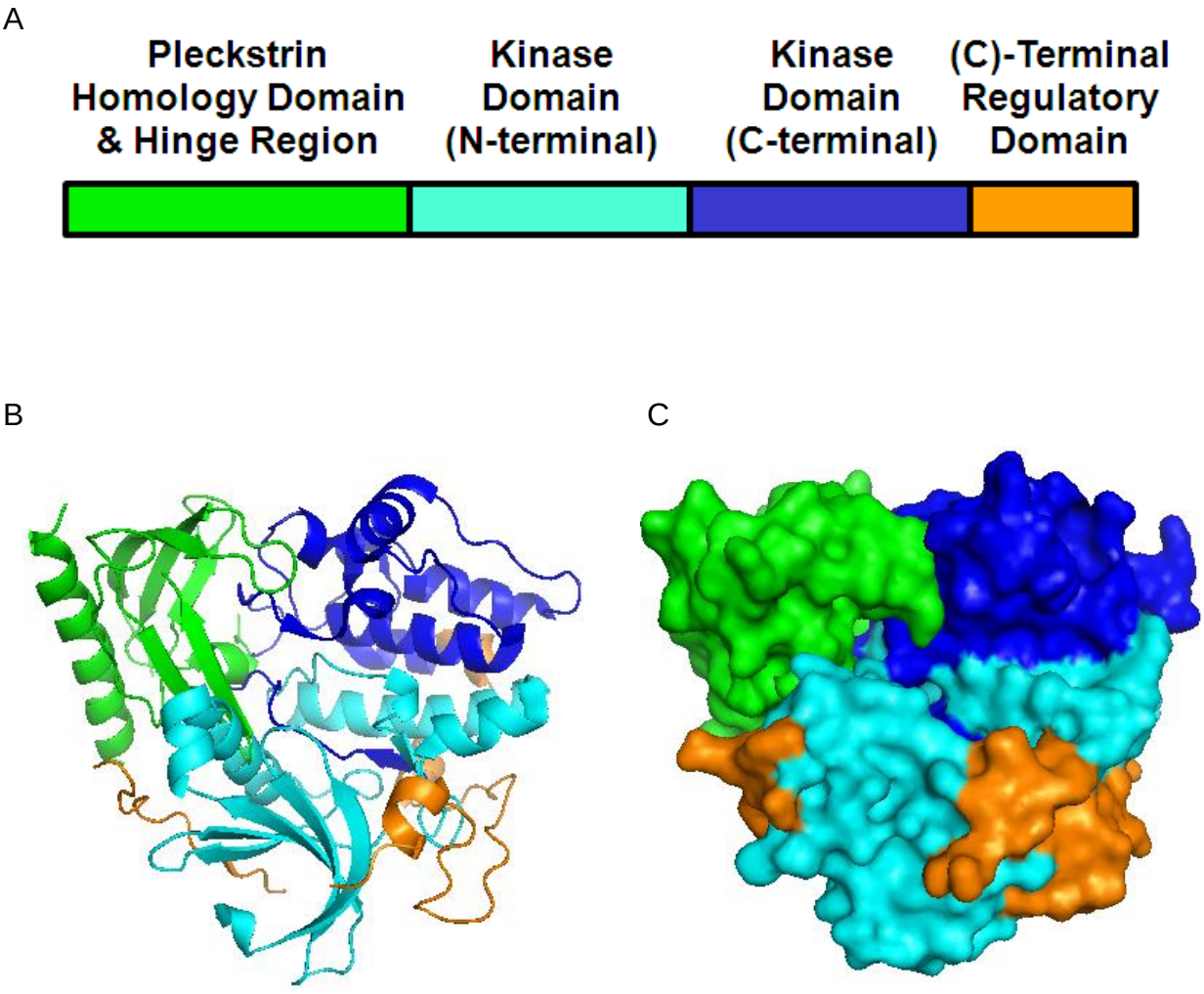


Figure 2.4. The Akt1 molecule
A diagram of the domains of Akt1 (A), a cartoon model of its secondary structures (B), and a filled model showing its surface features (C) were created in Pymol from the Protein Data Bank files 3CQW (active Akt1 with ATP-competitive inhibitor bound kinase) and 3O96 (cytoplasmic PH domain of Akt1).

requires concentrations of at 1, 10, and 50 μM, respectively (Heffler et al. 2013; Hochwald et al. 2009; Golubovskaya et al. 2008). The off-target effects of current FAK inhibitors and the general toxicity that arises from such non-specificity highlight the potential desirability of a therapeutic intervention that not only specifically targets FAK but even more specifically inhibits only certain deleterious aspects of FAK signaling in cancer cells.

From the Akt side of the issue, a similar outcome develops from the complete inhibition of the Akt1 kinase. Similar to the case with FAK, global Akt1 blockade also affects other signaling pathways. Not only are the general mechanics of cell-survival affected, which may be compensated for by parallel signaling cascades, but more specific response processes are also compromised. As a pro-anabolic kinase, Akt is well-known for its ability to rescue cells from detachment-induced apoptosis (Toker et al. 2017). In the context of mechanical forces, Akt has also been shown to be an upstream regulator of a process that combats disuse-atrophy of skeletal muscles, which is a central issue in the micrgravitational environment of space medicine but can also be applied to the challenges seen in rehabilitation medicine (Gwag et al. 2015). Outside of the non-specificity issue, current Akt inhibitors undergoing clinical trials are already showing deleterious effects when used in combination with well-established anti-neoplastic agents (Galvez-Peralta et al. 2014). Both novel anti-neoplastic drugs (such as the DNA intercalating agents tospyrquin and tosind) and conventional ones (such as the topoisomerase inhibitors camptothecin and etoposide) depend on DNA synthesis to achieve efficacy. Akt inhibition, on the other hand, fundamentally antagonizes these

therapies by causing a decrease in cell proliferation and thus active DNA replication (Tonton et al. 2013; Galvez-Peralta et al. 2014). The inability to use general Akt inhibitors in chemotherapeutic cocktails limits their usefulness and this problem can be avoided by designing drugs that target its inter-protein interactions and not its catalytic activity.

Our previous studies suggest that Akt-FAK interaction may therefore be a desirable target for the inhibition of this prometastatic-pathway, since Akt-FAK interaction could conceivably be blocked, and the force-activated signals that stimulate cancer cell adhesiveness inhibited, without altering many other activities of either FAK or Akt. This would require a precise understanding of the mechanism by which FAK and Akt interact. Coupling this knowledge with protein-model based drug discovery techniques, one could conceivably generate treatments aimed at blocking, competing with, or destabilizing the Akt-FAK interaction. To maximize the efficacy of these drugs, more studies are needed to delineate how Akt and FAK behave after force activation. For instance, the pressure-mediated adhesion returns to baseline by 60 minutes after extracellular pressure has returned to ambient, whereas pressure-mediated β1-integrin T788/9 phosphorylation is only measurably increased for 30 minutes, perhaps because the kinetics of the adhesion assay are slower and thus more sensitive to the adhesion that has occurred before the phosphorylation events receded (Craig et al. 2007). Our present results represent an initial step in this direction, focusing attention on the Akt kinase domain. Further studies to delineate more precisely the two specific FAK binding

sites we now postulate within this domain may ultimately lay the groundwork for the design of a specific therapeutic to block this pathway and inhibit metastasis.

In a broader context, 90% of cancer deaths are attributed not to the original tumor but to cancer metastases (Sporn et al. 1996). Central to metastatic development are processes of intravasation, extravasation, and implantation all of which require cell adhesion. Such adhesion could be modulated by pressurization from within the initial tumor environment itself (which range from 15-38 mmHg across a variety of tumors), pressures within the circulation (5-10 mmHg in venous and 90-120 mmHg in arterial circulation) or shear stresses within the same environment, or by iatrogenic manipulation such as the peritoneal insufflation in laparoscopic-assisted surgeries that generates 15 mmHg pressures, shear stress from peritoneal irrigation, or even direct surgical tumor manipulation (Thamilselvan et al. 2004; Less et al. 1992; Curti et al. 1993; Gutmann et al. 1992). Force-activated increased adhesiveness appears common to a variety of malignant cell types, including colon cancers, squamous head and neck cancers, breast cancers, and even sarcomas (Basson et al. 2000; Conway et al. 2006; Downey et al. 2006; Perry et al. 2010). By targeting pressure-mediated adhesion through Akt-FAK interaction we target an element shared across cancer pathology that seems less likely to be important in other biology since this Akt-FAK interaction has not previously been described. The ramifications of such an interaction may not be limited to adhesion and may in fact be far reaching. Indeed, FAK and Akt2 have been implicated in pressure-induced phagocytosis, and we have previously described in LPS-stimulated monocytes an inhibitory effect of pressure on the generation of IL-6 and

other inflammatory markers (Shiratsuchi and Basson 2005; Shiratsuchi and Basson 2007; Chaturvedi et al. 2011). In the setting of colorectal cancer, serum levels of IL-6 are also significantly elevated (Malicki et al. 2009). Connections like these not only broaden our view of the effects of mechanical forces on cell pathology and physiology, but also provide us with leads. Monocyte IL-6 synthesis can be countered by treatment with statins and such inhibition extends to colorectal cancer as well, which gives us a candidate pathway to study and possibly target (Malicki et al. 2009). Thus, it seems possible that in the future better understanding of this interaction will permit us to inhibit metastasis with a drug better tolerated than conventional cytotoxics, blocking this pathway with less systemic toxicity and facilitating longer term survival of patients with unresectable malignancy.

2.2 The C-terminal region of the FAK F1 domain binds Akt1 and inhibits pressure-induced cell adhesion

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2.2a Introduction

The metastatic spread of cancer from a primary tumor initially develops through an interplay between the dissemination and successful implantation of viable tumor cells. While a primary tumor of 1 cm in size is able to shed one million cells into circulation per day (Fidler et al. 2005), another important contributor is the release of tumor cells by physical manipulation during surgical interventions. Such iatrogenic dissemination not only dislodges tumor cells into the surgical site (Sugarbaker et al. 1999; Allardyce et al. 1996) but also substantially increases the number of circulating tumor cells (Weitz et al. 2000; Choy et al. 1996; Uchikura et al. 2002; Yamaguchi et al. 2000; Hayashi et al. 1999). How many of these iatrogenically dispersed tumor cells subsequently develop into metastatic growths is obscured by the population of existing, but undetectable, established metastases. However, up to 1% of curative cancer resections show verifiable signs of wound recurrence and many more develop signs consistent with peritoneal spreading (Nelson et al. 2004; Turnbull et al. 1967; Basson, Yu, and Herden 2000).

Physical forces such as pressure (Shiratsuchi and Basson 2004; Thubrikar and Robcsek 1995; Basson et al. 2015) and shear stress (Huynh et al. 2016; Thamilselvan

et al. 2004) have profound effects on cancer cell biology and are ubiquitous in the tumor environment. The increased pressure and shear stress within the circulatory system are well known. In the perioperative setting, tumor manipulation by the surgeon and surgical site irrigation subject tumor cells to pressure and shear stress, while laparoscopic surgery itself generally increases intraperitoneal pressure by 15 mmHg in order to create working space for surgery (O'Rourke and Kodali 2006; Daskalakis et al. 2009; Newdecker et al. 2002). Even relatively brief exposure to such pressures can trigger (Thamilselvan and Basson 2005; Craig, Haimovich, and Basson 2007) an intracellular signal cascade that increases the adhesive potential of suspended colon cancer (Basson 2008), breast cancer (Basson et al. 2015; Less et al. 1992), squamous cell carcinoma (Gutmann et al. 1992; Conway et al. 2006) and sarcoma cells (Perry, Wang, and Basson 2010). Although most such studies have focused on in vitro adhesion to purified matrix proteins such as collagen I (Basson et al. 2000; Thamilselvan, Craig, and Basson 2007), adhesion is also increased to more physiologically relevant substrates like endothelial cells (Thamilselvan and Basson 2004; van Zyp et al. 2006), and murine surgical wounds (van der Voort et al. 2005; Craig et al. 2008; Craig, Downey, and Basson 2008), and adversely impacts survival in mouse models (Craig, Downey, and Basson 2008). Parallel mechanosensitive pathways involving either the cytoskeleton and paxillin or Src and phosphatidylinositol 3-kinase (PI3K) detect the pressure stimulus (Basson 2008). These signaling pathways converge at an interaction between FAK and Akt1 in which Akt1 phosphorylates FAK at serines 517/601/695 to facilitate FAK activation at tyrosine 397 (Wang and Basson 2011). With FAK activation, the consequent increases in β1-integrin affinity and avidity reduce the

ligand threshold required for integrin-mediated cell adhesion thus providing a metastatic advantage for pressure-stimulated cells (Thamilselvan, Craig, and Basson 2007).

Integrin profiles are intimately tied to tumor metastasis. While the presence of specific integrins may direct the location of metastatic progression, as is the case with β3 and bone metastases in breast cancer (Pecheur et al. 2002), changes in integrin affinity and avidity dictate changes in cell adhesion. Additionally, decreased expression of β1 integrins is associated with the transition from epithelial to mesenchymal phenotypes that precedes the loss of cell adhesiveness necessary for pathological metastatic dissemination; the reverse process is initiated by the re-expression of these integrins as disseminated tumor cells adhere to distant organ parenchyma to create secondary tumors (Neal et al. 2011; Wang and Manning et al. 2002).

Under normal cell physiology, FAK and Akt influence essential cell functions, such as migration (Walsh et al. 2008; Gayer et al. 2009), survival, and protection against anokis (Walsh et al. 2003) (a mode of cell death to which cancer cells seem somewhat more resistant), which limits the utility of drugs that inhibit their catalytic abilities. However, the dearth of FAK-Akt binding outside the context of mechanical signaling (Wang and Basson 2011; Craig, Gayer, and Schaubert 2009) led us to seek to specifically target FAK-Akt interaction in order to prevent FAK activation without compromising alternative activities of either FAK or Akt1. The binding site for FAK on Akt1 seems quite large encompassing the kinase domain but not the Pleckstrin–homology or hinge regions (Basson et al. 2015). In the current investigation, we sought

to determine whether the converse binding site for Akt1 on FAK might be smaller and thus more amenable to future modeling and/or manipulation. Serial truncations isolated a relatively short 33 amino acid subdomain of FAK that is sufficient for Akt1 binding.

2.2b Materials and Methods

Materials

Human Caco-2 and murine CT-26 colon cancer cells were cultured according to American Type Culture Collection (Rockville, MD) recommendations. We obtained Lipofectamine 2000 and other transfection supplies from Invitrogen (Carlsbad, CA), Glutathione Sepharose 4B beads from GE Life Sciences (Pittsburg, PA), and Akt1 and GST antibodies from Cell Signaling Technology (Beverly, MA). All primers were purchased from Integrated DNA Technologies (Coralville, IA). QIAquick Gel Extraction, QIAprep spin Miniprep, QIAquick PCR purification and QIAfilter Plasmid Maxi kits were purchased from Qiagen (Valencia, CA).

Generation of Constructs

Mammalian expression vectors pGEX-4T-1 glutathione S-transferase (GST)-FAK-NT1 and its truncations were constructed via PCR of an HA-FAK(WT) plasmid template and introduced into the bacterial expression vector pGEX-4T1 (GE Healthcare, Munich, Germany) through 5’EcoRI and 3'XhoI cut sites. GST-Akt1 was generated by the same manner using a pcDNA3-myr-HA-Akt1 template (Addgene, Cambridge, MA). The HA-FAK(WT) itself, as well as the HA-FRNK (tagged at the COOH terminal), were gifts from Dr. David Schlaepfer. A similar protocol was used to generate the GFP-FAK-NT1

transient expression vector from the pEGFP-C1 vector Clontech (Mountain View, CA). Inducible GFP-FAK-NT1 expression was achieved using the pL6N2-RHS3H/ZF2-PL vector provided by the ARGENT regulated transcription retrovirus kit (now iDimerize inducible heterodimer system from Takara, Mountain View, CA).

Transfections

Caco-2 cells were plated on p100 dishes at 30–35% confluence one day prior to transfection. The constructed or empty plasmids were transfected into Caco-2 cells at final concentrations of 2 μg/ml plasmid and 5 μg/ml Lipofectamine 2000. Five hours after transfection, the medium was replaced with 15 ml pre-warmed Caco-2 media without antibiotics. Forty eight hours after DNA transfection, the cells were trypsinized for adhesion or pull-down experiments.

Inducible Expression

A stable CT-26 cell line was generated per ARIAD-ARGENT protocols and GFP-FAK-NT1 expression was induced using 50 nM of the provided non-immunosuppressive rapalog, AP21967 with an equal volume of ethanol used as the control vehicle. Forty eight hours after induction, the cells were trypsinized for adhesion experiments.

Glutathione S-transferase Pull-down

Glutathione-Sepharose 4B beads (15 μl) were washed twice in ice-cold PBS and resuspended in 400 μl PBS. Bacterial lysate containing GST-Akt1, GST-FAK-NT1 (truncations), or GST proteins (gift of Dr. J. Chen) were then added in excess and

incubated with the beads for 1 h. Glutathione-Sepharose 4B beads coupled to GST-Akt1, GST-FAK (truncations), or GST were then washed twice with PBS by centrifuge for 5 min at 500 g and incubated with nontransfected or transfected Caco-2 cell lysates (600–800 μg protein) overnight at 4°C. Transfected Caco-2 cells received plasmids encoding HA-WT-FAK or HA-FRNK. Nontransfected and transfected Caco-2 cell lysates were prepared in cell lysis buffer lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 50 mM NaF, 10 mM sodium pyrophosphate, 2 mg/ml aprotinin, and 2 mg/ml leupeptin (pH 7.4)]. Following incubation, beads were washed twice with lysis buffer without SDS and protease inhibitors. Proteins were eluted with Laemmli SDS sample dilution buffer, separated by 10% SDS-PAGE, and immunoblotted with GST, Akt1 (Cell Signaling Technology, Danvers, MA), or HA monoclonal antibodies (Covance, Chantilly, VA).

Western Blotting

Western blots were performed as previously described (Thamilselvan and Bassonb 2004). Eluate from the pull-downs were resolved by SDS-polyacrylamide gel electrophoresis and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were blotted with specific antibodies directed against either their wild-type structures or recombinant tags with the appropriate secondary antibody coupled to horseradish peroxidase. Bands were detected with enhanced chemiluminescence (Amersham) and analyzed with a Kodak

Image Station 440CF (Perkin Elmer, Boston, MA). All exposures were within the linear range.

Pressure Regulation

Experimental pressure conditions were controlled using an airtight box with inlet and outlet valves, thumb screws, a pressure gauge and an O-ring for an airtight seal as previously described in detail (Basson et al. 2000; van der Voort et al. 2005). The box was prewarmed to 37°C for 1 hour to minimize temperature fluctuations experienced by the cells. The gas used for pressurization was a filtered 5% CO2/95% air mixture consistent with the atmosphere in which the cells were routinely cultured. The temperature was maintained within ±2°C and the pressure within ±1.5 mmHg. Partial pressures of O2 and CO2 and pH do not change appreciably during pressurization (Basson et al. 2000). Cells were plated as for adhesion assays described below, and the six well plates were then placed into the prewarmed pressure box which was rapidly repressurized and monitored throughout the experiment with adjustment of the pressure if required. The pressure box was maintained in an incubator at 37ºC, and control cells, incubated at ambient pressure, were maintained in the same incubator. Pressure was maintained throughout the adhesion experiment, which lasted 30 minutes.

Cell Adhesion Studies

100,000 Caco-2 or CT-26 cells were seeded to collagen I coated 6-well plates under ambient or increased pressure conditions for 30 minutes. Non-adherent cells were washed away, and the remainder fixed with 0.01 M NaIO4, 0.75 M lysine, 0.0375

M sodium phosphate buffer, pH 7.4, and 2% paraformaldehyde on ice for 1 hour. The adherent cells were counted microscopically in at least 20 random high power fields per well under a fluorescent microscope.

FAK1 Structural Analysis

Structures were obtained from the Protein Data Bank (http://www.rcsb.org): 2AL6 (Ceccarelli et al. 2006). Structures were analyzed using Pymol from DeLano Scientific (San Carlos, CA).

Statistical analysis

Results were compared by Student's unpaired t-test and considered statistically significant when $p< 0.05$. All experiments were done independently at least three times unless indicated otherwise. All data are expressed as mean ± SEM.

2.2c Results

FRNK is not the primary binding site by which FAK binds to Akt1 in suspended Caco-2 cells

FRNK (FAK-related non-kinase, 67 kDa) is a segment from the COOH-terminal region of the FAK molecule, the C-terminal Focal Adhesion Targeting domain, which functions as an endogenous FAK inhibitor by competitively binding to focal contacts while lacking catalytic capability (Sieg et al. 1999). We transfected either HA-tagged wild type FAK or HA-tagged FRNK plasmids into Caco-2 cells, and incubated the resulting cell lysate with Sepharose beads conjugated to GST-Akt1 before Western blotting for

HA. Passage over the GST-Akt1 column enriched the resulting eluate for HA-conjugated wild type FAK while the amount of HA-conjugated FRNK was markedly reduced by this procedure. (Figure 2.5, 1 of 2 similar)

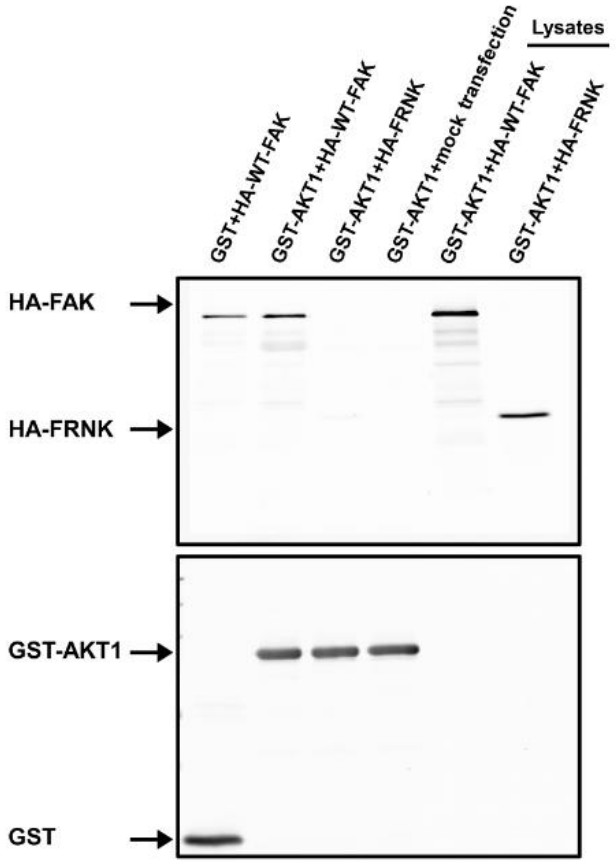


Figure 2.5. FRNK alone is not sufficient to bind Akt1
Pull-down assays used GST or GST-Akt1 (prey, bottom blot) to bind HA-FRNK or HA-WT-FAK (bait, top blot) found in the cell lysate of transiently transfected Caco-2 cells. Caco-2 cells expressing HA-FRNK control showed low levels of GST-Akt1 pull-down relative to those expressing HA-WT-FAK (One of two similar). Lysate from cells transfected with HA-WT-FAK or HA-FRNK were used as references.

Transient expression of FAK-NT1 inhibits pressure-induced adhesion

In contrast, previous preliminary observations suggested that the F1 lobe of FAK (herein referred to as the NT1 region) of wild-type FAK was sufficient to pull down Akt1

(Basson et al. 2015). To test whether this interaction of NT1 with Akt1 might have biological effects, we evaluated the effect of overexpressing NT1 in Caco-2 cells on the adhesive response to increased extracellular pressure, which requires FAK-Akt1 interaction (Thamilselvan and Basson 2004; Wang and Basson 2011). Caco2 cells transiently expressing the GFP-FAK-NT1 construct were therefore exposed to ambient or 15 mmHg increased pressure for 30 minutes. Pressure-induced adhesion was inhibited in the cells expressing GFP-FAK-NT1 but not in those expressing the control GFP alone (Fig. 2.6). Interestingly, the basal levels of adhesion were also reduced in the GFP-FAK-NT1 population.

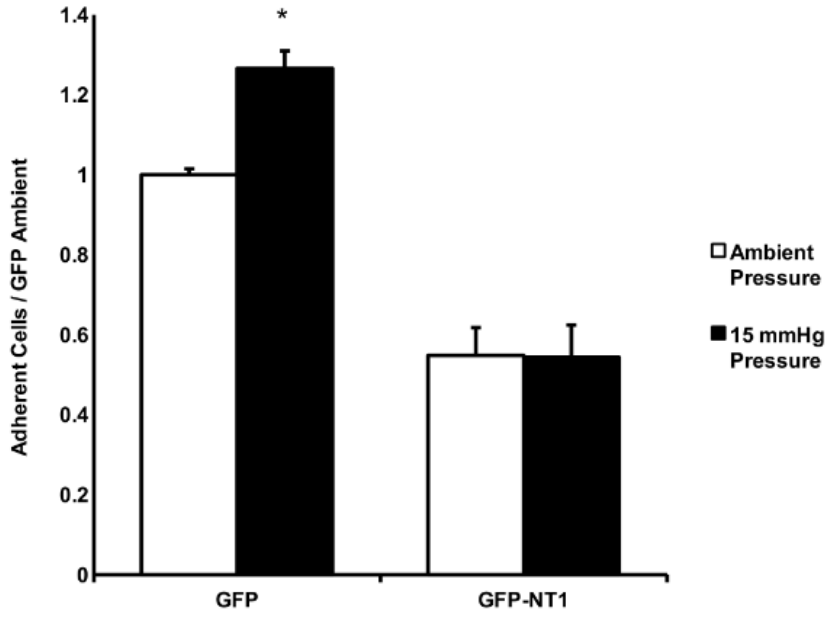


Figure 2.6. Transient expression of FAK-NT1 inhibits pressure-induced adhesion
Caco-2 cells transiently expressing the GFP control demonstrated increased cell adhesion after exposure to 15 mmHg pressure. Transient expression of GFP-FAK-NT1 blocked pressure-induced cell adhesion. Decreases in basal levels of cell adhesion were also seen in the cells expressing GFP-FAK-NT1 (N=6; *$p<0.05$ vs the ambient GFP control).

Inducible expression of FAK-NT1 inhibits pressure-induced adhesion

To establish the generalizability of this phenomenon, we constructed a stable murine CT26 colon cancer line which expressed GFP-FAK-NT1 on induction with rapalog, along with a control cell line which only expressed GFP on induction. Expression of the GFP-FAK-NT1 construct in CT26 cells was induced 48 hours prior to exposure to ambient or 15 mmHg increased pressure for 30 minutes. Inducing expression of the GFP-FAK-NT1 construct prevented the stimulation of cell adhesion by increased pressure. In contrast, cells in which only GFP was inducibly expressed did display increased adhesion in response to increased extracellular pressure. (Fig. 2.7). Basal levels of adhesion were reduced in the GFP-FAK-NT1 population.

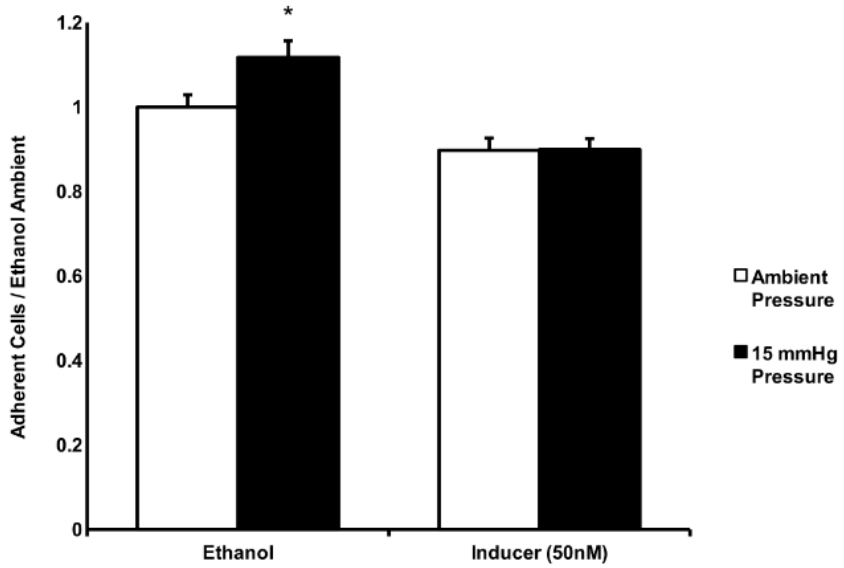


Figure 2.7. Inducible expression of FAK-NT1 inhibits pressure-induced adhesion
As with the transient expression model, CT-26 cells inducibly expressing the GFP control showed increased cell adhesion after exposure to 15 mmHg pressure. Inducible expression of GFP-FAK-NT1 blocked pressure-induced cell adhesion, however it did cause a larger decrease in basal cell adhesion (N=6; *$p<0.05$ vs the ambient control).

The FAK-NT1-2-2 region is sufficient to bind Akt1

Because the NT1 region of FAK is still quite large, we further truncated FAK-NT1 in an attempt to specify the region responsible for Akt1 binding. Five truncations were generated: NT1 (residues 1-126), NT1-1 (residues 1-60), NT1-2 (residues 61-126), NT1-1-2-1 (residues 61-93), and NT1-2-2 (residues 94-126) (Figure 2.8A). The truncations that contained the 33 amino acids found in the NT1-2-2 truncation (NT1, NT1-2, and NT1-2-2) were able to pull down more significantly more Akt1 than the constructs that did not (GST, NT1-1, and NT1-2-1) (Figure 2.8B, C; $p<0.05$).

A

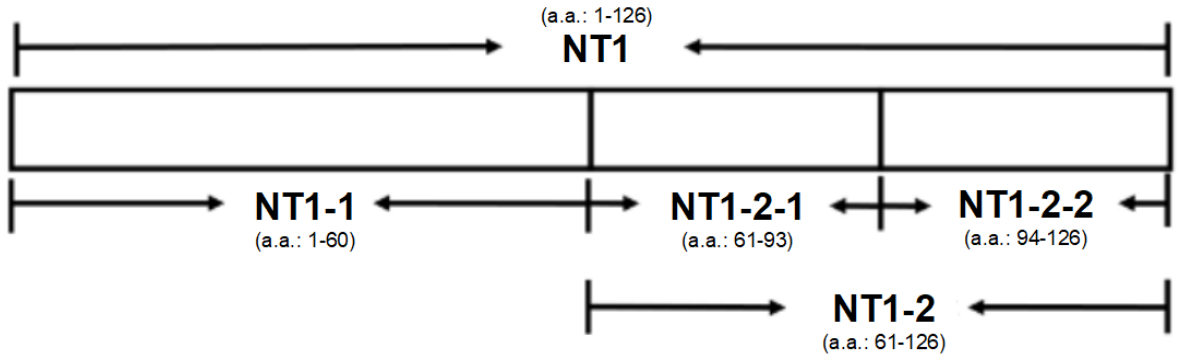
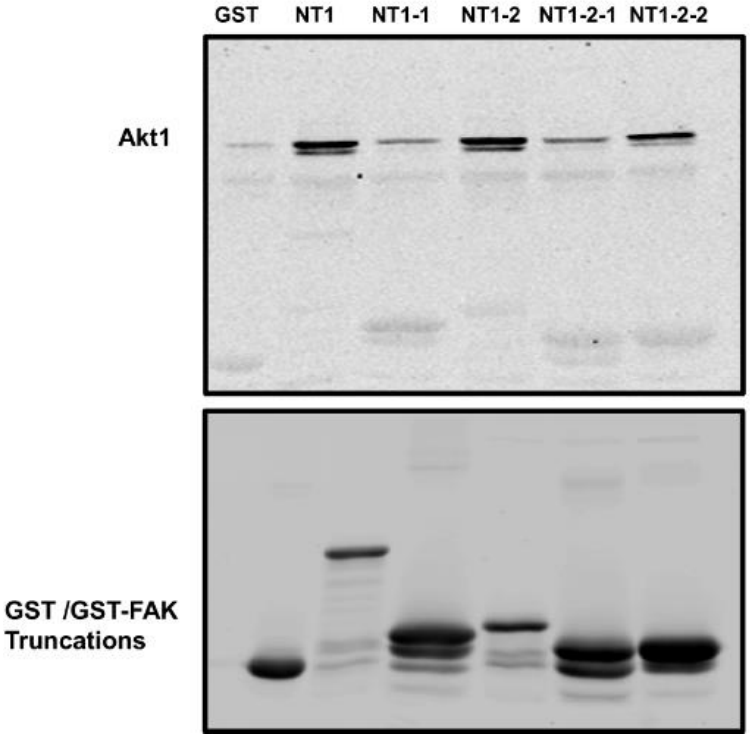


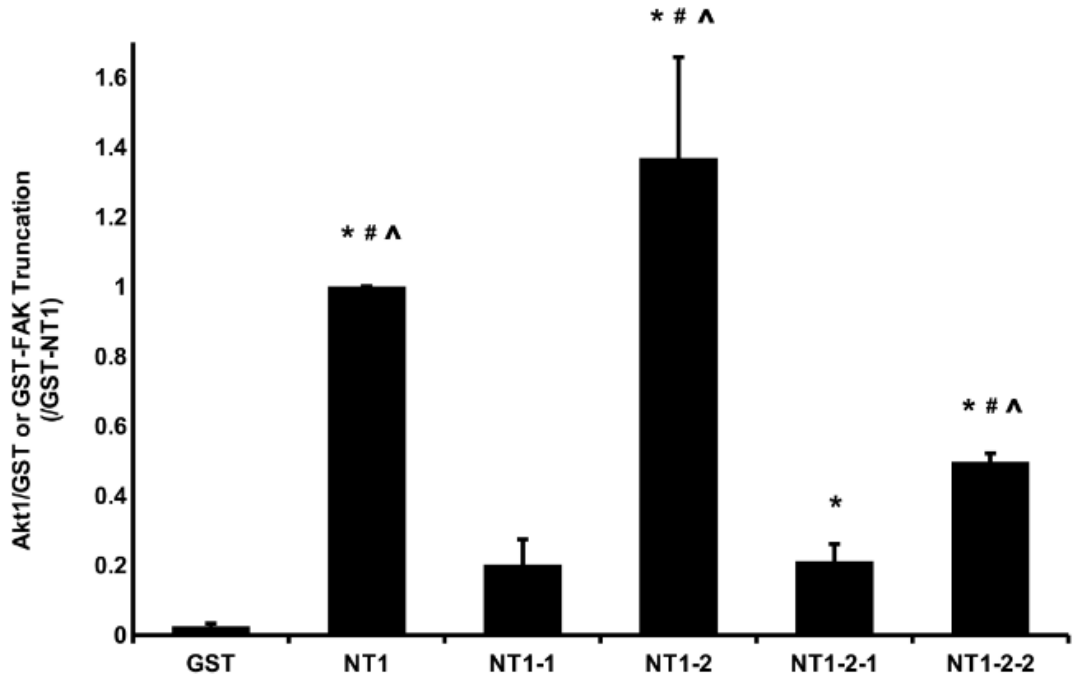
Figure 2.8. Akt1 / FAK binding requires the Akt1 kinase domain
(A) Recombinant GST-FAK truncations NT1 (residues 1-126), NT1-1 (residues 1-60), NT1-2 (residues 61-126), NT1-1-2-1 (residues 61-93), and NT1-2-2 (residues 94-126) were generated to test their ability to pull down Akt1. (B, C) All truncations containing the NT1-2-2 region (NT1, NT1-2, and NT1-2-2) pulled down Akt1. The constructs that did not (GST, NT1-1, and NT1-2-1) pulled down a significantly smaller amount (N=4; *$p<0.05$ vs GST control; #$p<0.05$ vs GST-NT1-1; ^$p<0.05$ vs GST-NT1-2-1).

Figure 2.8 (cont'd)

B



C



2.2d Discussion

Extracellular forces like pressure and shear stress increase the binding affinity of surface integrins effectively decreasing the ligand threshold required for cell adhesion (Thamilselvan et al. 2004; Basson 2008; Craig, Gayer, and Schaubert 2009). Pressure stimulation provides dislodged tumor cells with such a metastatic advantage through a mechanism that involves elements such as FAK, Akt1, and Src. Common signaling components, these kinases are well-defined pharmacologic targets but also essential to normal cell physiology. However, the relationship between FAK and Akt1 presents an opportunity to interfere with this protein-protein interaction without compromising other catalytic potentials of these kinases. A previous preliminary study suggested that the FAK F1 lobe can by itself associate with Akt1 (Basson et al. 2015). Here we demonstrate that overexpression of this FAK domain can itself inhibit pressure-induced adhesion. While the F1 lobe itself is still quite large, serial truncations demonstrated that the interaction between FAK and Akt1 depends upon a 33 amino acid region in the C-terminal of the FAK F1 lobe. These findings support the possibility of using therapeutics modeled after this much smaller region of FAK to disrupt the pressure signaling pathway.

In our model, 15mmHg increased extracellular pressure is achieved through the isothermic injection of a filtered 5% CO2/95% air mixture into airtight boxes; pressurization occurs at 37°C for 30 min. While CO2 was our gas of choice for its wide use in laparoscopic surgery, it should be noted that the exact effect of the type of gas is still debated: some groups show CO2 to produce less cell adhesion when compared to

nitrogen and helium (Tan et al. 2005) and others show no difference (Ludemann et al. 2003). Ma and colleagues reported that pure CO2 pressurization, particularly at high pressures, actually decreases both the expression of adhesion molecules and the adhesive and invasive potential of colon cancer cells (Ma et al. 2009). Our results here and in vivo (Craig, Owen, and Conway 2008), as well as those of others (Zhang et al. 2009) differ from the report by MA and colleagues. High pressure pure carbon dioxide gassing of cell suspensions with limited buffering capacity tends to be associated with substantial acidosis (Wildbrett et al. 2003), which is itself very toxic and may thus have effects beyond the signaling events studied here. The addition of the 95% room air has the added benefit of matching the atmosphere within most cell incubators which helps us focus on the impact of just the mechanical forces at work. Previous work from our laboratory has demonstrated that pressure stimulates adhesion even when the experiment is done in a pure nitrogen atmosphere (Basson et al. 2000).

Pressure-stimulated adhesion can be blocked and indeed survival improved in murine tumor models using either colchicine (Craig, Owen, and Conway 2008) or siRNA to alpha-actinin-1 (Craig, Downey, and Basson 2008) to interrupt cytoskeletal mechanotransduction, but the concentration of colchicine required to achieve these effects is substantially higher than that acceptable in humans, while molecular modification by siRNA techniques would be challenging in the clinical setting. Inhibitors that target FAK and Akt1 directly can also prevent pressure-stimulated adhesion in vitro (Thamilselvan et al. 2007; Craig, Downey, and Basson 2008; Craig, Gayer, and Schaubert 2009). However, these important kinases have diverse cell functions and

blocking either can produce substantial side effects. For instance, FAK inhibitors such as Y15 may cause peritonitis with fatal complications (Golubovskaya et al. 2015, Galvez-Peralta et al. 2014). Direct Akt1 blockade reduces cell proliferation in a way that diminishes the effect of chemotherapeutic agents reliant on DNA replication (Galvez-Peralta et al. 2014). Furthermore, agents that act upstream of these kinases may manifest varied, bordering unpredictable, consequences. In the case of Akt, the triterpene Celastrol decreases osteosarcoma invasion through inhibition of the PI3K/Akt pathway (Galvez-Peralta et al. 2014; Yu et al. 2016) yet it promotes myofibril hypertrophy by activating the Akt1/ERK1/2 pathway (Gwag et al. 2015). Such examples emphasize the benefit of targeting specific protein interactions over general function.

Mechanical forces recruit FAK and Akt1 to one another to stimulate cancer cell adhesion, an interaction not common among previously described signal pathways. This force-activated FAK-Akt1 interaction is therefore an enticing target because it may have less side effects. While the apparent novelty of this FAK-AKT1 interaction may reflect the limits of our knowledge about these kinases, preventing this specific interaction seems highly likely to have less off-target effects than the global consequences of blocking all catalytic functions of either kinase. We now show that such an interaction can be blocked by the expression of FAK fragments modeled after key components involved in the binding of these two kinases.

The FAK molecule is functionally divided into the N-terminal erythrocyte band four.1-ezrin-radixin-moesin (FERM) domain (residues 35-362), the central kinase

domain (residues 416-676), and the C-terminal focal adhesion targeting (FAT) domain (residues 677-1025) (Parsons et al. 2003). The FERM domain both connects components of the cell membrane to the cytoskeleton and regulates FAK activity (Parsons et al. 2003). The FERM domain is further divided into three lobes: F1 (residues 35-130), F2 (residues 131-255), and F3 (residues 256-362) (Fig. 2.9). The F2 lobe regulates FAK catalytic activity by binding the kinase domain and folding the FERM over it to physically occlude the active site (Lietha et al. 2007). The F3 lobe exhibits homology with regions of other FERM domains that bind the cytoplasmic tails of β integrins and ICAM-2 when activated (Ceccarelli et al. 2006). Compared to the rest of the FERM domain, less is known about the function of the F1 lobe. However, we have consistently shown, before with the F1 lobe in its entirety and here with the FAK NT1-2-2 region (residues 94-126), that the FAK F1 lobe is involved in Akt1 binding (Basson et al. 2015). Furthermore, while the activation site of FAK (tyrosine 397) is outside this region, changes to the residues within the F1 lobe, which do not physically contact tyrosine 397, are yet capable of triggering FAK activation (Ceccarelli et al. 2006). Such an allosteric activation may reconcile the importance of the F1 lobe with the Akt1-dependent FAK activation seen with pressure stimulation. Additionally, the specificity of this relationship is demonstrated by the inability of HA-FRNK (FAK-related non-kinase) to binds Akt1. This is noteworthy as FRNK is an established FAK truncation consisting of only the FAK C-terminal FAT domain and is often used for its ability to bind, but not phosphorylate, FAK targets (Sieg et al. 1999). Specifically, FRNK binds to focal adhesion complexes. This further coincides with our findings that pressure induces FAK-Akt1 association in suspended cells which have yet to form focal adhesion

complexes (Perry et al. 2010). Under this lens, we better understand the involvement of the F1 lobe in the FAK-Akt1 interaction.

The expression of the FAK-NT1 region successfully inhibited the stimulatory effect of pressure on cell adhesion in two different cell lines, using transient or stable inducible overexpression. This is consistent with previous observations showing binding between the NT1 region of FAK and Akt1 (Basson et al. 2015) as well as with the postulated necessity of FAK-Akt1 interactions for pressure-induced adhesion to occur (Wang and Basson 2011). Taken together, these observations in two different model systems strongly support the conclusion that FAK interacts with Akt1 in response to pressure stimulation via the FAK-NT1 region. Transient or induced overexpression of the GFP-FAK-NT1 plasmid in Caco-2 cells also decreased basal cell adhesion suggesting some tonic activity of this force-activated pathway even in the absence of increased pressure stimulation. However, FAK-NT1 is 126 amino acids in length. Such a large protein would be challenging either to dose pharmacologically or to mimic with small molecule analogs. A parallel set of studies examining Akt1 truncations was not able to narrow the Akt1 binding site for FAK down to a single small domain. The FAK binding site on Akt1 seems to span across the entirety of the Akt1 kinase domain as both N- and C- terminal based truncations of the region were equally capable of pulling down FAK (Basson et al. 2015). By contrast, our serial truncation studies of FAK were able to narrow down the region on FAK required for Akt1 binding to the 33 amino acid sequence in the FAK-NT1-2-2 truncation. Indeed, the FAK-NT1-2-2 region contains a segment that is surface accessible and may be responsible for orchestrating the Akt1

binding we observe through these pull-down assays (Figure 2.9). About one quarter the size of the larger FAK-NT1, this smaller subdomain may prove much easier to model or manipulate and may be an important target for future study.

Ninety percent of cancer deaths are attributed not to the original tumor but to metastatic growths (Sporn et al. 1996). Such metastasis requires many steps but one key step is the adhesion of disseminated or circulating tumor cells to a remote substrate. Exposure to increased extracellular pressure initiates changes that decrease cell motility and thus migratory potential (Kovalenko et al. 2012). At least some of the effect of pressure on cell motility may be its influence on β1 integrin subunit phosphorylation and thus cell adhesiveness (Flanigan et al. 2009). When this occurs in cancer cells after dissemination, the net effect may be to promote new tumor formation through the establishment of cell-cell and cell-matrix adhesions (Neal et al. 2011).

It is noteworthy in this regard that physical forces activate a very different pathway in cancer cells that are already adherent (Basson, Zeng, and Downey 2015; Walsh et al. 2004; Downet et al. 2011), so that the same stimuli that promote the adhesiveness of circulating cells would not be expected to prevent the motility and invasion of tumor cells within a primary tumor. The force-activated pro-adhesive pathway targeted here has been observed in malignant cell types including colon cancers (Basson et al. 2000), squamous head and neck cancers (Conway et al. 2006), breast cancers (Downey et al. 2006), and even sarcomas (Perry et al. 2010). Thus, inhibiting this pro-metastatic signal cascade might have substantial benefits for reducing

perioperative tumor dissemination and even longer term metastasis from unresectable tumors. Our results here raise the possibility that this pathway might be inhibited by interfering with FAK-Akt1 binding, in a fashion that may bypass the off-target effects common to currently available therapeutics.

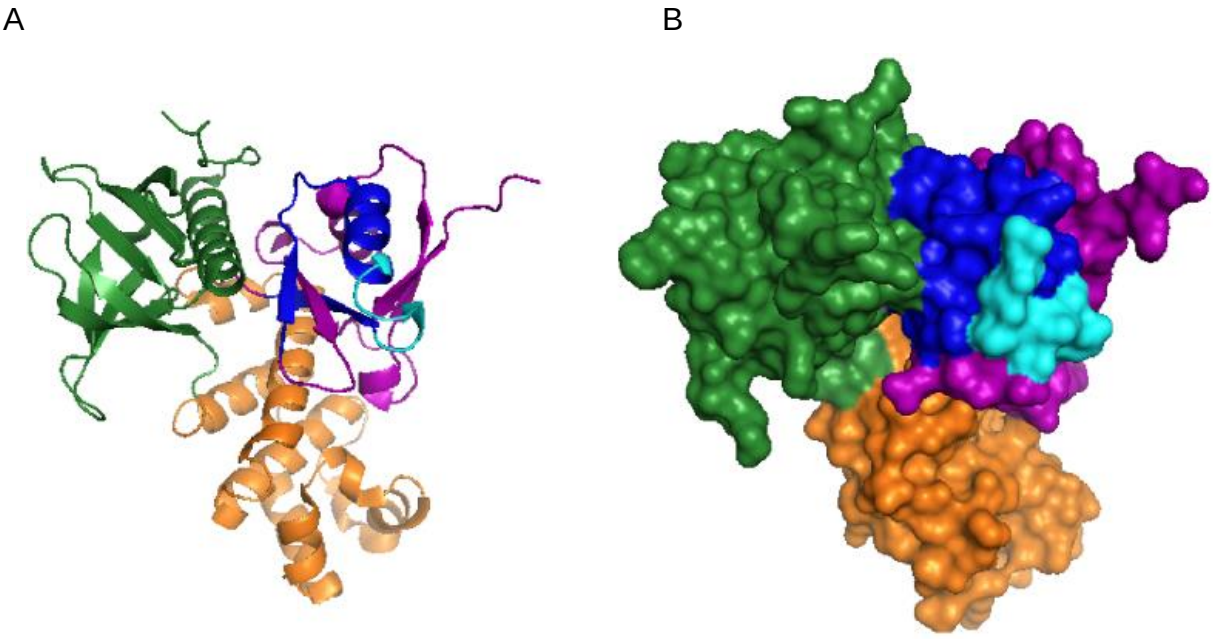


Figure 2.9. Models of the FAK molecule
Cartoon (A) and surface (B) depictions of the crystal structure of the FAK FERM domain containing the F1 (blue/purple/cyan), F2 (orange), and F3 (green) lobes (PDB code: 2AL6). The NT1-2-2 segment (blue) and its surface accessible region (cyan) are contained within the F1 lobe.

REFERENCES

REFERENCES

Allardyce, Randall A., Phil Morreau, and Philip F. Bagshaw. 1997. “Operative Factors Affecting Tumor Cell Distribution Following Laparoscopic Colectomy in a Porcine Model.” *Diseases of the Colon and Rectum*. https://doi.org/10.1007/BF02051202.

Basson, Marc D. 2008. “An Intracellular Signal Pathway That Regulates Cancer Cell Adhesion in Response to Extracellular Forces.” *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-07-2992.

Basson, Marc D., Cheng Fang Yu, Oliver Herden-Kirchoff, Marc Ellermeier, Matthew A. Sanders, Ronald C. Merrell, and Bauer E. Sumpio. 2000. “Effects of Increased Ambient Pressure on Colon Cancer Cell Adhesion.” *Journal of Cellular Biochemistry*. https://doi.org/10.1093/brain/114.3.1297.

Basson, Marc D., B. Zeng, and S. Wang. 2017. “The C-Terminal Region of the Focal Adhesion Kinase F1 Domain Binds Akt1 and Inhibits Pressure-Induced Cell Adhesion.” *Journal of Physiology and Pharmacology*.

Basson, Marc D., Bixi Zeng, Christina Downey, Madhu P. Sirivelu, and Jetze J. Tepe. 2015. “Increased Extracellular Pressure Stimulates Tumor Proliferation by a Mechanosensitive Calcium Channel and PKC-β.” *Molecular Oncology*. https://doi.org/10.1016/j.molonc.2014.10.008.

Ceccarelli, Derek F.J., Kyu Song Hyun, Florence Poy, Michael D. Schaller, and Michael J. Eck. 2006. “Crystal Structure of the FERM Domain of Focal Adhesion Kinase.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M509188200.

Chaturvedi, Lakshmi S., H. Michael Marsh, Xun Shang, Yi Zheng, and Marc D. Basson. 2007. “Repetitive Deformation Activates Focal Adhesion Kinase and ERK Mitogenic Signals in Human Caco-2 Intestinal Epithelial Cells through Src and Rac1.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M605817200.

Chaturvedi, Lakshmi S, Harold M Marsh, and Marc D Basson. 2007. “Src and Focal Adhesion Kinase Mediate Mechanical Strain-Induced Proliferation and ERK1/2 Phosphorylation in Human H441 Pulmonary Epithelial Cells.” *American Journal of Physiology-Cell Physiology*. https://doi.org/10.1152/ajpcell.00529.2006.

Chen, William S., Pei Zhang Xu, Kathrin Gottlob, Mei Ling Chen, Karen Sokol, Tanya Shiyanova, Igor Roninson, et al. 2001. “Growth Retardation and Increased Apoptosis in Mice with Homozygous Disruption of the Akt1 Gene.” *Genes and Development*. https://doi.org/10.1101/gad.913901.

Cheng, Zhaokang, Laura A. DiMichele, Mauricio Rojas, Cyrus Vaziri, Christopher P. Mack, and Joan M. Taylor. 2014. “Focal Adhesion Kinase Antagonizes Doxorubicin Cardiotoxicity via P21Cip1.” *Journal of Molecular and Cellular Cardiology*. https://doi.org/10.1016/j.yjmcc.2013.12.002.

Cho, H., J. Mu, J. K. Kim, J. L. Thorvaldsen, Q. Chu, E. B. Crenshaw, K. H. Kaestner, M. S. Bartolomei, G. I. Shulman, and M. J. Birnbaum. 2001. “Insulin Resistance and a Diabetes Mellitus-like Syndrome in Mice Lacking the Protein Kinase Akt2 (PKBβ).” *Science*. https://doi.org/10.1126/science.292.5522.1728.

Cohen, Lee Ann, and Jun Lin Guan. 2005. “Residues within the First Subdomain of the FERM-like Domain in Focal Adhesion Kinase Are Important in Its Regulation.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M412021200.

Conway, William C., Jochem Van Der Voort Van Zyp, Vijayalakshmi Thamilselvan, Mary F. Walsh, David L. Crowe, and Marc D. Basson. 2006. “Paxillin Modulates Squamous Cancer Cell Adhesion and Is Important in Pressure-Augmented Adhesion.” *Journal of Cellular Biochemistry*. https://doi.org/10.1002/jcb.20819.

Craig, D H, C P Gayer, K L Schaubert, Y Wei, J Li, Y Laouar, and M D Basson. 2009. “Increased Extracellular Pressure Enhances Cancer Cell Integrin-Binding Affinity through Phosphorylation of Beta1-Integrin at Threonine 788/789.” *American Journal of Physiology. Cell Physiology*. https://doi.org/10.1152/ajpcell.00355.2008.

Craig, David H., Cheri R. Owen, William C. Conway, Mary F. Walsh, Christina Downey, and Marc D. Basson. 2008. “Colchicine Inhibits Pressure-Induced Tumor Cell Implantation within Surgical Wounds and Enhances Tumor-Free Survival in Mice.” *Journal of Clinical Investigation*. https://doi.org/10.1172/JCI34279.

Craig, David H., Hiroe Shiratsuchi, and Marc D. Basson. 2009. “Increased Extracellular Pressure Provides a Novel Adjuvant Stimulus for Enhancement of Conventional Dendritic Cell Maturation Strategies.” *Biochemical and Biophysical Research Communications*. https://doi.org/10.1016/j.bbrc.2009.07.010.

Craig, David H, Christina Downey, and Marc D Basson. 2008. “SiRNA-Mediated Reduction of α-Actinin-1 Inhibits Pressure-Induced Murine Tumor Cell Wound Implantation and Enhances Tumor-Free Survival.” *Neoplasia*. https://doi.org/10.1593/neo.07945.

Craig, David H, Beatrice Haimovich, and Marc D Basson. 2007. “␣-Actinin-1 Phosphorylation Modulates Pressure-Induced Colon Cancer Cell Adhesion through Regulation of Focal Adhesion Kinase-Src Interaction.” *Am J Physiol Cell Physiol*. https://doi.org/10.1152/ajpcell.00118.2007.

Craig, Lisa, Paul C. Sanschagrin, Annett Rozek, Steve Lackie, Leslie A. Kuhn, and Jamie K. Scott. 1998. “The Role of Structure in Antibody Cross-Reactivity between

Peptides and Folded Proteins.” *Journal of Molecular Biology*. https://doi.org/10.1006/jmbi.1998.1907.

Datta, Sandeep Robert, Henryk Dudek, Tao Xu, Shane Masters, Fu Haian, Yukiko Gotoh, and Michael E. Greenberg. 1997. “Akt Phosphorylation of BAD Couples Survival Signals to the Cell- Intrinsic Death Machinery.” *Cell*. https://doi.org/10.1016/S0092-8674(00)80405-5.

DeLano, Warren L. 2002. “PyMOL: An Open-Source Molecular Graphics Tool.” *CCP4 Newsletter On Protein Crystallography*. https://doi.org/10.1038/s41598-017-03842-2.

Downey, Christina, Kamal Alwan, Vijayalakshmi Thamilselvan, Liyue Zhang, Yan Jiang, Arun K. Rishi, and Marc D. Basson. 2006. “Pressure Stimulates Breast Cancer Cell Adhesion Independently of Cell Cycle and Apoptosis Regulatory Protein (CARP)-1 Regulation of Focal Adhesion Kinase.” *American Journal of Surgery*. https://doi.org/10.1016/j.amjsurg.2006.08.006.

Dregelid, E., and E. Svendsen. 1988. “Endothelial Cell Injury in Human Saphenous Veins after Manipulation and Tweezer Grasping.” *Journal of Cardiovascular Surgery*.

Fidler, Isaiah J. 1970. “Metastasis: Quantitative Analysis of Distribution and Fate of Tumor Emboli Labeled with 125l-5-Lodo-2’-Deoxyuridine.” *Journal of the National Cancer Institute*. https://doi.org/10.1093/jnci/45.4.773.

Fuller, Jonathan C., Nicholas J. Burgoyne, and Richard M. Jackson. 2009. “Predicting Druggable Binding Sites at the Protein-Protein Interface.” *Drug Discovery Today*. https://doi.org/10.1016/j.drudis.2008.10.009.

Gayer, Christopher P., Lakshmi S. Chaturvedi, Shouye Wang, David H. Craig, Thomas Flanigan, and Marc D. Basson. 2009. “Strain-Induced Proliferation Requires the Phosphatidylinositol 3-Kinase/AKT/Glycogen Synthase Kinase Pathway.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M804576200.

Gupta, Gaorav P., and Joan Massagué. 2006. “Cancer Metastasis: Building a Framework.” *Cell*. https://doi.org/10.1016/j.cell.2006.11.001.

Han, Okhee, Guang Di Li, and Marc D Basson. 1998. “Strain Induces Caco-2 Intestinal Epithelial Proliferation and Differentiation via PKC and Tyrosine Kinase Signals.” *American Journal of Physiology*.

Hanahan, Douglas, Weinberg, Robert. 2000. “Hallmarks of Cancer.” *Cell*. https://doi.org/10.1007/s00262-010-0968-0.

Hartman, Z. R., M. D. Schaller, and Y. M. Agazie. 2013. “The Tyrosine Phosphatase SHP2 Regulates Focal Adhesion Kinase to Promote EGF-Induced Lamellipodia Persistence and Cell Migration.” *Molecular Cancer Research*. https://doi.org/10.1158/1541-7786.MCR-12-0578.

Hawkins, Paul C.D., and Anthony Nicholls. 2012. “Conformer Generation with OMEGA: Learning from the Data Set and the Analysis of Failures.” *Journal of Chemical Information and Modeling*. https://doi.org/10.1021/ci300314k.

Hawkins, Paul C.D., A. Geoffrey Skillman, and Anthony Nicholls. 2007. “Comparison of Shape-Matching and Docking as Virtual Screening Tools.” *Journal of Medicinal Chemistry*. https://doi.org/10.1021/jm0603365.

Hayashi, Naoko, Hiroshi Egami, Mikio Kai, Yuji Kurusu, Sadamu Takano, and Michio Ogawa. 1999. “No-Touch Isolation Technique Reduces Intraoperative Shedding of Tumor Cells into the Portal Vein during Resection of Colorectal Cancer.” *Surgery*. https://doi.org/10.1016/S0039-6060(99)70003-6.

Huynh, Khon C., Marianna Gyenes, Thi Hiep Nguyen, Toi Van Vo, and Volker R. Stoldt. 2017. “Impact of Shear Stress on Src and Focal Adhesion Kinase Phosphorylation in Fibrinogen-Adherent Platelets.” *Blood Coagulation and Fibrinolysis*. https://doi.org/10.1097/MBC.0000000000000593.

Hwang, Howook, Thom Vreven, Joël Janin, and Zhiping Weng. 2010. “Protein-Protein Docking Benchmark Version 4.0.” *Proteins: Structure, Function and Bioinformatics*. https://doi.org/10.1002/prot.22830.

Irwin, John J., and Brian K. Shoichet. 2005. “ZINC - A Free Database of Commercially Available Compounds for Virtual Screening.” *Journal of Chemical Information and Modeling*. https://doi.org/10.1021/ci049714+.

Jain, Rakesh K., Yves Boucher, and Norman Wolmark. 1992. “Interstitial Hypertension in Human Breast and Colorectal Tumors.” *Cancer Research*.

Jakalian, Araz, David B. Jack, and Christopher I. Bayly. 2002. “Fast, Efficient Generation of High-Quality Atomic Charges. AM1-BCC Model: II. Parameterization and Validation.” *Journal of Computational Chemistry*. https://doi.org/10.1002/jcc.10128.

Johnson, David K., and John Karanicolas. 2013. “Druggable Protein Interaction Sites Are More Predisposed to Surface Pocket Formation than the Rest of the Protein Surface.” *PLoS Computational Biology*. https://doi.org/10.1371/journal.pcbi.1002951.

Kao, Y. C., J. R. Jheng, H. J. Pan, W. Y. Liao, C. H. Lee, and P. L. Kuo. 2017. “Elevated Hydrostatic Pressure Enhances the Motility and Enlarges the Size of the Lung

Cancer Cells through Aquaporin Upregulation Mediated by Caveolin-1 and ERK1/2 Signaling." *Oncogene*. https://doi.org/10.1038/onc.2016.255.

Kleinschmidt, Elizabeth G., and David D. Schlaepfer. 2017. "Focal Adhesion Kinase Signaling in Unexpected Places." *Current Opinion in Cell Biology*. https://doi.org/10.1016/j.ceb.2017.01.003.

Klinghoffer, Richard A., Christoph Sachsenmaier, Jonathan A. Cooper, and Philippe Soriano. 1999. "Src Family Kinases Are Required for Integrin but Not PDGFR Signal Transduction." *EMBO Journal*. https://doi.org/10.1093/emboj/18.9.2459.

Ko, Hyo Rim, Il Sun Kwon, Inwoo Hwang, Eun Ju Jin, Joo Ho Shin, Angela M. Brennan-Minnella, Raymond Swanson, Sung Woo Cho, Kyung Hoon Lee, and Jee Yin Ahn. 2016. "Akt1-Inhibitor of DNA Binding2 Is Essential for Growth Cone Formation and Axon Growth and Promotes Central Nervous System Axon Regeneration." *ELife*. https://doi.org/10.7554/eLife.20799.

Koch, Moritz, Peter Kienle, Ulf Hinz, Dalibor Antolovic, Jan Schmidt, Christian Herfarth, Magnus Von Knebel Doeberitz, and Jürgen Weitz. 2005. "Detection of Hematogenous Tumor Cell Dissemination Predicts Tumor Relapse in Patients Undergoing Surgical Resection of Colorectal Liver Metastases." *Annals of Surgery*. https://doi.org/10.1097/01.sla.0000151795.15068.27.

Le Devedec, SE.,Geverts B, de Bont H, Yan K, Verbeek FJ, Houtsmuller AB, and van der Water B. 2018. "The Residence Time of Focal Adhesion Kinase (FAK) and Paxillin at Focal Adhesions in Renal Epithelial Cells is Determined by Adhesion Size, Strength, and Life Cycle Status." *J Cell Sci*.

Li, Wei, Aydin Duzgun, Bauer E. Sumpio, and Marc D. Basson. 2001. "Integrin and FAK-Mediated MAPK Activation Is Required for Cyclic Strain Mitogenic Effects in Caco-2 Cells." *Am J Physiol Gastrointest Liver Physiol*.

Li, Weiqun, Jiachang Zhang, Lawrence Flechner, Teresa Hyun, Alan Yam, Thomas F. Franke, and Jacalyn H. Pierce. 1999. "Protein Kinase C-α Overexpression Stimulates Akt Activity and Suppresses Apoptosis Induced by Interleukin 3 Withdrawal." *Oncogene*. https://doi.org/10.1038/sj.onc.1203065.

Lietha, Daniel, Xinming Cai, Derek F.J. Ceccarelli, Yiqun Li, Michael D. Schaller, and Michael J. Eck. 2007. "Structural Basis for the Autoinhibition of Focal Adhesion Kinase." *Cell*. https://doi.org/10.1016/j.cell.2007.05.041.

Liu, T.-J., T. LaFortune, T. Honda, O. Ohmori, S. Hatakeyama, T. Meyer, D. Jackson, J. de Groot, and W.K. A. Yung. 2007. "Inhibition of Both Focal Adhesion Kinase and Insulin-like Growth Factor-I Receptor Kinase Suppresses Glioma Proliferation in Vitro and in Vivo." *Molecular Cancer Therapeutics*. https://doi.org/10.1158/1535-7163.MCT-06-0476.

Liu, Weiguo, David A. Bloom, William G. Cance, Elena V. Kurenova, Vita M. Golubovskaya, and Steven N. Hochwald. 2008. “FAK and IGF-IR Interact to Provide Survival Signals in Human Pancreatic Adenocarcinoma Cells.” *Carcinogenesis*. https://doi.org/10.1093/carcin/bgn026.

Luzzi, Keith J., Ian C. MacDonald, Eric E. Schmidt, Nancy Kerkvliet, Vincent L. Morris, Ann F. Chambers, and Alan C. Groom. 1998. “Multistep Nature of Metastatic Inefficiency: Dormancy of Solitary Cells after Successful Extravasation and Limited Survival of Early Micrometastases.” *American Journal of Pathology*. https://doi.org/10.1016/S0002-9440(10)65628-3.

Parisi, Cristian, Vikesh V. Chandaria, and Niamh C. Nowlan. 2018. “Blocking Mechanosensitive Ion Channels Eliminates the Effects of Applied Mechanical Loading on Chick Joint Morphogenesis.” *Philosophical Transactions of the Royal Society B: Biological Sciences*. https://doi.org/10.1098/rstb.2017.0317.

Parsons, J. T. 2003. “Focal Adhesion Kinase: The First Ten Years.” *Journal of Cell Science*. https://doi.org/10.1242/jcs.00373.

Patel, Hitesh, Nadia Le Marer, Richard Q. Wharton, Zulfiqar A.J. Khan, Ruth Araia, Clare Glover, Michael M. Henry, and Timothy G. Allen-Mersh. 2002. “Clearance of Circulating Tumor Cells after Excision of Primary Colorectal Cancer.” *Annals of Surgery*. https://doi.org/10.1097/00000658-200202000-00010.

Perry, Brandon C., Shouye Wang, and Marc D. Basson. 2010. “Extracellular Pressure Stimulates Adhesion of Sarcoma Cells via Activation of Focal Adhesion Kinase and Akt.” *American Journal of Surgery*. https://doi.org/10.1016/j.amjsurg.2010.07.013.

Shiratsuchi, Hiroe, and Marc D. Basson. 2007. “Akt2, but Not Akt1 or Akt3 Mediates Pressure-Stimulated Serum-Opsonized Latex Bead Phagocytosis through Activating MTOR and P70 S6 Kinase.” *Journal of Cellular Biochemistry*. https://doi.org/10.1002/jcb.21295.

Shiratsuchi, Hiroe, and Marc D Basson. 2004. “Extracellular Pressure Stimulates Macrophage Phagocytosis by Inhibiting a Pathway Involving FAK and ERK.” *American Journal of Physiology. Cell Physiology*. https://doi.org/10.1152/ajpcell.00553.2003.

Thamilselvan, Vijayalakshmi, and Marc D. Basson. 2004. “Pressure Activates Colon Cancer Cell Adhesion by Inside-Out Focal Adhesion Complex and Actin Cytoskeletal Signaling.” *Gastroenterology*. https://doi.org/10.1053/j.gastro.2003.10.078.

Thamilselvan, Vijayalakshmi, David H Craig, and Marc D Basson. 2007. “FAK Association with Multiple Signal Proteins Mediates Pressure-Induced Colon Cancer

Cell Adhesion via a Src-Dependent PI3K/Akt Pathway." *The FASEB Journal : Research Communication*. https://doi.org/10.1096/fj.06-6545com.

Thomas, J W, M A Cooley, J M Broome, R Salgia, J D Griffin, C R Lombardo, and M D Schaller. 1999. "The Role of Focal Adhesion Kinase Binding in the Regulation of Tyrosine Phosphorylation of Paxillin." *The Journal of Biological Chemistry*. https://doi.org/10.1074/JBC.274.51.36684.

Thubrikar, Mano J., and Francis Robicsek. 1995. "Pressure-Induced Arterial Wall Stress and Atherosclerosis." *The Annals of Thoracic Surgery*. https://doi.org/10.1016/0003-4975(94)01037-D.

Toker, Alex, and Sandra Marmiroli. 2014. "Signaling Specificity in the Akt Pathway in Biology and Disease." *Advances in Biological Regulation*. https://doi.org/10.1016/j.jbior.2014.04.001.

Tschopp, O. 2005. "Essential Role of Protein Kinase B (PKB /Akt3) in Postnatal Brain Development but Not in Glucose Homeostasis." *Development*. https://doi.org/10.1242/dev.01864.

Turnbull, R. B. 1970. "Cancer of the Colon. The Five- and Ten-Year Survival Rates Following Resection Utilizing the Isolation Technique." *Annals of the Royal College of Surgeons of England*. https://doi.org/10.5833/jjgs.9.539.

Walsh, Mary F., Vijayalakshmi Thamilselvan, Rainer Grotelueschen, Lulu Farhana, and Marc D. Basson. 2003. "Absence of Adhesion Triggers Differential FAK and SAPKp38 Signals in SW620 Human Colon Cancer Cells That May Inhibit Adhesiveness and Lead to Cell Death." *Cellular Physiology and Biochemistry*. https://doi.org/10.1159/000071864.

Wang, S., and M. D. Basson. 2011. "Akt Directly Regulates Focal Adhesion Kinase through Association and Serine Phosphorylation: Implication for Pressure-Induced Colon Cancer Metastasis." *AJP: Cell Physiology*. https://doi.org/10.1152/ajpcell.00377.2010.

Wang, Shouye, and Marc D Basson. 2011. "Protein Kinase B/AKT And Focal Adhesion Kinase: Two Close Signaling Partners in Cancer." *Anti-Cancer Agents in Medicinal Chemistry*. https://doi.org/10.2174/187152011797927661.

Weitz, Jürgen, Moritz Koch, Peter Kienle, Andrea Schrödel, Frank Willeke, Axel Benner, Thomas Lehnert, Christian Herfarth, and Magnus Von Knebel Doeberitz. 2000. "Detection of Hematogenic Tumor Cell Dissemination in Patients Undergoing Resection of Liver Metastases of Colorectal Cancer." *Annals of Surgery*. https://doi.org/10.1097/00000658-200007000-00010.

Wells, James A., and Christopher L. McClendon. 2007. “Reaching for High-Hanging Fruit in Drug Discovery at Protein-Protein Interfaces.” *Nature*. https://doi.org/10.1038/nature06526.

Wen, Li, Tanveer A. Javed, Dean Yimlamai, Amitava Mukherjee, Xiangwei Xiao, and Sohail Z. Husain. 2018. “Transient High Pressure in Pancreatic Ducts Promotes Inflammation and Alters Tight Junctions via Calcineurin Signaling in Mice.” *Gastroenterology*. https://doi.org/10.1053/j.gastro.2018.06.036.

Wu, Meng Yao, Rong Rui Liang, Kai Chen, Meng Shen, Ya Li Tian, Dao Ming Li, Wei Ming Duan, et al. 2015. “FH535 Inhibited Metastasis and Growth of Pancreatic Cancer Cells.” *OncoTargets and Therapy*. https://doi.org/10.2147/OTT.S82718.

Wu, Rong, Xiao Meng Liu, Jian Guang Sun, Hong Chen, Jun Ma, Meng Dong, Shengyi Peng, et al. 2017. “DJ-1 Maintains Energy and Glucose Homeostasis by Regulating the Function of Brown Adipose Tissue.” *Cell Discovery*. https://doi.org/10.1038/celldisc.2016.54.

Yamaguchi, Kazuya, Yukihiro Takagi, Shinichirou Aoki, Manabu Futamura, and Shigetoyo Saji. 2000. “Significant Detection of Circulating Cancer Cells in the Blood by Reverse Transcriptase-Polymerase Chain Reaction during Colorectal Cancer Resection.” *Annals of Surgery*. https://doi.org/10.1097/00000658-200007000-00009.

Zeng, Bixi, Dinesh Devadoss, Shouye Wang, Emilie E. Vomhof-DeKrey, Leslie A. Kuhn, and Marc D. Basson. 2017. “Inhibition of Pressure-Activated Cancer Cell Adhesion by FAK-Derived Peptides.” *Oncotarget* 8 (58): 98051–67. https://doi.org/10.18632/oncotarget.20556.

Zhang, Jianhu, Cheri R. Owen, Matthew A. Sanders, Jerrold R. Turner, and Marc D. Basson. 2006. “The Motogenic Effects of Cyclic Mechanical Strain on Intestinal Epithelial Monolayer Wound Closure Are Matrix Dependent.” *Gastroenterology*. https://doi.org/10.1053/j.gastro.2006.08.007.

CHAPTER 3

Inhibition of pressure-activated cancer cell adhesion by FAK-derived peptides

The findings in this chapter have been previously published:

Bixi Zeng, Dinesh Devadoss, Shouye Wang, Emilie Vomhof-DeKrey, Leslie A. Kuhn, Marc D. Basson (2017). Inhibition of pressure-activated cancer cell adhesion by FAK-derived peptides. **Oncotarget**, 8(58):98051-98067.

3.1 Introduction

Disseminated tumor cells are pivotal in cancer metastasis. A one centimeter tumor sheds one million cells into the circulation daily (Fidler et al. 2005). Surgery may facilitate metastasis. Viable tumor cells (Umpleby et al. 1984) can frequently be recovered from the peritoneum (Allardyce et al. 1996) or portal or systemic circulation during colon cancer resections (Yamaguchi et al. 2000; Hayashi et al. 1999). Although it is difficult to distinguish undetected preoperative metastases from metastases arising from tumor cells dislodged by surgical manipulation, 0.2-0.5% of potentially curative surgical resections are marred by surgical wound recurrence (Nelson et al. 2004). Many more patients develop peritoneal dissemination or circulatory metastasis. 15% recurrence is seen in endometrial cancer, and a laparoscopic surgery is associated with 1.14% more wound recurrence than laparotomy (Walker et al. 2012). Since most cancer patients die of metastasis, not primary tumors, inhibition of metastasis is highly desirable. Perioperative tumor dissemination can turn curative cancer resections into a metastatic fatalities.

Exposing suspended colon cancer cells to 15 mmHg increased pressure promotes cell adhesion to collagen (Thamilselvan et al. 2007), endothelial monolayers, and murine surgical wounds (Craig, Owe, and Conway 2008; Craig et al. 2007). Pressure also activates adhesiveness in breast cancer (Downey et al. 2006), squamous cell carcinoma (Conway et al. 2006) and sarcoma cells (Perry et al. 2010). Activating this pathway potentiates peritoneal dissemination directly impacting survival in animal models (Craig, Owen, and Conway 2008; Downey et al. 2006). Shear similarly activates

cancer cell adhesion (Thamilselvan et al. 2007; Thamilselvan et al. 2004).

Disseminated tumor cells encounter such forces in various milieus. Circulatory pressures range from 5-10 mmHg in the portal vein to 90-120 mmHg in systemic arteries. (Pressures are referenced as gauge pressure, the excess beyond the 760 mmHg atmospheric pressure.) Tumors growing against constrictive stroma exhibit average 15-38 mmHg interstitial fluid pressures (Curti et al. 1993). Surgical tumor manipulation generates pressures of 1500 mmHg (Dregelid et al.1988) while irrigation causes shear. Laparoscopic insufflation elevates intra-abdominal pressure by 15 mmHg throughout surgery. Mechanical stimuli, such as pressure (Shiratsuchi and Basson 2004; Thubrikar and Robicsek 1995; Basson et al. 2015), shear (Thamilselvan et al. 2004; Huynh et al. 2016), and strain (Zhang et al. 2006; Sadoshima et al. 1997), influence physiological and pathological biology.

Pressure and shear activate dual mechanosensitive signaling pathways involving the cytoskeleton and paxillin or Src and phosphatidylinositol 3-kinase (PI3K) respectively (Basson 2008). These converge at FAK and Akt1, wherein Akt1 phosphorylates FAK at serines-517/601/695 facilitating FAK activation (Wang and Basson 2011). This increases β1-integrin affinity and avidity, lowers the ligand threshold required for integrin-mediated cell adhesion, and facilitates tumor dissemination (Thamilselvan et al. 2007). This signal pathway occurs in suspended cells. Adherent cancer cells, with a different cytoskeletal configuration, respond to increased pressure via a different pathway that triggers proliferation (Walsh et al. 2004). Because Akt

binding to FAK is uncommon in described signal pathways, we sought to prevent FAK activation by targeting this interaction, sparing conventional FAK-activating stimuli, and to inhibit this pathway in a more specific manner than typical FAK inhibitors.

The FAK molecule is divided into the N-terminal erythrocyte band four.1-ezrin-radixin-moesin (FERM) (residues 35-362), the kinase (residues 416-676), and the C-terminal focal adhesion targeting (FAT) (residues 677-1025) domains (Parsons 2003). The FERM domain provides an interface between membrane and cytoskeletal components and directs FAK activity (Parsons 2003). The conformation of the kinase domain exhibits little change in transition between active and inactive states and is therefore thought to be controlled through active site occlusion (Lietha et al. 2007). The FAT domain targets FAK to focal adhesions and is required for integrin-mediated FAK signaling (Thomas et al. 1999). We have previously shown that FAK-Akt1 binding requires neither the FAK kinase nor FAT domains; the highest Akt1 affinity depends on FERM domain residues 1-126 (Zeng et al. 2015; Basson et al. 2015). Using structural analysis and truncations of this fragment of human FAK as our starting point, we dissected the FAK-Akt1 interaction to define a short FAK-derived peptide that interrupts said interaction and demonstrated that this peptide inhibits cancer cell adhesion in vitro and in vivo and improves survival in a murine model of wound recurrence. 15 mmHg increased extracellular pressure was the prototypical force stimulus and wound adhesion the prototypical model for perioperative cancer cell metastasis.

3.2 Materials and Methods

Structure-based design of peptidyl epitopes to compete with FAK for binding Akt1

The crystal structure of human FAK (PDB 2AL6) and preliminary data showing Akt1 pulldown by a truncated 33 amino acid segment of the F1 lobe of FAK designated NT1-2-2 (Zeng et al. 2015) suggested that the NT1-2-2 region of FAK binds Akt1 through a short helical secondary structure accessible from the protein surface. The 33-residue peptide, NT1-2-2 (residues 94-126, EVHWVH....WKYELRI) includes the second and fourth strands from a small β sheet in FAK (labelled β4 and β5 in Figure 3.1D, based on their order in the sequence of PDB 2AL6 (Ceccarelli et al. 2006). This peptide does not include the third strand, which is needed for β-sheet integrity, so NT1-2-2 cannot mimic an intact β-sheet. However, the NT1-2-2 peptide does immunoprecipitate Akt1 (Figure 3.1A). This suggests that the structurally self-determinate helical region in the NT1-2-2 peptide is the epitope involved in Akt1 binding, formed by the α2 helix plus a single turn of helix formed by residues 116-118 (PPE) (Figure 3.7). Because hydrophobic interactions are important in protein-protein interfaces, we designed peptide variants centered on the hydrophobic C-terminal end of α2, followed by the PPE motif: LAHPPEE (residues 113-117). Consideration of statistical amino acid preferences to occur in α helices, β sheets and reverse (β) turns was augmented by Sequery and SSA analysis (Craig et al. 1998; Prevelige et al. 1989) of the preferred 3D conformations of tetrapeptide sequences in this region (e.g., LAHP, AHPP, HPPE, etc.) across a representative set of 4300 non-homologous structures in the Protein Data Bank. We designed mutants of FAK (shown as the LAHPPEE sequence for simplicity) or free peptides for competition with FAK as follows:

- L113A: AAHPPEE - Enhanced α helical preference
- P116N: LAHNPEE - Similar α helical, β turn preference in the PPE region, increased polarity
- P116C: LAHCPEE - Structurally labile, greater hydrophobicity
- P116G: LAHGPEE - Stronger turn preference, greater flexibility, less hydrophobicity
- P117K: LAHPKEE - Structurally labile, enhanced polarity
- P117S: LAHPSEE - More structurally labile and polar
- Triple mutant L113A, P116N, P117K: AAHNKEE - Enhanced helicity and polarity
- Triple mutant L113A, P116C, P117G: AAHCGEE - More structurally labile and hydrophobic
- Triple mutant: L113A, P116A, P117A: AAHAAEE - More helical and hydrophobic

Generation and expression of GST fusion proteins

Bacterial expression vector pGEX-4T1 (GE Healthcare, Munich, Germany) was used as a template to generate mutated and truncated human FAK as GST (Glutathione S-transferase) fusion proteins connected by a 12 residue linker. Point mutations (L113A, P116C, P116G, P116N, P117K, and P116S) and triple mutants (L113A/P116N/P117K, L113A/P116C/P117G, L113A/P116A/P117A) were generated using the Quick Change II XL Site-Directed Mutagenesis kit (Agilent Technologies, (Santa Clara, CA). Truncations were generated through PCR using forward and reverse primers to direct truncation (Table 1). PCR products were introduced into the pGEX-4T1 template between 5' EcoRI and 3'XhoI sites. Plasmids were purified via MiniPrep (QIAGEN, Valencia, CA) before

sequencing. BL21 competent E. coli (New England Biolabs, Ipswich, MA) were transformed with appropriate plasmids, and IPTG-induced.

Table 1: PCR forward primers for FAK truncations

Truncation name	Secondary-structure(s) truncated	Forward primer (*Eco*RI restriction site underlined)	Reverse primer (*Xho*I restriction site underlined)
Truncation 1	β-strand 4	5'-CCGGAATTCGTCTCCAGTGTGAGGGAGAAGTATGAGCTTGCTCACCCACCA-3'	5'-CCGCTCGAGAATTCTCAATTCATATTTCCACTCCTCTGGTGGGTGAGCAAG-3'
Truncation 2	β-strand 4, β-strand 5	5'-CCGGAATTCGTCTCCAGTGTGAGGGAGAAGTATGAGCTTGCT-3'	5'-CCGCTCGAGCTCCTCTGGTGGGTGAGCAAGCTCATACTTCTC-3'
Truncation 3	β-strand 4, α-helix 2	5'-CCGGAATTCCTTGCTCACCCACCAGAGGAGTGGAAATAT-3'	5'-CCGCTCGAGAATTCTCAATTCATATTTCCACTCCTCGGT-3'
Truncation 4	β-strand 4, β-strand 5 , α-helix 2	5'-CCGGAATTCCTTGCTCACCCACCAGAGGAG-3'	5'-CCGCTCGAGCTCCTCTGGTGGGTGAGCAAG-3'

Glutathione S-transferase pull-down

Glutathione-Sepharose-4B beads (30 µl) (GE Healthcare Life Science, Pittsburgh, PA) were conjugated with GST (expressed protein from a 250 µl bacterial pellet per 30 µl of beads) or recombinant GST-tagged (expressed protein from a 3 ml bacterial pellet per 30 µl of beads) (24) and incubated with lysate from $2x10^7$ Caco-2 or SW620 cells (1500 µg protein) or purified Akt1 (0.35 µg) (Origene, Rockville, MD) overnight at 4°C. Similar incubation for two hours at 4°C was performed with FAK-derived peptides (95% purity by HPLC) (Peptide 2.0, Chantilly, VA) reconstituted in sterile water and mixed with conjugated beads and cell lysate for a final concentration of 160mM before overnight Akt1 incubation. Bound protein was eluted for western analysis (Wang and Basson 2011).

Adenovirus vector construction and production

cDNA coding a seven amino acid segment from the F1 lobe of FAK (FAK-Helix, a.a. 113-119 LAHPPEE) and a scrambled version of this sequence (FAK-HelixScr, HPELAPE) were cloned in-frame into the MCS region of separate pShuttle-CMV vectors (Agilent, Santa Clara, CA) using forward primers that added 5'-NotI and reverse primers that added 3'-HindIII restriction sites (FAK-Helix forward, 5'-CCGTCGACGCGGCCGCATGCTTGCTCACCCACCAGAGGAGTAA-3' | FAK-Helix reverse, 5'-TCTTATCTAGAAGCTTTTACTCCTCTGGTGGGTGAGCAAGCAT-3') (FAK-HelixScr forward, 5'-CCGTCGACGCGGCCGCATGCACCCAGAGCTTGCTCCAGAGTAA-3' | FAK-HelixScr reverse, 5'-TCTTATCTAGAAGCTTTTACTCTGGAGCAAGCTCTGGGTGCAT-3'). The

PCR did not use template DNA as forward and reverse primers collectively spanned the entire product. Recombinants were generated per manufacturer's protocols (AdEasy, Agilent, Santa Clara, CA), selected using kanamycin resistance, confirmed by sequencing, amplified in XL10-gold ultracompetent cells, purified, PacI-linearized, and transfected into HEK293 cells to produce adenoviral vectors coding for FAK-Helix (Ad-FAK-Helix) and the FAK-HelixScr (Ad-FAK-HelixScr). Viral particles were expanded and collected per manufacturer's protocols, and passed through a Fast-Trap Adenovirus Purification and Concentration kit (EMD Millipore, Darmstadt, Germany) before reading OD at 260nm. Viral titer was calculated as one A260 unit to 1012 viral particles with a 50:1 ratio of particles to infectious particles via agarose overlay per manufacturer's protocols (not shown).

Pressure regulation

Pressure was controlled using an airtight apparatus previously described, pressurized with filtered 5% CO2/95%, and maintaining temperature, pressure, pO2, pCO2, and pH (Basson et al. 2000).

Cell adhesion studies

SW620 colorectal adenocarcinoma cells (ATCC, Manassas, VA) at 90% confluence were split 1:4 two days previously to achieve 50-60% confluence on the day of adhesion assay. SW620 cells were trypsinized, plated randomly at $5x10^4$ cells/well, and allowed to adhere to collagen-I-coated plates for 30 minutes at 37°C under ambient or 15mmHg increased pressure (Basson et al. 2000). After 30 minutes, non-adherent

cells were washed away with warm PBS. Ambient and pressure-treated plates were encoded to prevent treatment identification during washing. Adherent cells were incubated for 1 hour at 37°C with CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI), and absorbance measured at 490nm with an Epoch plate reader (Biotek, Winooski, VT). For adenoviral experiments, SW620 cells were grown to 90% confluence in a T25 flask (Corning, Corning, NY) before viral infection (13x 10^3 vp/cell) for 1 hour before replacing infection media with growth media. After 24 hours, infected SW620s were replated in new T25 flasks at a 1:4 ratio. At 72 hours, adhesion was assayed as above. Cells were used within ten passages and authenticated by ATCC.

Phosphorylation

SW620 cells were transfected with Ad-FAK-Helix or Ad-FAK-HelixScr for 72 hours, trypsinized, and exposed to ambient or 15mmHg increased pressure for 30 minutes at 37°C in growth media in 48 well plates pacificated with 1% heat-inactivated BSA in PBS (to prevent adhesion). SW620 cells allowed to adhere to collagen-I-coated plates for 30 minutes at 37°C were positive controls. Cells were lysed for western analysis.

Cell proliferation

SW620 colorectal adenocarcinoma cells were seeded at 10^4 cells/well on 96 well plates, recovered at 37°C for 12 hours, allowed 24 hours for proliferation, and counted using CellTiter 96 Aqueous One Solution Reagent as above. In adenoviral studies,

SW620 cells were transfected with Ad-FAK-Helix or Ad-FAK-HelixScr 60 hours before plating on 96 well plates. For nonsurvival wound adhesion studies, cells were dyed with 10μM Tag-it Violet proliferation and cell tracking dye (BioLegend, San Diego, Ca), or equivalent amounts of DMSO vehicle.

FAK-Akt1 coimmunoprecipitation

Coimmunoprecipitations were performed as previously (Craig et al. 2007) using mouse monoclonal antibodies to Akt1 (CST, Beverly, MA) and HA (Convance, Berkley, CA).

Transfections

SW620 cells were transfected using Lipofectamine 2000 reagent (ThermoFisher, Waltham, MA) and HA-FAK(WT) plasmid (from Dr. JL Guan). Cells were grown in T75 flasks until 80% confluent, replated into 6 well plates at 80% confluence, and transfected 12 hours later per manufacturer's protocols.

Western Blotting

Protein concentrations were determined by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Eluate from GST pull-downs or collected cell lysates was resolved by SDS-PAGE and transferred to Hybond P 0.45 PVDF blotting membrane (Amersham Life Science, Arlington Heights, IL). Membranes were blocked for 1 hour at room temperature with Odyssey TBS Blocking Buffer (Amersham Life Science, Arlington Heights, IL) and blotted overnight at 4°C with antibodies against FAK (#3285), Phospho-

FAK Tyr397 (#3283), Akt1 (#2967), Phospho-Akt1 Ser473 (#9271), GSK-3β (#9315), Phospho-GSK-3β Ser9 (#9315), or the GST tag (#2624) (CST, Beverly, MA). Membranes were visualized by the infrared fluorescent IRDye system (LI-COR Biosciences, Lincoln, NE) and analyzed on an Odyssey scanner (LI-COR Biosciences, Lincoln, NE) within the linear range. Results were normalized to the appropriate GST tag, GAPDH, or total protein (for phosphorylated proteins) and relative to associated ambient controls unless stated otherwise.

Akt1 frequently appears in published Western blots (Ko et al. 2016; Wu et al. 2017; Datta et al. 1997; Li et al. 1999) as a doublet. The reason for this is not entirely clear, but may represent altered phosphorylation states or other post-translational modifications of the molecule yet to be clarified. The cell line studied and its phenotype in the experiment in question, the amount of protein loaded and concentration of primary and secondary antibody, the percentage of the SDS-PAGE used to resolve the proteins, and numerous other factors may influence this. The densitometric data presented here considers Akt1 as the sum of the intensity of each of the two doublets per lane.

Wound implantation

SW620 colorectal adenocarcinoma cells were transfected with Ad-FAK-Helix or Ad-FAK-HelixScr. After 72 hours, cells for nonsurvival studies were trypsinized and dyed with 10μM Tag-it Violet (BioLegend, San Diego, Ca) per manufacturer's protocols; survival studies used undyed cells. Cells were incubated for 30 minutes at 37°C under

ambient or 15mmHg increased pressure in a 48 well plate pacificated with 1% heat inactivated BSA in PBS to prevent adhesion to the plate. These cells were then collected and washed in warm PBS. In nonsurvival studies, 1 cm groin incisions were made bilaterally in 6-7 week old 22.9-24.2 gram male BALB/cAnNHsd mice (Envigo, Haslett, MI) anesthetized i.p. with ketamine (100mg/kg), xylazine (10mg/kg), and acepromazine (3mg/kg). In survival studies, a single 1 cm groin incision was made in 6-7 week old 22.9-24.2 gram male athymic nude- Foxn1nu mice (Envigo, Haslett, MI) anesthetized with continuous inspired 1-2% IsoFlo (Abbott Laboratories, North Chicago, lL) in oxygen. A 50 µl suspension ambient or increased pressure of $5x10^5$ cells was randomly applied to the wounds. After 30 minutes, the fluid was aspirated and the wounds were washed with warm phosphate buffered saline six times to remove nonadherent cells. In nonsurvival studies, the mice were euthanized following wound irrigation, and wounds were excised to quantify tumor adhesion by fluorescence-activated cell sorting (FACS). The excised wound tissue was mechanically (paired scissors) and then enzymatically (3 ml/sample collagenase incubation for 1hour at 37°C with agitation) disaggregated before passage through a cell strainer and 20 minute room temperature incubation in BioLegend fixation buffer (BioLegend, San Diego, Ca). Fixed cells were resuspended in PBS with 5%FBS and Tag-IT dye. Fluorescence and cellular auto-fluorescence were detected using a LSR flow cytometer (BD Biosciences, San Jose, CA) with a filter for Pacific Blue (ex/em 410/455). In survival studies, the wounds were instead closed and followed as described (Craig, Own, and Conway 2008). Animal studies were sized to yield 95% confidence with 80% power and approved by the Institutional Animal Care and Use Committee of the University of North Dakota.

Statistics

Data are expressed as mean±SEM. Results were compared by Student's unpaired t-test and log-rank test as appropriate seeking 95% confidence. In vivo studies were analyzed by Mantel-Haenszel testing. Assays were within linear ranges.

3.3 Results

GST-FAK fusion proteins pull-down purified and endogenous Akt1, is modulated by mutations, and persists in NT1-2-2 truncations

Based on Western blots comparing band intensity of 0.001-0.05 micro gram/lane of purified Akt1 against the band intensity of 40 or 80 micro gram/lane of cell lysate (not shown), we used 0.3 µg of purified Akt1 in pull-down assays to approximate the Akt1 in 1,500 µg of whole cell lysate, the lysate from $1x10^7$ SW620 cells previously used in similar pull-down assays. GST-FAK-NT1-conjugated Sepharose beads pulled down Akt1 after overnight incubation with either cell lysate or purified Akt1 (Fig. 3.1A), suggesting that FAK and Akt1 bind directly without intermediary or scaffolding proteins.

We examined the role of a short helical secondary structure (LAHPPEE) using mutated variants of FAK-NT1 (Fig. 3.1B). FAK-NT1 is a larger truncation of FAK encompassing the NT1-2-2 region of interest; it is sufficient to pull down Akt1 and was chosen as a conservative platform that could support the native folding of the NT1-2-2 region in our mutation assays (Basson et al. 2015). Of the nine mutants studied, Akt1 binding affinity was significantly different from that of the wild-type F1 lobe for FAK(P117S) and (L113A/P116C/P117G) (Fig. 3.1B,C). FAK(P117S) was designed to

increase short helix rigidity and lowered Akt1 pull-down ($p < 0.005$, N=11).

A

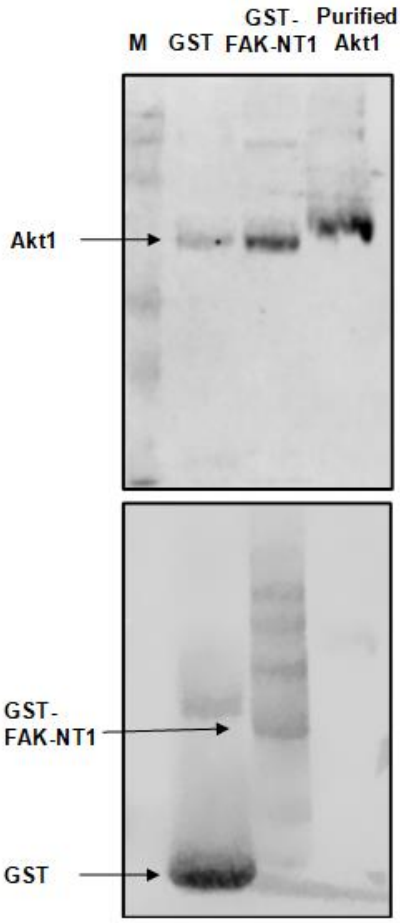
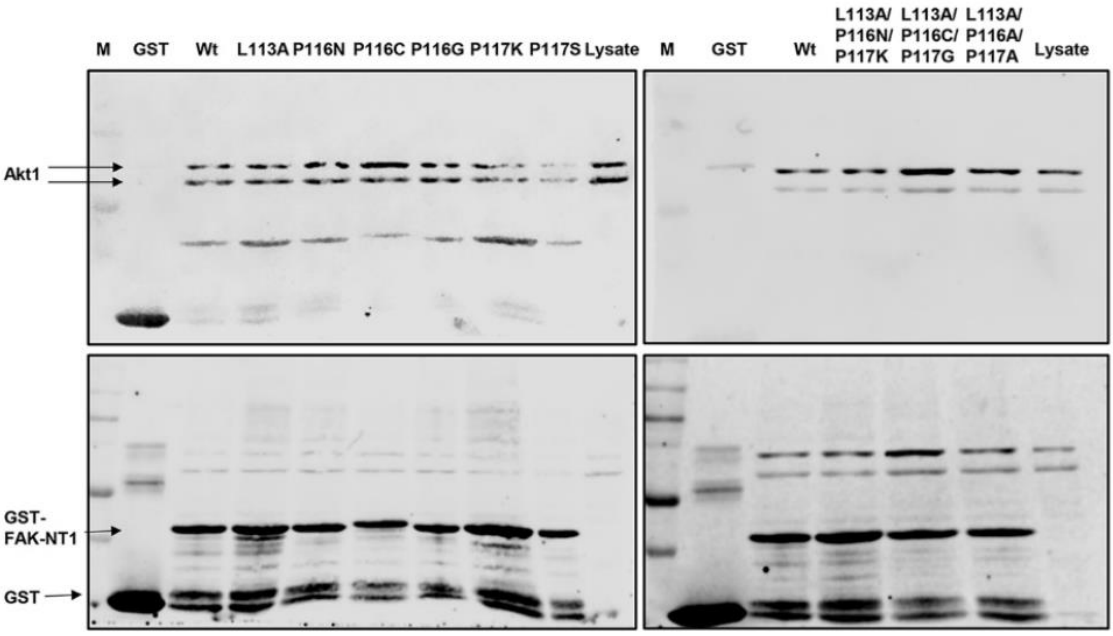


Figure 3.1. Interaction of Akt1 with FAK truncations
(Fig. 3.1A) GST-FAK-NT1 conjugated beads pulled down purified Akt1 (N=3). The western blot shows the amount of Akt1 (prey, 60 kDa) signal relative to the amount of GST-FAK-NT1 (bait, estimated 35 kDa) signal. (Fig. 3.1B) The western blot shows the amount of Akt1 (prey) signal relative to the amount of GST-FAK-NT1 or FAK mutant (bait) signal (representative blot). (Fig. 3.1C) Densitometric data was analyzed as the percentage of Akt1 signal over GST fusion protein signal, which was then normalized to the wild-type NT1. (n=8-19, * $p<0.005$ vs. the GST-FAK NT1 wild-type). (Fig. 3.1D) The amino acid sequence of the NT1-2-2 peptide with the corresponding secondary structures are shown; the β-helices are shown in green, the α-helix in gray, and the short helix in cyan. The cartoon representations of the secondary structures present in each truncation correspond to the constructs found in the table to the left. (Fig. 3.1E) The western blot shows the amount of Akt1 (prey) signal relative to the amount of GST/GST-FAK truncation (bait) signal. The low molecular weight of the truncations impedes the differentiation between GST-FAK truncations and unbound GST tags. (Fig. 3.1F) Densitometric data was analyzed as the percentage of Akt1 signal over GST fusion protein signal and then normalized to the Akt1 pulldown from the NT1-2-2

Figure 3.1 (cont'd)

construct. All truncations pulled down significantly more Akt1 than did the GST control (n = 6, * $p < 0.05$ vs. GST). Western blots were probed for Akt1 (top) and GST (bottom). A marker (M) and the amount of Akt1 signal produced by 40 µg of SW620 whole cell lysate control were used as a references.

B



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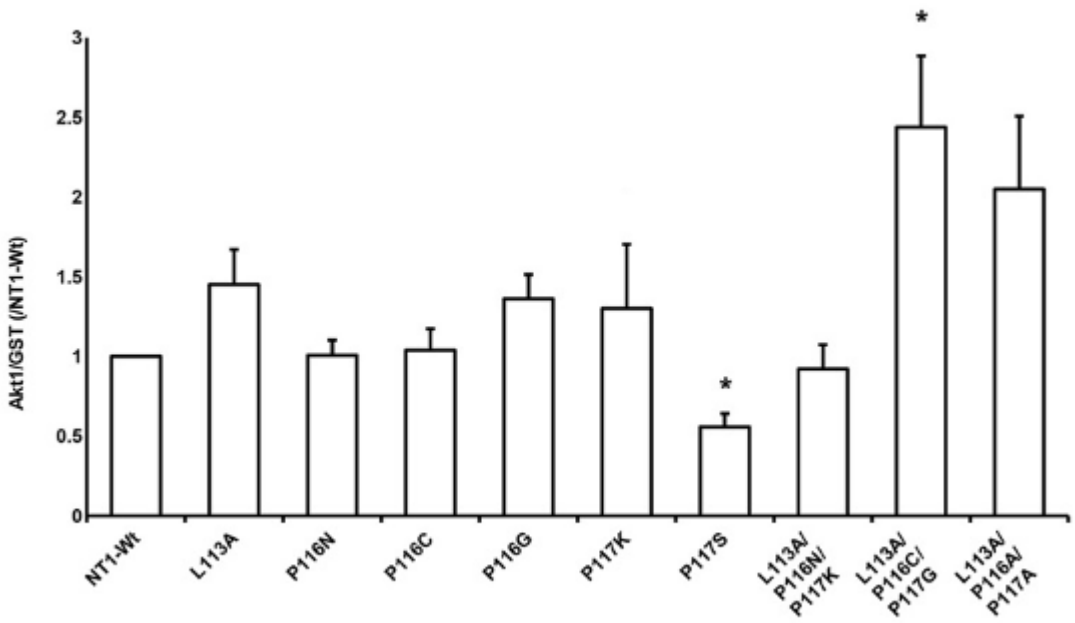

Figure 3.1 (cont'd)

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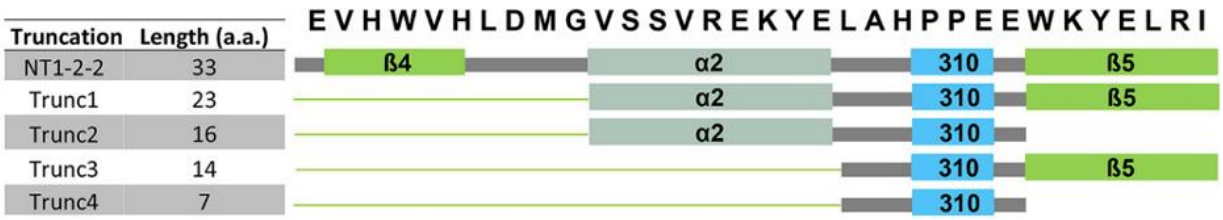

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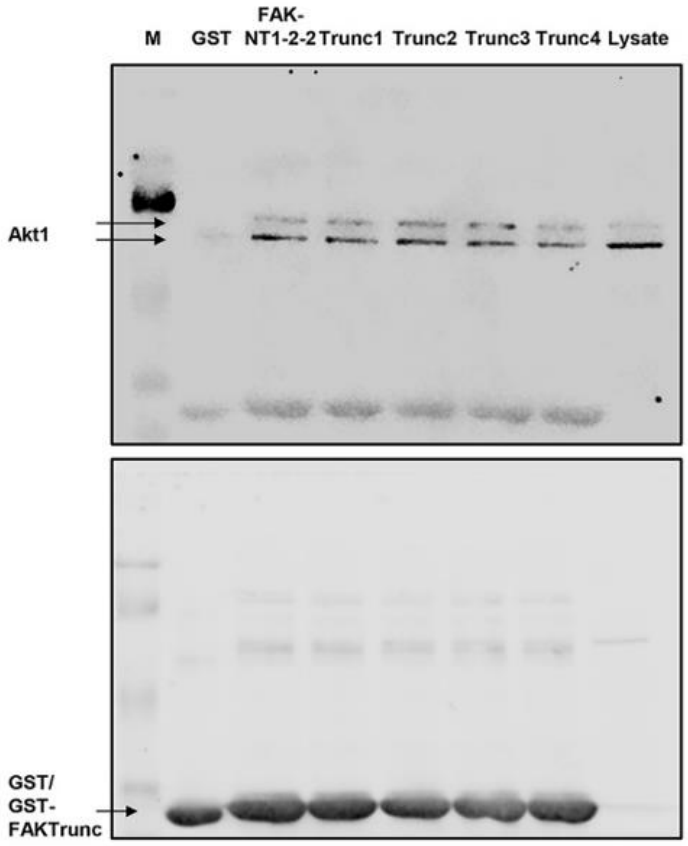

Figure 3.1 (cont'd)

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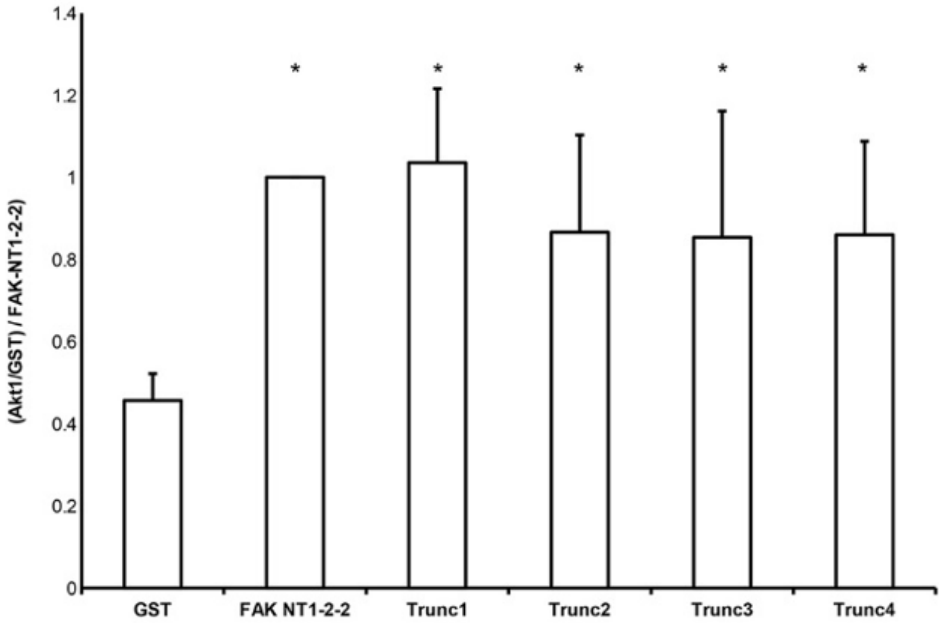


Conversely, the triple mutant FAK(L113A/P116C/P117G) aimed to destabilize the region and consequently increased Akt1 pull-down ($p < 0.005$, N=14) (Fig. 3.1C). Single mutants with increased helicity, FAK(L113A), or altered short helix hydrophobicity, FAK(P116N), (P116C), (P116G), and (P117K), did not change Akt1 pull-down. The triple mutants FAK(L113A/P116N/P117K) and FAK(L113A/P116A/P117A) that increased helicity did not significantly change Akt1 pull down. Altogether, FAK NT1 pull-down of Akt1 is altered by point mutations to this short helical region.

To investigate the importance of individual subdomain structures within NT1-2-2 in FAK-Akt1 binding, we generated four variants that successively excluded secondary structures in the N- and C-terminal of the short helical segment while preserving the

short helix itself (Fig. 3.1D). We challenged these truncated versions of NT1-2-2 by Akt1 pull-down. We observed some non-specific GST-binding of Akt1, but NT1-2-2 pulled down much more. Truncation did not interfere with the Akt1-binding of the larger sequence (Fig. 3.1E).

NT1-2-2 derived peptides inhibit FAK pull-down of Akt1

To minimize the effect of non-specific binding to GST, we next used the larger FAK-NT1 (relative to GST) as bait and assessed the effects of the NT1-2-2 FAK truncations as interfering peptides. The wild-type 33 amino acid peptide (Pep-FAK-NT1-2-2) reduced binding between GST-FAK-NT1 and Akt1. A scrambled 33 amino acid control peptide containing the same amino acids in a different order. (Pep-FAK-NT1-2-2Scr) did not (Fig. 3.2A, B). We subsequently used full length GST-FAK as bait to further validate the ability of the interfering peptide to block Akt1 interaction with the entire FAK molecule. Because the seven amino acid sequence from the short helix (LAHPPEE) seemed sufficient for Akt1 binding in figure 3.1E above, we focused on this seven amino acid sequence and mutants thereof. Bacterial production of GST and GST-FAK is not equal despite being under the control of identical promoters. In Figure 3.2C, lane 2 used GST alone as the bait protein and produced a large, low weight band while samples that used GST-FAK as bait (lanes 3-8) show multiple bands, which may be the breakdown products of the original GST-FAK construct. We matched the GST in all lanes to provide a conservative negative control. Despite the relatively higher amount of GST bait protein over GST-FAK, all GST-FAK constructs pulled down significantly more Akt1 than the GST alone. Wild-type LAHPPEE and mutant (LAHPSEE and AAHCGEE)

versions of the FAK peptide reduced Akt1 pulldown by human wild type GST-FAK in the presence of vehicle alone

A

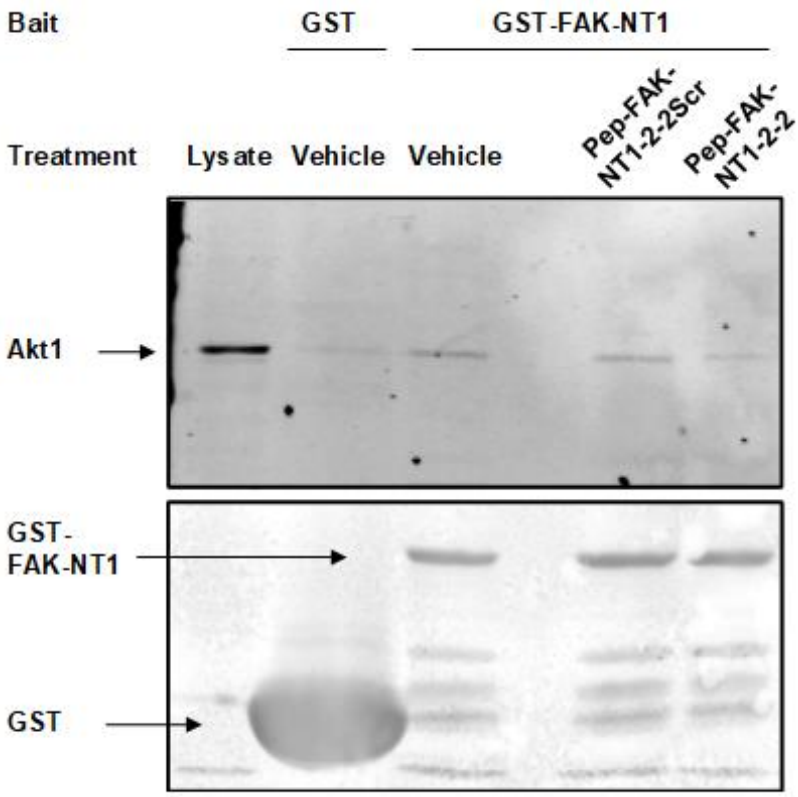
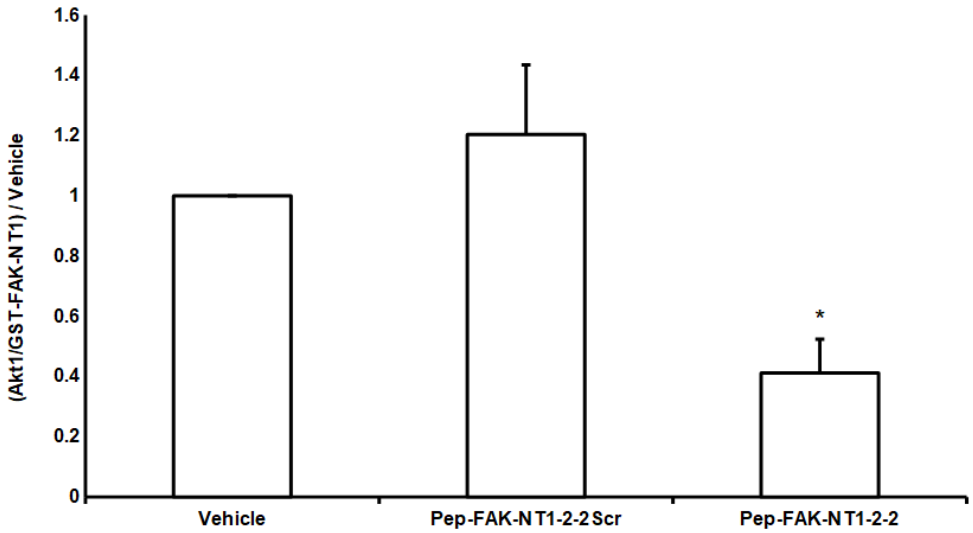


Figure 3.2. Truncated FAK mutants modulate pull down of Akt1
(Fig. 3.2A, B) Treatment with the wild-type 33 amino acid peptide (Pep-FAK-NT1-2-2) reduced the amount of Akt1 pulled down by GST-FAK-NT1 vs. vehicle; the scrambled peptide (Pep-FAK-NT1-2-2Scr) did not have any effect (n = 4, * $p < 0.05$ vs. vehicle-treated GST-FAK-NT1). (Fig. 3.2C) Studies parallel to those in Figure 3.2B, but which used GST-FAK as bait and interfering peptides 7 amino acids in length, showed decreased Akt1 pulldown with the addition of wild type or mutant FAK peptides. (Fig 3.2C)The GST probed western (bottom) shows more bands as the full-length GST-FAK (150 kDa) yields more break down products. (Fig. 3.2D) Incubation with the wild-type (LAHPPEE) or mutant (LAHPSEE and AAHCGEE) peptides reduced Akt1 pulldown compared to the vehicle. No such effect was seen after incubation with the scrambled (HPELAPE) or β-strand (WKYELRI) control (n = 12-14, * $p < 0.05$ vs. vehicle treated GST-FAK). All peptides were used at a concentration of 160 μM. Ribbon (Fig. 3.2E) and surface (Fig. 3.2F) depictions of the crystal structure of the FAK FERM domain

Figure 3.2 (cont'd)

containing the F1 (magenta, green, cyan and blue), F2 (orange), and F3 (yellow) lobes (PDB entry 2AL6 (30)), rendered by PyMOL (v. 1.8.2.2; Schrodinger LLC, NY). (Fig. 3.2G) Close-up of the FAK NT1-2-2 peptide region based on the crystal structure of chicken FAK, which is highly similar in sequence. Relative to (Fig. 3.2E) and (Fig. 3.2F), the view in panel (Fig. 3.2G) is rotated by 180° about the z-axis (perpendicular to the plane of the page), to better view the LAHPPEE epitope (residues 113-117). All renderings of the NT1-2-2 segment show secondary-structures β4 and β5 in green, α2 in cyan, and the short PPE helix in dark blue.

B



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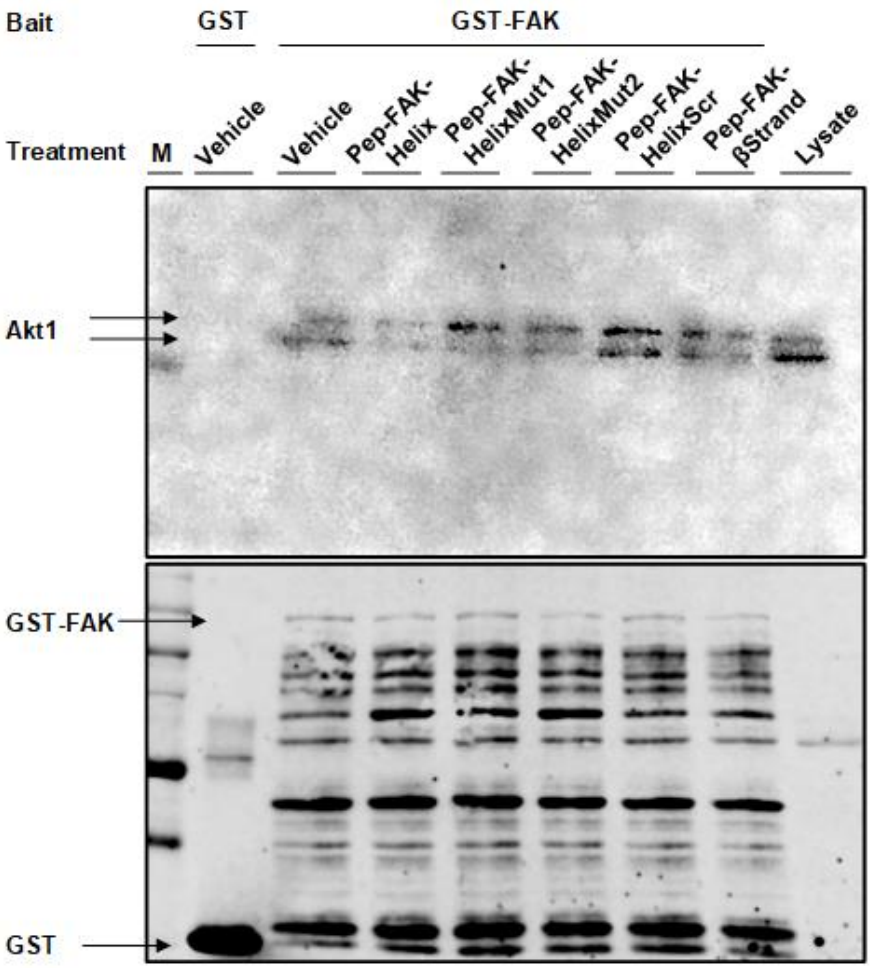

Figure 3.2 (cont'd)

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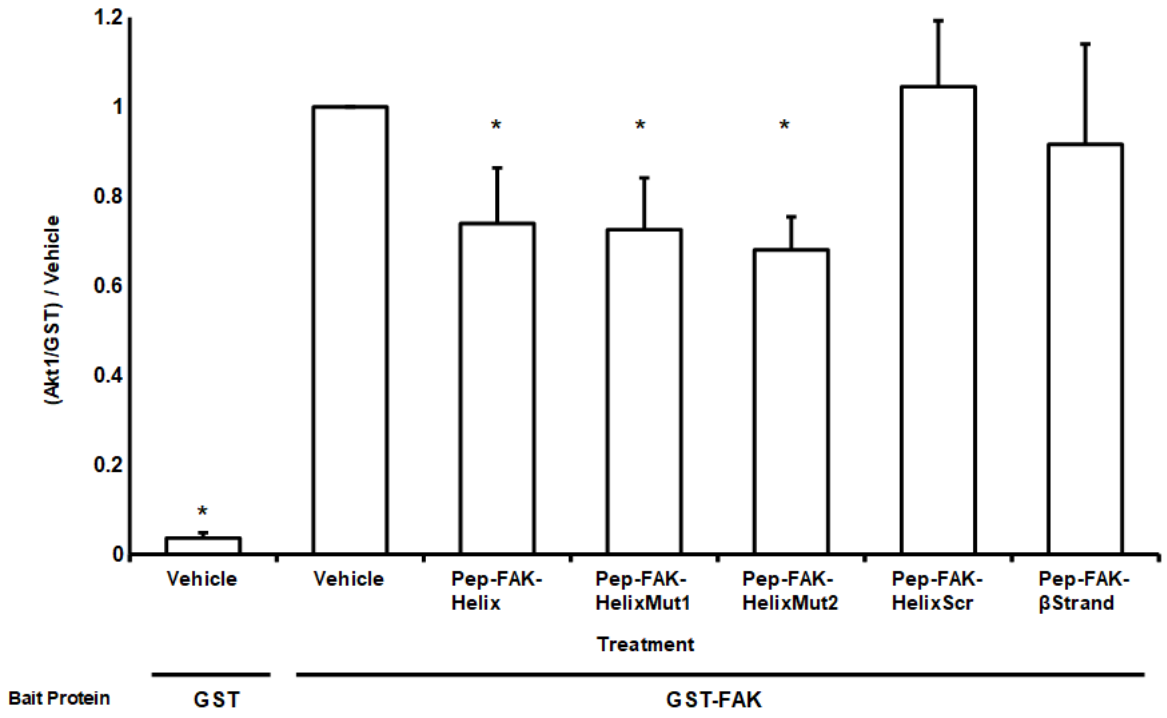

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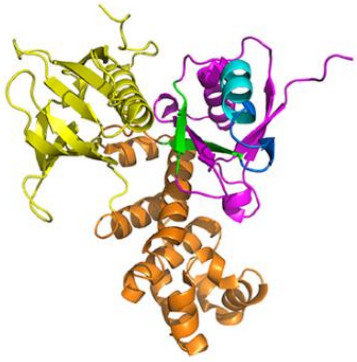

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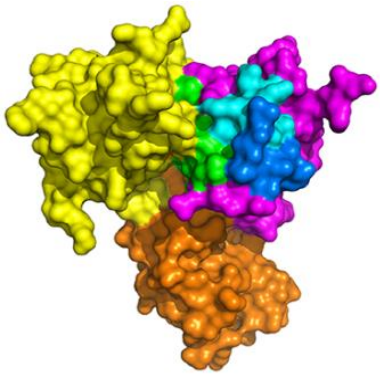

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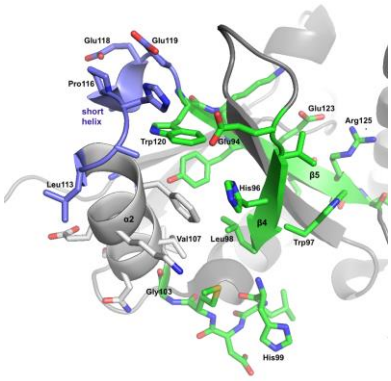


(Fig. 3.2C, D). Neither a scrambled version of the short helix (HPELAPE) nor a peptide derived from the β-strand secondary structure C-terminal adjacent to the short helix (WKYELRI) interfered with pulldown. The location of the LAHPPEE peptide within the

FAK molecule is shown in ribbon (Fig. 3.2E) and surface (Fig. 3.2F) renderings of the crystal structure of the FAK FERM domain (PDB entry 2AL6 (30)). The NT1-2-2 segment is located mostly on the surface of the F1 domain (magenta, green, cyan and blue) and is colored as shown in Fig. 4.1D, with β4 and β5 in green, α2 in cyan, and the short PPE helix in dark blue. A magnified version of the FAK NT1-2-2 peptide region (Fig. 3.2G) shows the β4 and β5 in green, α2 in gray (left), and the LAHPPEE short helix in dark blue (upper left).

Both pressure-induced phosphorylation of FAK, but not Akt1 or GSK-3β, and pressure-induced HA-FAK, Akt1 coimmunoprecipitation are inhibited in SW620 infected with adenovirus expressing FAK-derived peptides

We created adenoviral vectors to deliver the wild type sequence LAHPPEE (Ad-FAK-Helix) or the scrambled HPELAPE (Ad-FAK-HelixScr) into intact human SW620 colon cancer cells and assessed pressure-induced signaling. 15mmHg increased pressure stimulated FAK Tyr397 phosphorylation (145±10%) in cells infected with the scrambled Ad-FAK-HelixScr control vs. cells at ambient pressure (Fig. 3.3A, B). In contrast, pressure did not stimulate FAK Tyr397 phosphorylation in cells infected with Ad-FAK-Helix, overexpressing the native FAK-derived seven amino acid sequence. Pressure-induced FAK and Akt1 phosphorylation is initiated by cytoskeletal mechanosensing in suspended cells (Thamilselvan et al. 2007) independent of traditional adhesion-induced signaling, which begins with surface integrin binding and progresses inward activating associated proteins. Adhesion to collagen I induced FAK Tyr397 phosphorylation in both Ad-FAK-HelixScr and Ad-FAK-Helix infected cells

similarly (Fig. 3.3B), suggesting the specificity of the effect of LAHPPEE in inhibiting FAK-Akt1 interaction. Because pressure stimulates Akt1 Ser473 phosphorylation and Akt1 activation before Akt1 phosphorylates FAK (Wang and Basson 2011), we predicted that LAHPPEE would not interfere with other aspects of Akt1 signaling. Neither virus inhibited pressure-induced Akt1 Ser473 phosphorylation (consistent with the model that Akt1 activation by pressure occurs upstream of Akt1-FAK interaction) or adhesion-induced Akt1 Ser473 phosphorylation (Fig. 3.3C). We examined phosphorylation

A

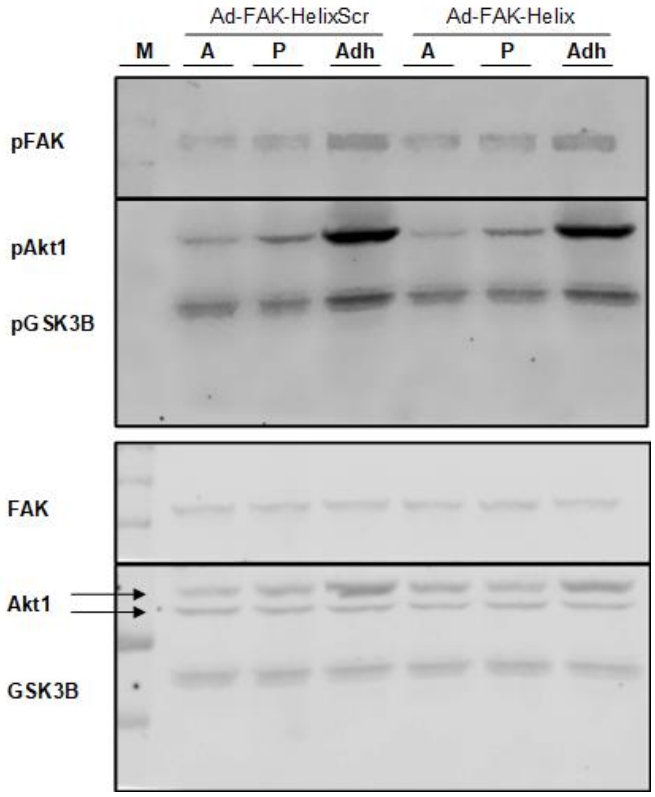
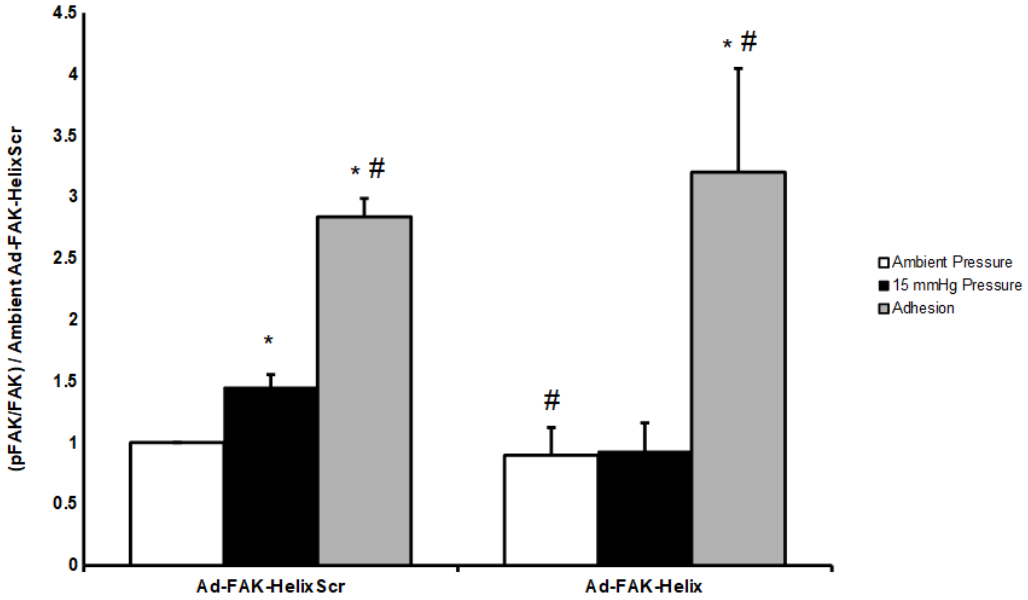


Figure 3.3. 7-Residue FAK peptide inhibits pressure-stimulated phosphorylation (Fig. 3.3A) Lysate from Ad-FAK-HelixScr and Ad-FAK-Helix infected cells exposed to ambient (A) or 15 mmHg (P) while in suspension, or allowed to adhere to collagen I (Adh) were probed for phospho- (top) and total (bottom) FAK, Akt1, and GSK3B. Blots

Figure 3.3 (cont'd)

were cut at the level of 75 kD, and the higher weight bands were incubated with pFAK/FAK (125 kDa) antibodies while the lower weight bands received pAkt1/Akt1 (60 kDa) and pGSK3B/GSK3B (46 kDa) probes. (Fig. 3.3B) In suspended cells, pFAK increased following 15 mmHg exposure vs. exposure to ambient atmosphere in Ad-FAK-HelixScr, but not Ad-FAK-Helix, infected cells. In contrast, adhesion increased FAK phosphorylation over suspended cells at ambient pressure in both Ad-FAK-HelixScr and Ad-FAK-Helix infected cells. (n = 4-8, * $p < 0.05$ vs. ambient Ad-FAK-HelixScr, # $p < 0.05$ vs. 15 mmHg Ad-FAK-HelixScr) (Fig. 3.3C) Both Ad-FAK-HelixScr and Ad-FAK-Helix virus infected cells exhibited increased Akt1 phosphorylation after exposure to 15 mmHg pressure as well as after adhesion. (n = 4-8, * $p < 0.05$ vs. ambient Ad-FAK-HelixScr, # $p < 0.05$ vs. 15 mmHg Ad-FAK-HelixScr) (Fig. 3.3D) GSK-3β phosphorylation also increased in both the virus treated cells in response to adhesion; however, in pressure treated groups, GSK-3β phosphorylation decreased in the Ad-FAK-HelixScr but not the Ad-FAK-Helix virus treated cells (n = 4-8, * $p < 0.05$ vs. ambient Ad-FAK-HelixScr). (Fig. 3.3E) HA/HA-FAK coimmunoprecipitated Akt1 (top) to produce a western signal which was normalized to the respective amount of FAK signal (bottom). The samples are grouped by viral infection, uninfected (Control), Ad-FAK-HelixScr, or Ad-FAK-Helix, and then subdivided by exposure to ambient (A) or 15 mmHg pressure (P). All cells were transfected with HA-FAK except the HA-Ctrl cells which were transfected with a plasmid expressing the HA tag alone. A marker (M) and 40 µg of SW620 whole cell lysate were used as a references for Akt1 and FAK. (Fig. 3.3F) Exposure to pressure increased Akt1 coimmunoprecipitation in control SW620 cells or cells infected with Ad-FAK-HelixScr. Pressure did not increase Akt1 coimmunoprecipitation in SW620 cells infected with Ad-FAK-Helix (n = 6, * $p < 0.05$ vs. ambient pressure, # $p < 0.05$ vs. 15 mmHg Ad-FAK-HelixScr).

B



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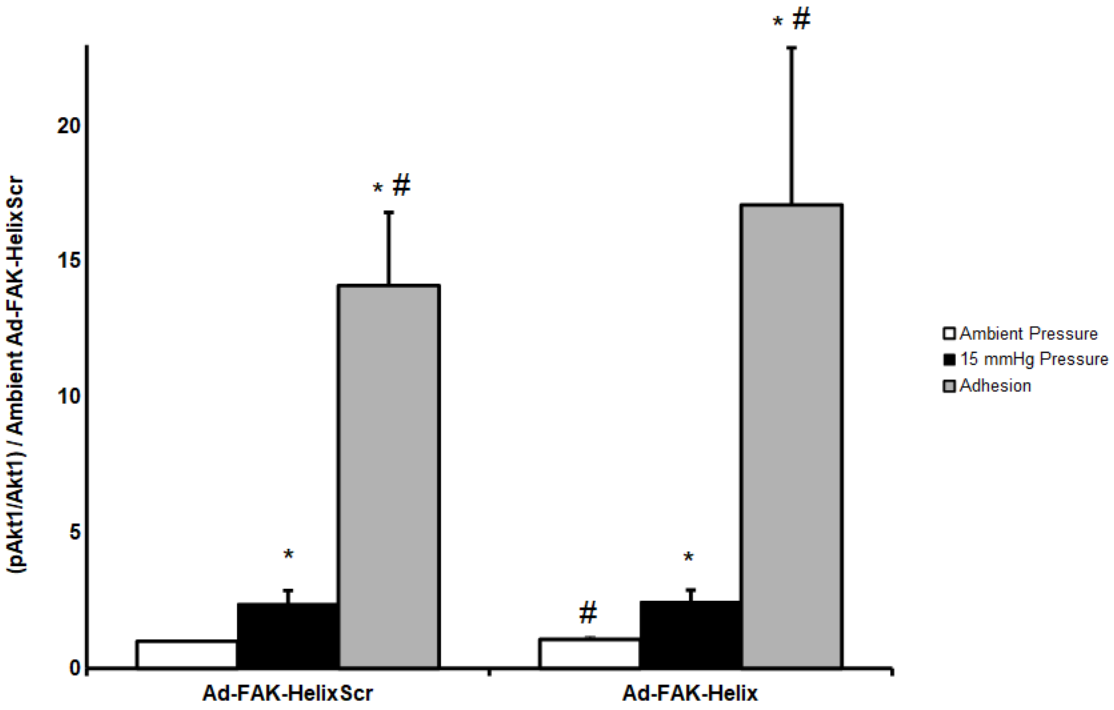

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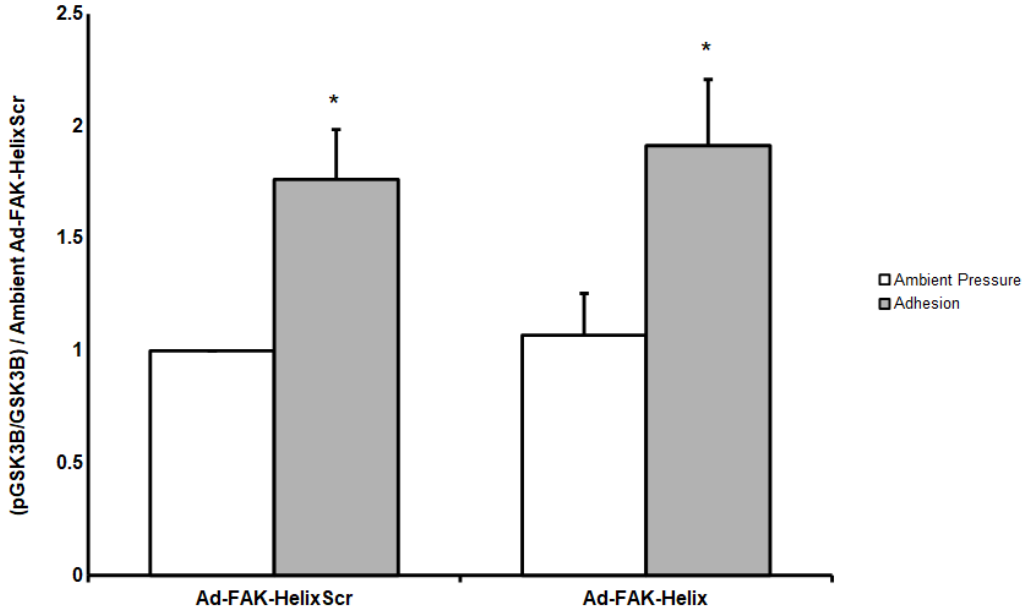

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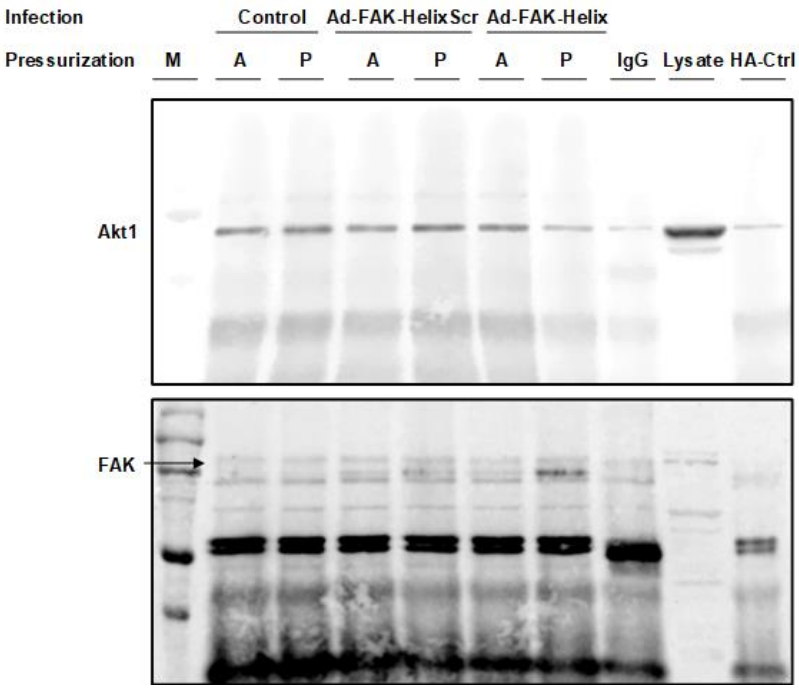

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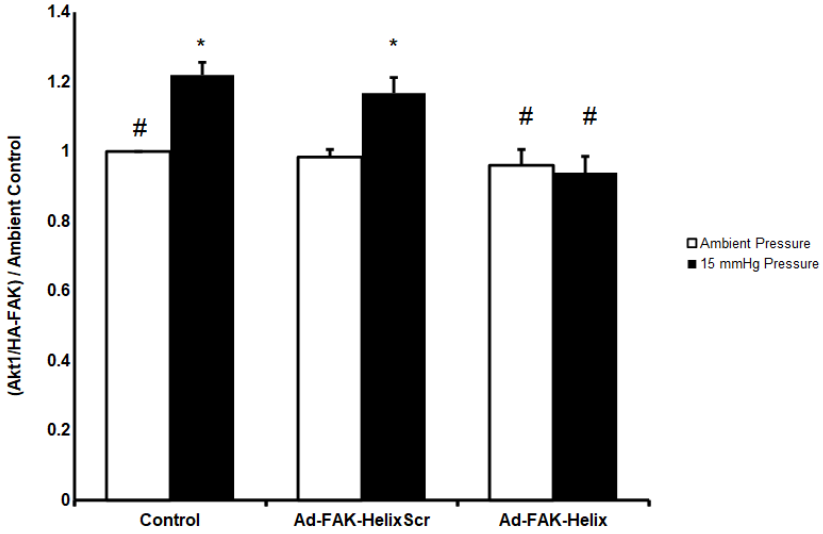

of the Akt target protein GSK-3β after adhesion to further evaluate the potential for peptide overexpression to modulate Akt1 downstream signaling. Cell adhesion stimulated GSK-3β Ser9 phosphorylation similarly in Ad-FAK-HelixScr-infected and Ad-

FAK-Helix infected cells (Fig. 3.3D). Interestingly, pressure had an unanticipated inhibitory effect on GSK-3β phosphorylation.

We have previously demonstrated that pressure stimulates FAK-Akt1 interaction in intact SW620 cells, transfected with HA-FAK before immunoprecipitation with anti-HA to amplify the signal produced by basal FAK-Akt1 interaction (Thamilselvan et al. 2007; Wang and Basson 2011). We now performed parallel studies to validate our in vitro findings in intact cells, using adenoviral infection to introduce FAK-derived peptides (Fig. 3.3E). Consistent with published observations (Wang and Basson 2011), pressure increased co-precipitating Akt1 from uninfected SW620 cells vs. cells at ambient atmospheric pressure. Infection with Ad-FAK-Helix blocked this effect. Ad-FAK-HelixScr did not (Fig. 3.3F). Due the size of the FAK-derived peptide as well as the region of FAK from which it was derived, Western blots using FAK antibody probes are unable to detect the presence of our peptide. However, q-RT-PCR analysis of the helical and scrambled peptide messages suggested similar expression in infected cells (not shown).

FAK-derived peptide overexpression prevents pressure-induced SW620 cell adhesion but does not affect proliferation

We hypothesized that peptide overexpression would similarly inhibit pressure-stimulated adhesion, the downstream consequence of pressure-activated FAK phosphorylation. Equal numbers of virus-treated cells were seeded onto collagen-I-coated plates under ambient or increased pressure for 30 minutes. The plates were

washed in blinded fashion to remove nonadherent cells. The remaining adherent cells were quantified by MTS assay. Pressure-induced adhesion was inhibited by Ad-FAK-Helix infection but not by Ad-FAK-HelixScr (Fig. 3.4A). Increased pressure stimulates cancer cell proliferation by a different mechanism (Craig et al. 2007; Basson et al. 2015;

A

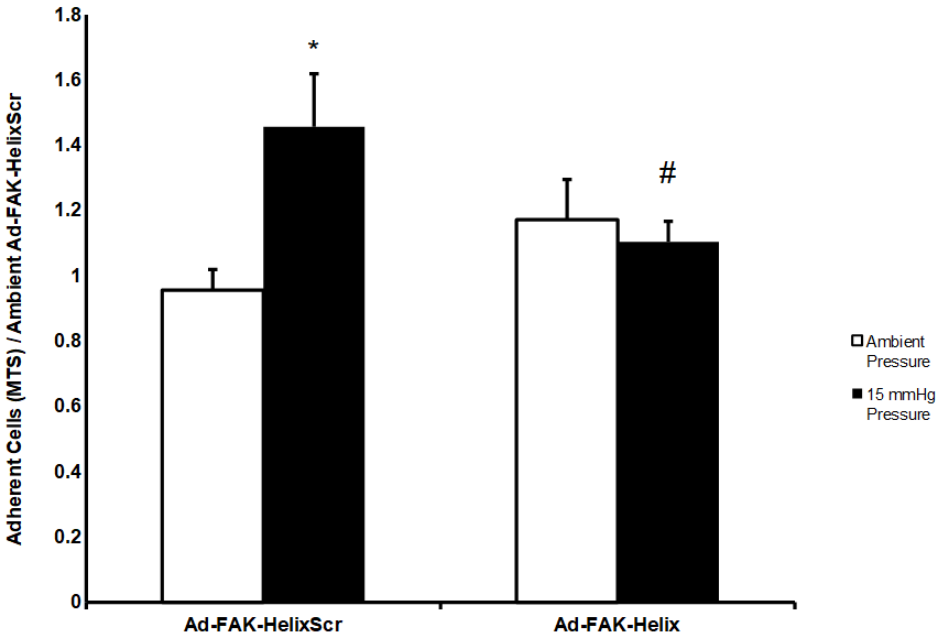

Figure 3.4. A FAK-derived peptide blocks pressure stimulation of adhesion but not pressure stimulation of proliferation
(Fig. 3.4A) Exposure to elevated pressure increased adhesion by SW620 cells infected with the Ad-FAK-HelixScr virus vs. ambient pressure. Adhesion by cells infected with the Ad-FAK-Helix virus did not change in response to increased pressure and was not different at ambient pressure from the adhesion of cells infected with Ad-FAK-HelixScr at ambient pressure. ($n = 8$, * $p < 0.05$ vs. the paired ambient pressure group, # $p < 0.05$ vs. 15 mmHg Ad-FAK-HelixScr). (Fig. 3.4B) In adherent cells, exposure to increased pressure stimulated cell proliferation in control (uninfected), Ad-FAK-HelixScr infected, and Ad-FAK-Helix infected SW620 cells ($n = 4$, * $p < 0.05$ vs. the paired ambient pressure group).

Figure 3.4 (cont'd)

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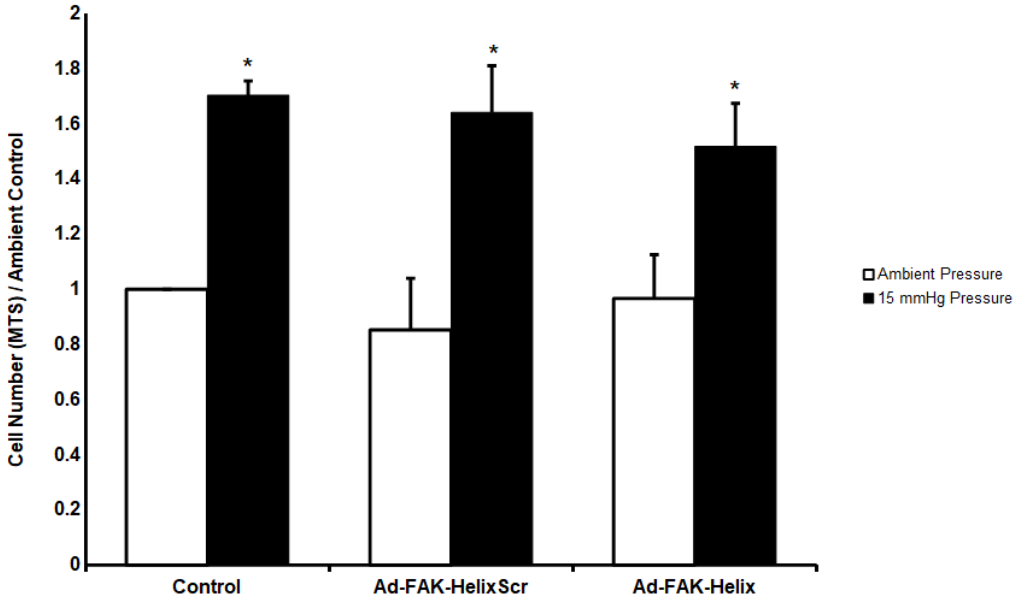

Walsh et al. 2004). We examined the effect of Ad-FAK-Helix on ambient and pressure-stimulated SW620 proliferation to determine whether observed changes in FAK signaling and adhesion might reflect non-specific disruption of cell physiology. Neither proliferation at ambient pressure nor the mitogenic effect of increased pressure was affected by either Ad-FAK-HelixScr or Ad-FAK-Helix. (Fig. 3.4B).

Infection with adenovirus expressing FAK-derived peptides inhibits pressure-stimulated wound-implantation

Since physiologic tissues are more complex than purified matrix proteins, we investigated SW620 cell adhesion to surgical wounds in BALB/c mice. SW620 cells infected with either Ad-FAK-HelixScr or Ad-FAK-Helix were labeled with Tag-it Violet

dye, exposed to ambient or 15 mmHg increased pressure for 30 minutes in suspension and seeded into standardized murine surgical wounds. After 30 minutes, copious irrigation removed non-adherent cells as in surgical settings. After sacrifice and wound

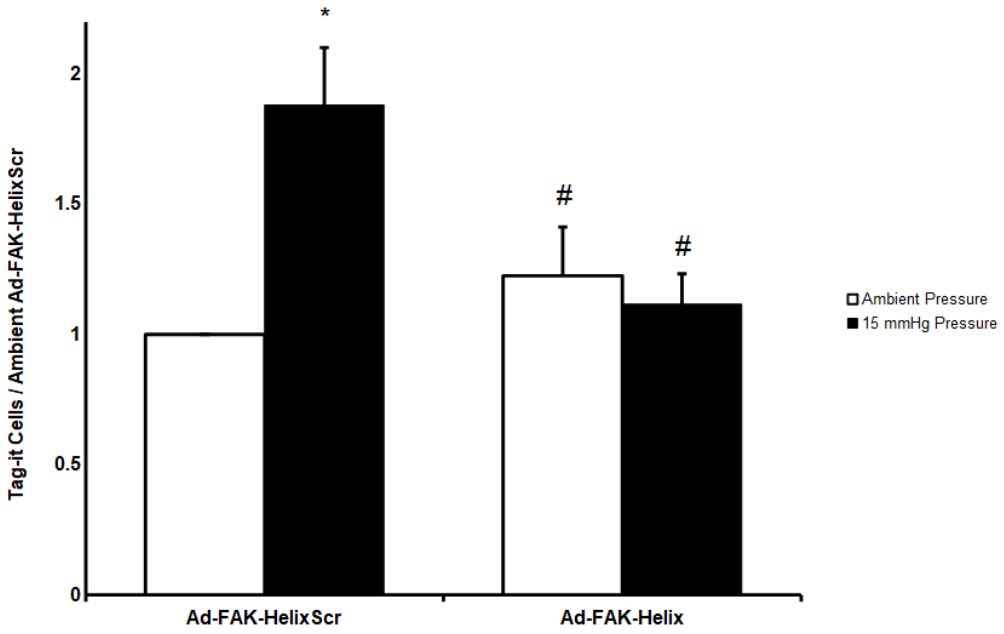

Figure 3.5. A FAK-derived peptide blocks pressure stimulation of adhesion of cancer cells to murine surgical wounds
Tag-it-labeled, Ad-FAK-HelixScr virus treated cells displayed increased wound implantation under elevated pressure conditions, after assay by complete excision of the wound and flow cytometric quantitation of labelled cells in wound tissues. Treatment with the Ad-FAK-Helix blocked this effect (n = 14, * $p < 0.05$ vs. the paired ambient pressure group, # $p < 0.05$ vs. 15 mmHg Ad-FAK-HelixScr).

excision, adherent SW620 cells were distinguished from mouse tissue by fluorescence-activated cell sorting (FACS) for the Tag-it Violet dye. The dye was non-toxic and did not alter proliferation (not shown). Pressure activation increased Ad-FAK-HelixScr-infected cell implantation into wound tissue. Ad-FAK-Helix infection blocked this effect (Fig. 3.5).

Infection with adenovirus expressing FAK-derived peptide reduces subsequent murine tumor development by pre-exposure of implanted tumor cells to elevated pressure

We next investigated whether such differences in cell adhesion alter tumor development. Using similar methodology, suspended cells from each of four conditions (ambient or increased pressure, infected with Ad-FAK-HelixScr or Ad-FAK-Helix) were relabeled to blind the surgical investigator and seeded into surgical wounds in mice. After 30 minutes, the wounds were washed six times with warm PBS and closed. The mice were observed for 90 days during which tumors were assessed as palpable or non-palpable (Fig. 3.6A) and palpable tumors were measured to provide objective data (Fig. 3.6B). Mice were euthanized at a 500 mg tumor burden per veterinary recommendations. In the mice implanted with Ad-FAK-HelixScr cells, 52% from the ambient pressure group eventually developed palpable tumors, with an average tumor-free survival time of 27 days, and a mean 500 mg tumor-burden by 58 days. In the mice that received pressure-activated Ad-FAK-HelixScr cells, 68% developed palpable tumors. Average tumor-free survival and time to maximum tumor burden decreased to 24 and 50 days, respectively. Log-rank analysis of both the time to palpable tumor and time to 500 mg tumor burden curves demonstrated statistically significant effects of pressure pre-activation in mice that received Ad-FAK-HelixScr cells ($p < 0.05$, N=83). In contrast, pressure pre-activation did not worsen survival in mice that received Ad-FAK-Helix cells. The mean average tumor-free survival remained at 27 days for mice receiving Ad-FAK-Helix cells previously exposed to either ambient or increased pressure. Indeed, the time till maximum tumor burden increased from 52 days in the

ambient pressure group to 55 days in the increased pressure group ($p < 0.05$, N=83) and the percentage of palpable tumors decreased (but not statistically significantly) from 60% in the ambient group to 46% in the increased pressure group.

A

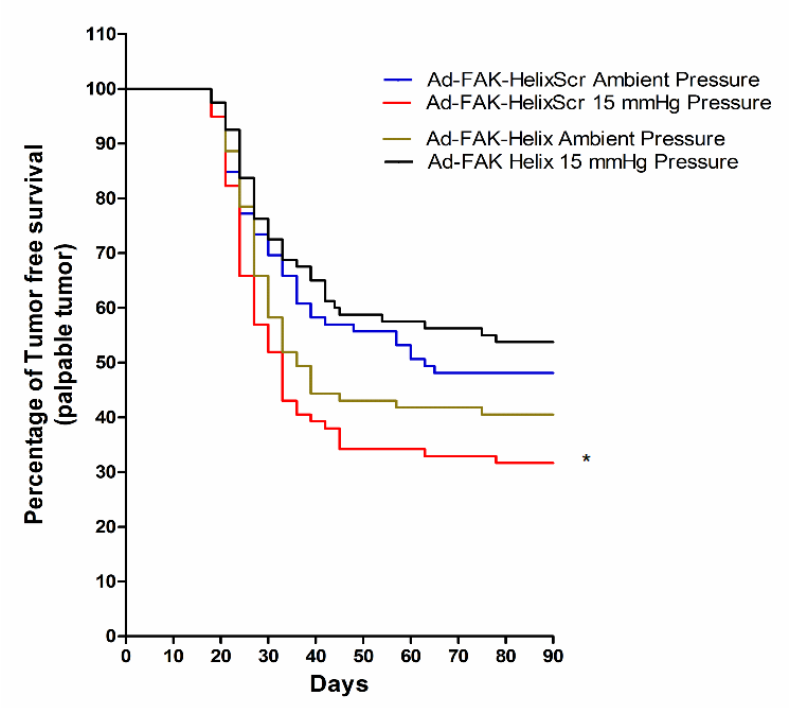

Figure 3.6. Effects of transient expression of a FAK-derived peptide on subsequent tumor development in a model of surgical wound occurrence
Murine tumor development and tumor-free survival were evaluated after exposing standardized surgical wounds to SW620 tumor cell suspensions for 30 minutes and then irrigating copiously before wound closure. Before implantation, the cells were infected with Ad-FAK-HelixScr or Ad-FAK-Helix and exposed to ambient pressure or 15 mmHg increased pressure. (Fig. 3.6A, B) The Kaplan-Meier graphs document palpable tumor development and population survival (as represented by the absence of tumors 500 mg in mass) over time. The data were analyzed by Mantel-Haenszel testing. * indicates $p < 0.05$ vs Ad-FAK-HelixScr infected ambient pressure control.

Figure 3.6 (cont'd)

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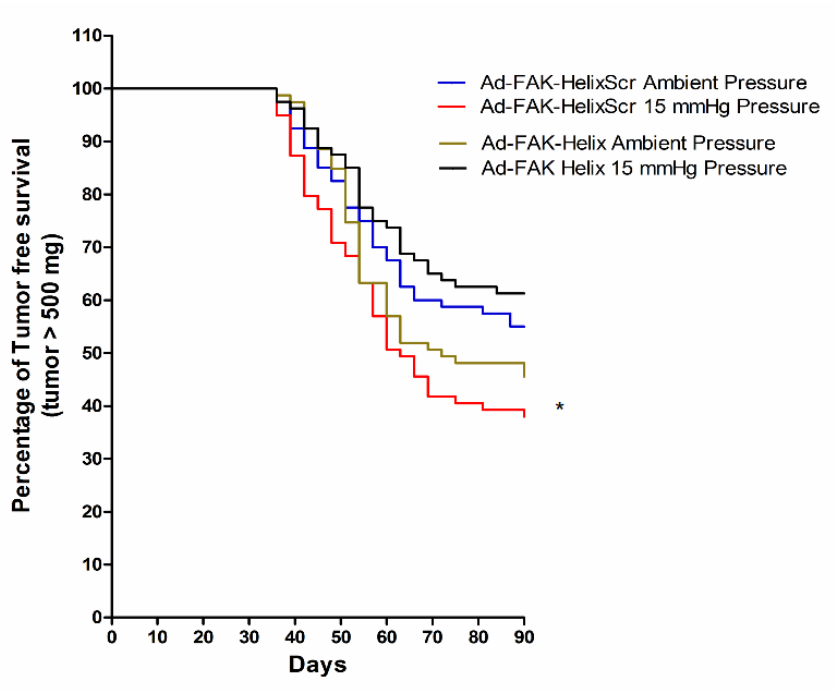


3.4 Discussion

Physical forces evoke signaling responses across diverse cells by different mechanisms. The potentiation of adhesion in suspended cancer cells by a force-activated pathway represents a target for inhibiting metastasis, and the uncommon FAK-Akt1 interaction essential for this pathway seems an attractive target because blocking it may not affect other FAK signaling. Our results demonstrate that Akt1 interacts with FAK directly without an intermediary protein, likely via a short helix on the surface of the FAK F1 lobe, and that this FAK-Akt1 interaction can be blocked by peptides derived from said F1 lobe. Indeed, adenoviral delivery of this peptide into intact cancer cells blocks both pressure-activated signaling and consequent increases in cell adhesion without interfering with other aspects of FAK or Akt1 signaling. Finally, while adenoviral peptide

delivery may not be a practical therapeutic modality, our findings suggest that interventions using or mimicking the FAK-derived peptide may translate to in vivo models and increase tumor-free survival by mitigating pressure-stimulated tumor adhesion.

FAK-Akt1 signaling has recently been described in several contexts with respect to malignancy. FAK (Leng et al. 2016) and Akt1 (Riggio et al. 2017) are important kinases in a common pathway in cancer cells, and increased extracellular pressure induces Akt1 to phosphorylate FAK at serines 517/601/695 and threonine 600 (Wang and Basson 2011). The GST-FAK pull-down of Akt1 from cell lysate and of purified Akt1, as well as co-precipitation of FAK and Akt1 from intact cells suggests a direct interaction between FAK and Akt1 independent of scaffolding proteins. Considered with our previous data characterizing FAK as a substrate of Akt1, this pull-down data provides the impetus behind our efforts to identify the region of FAK responsible for binding Akt1 (Wang and Basson 2011). Successive truncations revealed a seven residue sequence (residues 113-119 LAHPPEE) containing a short helix on the surface of the F1 lobe of the FAK FERM domain that was capable of pulling down Akt1. Furthermore, mutations in this region proved sufficient to alter Akt1 affinity. To address the size discrepancy between the GST tag and our shorter truncations, we reproduced the pull-down studies between wild-type GST-FAK and Akt1 with the addition of peptides modeled after the FAK short helix. The ability of this region to inhibit GST-FAK pull-down of Akt1 as an interfering peptide is consistent with its ability to bind Akt1 as a bait protein. The specificity of this interference is suggested by the inability to produce such effects using

either a scrambled version of this short helix or a FAK-derived β-strand, the secondary structure found COOH-terminally adjacent to the short helix in the wild-type FAK sequence.

Pressure requires FAK-Akt1 interaction to stimulate FAK tyrosine-397 autophosphorylation and activation (Wang and Basson 2011). The FAK F1 lobe appears sufficient to bind Akt1 (Basson et al. 2015). The FERM domain includes three lobes: F1 (residues 35-130), F2 (residues 131-255), and F3 (residues 256-362) (Fig. 3.2E, F). The F2 lobe binds the kinase domain to fold the entire FERM domain over the kinase, inhibiting FAK catalytic activity by active site denial (Lietha et al. 2007). The F3 lobe contains a site homologous to other FERM domains that, upon activation, binds cytoplasmic tails of β-integrins and ICAM-2 (Ceccarelli et al. 2006). It is interesting that the F1 lobe mediates FAK-Akt1 interaction, not the better characterized F2 and F3 lobes, because the F2 lobe regulates FAK activation *following* cell adhesion (Ceccarelli et al. 2006), while pressure activates FAK in suspended cells *before* adhesion (Thamilselvan and Basson 2004). Similarly, the potential protein interaction site of the F3 lobe is occluded in inactivated FAK, precluding it from participating in pressure-induced FAK-Akt1 interactions that cause FAK autophosphorylation (Thamilselvan et al. 2007). Changes to F1 lobe residues that do not physically contact tyrosine-397 can activate FAK (Ceccarelli et al. 2006). Mutation of lysine-38, which is topographically distant from tyrosine-397 in crystal structures, may promote tyrosine-397 phosphorylation by destabilizing the FERM-linker interaction (Cohen et al. 2005). These

data support a model in which the FAK-Akt1 interaction alters the F1 lobe to autophosphorylate tyrosine-397.

Consistent with the effects of pharmacologic Akt blockade (Thamilselvan et al. 2007; Wang and Basson 2001), adenoviral delivery of the short helix peptide blocked pressure-induced FAK tyrosine-397 phosphorylation and FAK-Akt1 coimmunoprecipitation. However, this short helix peptide interrupted FAK-Akt1 interaction while preserving Akt1 kinase activity and downstream GSK phosphorylation. Together with the decrease in pressure-induced FAK-Akt1 association and FAK activation, these observations suggest that the short helix peptide interferes with Akt1 binding to FAK, not Akt1's catalytic competence. We noted incidentally that pressure itself reduced levels of pGSK-3β Ser9 compared to the ambient pressure control. The cause and significance of this is outside the scope of the present study. However, metastatic dissemination is increasingly viewed through a lens of epithelial to mesenchymal and mesenchymal to epithelial transitions (EMT/MET), which has been reported to involve GSK-3β. Active GSK-3β phosphorylates the transcription factor Snail, marking Snail for degradation, and allowing the cell to express epithelial traits. Phosphorylated GSK-3β is inactive and unable to cause the degradation of Snail so that Snail is then free to drive the expression of the mesenchymal traits that allow the cell to migrate, invade, and survive anoikis (PMID:16940750, PMID:25124796). Thus, the pressure-stimulated decrease in pGSK-3β that we observed may then correspond to an increase in GSK-3β activity and the subsequent expression of epithelial markers such as E-cadherin which forms cell-cell adhesions (PMID:23900729). The ability of the short

helix peptide to block the pressure-induced inhibition of pGSK-3β could be attributed to the available pool of Akt1 generated by decreased FAK-Akt1 interactions. While the expression of mesenchymal traits aids tumor cells in the process of dissemination, the reexpression of epithelial markers may facilitate the subsequent attachment of disseminated tumor cells to surrounding organ parenchyma as they form secondary tumors. Further studies may examine how increased extracellular pressure interacts with this process.

As for the molecular data, the inhibition of pressure-induced cell adhesion by the short helix peptide was consistent with previous work using less specific pharmacologic agents (Thamilselvan et al. 2007; Craig, Owen, and Conway 2008, Wang and Basson 2011). Little difference was seen between the basal levels of ambient pressure cell adhesion to collagen I or murine surgical wounds in the groups that received the wild-type short helix peptide and those that received its scrambled control, but Ad-FAK-Helix prevented the stimulation of adhesion by pressure. Indeed, in the tumor progression model, treatment with the virally delivered peptide not only prevented the reduction of tumor-free survival by pressure but increased tumor-free survival in the mice implanted with Ad-FAK-Helix-infected SW620 cells activated with increased pressure compared to those preincubated only at ambient pressure. We previously observed a similar trend when blocking the pressure pathway with a high dose of colchicine (Craig, Owen, and Conway 2008). This possible reversal raises the possibilities of a minor counterregulatory pathway that awaits exploration.

Viral toxicity seems unlikely to explain our findings. Control cells were similarly infected, and Ad-FAK-Helix-infected adherent SW620 cells continued basal proliferation and responded to increased pressure with increased proliferation, similar to uninfected cancer cells (Basson et al. 2015; Walsh et al. 2004; Downey et al. 2001). This not only suggests the Ad-FAK-Helix-infected cells' continued viability but also their ability to respond to other mechanotransduced pathways. Because adenoviral infection was transient and proliferation unaffected, neither host effects nor long-term effects on the tumor cells seem likely to contribute to the effects of peptide delivery on tumor development that were constrained to the initial adhesive event.

To power our study, we sought a tumor prevalence of 50% in the ambient control group, which came at the expense of replicating the low baseline incidence of clinically significant tumor recurrence in surgical wounds. However, physical forces activate tumor cell adhesiveness not only in the context of wound implantation but also peritoneal implantation (Wu et al. 1997) and distant metastasis (Shen et al. 2008), which are much more common. This in vivo study explored pressure-induced adhesion in the context of wound recurrence following surgical resections, but raise the question of whether manipulation of FAK-AKt1 interaction might also influence metastasis to other sites. For instance, we have previously reported that this pathway potentiates metastatic adhesion in a model of peritoneal wound recurrence (Craig, Owen, and Conway 2008). This awaits further study beyond the scope of the current investigation. In addition, the magnitude of pressure effects on FAK phosphorylation and adhesion are admittedly small. However, others have previously studied signaling events and differences in

adhesion of similar magnitude in other contexts (Lee et al. 2014; Srinivas et al. 2001; Goreczny et al. 2017) More importantly, these relatively small changes in signaling or adhesion translate to potentially clinically relevant differences in percent tumor free survival that could substantially exceed the incremental benefit of some new antineoplastic cytotoxic agents.

These results suggest that perturbing FAK-Akt1 interaction, by mimicking the structure of a small segment of the F1 FAK lobe, can abate the sensitivity of suspended malignant cells to mechanical signals, potentially mitigating both the biochemical and the clinical consequences of this force-activated pathway. Such a resultant decrease in FAK and Akt1 activation and inhibition of cell adhesion could attenuate the metastatic potential of shed tumor cells during surgery and increase tumor-free survival. Potential toxicity and off-target effects limit the clinical utility of other methods to inhibit this force-activated adhesion pathway, such as high dose colchicine, disruption of the cytoarchitecture, or non-specific FAK and Akt inhibitors. Preventing FAK-Akt1 interaction without interfering with other aspects of FAK or Akt1 signaling might eventually achieve the desired effect with less compromise of other cell function and less toxicity.

REFERENCES

REFERENCES

Allardyce, Randall A., Phil Morreau, and Philip F. Bagshaw. 1997. “Operative Factors Affecting Tumor Cell Distribution Following Laparoscopic Colectomy in a Porcine Model.” *Diseases of the Colon and Rectum*. https://doi.org/10.1007/BF02051202.

Basson, Marc D. 2008. “An Intracellular Signal Pathway That Regulates Cancer Cell Adhesion in Response to Extracellular Forces.” *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-07-2992.

Basson, Marc D., Cheng Fang Yu, Oliver Herden-Kirchoff, Marc Ellermeier, Matthew A. Sanders, Ronald C. Merrell, and Bauer E. Sumpio. 2000. “Effects of Increased Ambient Pressure on Colon Cancer Cell Adhesion.” *Journal of Cellular Biochemistry*. https://doi.org/10.1093/brain/114.3.1297.

Basson, Marc D., B. Zeng, and S. Wang. 2017. “The C-Terminal Region of the Focal Adhesion Kinase F1 Domain Binds Akt1 and Inhibits Pressure-Induced Cell Adhesion.” *Journal of Physiology and Pharmacology*.

Basson, Marc D., Bixi Zeng, Christina Downey, Madhu P. Sirivelu, and Jetze J. Tepe. 2015. “Increased Extracellular Pressure Stimulates Tumor Proliferation by a Mechanosensitive Calcium Channel and PKC-β.” *Molecular Oncology*. https://doi.org/10.1016/j.molonc.2014.10.008.

Butler, Thomas P., and Pietro M. Gullino. 1975. “Quantitation of Cell Shedding Into Efferent Blood of Mammary Adenocarcinoma.” *Cancer Research*.

Ceccarelli, Derek F.J., Kyu Song Hyun, Florence Poy, Michael D. Schaller, and Michael J. Eck. 2006. “Crystal Structure of the FERM Domain of Focal Adhesion Kinase.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M509188200.

Chaturvedi, Lakshmi S., H. Michael Marsh, Xun Shang, Yi Zheng, and Marc D. Basson. 2007. “Repetitive Deformation Activates Focal Adhesion Kinase and ERK Mitogenic Signals in Human Caco-2 Intestinal Epithelial Cells through Src and Rac1.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M605817200.

Chaturvedi, Lakshmi S, Harold M Marsh, and Marc D Basson. 2007. “Src and Focal Adhesion Kinase Mediate Mechanical Strain-Induced Proliferation and ERK1/2 Phosphorylation in Human H441 Pulmonary Epithelial Cells.” *American Journal of Physiology-Cell Physiology*. https://doi.org/10.1152/ajpcell.00529.2006.

Chen, William S., Pei Zhang Xu, Kathrin Gottlob, Mei Ling Chen, Karen Sokol, Tanya Shiyanova, Igor Roninson, et al. 2001. “Growth Retardation and Increased

Apoptosis in Mice with Homozygous Disruption of the Akt1 Gene." *Genes and Development*. https://doi.org/10.1101/gad.913901.

Cheng, Zhaokang, Laura A. DiMichele, Mauricio Rojas, Cyrus Vaziri, Christopher P. Mack, and Joan M. Taylor. 2014. "Focal Adhesion Kinase Antagonizes Doxorubicin Cardiotoxicity via P21Cip1." *Journal of Molecular and Cellular Cardiology*. https://doi.org/10.1016/j.yjmcc.2013.12.002.

Cho, H., J. Mu, J. K. Kim, J. L. Thorvaldsen, Q. Chu, E. B. Crenshaw, K. H. Kaestner, M. S. Bartolomei, G. I. Shulman, and M. J. Birnbaum. 2001. "Insulin Resistance and a Diabetes Mellitus-like Syndrome in Mice Lacking the Protein Kinase Akt2 (PKBβ)." *Science*. https://doi.org/10.1126/science.292.5522.1728.

Cohen, Lee Ann, and Jun Lin Guan. 2005. "Residues within the First Subdomain of the FERM-like Domain in Focal Adhesion Kinase Are Important in Its Regulation." *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M412021200.

Conway, William C., Jochem Van Der Voort Van Zyp, Vijayalakshmi Thamilselvan, Mary F. Walsh, David L. Crowe, and Marc D. Basson. 2006. "Paxillin Modulates Squamous Cancer Cell Adhesion and Is Important in Pressure-Augmented Adhesion." *Journal of Cellular Biochemistry*. https://doi.org/10.1002/jcb.20819.

Craig, D H, C P Gayer, K L Schaubert, Y Wei, J Li, Y Laouar, and M D Basson. 2009. "Increased Extracellular Pressure Enhances Cancer Cell Integrin-Binding Affinity through Phosphorylation of Beta1-Integrin at Threonine 788/789." *American Journal of Physiology. Cell Physiology*. https://doi.org/10.1152/ajpcell.00355.2008.

Craig, David H., Cheri R. Owen, William C. Conway, Mary F. Walsh, Christina Downey, and Marc D. Basson. 2008. "Colchicine Inhibits Pressure-Induced Tumor Cell Implantation within Surgical Wounds and Enhances Tumor-Free Survival in Mice." *Journal of Clinical Investigation*. https://doi.org/10.1172/JCI34279.

Craig, David H., Hiroe Shiratsuchi, and Marc D. Basson. 2009. "Increased Extracellular Pressure Provides a Novel Adjuvant Stimulus for Enhancement of Conventional Dendritic Cell Maturation Strategies." *Biochemical and Biophysical Research Communications*. https://doi.org/10.1016/j.bbrc.2009.07.010.

Craig, David H, Christina Downey, and Marc D Basson. 2008. "SiRNA-Mediated Reduction of α-Actinin-1 Inhibits Pressure-Induced Murine Tumor Cell Wound Implantation and Enhances Tumor-Free Survival." *Neoplasia*. https://doi.org/10.1593/neo.07945.

Craig, David H, Beatrice Haimovich, and Marc D Basson. 2007. "␣-Actinin-1 Phosphorylation Modulates Pressure-Induced Colon Cancer Cell Adhesion through Regulation of Focal Adhesion Kinase-Src Interaction." *Am J Physiol Cell Physiol*. https://doi.org/10.1152/ajpcell.00118.2007.

Craig, Lisa, Paul C. Sanschagrin, Annett Rozek, Steve Lackie, Leslie A. Kuhn, and Jamie K. Scott. 1998. “The Role of Structure in Antibody Cross-Reactivity between Peptides and Folded Proteins.” *Journal of Molecular Biology*. https://doi.org/10.1006/jmbi.1998.1907.

Datta, Sandeep Robert, Henryk Dudek, Tao Xu, Shane Masters, Fu Haian, Yukiko Gotoh, and Michael E. Greenberg. 1997. “Akt Phosphorylation of BAD Couples Survival Signals to the Cell- Intrinsic Death Machinery.” *Cell*. https://doi.org/10.1016/S0092-8674(00)80405-5.

DeLano, Warren L. 2002. “PyMOL: An Open-Source Molecular Graphics Tool.” *CCP4 Newsletter On Protein Crystallography*. https://doi.org/10.1038/s41598-017-03842-2.

Downey, Christina, Kamal Alwan, Vijayalakshmi Thamilselvan, Liyue Zhang, Yan Jiang, Arun K. Rishi, and Marc D. Basson. 2006. “Pressure Stimulates Breast Cancer Cell Adhesion Independently of Cell Cycle and Apoptosis Regulatory Protein (CARP)-1 Regulation of Focal Adhesion Kinase.” *American Journal of Surgery*. https://doi.org/10.1016/j.amjsurg.2006.08.006.

Dregelid, E., and E. Svendsen. 1988. “Endothelial Cell Injury in Human Saphenous Veins after Manipulation and Tweezer Grasping.” *Journal of Cardiovascular Surgery*.

Fidler, Isaiah J. 1970. “Metastasis: Quantitative Analysis of Distribution and Fate of Tumor Emboli Labeled with 125l-5-Lodo-2’-Deoxyuridine.” *Journal of the National Cancer Institute*. https://doi.org/10.1093/jnci/45.4.773.

Gayer, Christopher P., Lakshmi S. Chaturvedi, Shouye Wang, David H. Craig, Thomas Flanigan, and Marc D. Basson. 2009. “Strain-Induced Proliferation Requires the Phosphatidylinositol 3-Kinase/AKT/Glycogen Synthase Kinase Pathway.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M804576200.

Han, Okhee, Guang Di Li, and Marc D Basson. 1998. “Strain Induces Caco-2 Intestinal Epithelial Proliferation and Differentiation via PKC and Tyrosine Kinase Signals.” *American Journal of Physiology*.

Hayashi, Naoko, Hiroshi Egami, Mikio Kai, Yuji Kurusu, Sadamu Takano, and Michio Ogawa. 1999. “No-Touch Isolation Technique Reduces Intraoperative Shedding of Tumor Cells into the Portal Vein during Resection of Colorectal Cancer.” *Surgery*. https://doi.org/10.1016/S0039-6060(99)70003-6.

Huynh, Khon C., Marianna Gyenes, Thi Hiep Nguyen, Toi Van Vo, and Volker R. Stoldt. 2017. “Impact of Shear Stress on Src and Focal Adhesion Kinase Phosphorylation in Fibrinogen-Adherent Platelets.” *Blood Coagulation and Fibrinolysis*. https://doi.org/10.1097/MBC.0000000000000593.

Hwang, Howook, Thom Vreven, Joël Janin, and Zhiping Weng. 2010. “Protein-Protein Docking Benchmark Version 4.0.” *Proteins: Structure, Function and Bioinformatics*. https://doi.org/10.1002/prot.22830.

Kao, Y. C., J. R. Jheng, H. J. Pan, W. Y. Liao, C. H. Lee, and P. L. Kuo. 2017. “Elevated Hydrostatic Pressure Enhances the Motility and Enlarges the Size of the Lung Cancer Cells through Aquaporin Upregulation Mediated by Caveolin-1 and ERK1/2 Signaling.” *Oncogene*. https://doi.org/10.1038/onc.2016.255.

Ko, Hyo Rim, Il Sun Kwon, Inwoo Hwang, Eun Ju Jin, Joo Ho Shin, Angela M. Brennan-Minnella, Raymond Swanson, Sung Woo Cho, Kyung Hoon Lee, and Jee Yin Ahn. 2016. “Akt1-Inhibitor of DNA Binding2 Is Essential for Growth Cone Formation and Axon Growth and Promotes Central Nervous System Axon Regeneration.” *ELife*. https://doi.org/10.7554/eLife.20799.

Koch, Moritz, Peter Kienle, Ulf Hinz, Dalibor Antolovic, Jan Schmidt, Christian Herfarth, Magnus Von Knebel Doeberitz, and Jürgen Weitz. 2005. “Detection of Hematogenous Tumor Cell Dissemination Predicts Tumor Relapse in Patients Undergoing Surgical Resection of Colorectal Liver Metastases.” *Annals of Surgery*. https://doi.org/10.1097/01.sla.0000151795.15068.27.

Krieger, Elmar, Tom Darden, Sander B. Nabuurs, Alexei Finkelstein, and Gert Vriend. 2004. “Making Optimal Use of Empirical Energy Functions: Force-Field Parameterization in Crystal Space.” *Proteins: Structure, Function and Genetics*. https://doi.org/10.1002/prot.20251.

Leunig, Michael, Rakesh K. Jain, Alwin E. Goetz, Konrad Messmer, and Ernst Kastenbauer. 1992. “Interstitial Hypertension in Head and Neck Tumors in Patients: Correlation with Tumor Size.” *Cancer Research*.

Li, Wei, Aydin Duzgun, Bauer E. Sumpio, and Marc D. Basson. 2001. “Integrin and FAK-Mediated MAPK Activation Is Required for Cyclic Strain Mitogenic Effects in Caco-2 Cells.” *Am J Physiol Gastrointest Liver Physiol*.

Li, Weiqun, Jiachang Zhang, Lawrence Flechner, Teresa Hyun, Alan Yam, Thomas F. Franke, and Jacalyn H. Pierce. 1999. “Protein Kinase C-α Overexpression Stimulates Akt Activity and Suppresses Apoptosis Induced by Interleukin 3 Withdrawal.” *Oncogene*. https://doi.org/10.1038/sj.onc.1203065.

Lietha, Daniel, Xinming Cai, Derek F.J. Ceccarelli, Yiqun Li, Michael D. Schaller, and Michael J. Eck. 2007. “Structural Basis for the Autoinhibition of Focal Adhesion Kinase.” *Cell*. https://doi.org/10.1016/j.cell.2007.05.041.

Liu, T.-J., T. LaFortune, T. Honda, O. Ohmori, S. Hatakeyama, T. Meyer, D. Jackson, J. de Groot, and W.K. A. Yung. 2007. “Inhibition of Both Focal Adhesion Kinase and Insulin-like Growth Factor-I Receptor Kinase Suppresses Glioma Proliferation in

Vitro and in Vivo.” *Molecular Cancer Therapeutics*. https://doi.org/10.1158/1535-7163.MCT-06-0476.

Liu, Weiguo, David A. Bloom, William G. Cance, Elena V. Kurenova, Vita M. Golubovskaya, and Steven N. Hochwald. 2008. “FAK and IGF-IR Interact to Provide Survival Signals in Human Pancreatic Adenocarcinoma Cells.” *Carcinogenesis*. https://doi.org/10.1093/carcin/bgn026.

Parsons, J. T. 2003. “Focal Adhesion Kinase: The First Ten Years.” *Journal of Cell Science*. https://doi.org/10.1242/jcs.00373.

Perry, Brandon C., Shouye Wang, and Marc D. Basson. 2010. “Extracellular Pressure Stimulates Adhesion of Sarcoma Cells via Activation of Focal Adhesion Kinase and Akt.” *American Journal of Surgery*. https://doi.org/10.1016/j.amjsurg.2010.07.013.

Prevelige, Peter, and Gerald D. Fasman. 1989. “Chou-Fasman Prediction of the Secondary Structure of Proteins.” In *Prediction of Protein Structure and the Principles of Protein Conformation*. https://doi.org/10.1007/978-1-4613-1571-1_9.

Sadoshima, Junichi, and Seigo Izumo. 1997. “THE CELLULAR AND MOLECULAR RESPONSE OF CARDIAC MYOCYTES TO MECHANICAL STRESS.” *Annual Review of Physiology*. https://doi.org/10.1146/annurev.physiol.59.1.551.

Shiratsuchi, Hiroe, and Marc D. Basson. 2007. “Akt2, but Not Akt1 or Akt3 Mediates Pressure-Stimulated Serum-Opsonized Latex Bead Phagocytosis through Activating MTOR and P70 S6 Kinase.” *Journal of Cellular Biochemistry*. https://doi.org/10.1002/jcb.21295.

Shiratsuchi, Hiroe, and Marc D Basson. 2004. “Extracellular Pressure Stimulates Macrophage Phagocytosis by Inhibiting a Pathway Involving FAK and ERK.” *American Journal of Physiology. Cell Physiology*. https://doi.org/10.1152/ajpcell.00553.2003.

Thamilselvan, Vijayalakshmi, and Marc D. Basson. 2004. “Pressure Activates Colon Cancer Cell Adhesion by Inside-Out Focal Adhesion Complex and Actin Cytoskeletal Signaling.” *Gastroenterology*. https://doi.org/10.1053/j.gastro.2003.10.078.

Thamilselvan, Vijayalakshmi, David H Craig, and Marc D Basson. 2007. “FAK Association with Multiple Signal Proteins Mediates Pressure-Induced Colon Cancer Cell Adhesion via a Src-Dependent PI3K/Akt Pathway.” *The FASEB Journal : Research Communication*. https://doi.org/10.1096/fj.06-6545com.

Thubrikar, Mano J., and Francis Robicsek. 1995. “Pressure-Induced Arterial Wall Stress and Atherosclerosis.” *The Annals of Thoracic Surgery*. https://doi.org/10.1016/0003-4975(94)01037-D.

Toker, Alex, and Sandra Marmiroli. 2014. “Signaling Specificity in the Akt Pathway in Biology and Disease.” *Advances in Biological Regulation*. https://doi.org/10.1016/j.jbior.2014.04.001.

Turnbull, R. B. 1970. “Cancer of the Colon. The Five- and Ten-Year Survival Rates Following Resection Utilizing the Isolation Technique.” *Annals of the Royal College of Surgeons of England*. https://doi.org/10.5833/jjgs.9.539.

Vassilev, Lyubomir T., Binh T. Vu, Bradford Graves, Daisy Carvajal, Frank Podlaski, Zoran Filipovic, Norman Kong, et al. 2004. “In Vivo Activation of the P53 Pathway by Small-Molecule Antagonists of MDM2.” *Science*. https://doi.org/10.1126/science.1092472.

Walsh, Mary F., Vijayalakshmi Thamilselvan, Rainer Grotelueschen, Lulu Farhana, and Marc D. Basson. 2003. “Absence of Adhesion Triggers Differential FAK and SAPKp38 Signals in SW620 Human Colon Cancer Cells That May Inhibit Adhesiveness and Lead to Cell Death.” *Cellular Physiology and Biochemistry*. https://doi.org/10.1159/000071864.

Wang, S., and M. D. Basson. 2011. “Akt Directly Regulates Focal Adhesion Kinase through Association and Serine Phosphorylation: Implication for Pressure-Induced Colon Cancer Metastasis.” *AJP: Cell Physiology*. https://doi.org/10.1152/ajpcell.00377.2010.

Wang, Shouye, and Marc D Basson. 2011. “Protein Kinase B/AKT And Focal Adhesion Kinase: Two Close Signaling Partners in Cancer.” *Anti-Cancer Agents in Medicinal Chemistry*. https://doi.org/10.2174/187152011797927661.

Weitz, Jürgen, Moritz Koch, Peter Kienle, Andrea Schrödel, Frank Willeke, Axel Benner, Thomas Lehnert, Christian Herfarth, and Magnus Von Knebel Doeberitz. 2000. “Detection of Hematogenic Tumor Cell Dissemination in Patients Undergoing Resection of Liver Metastases of Colorectal Cancer.” *Annals of Surgery*. https://doi.org/10.1097/00000658-200007000-00010.

Wells, James A., and Christopher L. McClendon. 2007. “Reaching for High-Hanging Fruit in Drug Discovery at Protein-Protein Interfaces.” *Nature*. https://doi.org/10.1038/nature06526.

Wen, Li, Tanveer A. Javed, Dean Yimlamai, Amitava Mukherjee, Xiangwei Xiao, and Sohail Z. Husain. 2018. “Transient High Pressure in Pancreatic Ducts Promotes Inflammation and Alters Tight Junctions via Calcineurin Signaling in Mice.” *Gastroenterology*. https://doi.org/10.1053/j.gastro.2018.06.036.

Wu, Meng Yao, Rong Rui Liang, Kai Chen, Meng Shen, Ya Li Tian, Dao Ming Li, Wei Ming Duan, et al. 2015. “FH535 Inhibited Metastasis and Growth of Pancreatic Cancer Cells.” *OncoTargets and Therapy*. https://doi.org/10.2147/OTT.S82718.

Wu, Rong, Xiao Meng Liu, Jian Guang Sun, Hong Chen, Jun Ma, Meng Dong, Shengyi Peng, et al. 2017. “DJ-1 Maintains Energy and Glucose Homeostasis by Regulating the Function of Brown Adipose Tissue.” *Cell Discovery*. https://doi.org/10.1038/celldisc.2016.54.

Yamaguchi, Kazuya, Yukihiro Takagi, Shinichirou Aoki, Manabu Futamura, and Shigetoyo Saji. 2000. “Significant Detection of Circulating Cancer Cells in the Blood by Reverse Transcriptase-Polymerase Chain Reaction during Colorectal Cancer Resection.” *Annals of Surgery*. https://doi.org/10.1097/00000658-200007000-00009.

Zahiri, Javad, Joseph Bozorgmehr, and Ali Masoudi-Nejad. 2013. “Computational Prediction of Protein–Protein Interaction Networks: Algorithms and Resources.” *Current Genomics*. https://doi.org/10.2174/1389202911314060004.

Zanotelli, Matthew R., and Cynthia A. Reinhart-King. 2018. “Mechanical Forces in Tumor Angiogenesis.” In *Advances in Experimental Medicine and Biology*, 1092:91–112. https://doi.org/10.1007/978-3-319-95294-9_6.

Zeng, Bixi, Dinesh Devadoss, Shouye Wang, Emilie E. Vomhof-DeKrey, Leslie A. Kuhn, and Marc D. Basson. 2017. “Inhibition of Pressure-Activated Cancer Cell Adhesion by FAK-Derived Peptides.” *Oncotarget* 8 (58): 98051–67. https://doi.org/10.18632/oncotarget.20556.

Zhang, Jianhu, Cheri R. Owen, Matthew A. Sanders, Jerrold R. Turner, and Marc D. Basson. 2006. “The Motogenic Effects of Cyclic Mechanical Strain on Intestinal Epithelial Monolayer Wound Closure Are Matrix Dependent.” *Gastroenterology*. https://doi.org/10.1053/j.gastro.2006.08.007.

CHAPTER 4

Inhibition of pressure-stimulated cell signaling through a FAK-derived small-molecule inhibitor

The following chapter outlines the steps and reasoning behind the development of the 7-residue FAK-derived peptide, from Chapter 3, into a small-molecule mimic. The information presented is a combination of my unpublished, in vitro experimental data and the virtual ligand-based screening performed by Dr. Sebastian Raschka and Dr. Leslie A. Kuhn. The culmination of our work can be found in the manuscript, "Identification of potential small-molecule protein-protein inhibitors of cancer metastasis by 3D epitope-based computational screening."

Sebastian Raschka, Shyam K. More, Dinesh Devadoss, **Bixi Zeng**, Leslie A. Kuhn, Marc D. Basson (2018). Identification of potential small-molecule protein-protein inhibitors of cancer metastasis by 3D epitope-based computational screening. **J Physiol Pharmacol**, 69(2).

4.1 Introduction

Pressure-stimulated cell adhesion may be successfully blocked with either siRNA to alpha-actinin-1 or high doses of colchicine, but this does so in a manner that indiscriminately jeopardizes cell adhesion (Craig, Downey, and Basson 2008; Craig et al. 2008). Similarly, FAK and Akt1 are each implicated in several signaling pathways in their own right, which make them poor targets for methods of inhibition aimed at compromising their ability to phosphorylate their respective substrates. In pressure-stimulated cell adhesion, increases in extracellular pressure are detected by two mechanosensitive branches of detection; together the cytoarchitecture and paxillin branch and the Src and phosphatidylinositol 3-kinase (PI3K) branch then activate FAK and Akt1 to increase β1-integrin affinity and avidity (Thamilselvan and Basson 2004; Basson 2008; Craig et al. 2009). These dual mechanosensory pathways converge at the binding of FAK and Akt1 which allows for the serine phosphorylation of FAK by Akt1. The phosphorylation of the serine sites on FAK is required for FAK-Tyr-397 tyrosine autophosphorylation and subsequent FAK activation in response to increased extracellular pressure (Wang and Basson 2011). This particular FAK-Akt1 interaction appears to be unique and is not found in other cell pathways (Kleinschmidt and Schlaepfer 2017). Furthermore, the FAK-Akt1 interaction is also not a universal element shared across other examples of mechanotransduction such as pressure-induced proliferation and strain-induced proliferation.

Unlike the mechanism seen in pressure-adhesion, the pressure-proliferation pathway is centered around the calcium channel Cav3.3 and PKC-B, and it is able to

progress despite treatment with FAK and Akt1 inhibitors (Basson et al. 2015). Neither FAK nor Akt1 are implicated in the pressure-proliferation pathway. FAK alone contributes to the mitogenic effects of cyclic strain in human Caco-2 colon cancer cells, H-441 lung epithelial cells, and non-malignant IEC-6 enterocytes (Basson et al. 2015; Wei Li et al. 2001; Chaturvedi et al. 2007; Chaturvedi, Marsh, and Basson 2007; Han, Li, and Basson 1998). The Akt1 isoform has not been implicated in other instances of mechanotransduction aside from pressure-stimulated adhesion and the related phenomenon of pressure-stimulated motility in lung cancer cells (Kao et al. 2017). Outside of Akt1, the Akt2 isoform meditates pressure-stimulated phagocytosis in macrophages and strain-induced cell proliferation in human Caco-2 and rat IEC-6 intestinal epithelial cells (Shiratsuchi and Basson 2007; Gayer et al. 2009). This adds a layer of isoform specificity to the varying classes of mechanotransduction. Admittedly, pressure-induced cell proliferation and adhesion are both implicated in cancer metastasis, and a single intervention that would inhibit both pathologies would be welcome. But, the loss of this additional application for a therapy targeting the FAK-Akt1 interaction ultimately helps to strengthen the notion that the FAK-Akt1 interaction is unique to pressure-stimulated cell adhesion and that its inhibition would generate relatively fewer side effects than the direct blockage of FAK or Akt1 kinase activity.

Continuing in this vein, the peptide-based inhibition of the FAK-Akt1 interaction provides promise for the development of other modalities of inhibition. The ability of the FAK-derived peptide to block pressure-stimulated cell adhesion both in vitro and in vivo rests upon on the ability of the peptide to access the intracellular environment. Methods

of delivery include iron oxide (Fe_3O_4) nanoparticles, cell-penetrating peptide tags, and the use of adenoviral vectors. However, a collaboration with Dr. Leslie Kuhn allowed us to make use of 3D epitope-based computational screening to identify small-molecule mimics of the FAK-derived peptide (LAHPPEE) and bypass the issue of peptide delivery. Peptides exhibit low membrane permeability and may also have poor chemical and physical stability that translate into obstacles within the realm of laboratory work. They are prone to proteolytic degradation in the gut and elsewhere leading to a short circulating plasma half-life that limits their clinical viability (Fosgerau and Hoffmann 2015). Further development of the peptide-based drug could involve neutralization of hydrophobic patches that promote aggregation, the addition of chemical modifications to preserve desired physicochemical properties, and the adjustment of charge distribution or the isoelectric point to counter solubility issues (Fosgerau and Hoffmann 2015). As it is now in its 7-residue state, the size and make-up of the FAK-derived peptide does not require hydrophobic patch corruption or the addition of stabilizing modifications; it is also fairly water soluble and has a pI of 4.14 with a net charge of -1.9 at pH 7. Still, these interventions could be needed depending on the final state of the peptide drug after the selection of a carrier. It is undeniable that there have been major successes in the field of therapeutic peptide research. The prostate cancer treatment, Lupron TM, and the type 2 diabetes mellitus treatment, glucagon-like peptide-1 (GLP-1) agonists, are just two such examples that have found widespread acceptance in clinical practice (Kaspar and Reichert 2013). Even so, our use of virtual screening and small-molecule mimics represents one path towards drug development and does not discount the possibility of more peptide work in the future.

Initial strategies considered utilizing high-throughput screening, however there are known issues with high rates of false positives and negatives (Zahiri, Bozorgmehr, and Masoudi-Nejad 2013). Another potential pitfall of high-throughput screening is that library of compounds available may not possess the attributes needed to replicate the protein-protein interaction epitope. In most protein-protein interactions, the main site of binding can be divided into sub-pockets that contain key, “hot-spot” residues which facilitate binding to complimentary residues on the partner protein (Fuller, Burgoyne, and Jackson 2009). These sub-pockets may not be contiguous and thus require the protein-protein interaction inhibitor to stretch across the separate sites. Such an inhibitor could possess a degree of three-dimensionality that is not common to screening libraries (Basse et al. 2016; Labbé et al. 2013). Said complications were avoided through the characterization the section of the FAK molecule responsible for facilitating the binding interaction with Akt1. This defined epitope then becomes the basis for virtual screening (London, Raveh, and Schueler-Furman 2013; Raschka et al. 2018).

Virtual screening centered on docking algorithms rely on the accuracy of the models involved; large binding sites on both partners create a greater number of opportunities for misstep. Ligand-based computational screening bypasses this with its focus on matching, not docking, the target molecule, and it has been shown to outperform docking screens (McGaughey et al. 2007; Hawkins, Skillman, and Nicholls 2007). Although the target in ligand-based screening is usually a kinase substrate or metabolite, our identification of a 7-residue binding epitope on FAK combined with a complementary binding site on Akt1 located within the 260 residue kinase domain

makes ligand-based screening more feasible than the docking option. The FAK-derived query molecule, LAHPPEE, was screened against a virtual library of small molecules and the resulting matches were assessed for their ability to block FAK-Akt1 interaction, inhibit force-activated FAK phosphorylation by AKT1, and reduce pressure-stimulated cancer cell adhesion.

4.2 Materials and Methods

Computational screening

The following outline of the three-dimensional ligand-based screening methodology used to identify small-molecule mimics of the FAK-derived peptidyl epitopes was conducted by Dr. Leslie A. Kuhn and Dr. Sebastian Raschka (Raschka et al. 2018).

The ligand-based virtual screen was based on the 7-residue FAK-derived peptide (LAHPPEE) and a two-site mutant analog (AAHPSEE) designed to incorporate greater helicity (Zeng et al. 2017). The LAHPPEE peptide was further shortened to LAHPP (residues 113-117) to emphasize the rigid, helix-turn secondary structure; the 3D atomic coordinates are in reference to the crystal structure of FAK (PDB entry: 2al6) (Ceccarelli et al. 2006). PyMOL v. 1.8.2.2 (Schroedinger, LLC) was used to create eight structures that demonstrated alternative positions for Leu (DeLano 2002). This was to account for the rotational flexibility of the Leu side-chain and was accomplished through backbone-dependent rotamer sampling; a similar assessment of the His yielded no favorable alternatives (Shapovalov and Dunbrack 2011). To recreate the charge distribution of the

peptidyl epitope within native FAK, the N and C-termini were capped to a neutral net charge. Partial atomic charges were set using molcharge (QUACPAC v. 1.6.3.1; https://www.eyesopen.com /quacpac; OpenEye Scientific Software, Santa Fe, NM) with the AM1BCC force-field, extra protons were removed from the C- and N-terminal nitrogens, and nitrogen charges were set to –0.55 to reflect conditions within FAK at physiological pH (Jakalian, Jack, and Bayly 2002).

The AAHPSEE peptide (residues 113-119) is a variant of human FAK. In the crystal structure of FAK (PDB entry: 2al6), the LAHPPEE region forms a helix followed by a 310 turn. It is possible that the FAK-Akt1 interaction alters these distinct secondary structures into a single, continuous helix. Were this the case, the binding site on Akt1 would display less affinity for small-molecule mimics that rigidly imitated the helix-turn formation found in wild-type FAK. Ala is known to form helices and the Pro-Ser combination forms a straighter structure than the bent Pro-Pro segment of the helix found in wild-type FAK. The AAHPSEE exploits these tendencies to create a mutant that better resembles a single linear helix; this was confirmed by using Sequery and Superpositional Structure Assignment to assess the helicity of corresponding AAHPSEE sequences in the Protein Data Bank (Collawn et al. 1990; L. Craig et al. 1998; Prevelige and Fasman 1989). As a purified peptide, AAHPSEE was able to block FAK pulldown of Akt1 (Zeng et al. 2017). Representations of AAHPSEE built in PyMOL appeared as an alpha-helix with the conformation of the Ser oriented in the manner of the Pro it replaced (DeLano 2002). Charges were assigned as with LAHPP, and energy-minimization was computed through YASARA (http://www.yasara.org

/minimizationserver.htm) (Krieger et al. 2004).

The LAHPP and the AAHPSEE query peptides were then screened against a library of 10,639,555 3D structure files, in MOL2 format, of commercially available molecules with drug-like properties provided by ZINC (http://zinc.docking.org) (Irwin and Shoichet 2005). Molecules below 250 Da were filtered; the remaining ZINC molecules were then overlaid on the query peptides in ROCS (version 2.4.6; https://www.eyesopen.com/rocs; OpenEye Scientific Software, Santa Fe, NM) (Hawkins, Skillman, and Nicholls 2007). The structural mimics were organized by volumetric and chemical similarity via TanimotoCombo scoring; LAHPP matches were gated for similarity scores 2 standard deviations above the mean and AAHPSEE for 3 standard deviations above the mean. Omega (version 2.4.1; https://www.eyesopen.com/omega; OpenEye Scientific Software, Santa Fe, NM) was used to generate up to 200 favorable, low-energy 3D conformations for each of the eligible ZINC candidates (Hawkins and Nicholls 2012). These conformers were overlaid on the query peptides a second time, and the top 500 overlays were selected. From this group, known pan-assay interference compounds, were removed using PAINSRemover (http://cbligand.org/PAINS/) (Baell and Holloway 2010). Assay candidates were then evaluated through visual inspection in PyMOL for clear alignment between the scaffold of the ZINC molecule and the peptide backbone as well as chemical and volumetric similarity with surface-accessible side chains in the peptide (Raschka et al. 2018). ZINC31501681, a small-molecule from the LAHPP overlay branch, was selected for further testing (Fig. 4.1).

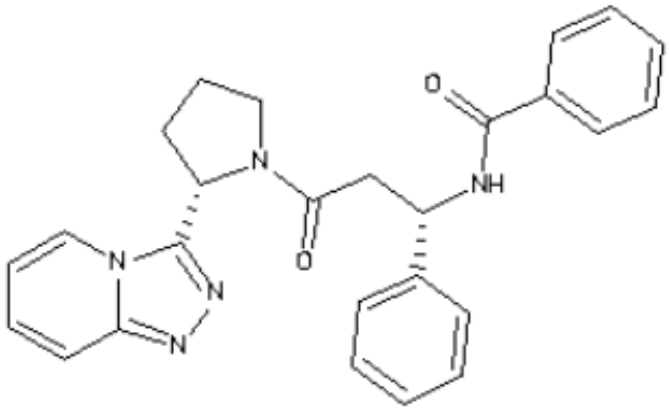


Figure 4.1. ZINC31501681
Depicted is the structure of ZINC31501681**,** N-[(1S)-3-oxo-1-phenyl-3-[(2S)-2-([1,2,4]triazolo[4,3-a]pyridin-3-yl)pyrrolidin-1-yl]propyl]benzamid

Glutathione S-transferase pull-down

Glutathione-Sepharose-4B beads (30 µl) (GE Healthcare Life Science, Pittsburgh, PA) were conjugated with GST (expressed protein from a 250 µl bacterial pellet per 30 µl of beads) or recombinant GST-tagged (expressed protein from a 3 ml bacterial pellet per 30 µl of beads) and incubated with lysate from $2x10^7$ SW620 cells (1500 µg protein) or purified Akt1 (0.35 µg) (Origene, Rockville, MD) overnight at 4°C. Similar incubation for two hours at 4°C was performed with FAK-derived peptides (95% purity by HPLC) (Peptide 2.0, Chantilly, VA) reconstituted in sterile water and mixed with conjugated beads and cell lysate for a final concentration of 160mM before overnight Akt1 incubation (S. Wang and Basson 2011). Bound protein was eluted for western analysis (S. Wang and Basson 2011).

Cell adhesion studies

SW620 colorectal adenocarcinoma cells (ATCC, Manassas, VA) at 90% confluence were split 1:4 two days previously to achieve 50-60% confluence on the day of adhesion assay. SW620 cells were trypsinized, treated as described for 1 hr, plated randomly at $5x10^4$ cells/well, and allowed to adhere to collagen-I-coated plates for 30 minutes at 37°C under ambient or 15mmHg increased pressure (Basson et al. 2000). After 30 minutes, non-adherent cells were washed away with warm PBS. Ambient and pressure-treated plates were encoded to prevent treatment identification during washing. Adherent cells were incubated for 1 hour at 37°C with CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI), and absorbance measured at 490nm with an Epoch plate reader (Biotek, Winooski, VT).

Phosphorylation

SW620 cells were trypsinized, treated as described for 1hr, and exposed to ambient or 15mmHg increased pressure for 30 minutes at 37°C in growth media in 48 well plates pacificated with 1% heat-inactivated BSA in PBS (to prevent adhesion). SW620 cells allowed to adhere to collagen-I-coated plates for 30 minutes at 37°C were positive controls. Cells were lysed for western analysis.

Western Blotting

Protein concentrations were determined by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Eluate from GST pull-downs or collected cell lysates was resolved by SDS-PAGE and transferred to Hybond P 0.45 PVDF blotting membrane

(Amersham Life Science, Arlington Heights, IL). Membranes were blocked for 1 hour at room temperature with Odyssey TBS Blocking Buffer (Amersham Life Science, Arlington Heights, IL) and blotted overnight at 4°C with antibodies against FAK (#3285), Phospho-FAK Tyr397 (#3283), Akt1 (#2967), Phospho-Akt1 Ser473 (#9271), or the GST tag (#2624) (CST, Beverly, MA). Membranes were visualized by the infrared fluorescent IRDye system (LI-COR Biosciences, Lincoln, NE) and analyzed on an Odyssey scanner (LI-COR Biosciences, Lincoln, NE) within the linear range. Results were normalized to the appropriate GST tag, GAPDH, or total protein (for phosphorylated proteins) and relative to associated ambient controls unless stated otherwise. Akt1 frequently appears in published Western blots as a doublet (Ko et al. 2016; R. Wu et al. 2017; Datta et al. 1997; Weiqun Li et al. 1999). The reason for this is not entirely clear, but may represent altered phosphorylation states or other post-translational modifications of the molecule yet to be clarified. The cell line studied and its phenotype in the experiment in question, the amount of protein loaded and concentration of primary and secondary antibody, the percentage of the SDS-PAGE used to resolve the proteins, and numerous other factors may influence this. The densitometric data presented here considers Akt1 as the sum of the intensity of each of the two doublets per lane.

Statistics

Data are expressed as mean±SEM. Results were compared by Student's unpaired t-test and log-rank test as appropriate seeking 95% confidence.

4.3 Results

ZINC31501681 inhibits GST-FAK fusion protein pull-down of purified Akt1

In pull-down assays, purified full-length Akt1 (10nM) was used to approximate the Akt1 in 1,500 µg of whole cell lysate, i.e. the lysate from approximately $1x10^7$ SW620 cells (Zeng et al. 2017). We used full-length GST-FAK conjugated to Sepharose beads as bait against purified Akt1 and assessed the effects of varying concentrations of ZINC31501681 as a potential protein-protein interaction inhibitor. ZINC31501681 at 10 µM, 100 µM, and 1000 µM reduced binding between GST-FAK-NT1 and purified

A

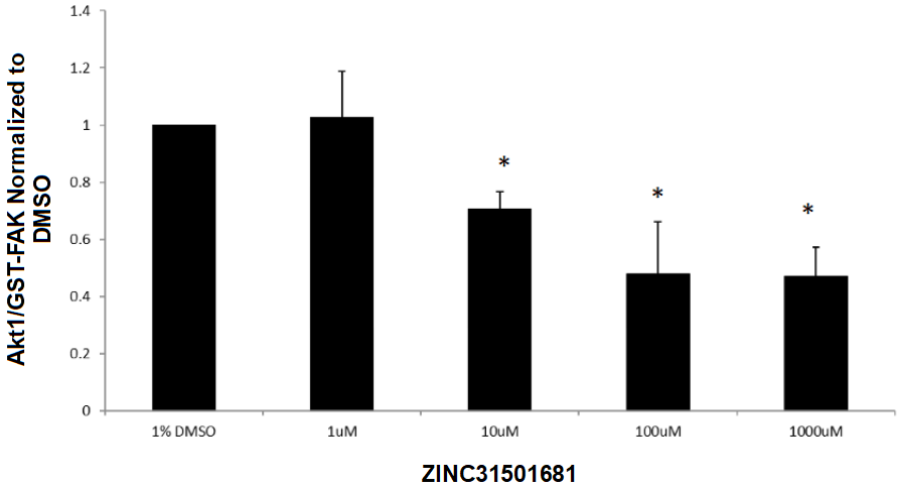
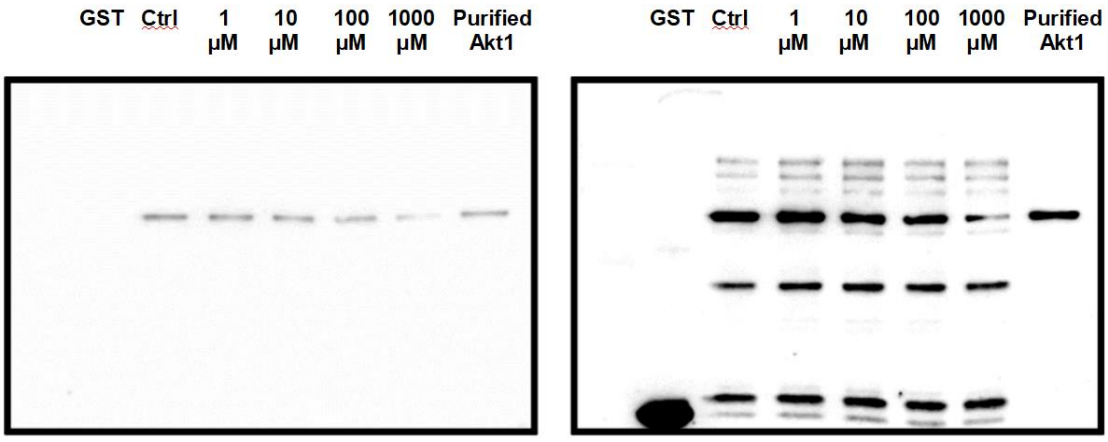


Figure 4.2. ZINC31501681 Disrupts FAK pull down of Akt1
(Fig. 4.2A) GST-FAK conjugated beads pulled down decreasing levels of purified Akt1 in the presence of ZINC31501681 (n = 4). The left blot was probed first for Akt1, and the right blot was probed second for GST without being stripped beforehand. The western blot shows the amount of Akt1 (prey, 60 kDa) signal relative to the amount of GST-FAK (bait, estimated 150 kDa) signal. (Fig. 4.2B) Densitometric data was analyzed as the percentage of Akt1 signal over GST fusion protein signal, which was then normalized to the 1% DMSO group. Treatment with ZINC31501681 reduced the amount of Akt1 pulled down by GST-FAK vs. 1% DMSO (n = 4, * $p < 0.05$ vs. vehicle-treated GST-FAK).

Figure 4.2 (cont'd)

B



Akt1; the 1 µM concentration of ZINC31501681 failed to significantly inhibit the GST-FAK pulldown of Akt1 (Fig. 4.2A, B). The densitometric values of the Akt1 band were normalized to their respective FAK bands. All ratios were then normalized to the 0.1% DMSO control.

Pressure-induced phosphorylation of FAK, but not Akt1, is inhibited in SW620 treated with ZINC31501681

SW620 colorectal adenocarcinoma cells were pretreated with either 1% DMSO or ZINC31501681 (300 µM) for 1hr before being exposed to 15mmHg increased extracellular pressure for 30 minutes at 37°C. The pressurized incubation was performed in 1% heat-inactivated BSA pacificated plates to prevent the generation of pFAK Tyr397 through regular cell adhesion. Pressure-stimulated FAK Tyr397 phosphorylation was observed in cells treated with 0.1% DMSO but not in those treated

with 300 μM ZINC31501681 (Fig. 4.3A, B). In contrast, 15mmHg pressure did not stimulate Akt1 Ser473 phosphorylation in either group of cells (Fig. 4.3C, D). Increases in extracellular pressure trigger a cascade that activates Akt1 at Ser473 so that it may phosphorylate FAK at Ser517/601/695. Phosphorylation at these Ser sites allows FAK to autophosphorylate at Tyr397. By inhibiting the FAK-Akt1 interaction with LAHPP mimic, ZINC31501681, we see the kinetically competent Akt1 is unable to activate FAK in a manner previously seen using peptide-based inhibitors (Zeng et al. 2017).

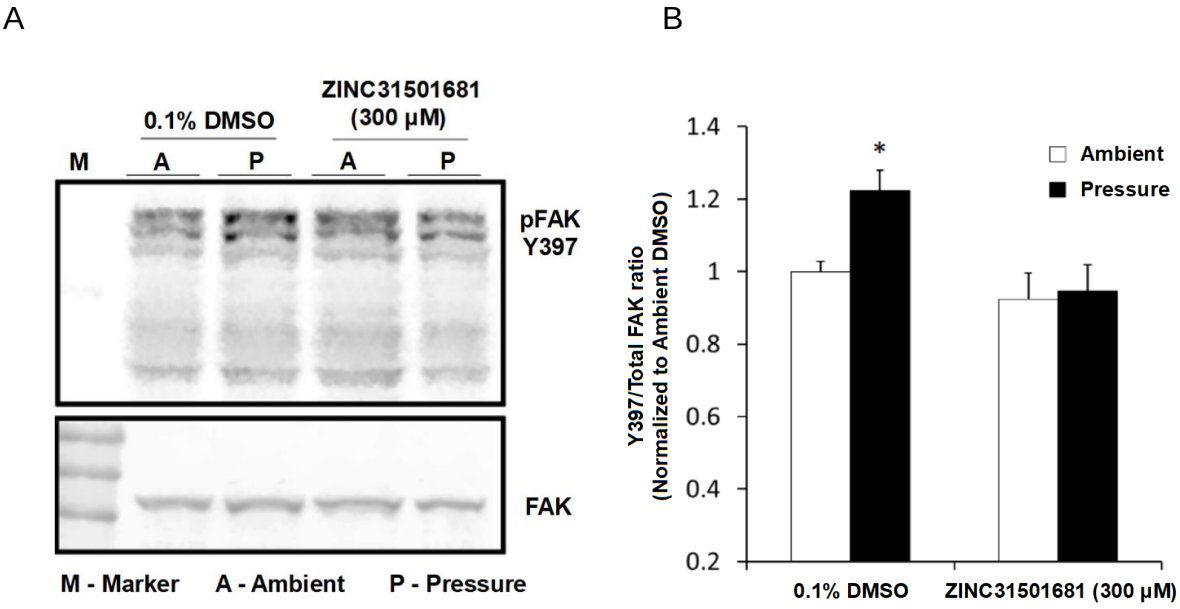
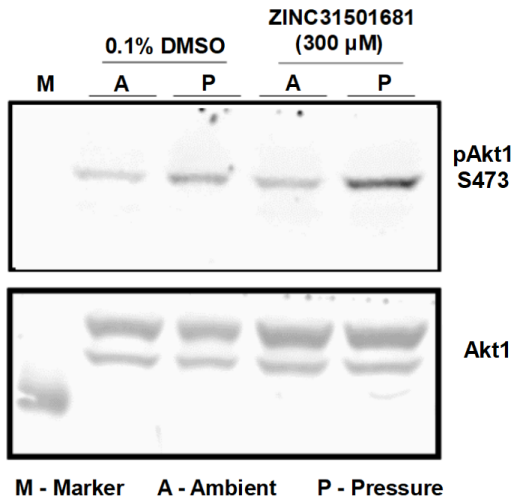


Figure 4.3. ZINC31501681 Disrupts pressure-induced phosphorylation
(Fig. 4.3A) Lysate from SW620 cells exposed to ambient (A) or 15 mmHg (P) while in suspension were probed for phospho- (top) and total (bottom) FAK. ZINC31501681 blocks pressure stimulation of FAK Typ397 phosphorylation. (Fig. 4.3B) Densitometric data was analyzed as the percentage of pFAK Tyr397 signal over total FAK protein signal, which was then normalized to the 0.1% DMSO ambient control. Treatment with ZINC31501681 inhibited pressure-stimulated FAK Tyr397 phosphorylation (n = 3, * $p < 0.05$ vs. vehicle-treated, ambient control). (Fig. 4.3C) Lysate from SW620 cells exposed to ambient (A) or 15 mmHg (P) while in suspension were probed for phospho- (top) and total (bottom) Akt1. ZINC31501681 did not block pressure stimulation of Akt1 Ser473 phosphorylation. (Fig. 4.3D) The densitometry was analyzed by the same methodology

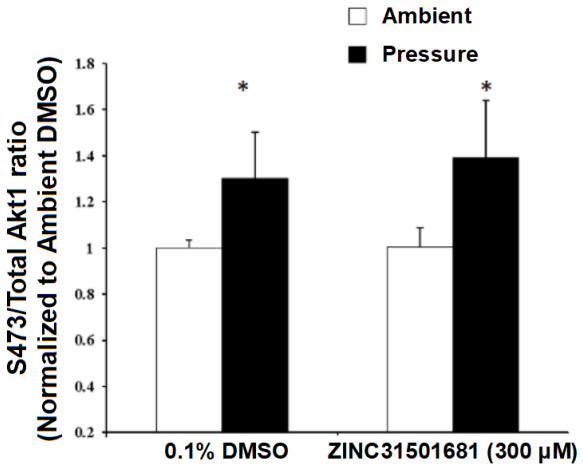
Figure 4.3 (cont'd)

as the FAK/pFAK blots. Treatment with ZINC31501681 did not block pressure-stimulated Akt1 Ser397 phosphorylation (n = 3, * p < 0.05 vs. vehicle-treated, ambient control).

C



D



ZINC31501681 prevents pressure-induced SW620 cell adhesion

ZINC31501681 similarly inhibited pressure-stimulated adhesion. SW60 cells were pretreated with either 0.1% DMSO or ZINC31501681 (300 μM) for 1hr and then seeded on collagen-I-coated plates under ambient or increased pressure for 30 minutes. The plates were washed in a blinded fashion to remove nonadherent cells. The remaining adherent cells were quantified by MTS assay. Pressure-induced adhesion was inhibited by ZINC31501681 (300 μM) but not 0.1#DMSO (Fig. 4.4). Additionally, treatment with ZINC31501681 did not produce any significant differences in the basal levels of cell adhesion within the groups exposed to ambient pressure.

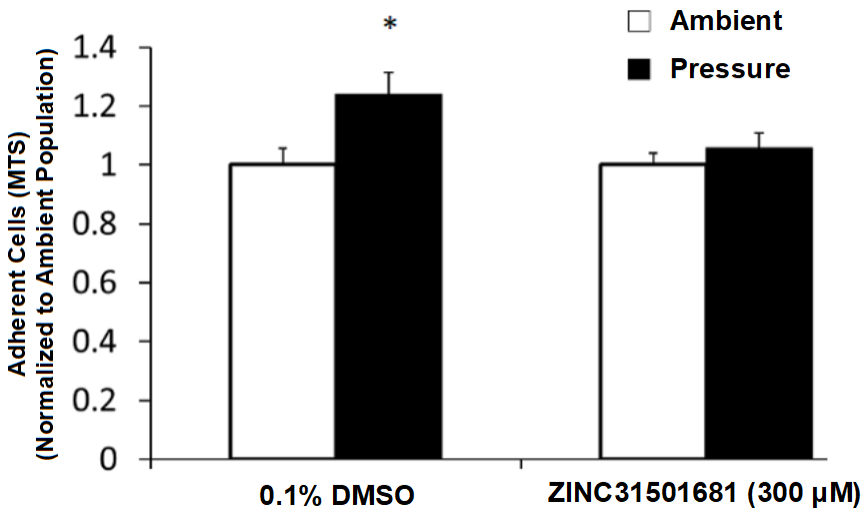


Figure 4.4. ZINC31501681 prevents pressure-induced SW620 cell adhesion
Exposure to elevated pressure increased adhesion by SW620 cells treated with 0.1% DMSO vs. ambient pressure. Adhesion by cells treated with ZINC31501681 did not change in response to increased pressure and was not different at ambient pressure from the group treated with 0.1% DMSO and ambient pressure. (n = 4, * $p < 0.05$ vs. the 0.1% DMSO, ambient control).

4.4 Discussion

Our study of the small-molecule mimic, ZINC31501681, showed it to behave in a manner similar to the 7-residue FAK-derived peptide it was modeled after (Zeng et al. 2017). Like the FAK-derived peptide, the ability of ZINC31501681 to inhibit FAK pull-down of Akt1 translated into corresponding changes in cell behavior when used to treat pressure-stimulated SW620 cells. ZINC31501681 successfully blocked pressure-stimulated increases in FAK Try397 phosphorylation and the resultant increases in cell adhesion. Pressure-induced phosphorylation of Akt1 Ser73 however was not affected by ZINC31501681, which is consistent with other peptide and siRNA based interventions (Zeng et al. 2017; S. Wang and Basson 2011). The nature of the signaling

pathway allows for Akt1 to be phosphorylated at S473 in response to pressure stimulation by a FAK-independent mechanism that involves the actin cross-linking protein alpha-actinin-4. While these results suggest inhibition of Akt1 by ZINC31501681, confirmation of direct binding between the two could be helpful. Due to the small size of ZINC31501681, label-free techniques such as surface plasmon resonance or isothermal titration calorimetry, or techniques that only require the Akt1 to be labeled such as microscale thermophoresis, could be employed.

In these studies, ZINC31501681 was able to prevent pressure-stimulation from increasing FAK pTyr397 without altering the basal levels of FAK pTyr397. However, in hands of others, ZINC31501681 inhibition of pressure-stimulated FAK phosphorylation has been associated with increases in basal FAK pTyr397 (Raschka et al. 2018). Whether this disparity is due to differences in the concentrations of ZINC31501681 used (300 μM vs 1-100 pM), in the elapsed storage time of the ZINC31501681 stock, or in the idiosyncrasies of western blot development and analysis, the issue may best be resolved by a larger sample size. The limited scope of these initial assays testing the small-molecule mimics found by the ligand-based virtual screen is addressed by the large number of ZINC molecules tested by Raschka et al.; 11 ZINC compounds in total, 4 compounds modeled after the LAHPP query molecule (ZINC31501681 included) and 7 compounds modeled after the AAHPSEE query molecule, were tested to gauge their effects on pressure signaling (Raschka et al. 2018). These ZINC small-molecule candidates were generated through the three-dimensional ligand-based screening methodology outlined above. Raschka et al. found the ZINC small-molecules based off

of LAHPP were generally able to block pressure-stimulated FAK Tyr397 phosphorylation, but not without increasing the basal levels of FAK pTyr397. On the other hand, the AAHPSEE mimicking ZINC compounds generally could neither inhibit pressure-stimulated FAK Tyr397 phosphorylation nor could they elevate basal levels of FAK pTyr397 (Raschka et al. 2018). The two exceptions were ZINC04085549 and ZINC4085554, again from the AAHPSEE branch, which both blocked pressure-stimulated FAK Tyr397 phosphorylation and did not alter the level of basal phosphorylation. Like ZINC31501681, ZINC04085549 also demonstrated an ability to inhibit pressure-stimulated cell adhesion (Raschka et al. 2018).

It is promising is that there indeed are small-molecule mimics of the FAK-derived peptide that can inhibit the pressure-stimulated FAK phosphorylation and cell adhesion without increasing the basal levels of either. The search for such a compound through a means other than in vitro high-throughput screening is less common, even more so is the use of virtual ligand-based screening. Virtual ligand-based screening was intended to use bioactive molecules, such as substrates and metabolites, as query molecules, but it may now be used to overlay compounds against protein epitopes (Raschka et al. 2018). The efficacy of the compounds discovered is still unproven, but the leads generated will provide a wide selection of candidates for future work. In this study we tested the top scoring compounds found among all that were searched. This conservative approach was chosen to maximize our chances of finding a small-molecule that behaved like the 7-residue FAK-derived peptide and thus validate the process. Alternatively, the compounds, post PAINS removal, could be reorganized by

physical or chemical traits; the in vitro assays could be used to test not only the top scoring candidates among all small-molecules but the top few from each group. Such an approach could reveal more information about the chemical and steric traits that drive the interaction and ultimately lessen the total in vitro work needed.

REFERENCES

REFERENCES

Arkin, Michelle R., Yinyan Tang, and James A. Wells. 2014. “Small-Molecule Inhibitors of Protein-Protein Interactions: Progressing toward the Reality.” *Chemistry and Biology*. https://doi.org/10.1016/j.chembiol.2014.09.001.

Arkin, Michelle R., and James A Wells. 2004. “Small-Molecule Inhibitors of Protein-Protein-Interactions: Progression Towards the Dream.” *Nature Reviews*. https://doi.org/10.1038/nrd1343.

Baell, Jonathan B., and Georgina A. Holloway. 2010. “New Substructure Filters for Removal of Pan Assay Interference Compounds (PAINS) from Screening Libraries and for Their Exclusion in Bioassays.” *Journal of Medicinal Chemistry*. https://doi.org/10.1021/jm901137j.

Baeyens, Nicolas. 2018. “Fluid Shear Stress Sensing in Vascular Homeostasis and Remodeling: Towards the Development of Innovative Pharmacological Approaches to Treat Vascular Dysfunction.” *Biochemical Pharmacology* 158 (October): 185–91. https://doi.org/10.1016/j.bcp.2018.10.023.

Basse, Marie Jeanne, Stéphane Betzi, Xavier Morelli, and Philippe Roche. 2016. “2P2Idb v2: Update of a Structural Database Dedicated to Orthosteric Modulation of Protein-Protein Interactions.” *Database*. https://doi.org/10.1093/database/baw007.

Basson, Marc D. 2008. “An Intracellular Signal Pathway That Regulates Cancer Cell Adhesion in Response to Extracellular Forces.” *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-07-2992.

Basson, Marc D., Cheng Fang Yu, Oliver Herden-Kirchoff, Marc Ellermeier, Matthew A. Sanders, Ronald C. Merrell, and Bauer E. Sumpio. 2000. “Effects of Increased Ambient Pressure on Colon Cancer Cell Adhesion.” *Journal of Cellular Biochemistry*. https://doi.org/10.1093/brain/114.3.1297.

Basson, Marc D., B. Zeng, and S. Wang. 2017. “The C-Terminal Region of the Focal Adhesion Kinase F1 Domain Binds Akt1 and Inhibits Pressure-Induced Cell Adhesion.” *Journal of Physiology and Pharmacology*.

Basson, Marc D., Bixi Zeng, Christina Downey, Madhu P. Sirivelu, and Jetze J. Tepe. 2015. “Increased Extracellular Pressure Stimulates Tumor Proliferation by a Mechanosensitive Calcium Channel and PKC-β.” *Molecular Oncology*. https://doi.org/10.1016/j.molonc.2014.10.008.

Ceccarelli, Derek F.J., Kyu Song Hyun, Florence Poy, Michael D. Schaller, and Michael J. Eck. 2006. “Crystal Structure of the FERM Domain of Focal Adhesion Kinase.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M509188200.

Chaturvedi, Lakshmi S., H. Michael Marsh, Xun Shang, Yi Zheng, and Marc D. Basson. 2007. “Repetitive Deformation Activates Focal Adhesion Kinase and ERK Mitogenic Signals in Human Caco-2 Intestinal Epithelial Cells through Src and Rac1.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M605817200.

Chaturvedi, Lakshmi S, Harold M Marsh, and Marc D Basson. 2007. “Src and Focal Adhesion Kinase Mediate Mechanical Strain-Induced Proliferation and ERK1/2 Phosphorylation in Human H441 Pulmonary Epithelial Cells.” *American Journal of Physiology-Cell Physiology*. https://doi.org/10.1152/ajpcell.00529.2006.

Cheng, Zhaokang, Laura A. DiMichele, Mauricio Rojas, Cyrus Vaziri, Christopher P. Mack, and Joan M. Taylor. 2014. “Focal Adhesion Kinase Antagonizes Doxorubicin Cardiotoxicity via P21Cip1.” *Journal of Molecular and Cellular Cardiology*. https://doi.org/10.1016/j.yjmcc.2013.12.002.

Cho, H., J. Mu, J. K. Kim, J. L. Thorvaldsen, Q. Chu, E. B. Crenshaw, K. H. Kaestner, M. S. Bartolomei, G. I. Shulman, and M. J. Birnbaum. 2001. “Insulin Resistance and a Diabetes Mellitus-like Syndrome in Mice Lacking the Protein Kinase Akt2 (PKBβ).” *Science*. https://doi.org/10.1126/science.292.5522.1728.

Clackson, Tim, and James A. Wells. 1995. “A Hot Spot of Binding Energy in a Hormone-Receptor Interface.” *Science*. https://doi.org/10.1126/science.7529940.

Cohen, Lee Ann, and Jun Lin Guan. 2005. “Residues within the First Subdomain of the FERM-like Domain in Focal Adhesion Kinase Are Important in Its Regulation.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M412021200.

Collawn, James F., Martin Stangel, Leslie A. Kuhn, Victor Esekogwu, Shuqian Jing, Ian S. Trowbridge, and John A. Tainer. 1990. “Transferrin Receptor Internalization Sequence YXRF Implicates a Tight Turn as the Structural Recognition Motif for Endocytosis.” *Cell*. https://doi.org/10.1136/jnnp.s1-12.48.320.

Craig, D H, C P Gayer, K L Schaubert, Y Wei, J Li, Y Laouar, and M D Basson. 2009. “Increased Extracellular Pressure Enhances Cancer Cell Integrin-Binding Affinity through Phosphorylation of Beta1-Integrin at Threonine 788/789.” *American Journal of Physiology. Cell Physiology*. https://doi.org/10.1152/ajpcell.00355.2008.

Craig, David H., Cheri R. Owen, William C. Conway, Mary F. Walsh, Christina Downey, and Marc D. Basson. 2008. “Colchicine Inhibits Pressure-Induced Tumor Cell Implantation within Surgical Wounds and Enhances Tumor-Free Survival in Mice.” *Journal of Clinical Investigation*. https://doi.org/10.1172/JCI34279.

Craig, David H., Hiroe Shiratsuchi, and Marc D. Basson. 2009. “Increased Extracellular Pressure Provides a Novel Adjuvant Stimulus for Enhancement of Conventional Dendritic Cell Maturation Strategies.” *Biochemical and Biophysical Research Communications*. https://doi.org/10.1016/j.bbrc.2009.07.010.

Craig, David H, Christina Downey, and Marc D Basson. 2008. “SiRNA-Mediated Reduction of α-Actinin-1 Inhibits Pressure-Induced Murine Tumor Cell Wound Implantation and Enhances Tumor-Free Survival.” *Neoplasia*. https://doi.org/10.1593/neo.07945.

DeLano, Warren L. 2002. “PyMOL: An Open-Source Molecular Graphics Tool.” *CCP4 Newsletter On Protein Crystallography*. https://doi.org/10.1038/s41598-017-03842-2.

Dorlo, Thomas P.C., Manica Balasegaram, Jos H. Beijnen, and Peter J. de vries. 2012. “Miltefosine: A Review of Its Pharmacology and Therapeutic Efficacy in the Treatment of Leishmaniasis.” *Journal of Antimicrobial Chemotherapy*. https://doi.org/10.1093/jac/dks275.

Downey, Christina, Kamal Alwan, Vijayalakshmi Thamilselvan, Liyue Zhang, Yan Jiang, Arun K. Rishi, and Marc D. Basson. 2006. “Pressure Stimulates Breast Cancer Cell Adhesion Independently of Cell Cycle and Apoptosis Regulatory Protein (CARP)-1 Regulation of Focal Adhesion Kinase.” *American Journal of Surgery*. https://doi.org/10.1016/j.amjsurg.2006.08.006.

Fosgerau, Keld, and Torsten Hoffmann. 2015. “Peptide Therapeutics: Current Status and Future Directions.” *Drug Discovery Today*. https://doi.org/10.1016/j.drudis.2014.10.003.

Fuller, Jonathan C., Nicholas J. Burgoyne, and Richard M. Jackson. 2009. “Predicting Druggable Binding Sites at the Protein-Protein Interface.” *Drug Discovery Today*. https://doi.org/10.1016/j.drudis.2008.10.009.

Gayer, Christopher P., Lakshmi S. Chaturvedi, Shouye Wang, David H. Craig, Thomas Flanigan, and Marc D. Basson. 2009. “Strain-Induced Proliferation Requires the Phosphatidylinositol 3-Kinase/AKT/Glycogen Synthase Kinase Pathway.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M804576200.

Han, Okhee, Guang Di Li, and Marc D Basson. 1998. “Strain Induces Caco-2 Intestinal Epithelial Proliferation and Differentiation via PKC and Tyrosine Kinase Signals.” *American Journal of Physiology*.

Hawkins, Paul C.D., and Anthony Nicholls. 2012. “Conformer Generation with OMEGA: Learning from the Data Set and the Analysis of Failures.” *Journal of Chemical Information and Modeling*. https://doi.org/10.1021/ci300314k.

Hawkins, Paul C.D., A. Geoffrey Skillman, and Anthony Nicholls. 2007. “Comparison of Shape-Matching and Docking as Virtual Screening Tools.” *Journal of Medicinal Chemistry*. https://doi.org/10.1021/jm0603365.

Huynh, Khon C., Marianna Gyenes, Thi Hiep Nguyen, Toi Van Vo, and Volker R. Stoldt. 2017. “Impact of Shear Stress on Src and Focal Adhesion Kinase Phosphorylation in Fibrinogen-Adherent Platelets.” *Blood Coagulation and Fibrinolysis*. https://doi.org/10.1097/MBC.0000000000000593.

Hwang, Howook, Thom Vreven, Joël Janin, and Zhiping Weng. 2010. “Protein-Protein Docking Benchmark Version 4.0.” *Proteins: Structure, Function and Bioinformatics*. https://doi.org/10.1002/prot.22830.

Irwin, John J., and Brian K. Shoichet. 2005. “ZINC - A Free Database of Commercially Available Compounds for Virtual Screening.” *Journal of Chemical Information and Modeling*. https://doi.org/10.1021/ci049714+.

Jain, Rakesh K., Yves Boucher, and Norman Wolmark. 1992. “Interstitial Hypertension in Human Breast and Colorectal Tumors.” *Cancer Research*.

Jakalian, Araz, David B. Jack, and Christopher I. Bayly. 2002. “Fast, Efficient Generation of High-Quality Atomic Charges. AM1-BCC Model: II. Parameterization and Validation.” *Journal of Computational Chemistry*. https://doi.org/10.1002/jcc.10128.

Johnson, David K., and John Karanicolas. 2013. “Druggable Protein Interaction Sites Are More Predisposed to Surface Pocket Formation than the Rest of the Protein Surface.” *PLoS Computational Biology*. https://doi.org/10.1371/journal.pcbi.1002951.

Kaspar, Allan A., and Janice M. Reichert. 2013. “Future Directions for Peptide Therapeutics Development.” *Drug Discovery Today*. https://doi.org/10.1016/j.drudis.2013.05.011.

Kleinschmidt, Elizabeth G., and David D. Schlaepfer. 2017. “Focal Adhesion Kinase Signaling in Unexpected Places.” *Current Opinion in Cell Biology*. https://doi.org/10.1016/j.ceb.2017.01.003.

Klinghoffer, Richard A., Christoph Sachsenmaier, Jonathan A. Cooper, and Philippe Soriano. 1999. “Src Family Kinases Are Required for Integrin but Not PDGFR Signal Transduction.” *EMBO Journal*. https://doi.org/10.1093/emboj/18.9.2459.

Ko, Hyo Rim, Il Sun Kwon, Inwoo Hwang, Eun Ju Jin, Joo Ho Shin, Angela M. Brennan-Minnella, Raymond Swanson, Sung Woo Cho, Kyung Hoon Lee, and Jee Yin Ahn. 2016. “Akt1-Inhibitor of DNA Binding2 Is Essential for Growth Cone Formation and Axon Growth and Promotes Central Nervous System Axon Regeneration.” *ELife*. https://doi.org/10.7554/eLife.20799.

Koch, Moritz, Peter Kienle, Ulf Hinz, Dalibor Antolovic, Jan Schmidt, Christian Herfarth, Magnus Von Knebel Doeberitz, and Jürgen Weitz. 2005. “Detection of Hematogenous Tumor Cell Dissemination Predicts Tumor Relapse in Patients Undergoing Surgical Resection of Colorectal Liver Metastases.” *Annals of Surgery*. https://doi.org/10.1097/01.sla.0000151795.15068.27.

Krieger, Elmar, Tom Darden, Sander B. Nabuurs, Alexei Finkelstein, and Gert Vriend. 2004. “Making Optimal Use of Empirical Energy Functions: Force-Field Parameterization in Crystal Space.” *Proteins: Structure, Function and Genetics*. https://doi.org/10.1002/prot.20251.

Labbé, Céline M., Guillaume Laconde, Mélaine A. Kuenemann, Bruno O. Villoutreix, and Olivier Sperandio. 2013. “IPPI-DB: A Manually Curated and Interactive Database of Small Non-Peptide Inhibitors of Protein-Protein Interactions.” *Drug Discovery Today*. https://doi.org/10.1016/j.drudis.2013.05.003.

Lamore, Sarah D., Ernst Ahlberg, Scott Boyer, Michelle L. Lamb, Maria P. Hortigon-Vinagre, Victor Rodriguez, Godfrey L. Smith, et al. 2017. “Deconvoluting Kinase Inhibitor Induced Cardiotoxicity.” *Toxicological Sciences*. https://doi.org/10.1093/toxsci/kfx082.

Lipinski, Christopher A., Franco Lombardo, Beryl W. Dominy, and Paul J. Feeney. 2012. “Experimental and Computational Approaches to Estimate Solubility and Permeability in Drug Discovery and Development Settings.” *Advanced Drug Delivery Reviews*. https://doi.org/10.1016/j.addr.2012.09.019.

Liu, T.-J., T. LaFortune, T. Honda, O. Ohmori, S. Hatakeyama, T. Meyer, D. Jackson, J. de Groot, and W.K. A. Yung. 2007. “Inhibition of Both Focal Adhesion Kinase and Insulin-like Growth Factor-I Receptor Kinase Suppresses Glioma Proliferation in Vitro and in Vivo.” *Molecular Cancer Therapeutics*. https://doi.org/10.1158/1535-7163.MCT-06-0476.

Liu, Weiguo, David A. Bloom, William G. Cance, Elena V. Kurenova, Vita M. Golubovskaya, and Steven N. Hochwald. 2008. “FAK and IGF-IR Interact to Provide Survival Signals in Human Pancreatic Adenocarcinoma Cells.” *Carcinogenesis*. https://doi.org/10.1093/carcin/bgn026.

London, Nir, Barak Raveh, and Ora Schueler-Furman. 2013. “Druggable Protein-Protein Interactions - from Hot Spots to Hot Segments.” *Current Opinion in Chemical Biology*. https://doi.org/10.1016/j.cbpa.2013.10.011.

Luzzi, Keith J., Ian C. MacDonald, Eric E. Schmidt, Nancy Kerkvliet, Vincent L. Morris, Ann F. Chambers, and Alan C. Groom. 1998. “Multistep Nature of Metastatic Inefficiency: Dormancy of Solitary Cells after Successful Extravasation and Limited Survival of Early Micrometastases.” *American Journal of Pathology*. https://doi.org/10.1016/S0002-9440(10)65628-3.

Madara, Karen, John E. Janik, John W. Smith, and Dan L. Longo. 1993. “Interstitial Pressure of Subcutaneous Nodules in Melanoma and Lymphoma Patients: Changes during Treatment1.” *Cancer Research*.

Mastroleo, Felice, Rob van Houdt, Steve Atkinson, Max Mergeay, Larissa Hendrickx, Ruddy Wattiez, and Natalie Leys. 2013. “Modelled Microgravity Cultivation Modulates N-Acylhomoserine Lactone Production in Rhodospirillum Rubrum S1H Independently of Cell Density.” *Microbiology (United Kingdom)*. https://doi.org/10.1099/mic.0.066415-0.

McGaughey, Georgia B., Robert P. Sheridan, Christopher I. Bayly, J. Chris Culberson, Constantine Kreatsoulas, Stacey Lindsley, Vladimir Maiorov, Jean Francois Truchon, and Wendy D. Cornell. 2007. “Comparison of Topological, Shape, and Docking Methods in Virtual Screening.” *Journal of Chemical Information and Modeling*. https://doi.org/10.1021/ci700052x.

Miller, Kimberly D., Rebecca L. Siegel, Chun Chieh Lin, Angela B. Mariotto, Joan L. Kramer, Julia H. Rowland, Kevin D. Stein, Rick Alteri, and Ahmedin Jemal. 2016. “Cancer Treatment and Survivorship Statistics, 2016.” *CA: A Cancer Journal for Clinicians*. https://doi.org/10.3322/caac.21349.

Molladavoodi, Sara, Matthew Robichaud, David Wulff, and Maud Gorbet. 2017. “Corneal Epithelial Cells Exposed to Shear Stress Show Altered Cytoskeleton and Migratory Behaviour.” *PLoS ONE*. https://doi.org/10.1371/journal.pone.0178981.

Moran-Jones, Kim, Laura M. Brown, and Goli Samimi. 2015. “INC280, an Orally Available Small Molecule Inhibitor of c-MET, Reduces Migration and Adhesion in Ovarian Cancer Cell Models.” *Scientific Reports*. https://doi.org/10.1038/srep11749.

Motro, René. 2003. *Tensegrity: Structural Systems for the Future*. *Tensegrity: Structural Systems for the Future*. https://doi.org/10.1016/B978-1-903996-37-9.X5028-8.

Nishizaki, Takashi, Takashi Matsumata, Takashi Kanematsu, Chikao Yasunaga, and Keizo Sugimachi. 1990. “Surgical Manipulation of VX2 Carcinoma in the Rabbit Liver Evokes Enhancement of Metastasis.” *Journal of Surgical Research*. https://doi.org/10.1016/0022-4804(90)90116-J.

Oki, Yasuhiro, Michelle Fanale, Jorge Romaguera, Luis Fayad, Nathan Fowler, Amanda Copeland, Felipe Samaniego, et al. 2015. “Phase II Study of an AKT Inhibitor MK2206 in Patients with Relapsed or Refractory Lymphoma.” *British Journal of Haematology*. https://doi.org/10.1111/bjh.13603.

Oltersdorf, Tilman, Steven W. Elmore, Alexander R. Shoemaker, Robert C. Armstrong, David J. Augeri, Barbara A. Belli, Milan Bruncko, et al. 2005. “An Inhibitor of Bcl-2 Family Proteins Induces Regression of Solid Tumours.” *Nature*. https://doi.org/10.1038/nature03579.

Pachioni, Juliana de Almeida, Juliana Gallottini Magalhães, Elys Juliane Cardoso Lima, Luciana de Moura Bueno, Joyce Ferreira Barbosa, Matheus Malta de Sá, and Carlota de Oliveira Rangel-Yagui. 2013. “Alkylphospholipids - A Promising Class of Chemotherapeutic Agents with a Broad Pharmacological Spectrum.” *Journal of Pharmacy and Pharmaceutical Sciences*. https://doi.org/10.18433/J3CW23.

Parisi, Cristian, Vikesh V. Chandaria, and Niamh C. Nowlan. 2018. “Blocking Mechanosensitive Ion Channels Eliminates the Effects of Applied Mechanical Loading on Chick Joint Morphogenesis.” *Philosophical Transactions of the Royal Society B: Biological Sciences*. https://doi.org/10.1098/rstb.2017.0317.

Parsons, J. T. 2003. “Focal Adhesion Kinase: The First Ten Years.” *Journal of Cell Science*. https://doi.org/10.1242/jcs.00373.

Patel, Hitesh, Nadia Le Marer, Richard Q. Wharton, Zulfiqar A.J. Khan, Ruth Araia, Clare Glover, Michael M. Henry, and Timothy G. Allen-Mersh. 2002. “Clearance of Circulating Tumor Cells after Excision of Primary Colorectal Cancer.” *Annals of Surgery*. https://doi.org/10.1097/00000658-200202000-00010.

Petsalaki, Evangelia, and Robert B. Russell. 2008. “Peptide-Mediated Interactions in Biological Systems: New Discoveries and Applications.” *Current Opinion in Biotechnology*. https://doi.org/10.1016/j.copbio.2008.06.004.

Prevelige, Peter, and Gerald D. Fasman. 1989. “Chou-Fasman Prediction of the Secondary Structure of Proteins.” In *Prediction of Protein Structure and the Principles of Protein Conformation*. https://doi.org/10.1007/978-1-4613-1571-1_9.

Raj, Monika, Brooke N. Bullock, and Paramjit S. Arora. 2013. “Plucking the High Hanging Fruit: A Systematic Approach for Targeting Protein-Protein Interactions.” *Bioorganic and Medicinal Chemistry*. https://doi.org/10.1016/j.bmc.2012.11.023.

Raschka, S, S K More, D Devadoss, B Zeng, L A Kuhn, and M D Basson. 2018. “Identification of Potential Small-Molecule Protein-Protein Inhibitors of Cancer Metastasis by 3D Epitope-Based Computational Screening.” *Journal of Physiology and Pharmacology : An Official Journal of the Polish Physiological Society* 69 (2). https://doi.org/10.26402/jpp.2018.2.11.

Sadoshima, Junichi, and Seigo Izumo. 1997. “THE CELLULAR AND MOLECULAR RESPONSE OF CARDIAC MYOCYTES TO MECHANICAL STRESS.” *Annual Review of Physiology*. https://doi.org/10.1146/annurev.physiol.59.1.551.

Shapovalov, Maxim V., and Roland L. Dunbrack. 2011. “A Smoothed Backbone-Dependent Rotamer Library for Proteins Derived from Adaptive Kernel Density Estimates and Regressions.” *Structure*. https://doi.org/10.1016/j.str.2011.03.019.

Souers, Andrew J., Joel D. Leverson, Erwin R. Boghaert, Scott L. Ackler, Nathaniel D. Catron, Jun Chen, Brian D. Dayton, et al. 2013. “ABT-199, a Potent and Selective BCL-2 Inhibitor, Achieves Antitumor Activity While Sparing Platelets.” *Nature Medicine*. https://doi.org/10.1038/nm.3048.

Tamura, Masahito, Jianguo Gu, Erik H J Danen, Takahisa Takino, Shingo Miyamoto, and Kenneth M. Yamada. 1999. “PTEN Interactions with Focal Adhesion Kinase and Suppression of the Extracellular Matrix-Dependent Phosphatidylinositol 3-Kinase/Akt Cell Survival Pathway.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.274.29.20693.

Tatsumi, Hitoshi, Masatsugu Toyota, Takuya Furuichi, and Masahiro Sokabe. 2014. “Calcium Mobilizations in Response to Changes in the Gravity Vector in Arabidopsis Seedlings Possible Cellular Mechanisms.” *Plant Signaling and Behavior*. https://doi.org/10.4161/psb.29099.

Thamilselvan, Vijayalakshmi, and Marc D. Basson. 2004. “Pressure Activates Colon Cancer Cell Adhesion by Inside-Out Focal Adhesion Complex and Actin Cytoskeletal Signaling.” *Gastroenterology*. https://doi.org/10.1053/j.gastro.2003.10.078.

Thamilselvan, Vijayalakshmi, David H Craig, and Marc D Basson. 2007. “FAK Association with Multiple Signal Proteins Mediates Pressure-Induced Colon Cancer Cell Adhesion via a Src-Dependent PI3K/Akt Pathway.” *The FASEB Journal : Research Communication*. https://doi.org/10.1096/fj.06-6545com.

Thubrikar, Mano J., and Francis Robicsek. 1995. “Pressure-Induced Arterial Wall Stress and Atherosclerosis.” *The Annals of Thoracic Surgery*. https://doi.org/10.1016/0003-4975(94)01037-D.

Vassilev, Lyubomir T., Binh T. Vu, Bradford Graves, Daisy Carvajal, Frank Podlaski, Zoran Filipovic, Norman Kong, et al. 2004. “In Vivo Activation of the P53 Pathway by Small-Molecule Antagonists of MDM2.” *Science*. https://doi.org/10.1126/science.1092472.

Walsh, Mary F., Vijayalakshmi Thamilselvan, Rainer Grotelueschen, Lulu Farhana, and Marc D. Basson. 2003. “Absence of Adhesion Triggers Differential FAK and SAPKp38 Signals in SW620 Human Colon Cancer Cells That May Inhibit Adhesiveness and Lead to Cell Death.” *Cellular Physiology and Biochemistry*. https://doi.org/10.1159/000071864.

Wang, S., and M. D. Basson. 2011. “Akt Directly Regulates Focal Adhesion Kinase through Association and Serine Phosphorylation: Implication for Pressure-Induced Colon Cancer Metastasis.” *AJP: Cell Physiology*. https://doi.org/10.1152/ajpcell.00377.2010.

Wang, Shouye, and Marc D Basson. 2011. “Protein Kinase B/AKT And Focal Adhesion Kinase: Two Close Signaling Partners in Cancer.” *Anti-Cancer Agents in Medicinal Chemistry*. https://doi.org/10.2174/187152011797927661.

Weitz, Jürgen, Moritz Koch, Peter Kienle, Andrea Schrödel, Frank Willeke, Axel Benner, Thomas Lehnert, Christian Herfarth, and Magnus Von Knebel Doeberitz. 2000. “Detection of Hematogenic Tumor Cell Dissemination in Patients Undergoing Resection of Liver Metastases of Colorectal Cancer.” *Annals of Surgery*. https://doi.org/10.1097/00000658-200007000-00010.

Wells, James A., and Christopher L. McClendon. 2007. “Reaching for High-Hanging Fruit in Drug Discovery at Protein-Protein Interfaces.” *Nature*. https://doi.org/10.1038/nature06526.

Wen, Li, Tanveer A. Javed, Dean Yimlamai, Amitava Mukherjee, Xiangwei Xiao, and Sohail Z. Husain. 2018. “Transient High Pressure in Pancreatic Ducts Promotes Inflammation and Alters Tight Junctions via Calcineurin Signaling in Mice.” *Gastroenterology*. https://doi.org/10.1053/j.gastro.2018.06.036.

Wolfenson, Haguy, Bo Yang, and Michael P Sheetz. 2018. “Steps in Mechanotransduction Pathways That Control Cell Morphology.” *Annual Review of Physiology* 81 (1): annurev-physiol-021317-121245. https://doi.org/10.1146/annurev-physiol-021317-121245.

Wu, Meng Yao, Rong Rui Liang, Kai Chen, Meng Shen, Ya Li Tian, Dao Ming Li, Wei Ming Duan, et al. 2015. “FH535 Inhibited Metastasis and Growth of Pancreatic Cancer Cells.” *OncoTargets and Therapy*. https://doi.org/10.2147/OTT.S82718.

Wu, Rong, Xiao Meng Liu, Jian Guang Sun, Hong Chen, Jun Ma, Meng Dong, Shengyi Peng, et al. 2017. “DJ-1 Maintains Energy and Glucose Homeostasis by Regulating the Function of Brown Adipose Tissue.” *Cell Discovery*. https://doi.org/10.1038/celldisc.2016.54.

Yamaguchi, Kazuya, Yukihiro Takagi, Shinichirou Aoki, Manabu Futamura, and Shigetoyo Saji. 2000. “Significant Detection of Circulating Cancer Cells in the Blood by Reverse Transcriptase-Polymerase Chain Reaction during Colorectal Cancer Resection.” *Annals of Surgery*. https://doi.org/10.1097/00000658-200007000-00009.

Zahiri, Javad, Joseph Bozorgmehr, and Ali Masoudi-Nejad. 2013. “Computational Prediction of Protein–Protein Interaction Networks: Algorithms and Resources.” *Current Genomics*. https://doi.org/10.2174/1389202911314060004.

Zanotelli, Matthew R., and Cynthia A. Reinhart-King. 2018. “Mechanical Forces in Tumor Angiogenesis.” In *Advances in Experimental Medicine and Biology*, 1092:91–112. https://doi.org/10.1007/978-3-319-95294-9_6.

Zeng, Bixi, Dinesh Devadoss, Shouye Wang, Emilie E. Vomhof-DeKrey, Leslie A. Kuhn, and Marc D. Basson. 2017. “Inhibition of Pressure-Activated Cancer Cell Adhesion by FAK-Derived Peptides.” *Oncotarget* 8 (58): 98051–67. https://doi.org/10.18632/oncotarget.20556.

CHAPTER 5

Conclusion

Relative the prevalence of physical stimuli in the world, surprisingly little is known about pressure-stimulated adhesion. However, progress has been made in the understanding of how physical forces can be translated into chemical signals and how the signals can then go on to regulate normal cell physiology or invoke unwanted pathologies (Wolfenson, Yang, and Sheetz 2018; Zanotelli and Reinhart-King 2018). As the body of knowledge around the components of mechanotransduction grows, the trailing wake of applied research similarly develops. Already there are studies aimed at targeting pathways triggered by accumulated fluid sheer stress, morphogenic mechanical loading, and transient increases in hydrostatic pressure; if successful, these interventions could forestall lethal cardiac events, prevent osteoporosis at the embryonic level, and treat acute pancreatitis, respectively (Baeyens 2018; Parisi, Chandaria, and Nowlan 2018; Wen et al. 2018). A common theme behind these interventions, and indeed the majority of pharmacologic therapies, is the targeting of a key component that may or may not be unique to the pathway in question. With the aforementioned examples the targets are VEGF, calcium ion channels, and calcineurin (Baeyens 2018; Parisi, Chandaria, and Nowlan 2018; Wen et al. 2018). Our study of pressure-stimulated cell adhesion is within the context of cancer metastasis, which provides a convenient, if not extreme, example of the trade-off between efficacy and side-effects associated with drug treatments. With chemotherapy, which targets the mitotic process, the cells most effected are those that have a high rate of division. The balance needed to be struck is between the rate of death of rapidly dividing cancer cells and the similarly prolific cells of our gastrointestinal and vascular systems. Weighted in this balance is the distribution of the target between pathologic and normal cells, the burden of the problem, and the size

of the treatment effect. The combination of the effect size of the pressure-stimulated cell adhesion (10-20%), the pervasive nature of FAK and Akt1, and the promiscuity of kinase inhibitors challenged us to pursue a method of inhibition that did not focus on disrupting kinase activity (S. Wang and Basson 2011; Zeng et al. 2017; Thamilselvan and Basson 2004).

The FAK-Akt1 interaction as a possible target for drug inhibition is a mixed-blessing. There is a dearth of knowledge concerning the FAK-Akt1 interaction outside the context of pressure-stimulated cell adhesion, and while this does not preclude the possibility of side effects from inhibitors targeting this protein-protein interaction, it is promising that such a target would not come with the known and considerable problems accompanying the existing drugs that can inhibit pressure-stimulated cell adhesion. Colchicine disrupts the pressure-adhesion signaling pathway by inhibiting microtubule polymerization but requires concentrations not feasible for human use (Craig et al. 2008). FAK kinase activity is also a risky target as FAK itself is used as a sentinel kinase to predict the development of cardiotoxicity by untested kinase inhibitors (Lamore et al. 2017). This is particularly salient for cancer treatment as FAK has been shown to exhibit protective qualities against doxorubicin-induced cardiotoxicity (Cheng et al. 2014). Akt1 inhibitors are accompanied by high general toxicity and low specificity (edelfosine), gastrointestinal toxicity (ilmofosine), and hemolytic toxicity (miltefosine); however there have been some encouraging results with MK2206 (Pachioni et al. 2013; Giantonio et al. 2004; Dorlo et al. 2012; Oki et al. 2015). These problems make clear the advantages of producing treatments that are more selective. However, approaching protein-protein

interactions as a therapeutic target is not easy and was more-or-less dismissed when initial high-resolution structures revealed protein-protein interaction interfaces to be flat spans 1000–2000 Å2 in size (Arkin, Tang, and Wells 2014).

It is now known that protein-protein interactions do not involve all residues present at the interface, but rather specific “hot spots” that reside in the sub-pockets of binding grooves (Arkin and Wells 2004; Clackson and Wells 1995). These sub-pockets may shift during binding and displace the hot-spot from its coordinates in the crystal structure; this is one of the reasons behind engineering flexibility into our ligand models (Johnson and Karanicolas 2013; Wells and McClendon 2007; Raschka et al. 2018). Protein-protein interactions can be separated into those that include a linear protein sequence, those that contain one element of secondary-structure, and those that involve several elements of secondary-structure (Arkin, Tang, and Wells 2014; Hwang et al. 2010). The 7-residue FAK-derived peptidyl epitope more similarly resembles the second category, and even more so the straight alpha-helix of AAHPSEE. This constellation of characteristics fits well into the statistics describing protein-protein interactions; 40% of protein-protein interactions rely solely on a one peptide-one groove basis, and 60% involve an alpha-helix (Petsalaki and Russell 2008; Raj, Bullock, and Arora 2013; Arkin, Tang, and Wells 2014; Hwang et al. 2010). Noteworthy examples of protein-protein interaction inhibitors that share this single-secondary structure motif are the cancer drugs Ro8994 and ABT-199. Ro8994 is the latest in a line of inhibitors that target MDM2 binding to the tumor suppressor p53; MDM2 is a ubiquitin E3 ligase that degrades p53, and the inhibition of this interaction increases the amount of functional

p53 for cell-cycle regulation (Arkin, Tang, and Wells 2014). Ro8994 has its origins in high-throughput screening and is now in clinical trials (Vassilev et al. 2004). ABT-199, an inhibitor of the anti-apoptotic protein BCL2, was created through breakthroughs made via NMR-based fragment discovery and is also currently in phase 1 trials (Oltersdorf et al. 2005; Souers et al. 2013). However, as we learn more about protein-protein interactions and the existence of multiple hot-spots spread across large binding interfaces, future development of small-molecule inhibitors of protein-protein interactions could necessitate the use of small-molecule drug cocktails

Our dual-pronged approach towards disrupting the FAK-Akt1 interaction revealed both peptide and small-molecule based interventions to be capable of inhibiting pressure-stimulated cell adhesion (Zeng et al. 2017; Raschka et al. 2018). Staggered in time, the initial foray into peptide-mediated treatments laid the groundwork needed to generate the query models needed for the virtual ligand-based screening. The successes seen blocking pressure-stimulated cell adhesion in vitro and in vivo with the adenoviral FAK-derived peptide were limited by their limited application in the clinical setting. A return to earlier attempts at using a nanoparticle delivery system for the FAK-derived peptide was possible but unlikely due to logistical barriers. However, as the proof-of-concept peptide-work concluded, the virtual screening was able to identify several small-molecule leads ready for in vitro testing. Despite discrepancies seen in how ZINC31501681 affects basal levels of FAK Tyr397 between the unpublished results shown here and the work of Raschka et al., the general trend of the work as a whole is one that supports the continued development of small-molecule inhibitors against the

FAK-Akt1 interaction. Furthermore, the clinical applications for these small-molecules are much more favorable. By design, the library of small-molecules screened were ones that tended to demonstrate the drug-like attributes outlined in Lipinski's Rule of 5: ≤ 5 H-bond donors, ≤ 10 H-bond acceptors, molecular weight ≤ 500D, and predicted aqueous solubility, as estimated by clogP ≤ 5 (Lipinski et al. 2012; Raschka et al. 2018).

The basal level of FAK Tyr397 phosphorylation was increased by mimics modeled after the native LAHPP (Raschka et al. 2018). While they were also able to inhibit and further increases in pFAK due to pressure stimulation, the effect of this cannot be extricated from the possibility that the increase in basal phosphorylation overwhelmed the ability of the pressure-stimulation to activate FAK phosphorylation any further. Due to this the use of those particular FAK mimics were deemed unfeasible. The use of ZINC small-molecules modeled after AAHPSEE showed more promise as ZINC04085549 and ZINC4085554 were able to prevent pressure-stimulated FAK phosphorylation and cell adhesion without affecting their basal levels. It is unclear whether the LAHPP mimics increase FAK Tyr397 phosphorylation by facilitating autophosphorylation or by inhibiting SHP2- or PTEN-mediated dephosphorylation, and it is not yet understood how the AAHPSEE mimics avoid this (Hartman, Schaller, and Agazie 2013; Tamura et al. 1999). Aside from the need to continue testing ZINC04085549 and ZINC4085554, future studies should also include the testing of other, lower-scoring ZINC candidates. By reorganizing the 500 ZINC small-molecules found through the virtual screen by shared physical or chemical traits and then assaying select compounds from these groups, we could identify mimics that bind Akt1 with low-

affinity. Analysis of these compounds could provide insight into the binding of ZINC04085549 and ZINC4085554 as well as help to reverse engineer elements of the Akt1 binding groove.

The work that went into the discovery of pressure-stimulated cell adhesion, the detailing of the mechanotransductive pathway, and the identification of a FAK domain that could pulldown Akt1 was made possible the work presented here in the development of a FAK-derived peptide and its small-molecule mimic. While the conservative purpose at the beginning of the project was to determine whether the relationship between FAK and Akt1 was a targetable interaction, the unspoken but loudly thought aspiration was one of drug discovery. These steps towards that goal help to address the issues surrounding cancer metastasis. Again, the conservative application of such a treatment would be aimed at preventing the iatrogenic seeding of tumor cells by laparoscopic insufflation. An ambitious follow-up would be whether this therapy could have an effect on the lesser studied phenomenon of pressure-stimulation linked to cell intravasation, extravasation, and flow through the circulation; still other applications related to force-activated phenomena could also arise. With all there is still left to learn about cellular mechanosensitivity, the optimism may not be undue.

REFERENCES

REFERENCES

Arkin, Michelle R., Yinyan Tang, and James A. Wells. 2014. “Small-Molecule Inhibitors of Protein-Protein Interactions: Progressing toward the Reality.” *Chemistry and Biology*. https://doi.org/10.1016/j.chembiol.2014.09.001.

Arkin, Michelle R., and James A Wells. 2004. “Small-Molecule Inhibitors of Protein-Protein-Interactions: Progression Towards the Dream.” *Nature Reviews*. https://doi.org/10.1038/nrd1343.

Baell, Jonathan B., and Georgina A. Holloway. 2010. “New Substructure Filters for Removal of Pan Assay Interference Compounds (PAINS) from Screening Libraries and for Their Exclusion in Bioassays.” *Journal of Medicinal Chemistry*. https://doi.org/10.1021/jm901137j.

Baeyens, Nicolas. 2018. “Fluid Shear Stress Sensing in Vascular Homeostasis and Remodeling: Towards the Development of Innovative Pharmacological Approaches to Treat Vascular Dysfunction.” *Biochemical Pharmacology* 158 (October): 185–91. https://doi.org/10.1016/j.bcp.2018.10.023.

Basse, Marie Jeanne, Stéphane Betzi, Xavier Morelli, and Philippe Roche. 2016. “2P2Idb v2: Update of a Structural Database Dedicated to Orthosteric Modulation of Protein-Protein Interactions.” *Database*. https://doi.org/10.1093/database/baw007.

Basson, Marc D. 2008. “An Intracellular Signal Pathway That Regulates Cancer Cell Adhesion in Response to Extracellular Forces.” *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-07-2992.

Basson, Marc D., Cheng Fang Yu, Oliver Herden-Kirchoff, Marc Ellermeier, Matthew A. Sanders, Ronald C. Merrell, and Bauer E. Sumpio. 2000. “Effects of Increased Ambient Pressure on Colon Cancer Cell Adhesion.” *Journal of Cellular Biochemistry*. https://doi.org/10.1093/brain/114.3.1297.

Basson, Marc D., B. Zeng, and S. Wang. 2017. “The C-Terminal Region of the Focal Adhesion Kinase F1 Domain Binds Akt1 and Inhibits Pressure-Induced Cell Adhesion.” *Journal of Physiology and Pharmacology*.

Basson, Marc D., Bixi Zeng, Christina Downey, Madhu P. Sirivelu, and Jetze J. Tepe. 2015. “Increased Extracellular Pressure Stimulates Tumor Proliferation by a Mechanosensitive Calcium Channel and PKC-β.” *Molecular Oncology*. https://doi.org/10.1016/j.molonc.2014.10.008.

Bonjer, H. Jacob, Wim C J Hop, Heidi Nelson, Daniel J. Sargent, Antonio M. Lacy, Antoni Castells, Pierre J. Guillou, et al. 2007. “Laparoscopically Assisted vs Open

Colectomy for Colon Cancer: A Meta-Analysis.” *Archives of Surgery*. https://doi.org/10.1001/archsurg.142.3.298.

Buckley, M J, A J Banes, L G Levin, B E Sumpio, M Sato, R Jordan, J Gilbert, G W Link, and R Tran Son Tay. 1988. “Osteoblasts Increase Their Rate of Division and Align in Response to Cyclic, Mechanical Tension in Vitro.” *Bone & Mineral*.

Butler, Thomas P., and Pietro M. Gullino. 1975. “Quantitation of Cell Shedding Into Efferent Blood of Mammary Adenocarcinoma.” *Cancer Research*.

Ceccarelli, Derek F.J., Kyu Song Hyun, Florence Poy, Michael D. Schaller, and Michael J. Eck. 2006. “Crystal Structure of the FERM Domain of Focal Adhesion Kinase.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M509188200.

Chaturvedi, Lakshmi S., H. Michael Marsh, Xun Shang, Yi Zheng, and Marc D. Basson. 2007. “Repetitive Deformation Activates Focal Adhesion Kinase and ERK Mitogenic Signals in Human Caco-2 Intestinal Epithelial Cells through Src and Rac1.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M605817200.

Chaturvedi, Lakshmi S, Harold M Marsh, and Marc D Basson. 2007. “Src and Focal Adhesion Kinase Mediate Mechanical Strain-Induced Proliferation and ERK1/2 Phosphorylation in Human H441 Pulmonary Epithelial Cells.” *American Journal of Physiology-Cell Physiology*. https://doi.org/10.1152/ajpcell.00529.2006.

Chen, William S., Pei Zhang Xu, Kathrin Gottlob, Mei Ling Chen, Karen Sokol, Tanya Shiyanova, Igor Roninson, et al. 2001. “Growth Retardation and Increased Apoptosis in Mice with Homozygous Disruption of the Akt1 Gene.” *Genes and Development*. https://doi.org/10.1101/gad.913901.

Cheng, Zhaokang, Laura A. DiMichele, Mauricio Rojas, Cyrus Vaziri, Christopher P. Mack, and Joan M. Taylor. 2014. “Focal Adhesion Kinase Antagonizes Doxorubicin Cardiotoxicity via P21Cip1.” *Journal of Molecular and Cellular Cardiology*. https://doi.org/10.1016/j.yjmcc.2013.12.002.

Cho, H., J. Mu, J. K. Kim, J. L. Thorvaldsen, Q. Chu, E. B. Crenshaw, K. H. Kaestner, M. S. Bartolomei, G. I. Shulman, and M. J. Birnbaum. 2001. “Insulin Resistance and a Diabetes Mellitus-like Syndrome in Mice Lacking the Protein Kinase Akt2 (PKBβ).” *Science*. https://doi.org/10.1126/science.292.5522.1728.

Clackson, Tim, and James A. Wells. 1995. “A Hot Spot of Binding Energy in a Hormone-Receptor Interface.” *Science*. https://doi.org/10.1126/science.7529940.

Cohen, Lee Ann, and Jun Lin Guan. 2005. “Residues within the First Subdomain of the FERM-like Domain in Focal Adhesion Kinase Are Important in Its Regulation.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M412021200.

Collawn, James F., Martin Stangel, Leslie A. Kuhn, Victor Esekogwu, Shuqian Jing, Ian S. Trowbridge, and John A. Tainer. 1990. “Transferrin Receptor Internalization Sequence YXRF Implicates a Tight Turn as the Structural Recognition Motif for Endocytosis.” *Cell*. https://doi.org/10.1136/jnnp.s1-12.48.320.

Conway, William C., Jochem Van Der Voort Van Zyp, Vijayalakshmi Thamilselvan, Mary F. Walsh, David L. Crowe, and Marc D. Basson. 2006. “Paxillin Modulates Squamous Cancer Cell Adhesion and Is Important in Pressure-Augmented Adhesion.” *Journal of Cellular Biochemistry*. https://doi.org/10.1002/jcb.20819.

Craig, D H, C P Gayer, K L Schaubert, Y Wei, J Li, Y Laouar, and M D Basson. 2009. “Increased Extracellular Pressure Enhances Cancer Cell Integrin-Binding Affinity through Phosphorylation of Beta1-Integrin at Threonine 788/789.” *American Journal of Physiology. Cell Physiology*. https://doi.org/10.1152/ajpcell.00355.2008.

Craig, David H., Cheri R. Owen, William C. Conway, Mary F. Walsh, Christina Downey, and Marc D. Basson. 2008. “Colchicine Inhibits Pressure-Induced Tumor Cell Implantation within Surgical Wounds and Enhances Tumor-Free Survival in Mice.” *Journal of Clinical Investigation*. https://doi.org/10.1172/JCI34279.

Craig, David H., Hiroe Shiratsuchi, and Marc D. Basson. 2009. “Increased Extracellular Pressure Provides a Novel Adjuvant Stimulus for Enhancement of Conventional Dendritic Cell Maturation Strategies.” *Biochemical and Biophysical Research Communications*. https://doi.org/10.1016/j.bbrc.2009.07.010.

Craig, David H, Christina Downey, and Marc D Basson. 2008. “SiRNA-Mediated Reduction of α-Actinin-1 Inhibits Pressure-Induced Murine Tumor Cell Wound Implantation and Enhances Tumor-Free Survival.” *Neoplasia*. https://doi.org/10.1593/neo.07945.

Craig, David H, Beatrice Haimovich, and Marc D Basson. 2007. “␣-Actinin-1 Phosphorylation Modulates Pressure-Induced Colon Cancer Cell Adhesion through Regulation of Focal Adhesion Kinase-Src Interaction.” *Am J Physiol Cell Physiol*. https://doi.org/10.1152/ajpcell.00118.2007.

Craig, Lisa, Paul C. Sanschagrin, Annett Rozek, Steve Lackie, Leslie A. Kuhn, and Jamie K. Scott. 1998. “The Role of Structure in Antibody Cross-Reactivity between Peptides and Folded Proteins.” *Journal of Molecular Biology*. https://doi.org/10.1006/jmbi.1998.1907.

Datta, Sandeep Robert, Henryk Dudek, Tao Xu, Shane Masters, Fu Haian, Yukiko Gotoh, and Michael E. Greenberg. 1997. “Akt Phosphorylation of BAD Couples Survival Signals to the Cell- Intrinsic Death Machinery.” *Cell*. https://doi.org/10.1016/S0092-8674(00)80405-5.

Downey, Christina, Kamal Alwan, Vijayalakshmi Thamilselvan, Liyue Zhang, Yan Jiang, Arun K. Rishi, and Marc D. Basson. 2006. “Pressure Stimulates Breast Cancer Cell Adhesion Independently of Cell Cycle and Apoptosis Regulatory Protein (CARP)-1 Regulation of Focal Adhesion Kinase.” *American Journal of Surgery*. https://doi.org/10.1016/j.amjsurg.2006.08.006.

Dregelid, E., and E. Svendsen. 1988. “Endothelial Cell Injury in Human Saphenous Veins after Manipulation and Tweezer Grasping.” *Journal of Cardiovascular Surgery*.

Fidler, Isaiah J. 1970. “Metastasis: Quantitative Analysis of Distribution and Fate of Tumor Emboli Labeled with 125I-5-Lodo-2’-Deoxyuridine.” *Journal of the National Cancer Institute*. https://doi.org/10.1093/jnci/45.4.773.

Fosgerau, Keld, and Torsten Hoffmann. 2015. “Peptide Therapeutics: Current Status and Future Directions.” *Drug Discovery Today*. https://doi.org/10.1016/j.drudis.2014.10.003.

Fuller, Jonathan C., Nicholas J. Burgoyne, and Richard M. Jackson. 2009. “Predicting Druggable Binding Sites at the Protein-Protein Interface.” *Drug Discovery Today*. https://doi.org/10.1016/j.drudis.2008.10.009.

Gayer, Christopher P., Lakshmi S. Chaturvedi, Shouye Wang, David H. Craig, Thomas Flanigan, and Marc D. Basson. 2009. “Strain-Induced Proliferation Requires the Phosphatidylinositol 3-Kinase/AKT/Glycogen Synthase Kinase Pathway.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M804576200.

Giantonio, Bruce J., Christine Derry, Cecilia McAleer, Joseph J. McPhillips, and Peter J. O’Dwyer. 2004. “Phase I and Pharmacokinetic Study of the Cytotoxic Ether Lipid Ilmofosine Administered by Weekly Two-Hour Infusion in Patients with Advanced Solid Tumors.” *Clinical Cancer Research*. https://doi.org/10.1158/1078-0432.CCR-0837-02.

Gupta, Gaorav P., and Joan Massagué. 2006. “Cancer Metastasis: Building a Framework.” *Cell*. https://doi.org/10.1016/j.cell.2006.11.001.

Han, Okhee, Guang Di Li, and Marc D Basson. 1998. “Strain Induces Caco-2 Intestinal Epithelial Proliferation and Differentiation via PKC and Tyrosine Kinase Signals.” *American Journal of Physiology*.

Hanahan, Douglas, Weinberg, Robert. 2000. “Hallmarks of Cancer.” *Cell*. https://doi.org/10.1007/s00262-010-0968-0.

Hartman, Z. R., M. D. Schaller, and Y. M. Agazie. 2013. “The Tyrosine Phosphatase SHP2 Regulates Focal Adhesion Kinase to Promote EGF-Induced Lamellipodia

Persistence and Cell Migration.” *Molecular Cancer Research*. https://doi.org/10.1158/1541-7786.MCR-12-0578.

Hawkins, Paul C.D., and Anthony Nicholls. 2012. “Conformer Generation with OMEGA: Learning from the Data Set and the Analysis of Failures.” *Journal of Chemical Information and Modeling*. https://doi.org/10.1021/ci300314k.

Hawkins, Paul C.D., A. Geoffrey Skillman, and Anthony Nicholls. 2007. “Comparison of Shape-Matching and Docking as Virtual Screening Tools.” *Journal of Medicinal Chemistry*. https://doi.org/10.1021/jm0603365.

Hayashi, Naoko, Hiroshi Egami, Mikio Kai, Yuji Kurusu, Sadamu Takano, and Michio Ogawa. 1999. “No-Touch Isolation Technique Reduces Intraoperative Shedding of Tumor Cells into the Portal Vein during Resection of Colorectal Cancer.” *Surgery*. https://doi.org/10.1016/S0039-6060(99)70003-6.

Huynh, Khon C., Marianna Gyenes, Thi Hiep Nguyen, Toi Van Vo, and Volker R. Stoldt. 2017. “Impact of Shear Stress on Src and Focal Adhesion Kinase Phosphorylation in Fibrinogen-Adherent Platelets.” *Blood Coagulation and Fibrinolysis*. https://doi.org/10.1097/MBC.0000000000000593.

Hwang, Howook, Thom Vreven, Joël Janin, and Zhiping Weng. 2010. “Protein-Protein Docking Benchmark Version 4.0.” *Proteins: Structure, Function and Bioinformatics*. https://doi.org/10.1002/prot.22830.

Ingber, D. E. 2006. “Cellular Mechanotransduction: Putting All the Pieces Together Again.” *The FASEB Journal*. https://doi.org/10.1096/fj.05-5424rev.

Ingber, Donald E., Ning Wang, and Dimitrije Stamenović. 2014. “Tensegrity, Cellular Biophysics, and the Mechanics of Living Systems.” *Reports on Progress in Physics*. https://doi.org/10.1088/0034-4885/77/4/046603.

Irwin, John J., and Brian K. Shoichet. 2005. “ZINC - A Free Database of Commercially Available Compounds for Virtual Screening.” *Journal of Chemical Information and Modeling*. https://doi.org/10.1021/ci049714+.

Jain, Rakesh K., Yves Boucher, and Norman Wolmark. 1992. “Interstitial Hypertension in Human Breast and Colorectal Tumors.” *Cancer Research*.

Jakalian, Araz, David B. Jack, and Christopher I. Bayly. 2002. “Fast, Efficient Generation of High-Quality Atomic Charges. AM1-BCC Model: II. Parameterization and Validation.” *Journal of Computational Chemistry*. https://doi.org/10.1002/jcc.10128.

Johnson, David K., and John Karanicolas. 2013. “Druggable Protein Interaction Sites Are More Predisposed to Surface Pocket Formation than the Rest of the Protein

Surface.” *PLoS Computational Biology*. https://doi.org/10.1371/journal.pcbi.1002951.

Kao, Y. C., J. R. Jheng, H. J. Pan, W. Y. Liao, C. H. Lee, and P. L. Kuo. 2017. “Elevated Hydrostatic Pressure Enhances the Motility and Enlarges the Size of the Lung Cancer Cells through Aquaporin Upregulation Mediated by Caveolin-1 and ERK1/2 Signaling.” *Oncogene*. https://doi.org/10.1038/onc.2016.255.

Kaspar, Allan A., and Janice M. Reichert. 2013. “Future Directions for Peptide Therapeutics Development.” *Drug Discovery Today*. https://doi.org/10.1016/j.drudis.2013.05.011.

Kleinschmidt, Elizabeth G., and David D. Schlaepfer. 2017. “Focal Adhesion Kinase Signaling in Unexpected Places.” *Current Opinion in Cell Biology*. https://doi.org/10.1016/j.ceb.2017.01.003.

Klinghoffer, Richard A., Christoph Sachsenmaier, Jonathan A. Cooper, and Philippe Soriano. 1999. “Src Family Kinases Are Required for Integrin but Not PDGFR Signal Transduction.” *EMBO Journal*. https://doi.org/10.1093/emboj/18.9.2459.

Ko, Hyo Rim, Il Sun Kwon, Inwoo Hwang, Eun Ju Jin, Joo Ho Shin, Angela M. Brennan-Minnella, Raymond Swanson, Sung Woo Cho, Kyung Hoon Lee, and Jee Yin Ahn. 2016. “Akt1-Inhibitor of DNA Binding2 Is Essential for Growth Cone Formation and Axon Growth and Promotes Central Nervous System Axon Regeneration.” *ELife*. https://doi.org/10.7554/eLife.20799.

Koch, Moritz, Peter Kienle, Ulf Hinz, Dalibor Antolovic, Jan Schmidt, Christian Herfarth, Magnus Von Knebel Doeberitz, and Jürgen Weitz. 2005. “Detection of Hematogenous Tumor Cell Dissemination Predicts Tumor Relapse in Patients Undergoing Surgical Resection of Colorectal Liver Metastases.” *Annals of Surgery*. https://doi.org/10.1097/01.sla.0000151795.15068.27.

Krieger, Elmar, Tom Darden, Sander B. Nabuurs, Alexei Finkelstein, and Gert Vriend. 2004. “Making Optimal Use of Empirical Energy Functions: Force-Field Parameterization in Crystal Space.” *Proteins: Structure, Function and Genetics*. https://doi.org/10.1002/prot.20251.

Labbé, Céline M., Guillaume Laconde, Mélaine A. Kuenemann, Bruno O. Villoutreix, and Olivier Sperandio. 2013. “IPPI-DB: A Manually Curated and Interactive Database of Small Non-Peptide Inhibitors of Protein-Protein Interactions.” *Drug Discovery Today*. https://doi.org/10.1016/j.drudis.2013.05.003.

Lamore, Sarah D., Ernst Ahlberg, Scott Boyer, Michelle L. Lamb, Maria P. Hortigon-Vinagre, Victor Rodriguez, Godfrey L. Smith, et al. 2017. “Deconvoluting Kinase Inhibitor Induced Cardiotoxicity.” *Toxicological Sciences*. https://doi.org/10.1093/toxsci/kfx082.

Le Devedec, SE.,Geverts B, de Bont H, Yan K, Verbeek FJ, Houtsmuller AB, and van der Water B. 2018. “The Residence Time of Focal Adhesion Kinase (FAK) and Paxillin at Focal Adhesions in Renal Epithelial Cells is Determined by Adhesion Size, Strength, and Life Cycle Status.” *J Cell Sci*.

Leunig, Michael, Rakesh K. Jain, Alwin E. Goetz, Konrad Messmer, and Ernst Kastenbauer. 1992. “Interstitial Hypertension in Head and Neck Tumors in Patients: Correlation with Tumor Size.” *Cancer Research*.

Li, Wei, Aydin Duzgun, Bauer E. Sumpio, and Marc D. Basson. 2001. “Integrin and FAK-Mediated MAPK Activation Is Required for Cyclic Strain Mitogenic Effects in Caco-2 Cells.” *Am J Physiol Gastrointest Liver Physiol*.

Li, Weiqun, Jiachang Zhang, Lawrence Flechner, Teresa Hyun, Alan Yam, Thomas F. Franke, and Jacalyn H. Pierce. 1999. “Protein Kinase C-α Overexpression Stimulates Akt Activity and Suppresses Apoptosis Induced by Interleukin 3 Withdrawal.” *Oncogene*. https://doi.org/10.1038/sj.onc.1203065.

Lietha, Daniel, Xinming Cai, Derek F.J. Ceccarelli, Yiqun Li, Michael D. Schaller, and Michael J. Eck. 2007. “Structural Basis for the Autoinhibition of Focal Adhesion Kinase.” *Cell*. https://doi.org/10.1016/j.cell.2007.05.041.

Lipinski, Christopher A., Franco Lombardo, Beryl W. Dominy, and Paul J. Feeney. 2012. “Experimental and Computational Approaches to Estimate Solubility and Permeability in Drug Discovery and Development Settings.” *Advanced Drug Delivery Reviews*. https://doi.org/10.1016/j.addr.2012.09.019.

Liu, T.-J., T. LaFortune, T. Honda, O. Ohmori, S. Hatakeyama, T. Meyer, D. Jackson, J. de Groot, and W.K. A. Yung. 2007. “Inhibition of Both Focal Adhesion Kinase and Insulin-like Growth Factor-I Receptor Kinase Suppresses Glioma Proliferation in Vitro and in Vivo.” *Molecular Cancer Therapeutics*. https://doi.org/10.1158/1535-7163.MCT-06-0476.

Liu, Weiguo, David A. Bloom, William G. Cance, Elena V. Kurenova, Vita M. Golubovskaya, and Steven N. Hochwald. 2008. “FAK and IGF-IR Interact to Provide Survival Signals in Human Pancreatic Adenocarcinoma Cells.” *Carcinogenesis*. https://doi.org/10.1093/carcin/bgn026.

London, Nir, Barak Raveh, and Ora Schueler-Furman. 2013. “Druggable Protein-Protein Interactions - from Hot Spots to Hot Segments.” *Current Opinion in Chemical Biology*. https://doi.org/10.1016/j.cbpa.2013.10.011.

Luzzi, Keith J., Ian C. MacDonald, Eric E. Schmidt, Nancy Kerkvliet, Vincent L. Morris, Ann F. Chambers, and Alan C. Groom. 1998. “Multistep Nature of Metastatic Inefficiency: Dormancy of Solitary Cells after Successful Extravasation and Limited

Survival of Early Micrometastases.” *American Journal of Pathology*. https://doi.org/10.1016/S0002-9440(10)65628-3.

Madara, Karen, John E. Janik, John W. Smith, and Dan L. Longo. 1993. “Interstitial Pressure of Subcutaneous Nodules in Melanoma and Lymphoma Patients: Changes during Treatment1.” *Cancer Research*.

Mastroleo, Felice, Rob van Houdt, Steve Atkinson, Max Mergeay, Larissa Hendrickx, Ruddy Wattiez, and Natalie Leys. 2013. “Modelled Microgravity Cultivation Modulates N-Acylhomoserine Lactone Production in Rhodospirillum Rubrum S1H Independently of Cell Density.” *Microbiology (United Kingdom)*. https://doi.org/10.1099/mic.0.066415-0.

McGaughey, Georgia B., Robert P. Sheridan, Christopher I. Bayly, J. Chris Culberson, Constantine Kreatsoulas, Stacey Lindsley, Vladimir Maiorov, Jean Francois Truchon, and Wendy D. Cornell. 2007. “Comparison of Topological, Shape, and Docking Methods in Virtual Screening.” *Journal of Chemical Information and Modeling*. https://doi.org/10.1021/ci700052x.

Miller, Kimberly D., Rebecca L. Siegel, Chun Chieh Lin, Angela B. Mariotto, Joan L. Kramer, Julia H. Rowland, Kevin D. Stein, Rick Alteri, and Ahmedin Jemal. 2016. “Cancer Treatment and Survivorship Statistics, 2016.” *CA: A Cancer Journal for Clinicians*. https://doi.org/10.3322/caac.21349.

Molladavoodi, Sara, Matthew Robichaud, David Wulff, and Maud Gorbet. 2017. “Corneal Epithelial Cells Exposed to Shear Stress Show Altered Cytoskeleton and Migratory Behaviour.” *PLoS ONE*. https://doi.org/10.1371/journal.pone.0178981.

Moran-Jones, Kim, Laura M. Brown, and Goli Samimi. 2015. “INC280, an Orally Available Small Molecule Inhibitor of c-MET, Reduces Migration and Adhesion in Ovarian Cancer Cell Models.” *Scientific Reports*. https://doi.org/10.1038/srep11749.

Motro, René. 2003. *Tensegrity: Structural Systems for the Future*. *Tensegrity: Structural Systems for the Future*. https://doi.org/10.1016/B978-1-903996-37-9.X5028-8.

Nishizaki, Takashi, Takashi Matsumata, Takashi Kanematsu, Chikao Yasunaga, and Keizo Sugimachi. 1990. “Surgical Manipulation of VX2 Carcinoma in the Rabbit Liver Evokes Enhancement of Metastasis.” *Journal of Surgical Research*. https://doi.org/10.1016/0022-4804(90)90116-J.

Oki, Yasuhiro, Michelle Fanale, Jorge Romaguera, Luis Fayad, Nathan Fowler, Amanda Copeland, Felipe Samaniego, et al. 2015. “Phase II Study of an AKT Inhibitor MK2206 in Patients with Relapsed or Refractory Lymphoma.” *British Journal of Haematology*. https://doi.org/10.1111/bjh.13603.

Oltersdorf, Tilman, Steven W. Elmore, Alexander R. Shoemaker, Robert C. Armstrong, David J. Augeri, Barbara A. Belli, Milan Bruncko, et al. 2005. “An Inhibitor of Bcl-2 Family Proteins Induces Regression of Solid Tumours.” *Nature*. https://doi.org/10.1038/nature03579.

Pachioni, Juliana de Almeida, Juliana Gallottini Magalhães, Elys Juliane Cardoso Lima, Luciana de Moura Bueno, Joyce Ferreira Barbosa, Matheus Malta de Sá, and Carlota de Oliveira Rangel-Yagui. 2013. “Alkylphospholipids - A Promising Class of Chemotherapeutic Agents with a Broad Pharmacological Spectrum.” *Journal of Pharmacy and Pharmaceutical Sciences*. https://doi.org/10.18433/J3CW23.

Parisi, Cristian, Vikesh V. Chandaria, and Niamh C. Nowlan. 2018. “Blocking Mechanosensitive Ion Channels Eliminates the Effects of Applied Mechanical Loading on Chick Joint Morphogenesis.” *Philosophical Transactions of the Royal Society B: Biological Sciences*. https://doi.org/10.1098/rstb.2017.0317.

Parsons, J. T. 2003. “Focal Adhesion Kinase: The First Ten Years.” *Journal of Cell Science*. https://doi.org/10.1242/jcs.00373.

Patel, Hitesh, Nadia Le Marer, Richard Q. Wharton, Zulfiqar A.J. Khan, Ruth Araia, Clare Glover, Michael M. Henry, and Timothy G. Allen-Mersh. 2002. “Clearance of Circulating Tumor Cells after Excision of Primary Colorectal Cancer.” *Annals of Surgery*. https://doi.org/10.1097/00000658-200202000-00010.

Perry, Brandon C., Shouye Wang, and Marc D. Basson. 2010. “Extracellular Pressure Stimulates Adhesion of Sarcoma Cells via Activation of Focal Adhesion Kinase and Akt.” *American Journal of Surgery*. https://doi.org/10.1016/j.amjsurg.2010.07.013.

Petsalaki, Evangelia, and Robert B. Russell. 2008. “Peptide-Mediated Interactions in Biological Systems: New Discoveries and Applications.” *Current Opinion in Biotechnology*. https://doi.org/10.1016/j.copbio.2008.06.004.

Prevelige, Peter, and Gerald D. Fasman. 1989. “Chou-Fasman Prediction of the Secondary Structure of Proteins.” In *Prediction of Protein Structure and the Principles of Protein Conformation*. https://doi.org/10.1007/978-1-4613-1571-1_9.

Raj, Monika, Brooke N. Bullock, and Paramjit S. Arora. 2013. “Plucking the High Hanging Fruit: A Systematic Approach for Targeting Protein-Protein Interactions.” *Bioorganic and Medicinal Chemistry*. https://doi.org/10.1016/j.bmc.2012.11.023.

Raschka, S, S K More, D Devadoss, B Zeng, L A Kuhn, and M D Basson. 2018. “Identification of Potential Small-Molecule Protein-Protein Inhibitors of Cancer Metastasis by 3D Epitope-Based Computational Screening.” *Journal of Physiology and Pharmacology : An Official Journal of the Polish Physiological Society* 69 (2). https://doi.org/10.26402/jpp.2018.2.11.

Sadoshima, Junichi, and Seigo Izumo. 1997. “THE CELLULAR AND MOLECULAR RESPONSE OF CARDIAC MYOCYTES TO MECHANICAL STRESS.” *Annual Review of Physiology*. https://doi.org/10.1146/annurev.physiol.59.1.551.

Shapovalov, Maxim V., and Roland L. Dunbrack. 2011. “A Smoothed Backbone-Dependent Rotamer Library for Proteins Derived from Adaptive Kernel Density Estimates and Regressions.” *Structure*. https://doi.org/10.1016/j.str.2011.03.019.

Shiratsuchi, Hiroe, and Marc D. Basson. 2007. “Akt2, but Not Akt1 or Akt3 Mediates Pressure-Stimulated Serum-Opsonized Latex Bead Phagocytosis through Activating MTOR and P70 S6 Kinase.” *Journal of Cellular Biochemistry*. https://doi.org/10.1002/jcb.21295.

Shiratsuchi, Hiroe, and Marc D Basson. 2004. “Extracellular Pressure Stimulates Macrophage Phagocytosis by Inhibiting a Pathway Involving FAK and ERK.” *American Journal of Physiology. Cell Physiology*. https://doi.org/10.1152/ajpcell.00553.2003.

Souers, Andrew J., Joel D. Leverson, Erwin R. Boghaert, Scott L. Ackler, Nathaniel D. Catron, Jun Chen, Brian D. Dayton, et al. 2013. “ABT-199, a Potent and Selective BCL-2 Inhibitor, Achieves Antitumor Activity While Sparing Platelets.” *Nature Medicine*. https://doi.org/10.1038/nm.3048.

Tamura, Masahito, Jianguo Gu, Erik H J Danen, Takahisa Takino, Shingo Miyamoto, and Kenneth M. Yamada. 1999. “PTEN Interactions with Focal Adhesion Kinase and Suppression of the Extracellular Matrix-Dependent Phosphatidylinositol 3-Kinase/Akt Cell Survival Pathway.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.274.29.20693.

Tatsumi, Hitoshi, Masatsugu Toyota, Takuya Furuichi, and Masahiro Sokabe. 2014. “Calcium Mobilizations in Response to Changes in the Gravity Vector in Arabidopsis Seedlings Possible Cellular Mechanisms.” *Plant Signaling and Behavior*. https://doi.org/10.4161/psb.29099.

Thamilselvan, Vijayalakshmi, and Marc D. Basson. 2004. “Pressure Activates Colon Cancer Cell Adhesion by Inside-Out Focal Adhesion Complex and Actin Cytoskeletal Signaling.” *Gastroenterology*. https://doi.org/10.1053/j.gastro.2003.10.078.

Thamilselvan, Vijayalakshmi, David H Craig, and Marc D Basson. 2007. “FAK Association with Multiple Signal Proteins Mediates Pressure-Induced Colon Cancer Cell Adhesion via a Src-Dependent PI3K/Akt Pathway.” *The FASEB Journal : Research Communication*. https://doi.org/10.1096/fj.06-6545com.

Thomas, J W, M A Cooley, J M Broome, R Salgia, J D Griffin, C R Lombardo, and M D Schaller. 1999. “The Role of Focal Adhesion Kinase Binding in the Regulation of

Tyrosine Phosphorylation of Paxillin." *The Journal of Biological Chemistry*. https://doi.org/10.1074/JBC.274.51.36684.

Thubrikar, Mano J., and Francis Robicsek. 1995. "Pressure-Induced Arterial Wall Stress and Atherosclerosis." *The Annals of Thoracic Surgery*. https://doi.org/10.1016/0003-4975(94)01037-D.

Tschopp, O. 2005. "Essential Role of Protein Kinase B (PKB /Akt3) in Postnatal Brain Development but Not in Glucose Homeostasis." *Development*. https://doi.org/10.1242/dev.01864.

Turnbull, R. B. 1970. "Cancer of the Colon. The Five- and Ten-Year Survival Rates Following Resection Utilizing the Isolation Technique." *Annals of the Royal College of Surgeons of England*. https://doi.org/10.5833/jjgs.9.539.

Vassilev, Lyubomir T., Binh T. Vu, Bradford Graves, Daisy Carvajal, Frank Podlaski, Zoran Filipovic, Norman Kong, et al. 2004. "In Vivo Activation of the P53 Pathway by Small-Molecule Antagonists of MDM2." *Science*. https://doi.org/10.1126/science.1092472.

Walsh, Mary F., Vijayalakshmi Thamilselvan, Rainer Grotelueschen, Lulu Farhana, and Marc D. Basson. 2003. "Absence of Adhesion Triggers Differential FAK and SAPKp38 Signals in SW620 Human Colon Cancer Cells That May Inhibit Adhesiveness and Lead to Cell Death." *Cellular Physiology and Biochemistry*. https://doi.org/10.1159/000071864.

Wang, S., and M. D. Basson. 2011. "Akt Directly Regulates Focal Adhesion Kinase through Association and Serine Phosphorylation: Implication for Pressure-Induced Colon Cancer Metastasis." *AJP: Cell Physiology*. https://doi.org/10.1152/ajpcell.00377.2010.

Wang, Shouye, and Marc D Basson. 2011. "Protein Kinase B/AKT And Focal Adhesion Kinase: Two Close Signaling Partners in Cancer." *Anti-Cancer Agents in Medicinal Chemistry*. https://doi.org/10.2174/187152011797927661.

Weitz, Jürgen, Moritz Koch, Peter Kienle, Andrea Schrödel, Frank Willeke, Axel Benner, Thomas Lehnert, Christian Herfarth, and Magnus Von Knebel Doeberitz. 2000. "Detection of Hematogenic Tumor Cell Dissemination in Patients Undergoing Resection of Liver Metastases of Colorectal Cancer." *Annals of Surgery*. https://doi.org/10.1097/00000658-200007000-00010.

Wells, James A., and Christopher L. McClendon. 2007. "Reaching for High-Hanging Fruit in Drug Discovery at Protein-Protein Interfaces." *Nature*. https://doi.org/10.1038/nature06526.

Wen, Li, Tanveer A. Javed, Dean Yimlamai, Amitava Mukherjee, Xiangwei Xiao, and Sohail Z. Husain. 2018. “Transient High Pressure in Pancreatic Ducts Promotes Inflammation and Alters Tight Junctions via Calcineurin Signaling in Mice.” *Gastroenterology*. https://doi.org/10.1053/j.gastro.2018.06.036.

Wolfenson, Haguy, Bo Yang, and Michael P Sheetz. 2018. “Steps in Mechanotransduction Pathways That Control Cell Morphology.” *Annual Review of Physiology* 81 (1): annurev-physiol-021317-121245. https://doi.org/10.1146/annurev-physiol-021317-121245.

Yamaguchi, Kazuya, Yukihiro Takagi, Shinichirou Aoki, Manabu Futamura, and Shigetoyo Saji. 2000. “Significant Detection of Circulating Cancer Cells in the Blood by Reverse Transcriptase-Polymerase Chain Reaction during Colorectal Cancer Resection.” *Annals of Surgery*. https://doi.org/10.1097/00000658-200007000-00009.

Zahiri, Javad, Joseph Bozorgmehr, and Ali Masoudi-Nejad. 2013. “Computational Prediction of Protein–Protein Interaction Networks: Algorithms and Resources.” *Current Genomics*. https://doi.org/10.2174/1389202911314060004.

Zanotelli, Matthew R., and Cynthia A. Reinhart-King. 2018. “Mechanical Forces in Tumor Angiogenesis.” In *Advances in Experimental Medicine and Biology*, 1092:91–112. https://doi.org/10.1007/978-3-319-95294-9_6.

Zeng, Bixi, Dinesh Devadoss, Shouye Wang, Emilie E. Vomhof-DeKrey, Leslie A. Kuhn, and Marc D. Basson. 2017. “Inhibition of Pressure-Activated Cancer Cell Adhesion by FAK-Derived Peptides.” *Oncotarget* 8 (58): 98051–67. https://doi.org/10.18632/oncotarget.20556.

Zhang, Jianhu, Cheri R. Owen, Matthew A. Sanders, Jerrold R. Turner, and Marc D. Basson. 2006. “The Motogenic Effects of Cyclic Mechanical Strain on Intestinal Epithelial Monolayer Wound Closure Are Matrix Dependent.” *Gastroenterology*. https://doi.org/10.1053/j.gastro.2006.08.007.

APPENDIX

Increased extracellular pressure stimulates tumor proliferation by a mechanosensitive calcium channel and PKC-ß

Marc D. Basson, **Bixi Zeng**, Christina Downey, Madhu Siriveluprabhakar, Jetze Tepe (2015). Increased extracellular pressure stimulates tumor proliferation by a mechanosensitive calcium channel and PKC-ß. **Mol Onc**, 9(2):513-26.

The findings in this chapter have been previously published. The following paper was authored by me, and I contributed to the work shown in figures A.1 and A.4. This paper provides a different force-activated cellular pathway that does not implicate FAK or Akt1. This is to establish background for future comparisons highlighting the specificity of the FAK-Akt1 interaction even in the realm of mechanotransduction.

Introduction

Malignant tumor extracellular matrix is often stiffer than the matrix surrounding adjacent non-malignant cells (Ingber, 2008). As solid tumors expand against constraining stroma, interstitial pressure increases by 4-50mmHg relative to pressure within normal surrounding tissues (Gutmann et al., 1992; Less et al., 1992; Raju et al., 2008). Mathematical models (Sarntinoranont et al., 2003) and direct observation suggest higher pressures within large tumors' centers decrease toward their peripheries (Boucher et al., 1990). Such increased pressure impedes perfusion and delivery of chemotherapy to tumors (Navalitloha et al., 2006), but the direct effects of increased extracellular pressure on the tumor cells themselves are less clear.

Prolonged pressures similar to those in tumors stimulate proliferation in mesangial cells during glomerular hypertension, in cardiac myocytes after abdominal aortic constriction, and in endothelial cells (Bevan, 1976; Kawata et al., 1998; Schwartz et al., 1999). Our preliminary study found that 15mmHg increased pressure stimulates SW620 and HCT-116 colon cancer cell proliferation but did not define the mechanism of this effect (Walsh et al., 2004). Substrate stiffness and substrate deformation also influence cell growth in vitro (S. Kumar and Weaver, 2009; Paszek et al., 2005). This may occur through mechanosensitive ion channels, which influence processes ranging from bacterial turgor to growth in cardiac myocytes and epithelial cells (Hamill and Martinac, 2001).

Calcium is commonly transported by mechanosensitive ion channels and

necessary for several cell processes (Hamill and Martinac, 2001). $[Ca^{2+}]i$ increases transiently in the G1/S transition of normal cells (Capiod et al., 2007) while sustained $[Ca^{2+}]i$, due to T-type channel over-expression, causes androgen-dependent LNCaP prostate cancer to assume a malignant apoptosis-resistant neuroendocrine phenotype (Mariot et al., 2002). We sought to explore whether increased extracellular pressure stimulates proliferation in cancer cells by activating a mechanosensitive calcium channel. We then further investigated calcium-sensitive mediators that modulate proliferation. This led us to the serine/threonine kinase PKC and the transcription factor NF-kB. Our preliminary work suggested that mitogenic effects of pressure in colon cancer cells require PKC and are associated with PKCα membrane translocation (Walsh et al., 2004). NF-kB modulates gene transcription in cell-cycle regulation, apoptosis, and proliferation and is activated by high pressures in the vasculature (Lemarie et al., 2003), mechanical stretch in myocytes (A. Kumar and Boriek, 2003), and low amplitude cyclic strain in osteoblast-like MF-63 cells (J. Liu et al., 2007). Furthermore, direct links between PKC and NF-kB activation have been documented in several cell lines (Sun and Yang). We hypothesized that there is a link between extracellular pressure, calcium, and tumor proliferation.

We demonstrated that increased extracellular pressure stimulated proliferation in 3 colon cancer, a breast cancer, and 2 prostate cancer cell lines. The SW620 colon cancer cell line was chosen as a typical model for further study, and the studies were repeated after treatment with calcium chelators and calcium-channel blockers. We identified a novel pressure-sensitive calcium-channel, Cav3.3, that drives proliferation

by increasing [Ca^{2+}]i. This Cav3.3-dependent Ca^{2+} influx promotes proliferation through PKC-β activation (not PKC-□ as previously suspected), which in turn mobilizes NF-kB through the classical IKK- IkB pathway. Pressure-induced activation of these elements was Cav3.3-dependent and ultimately increased cyclin D and proliferation. To assess the clinical relevance of our findings, we compared the lower pressure peripheries to the relatively higher pressure centers of 28 large primary human tumors and demonstrated gradients in IkB phosphorylation, NF-kB, cyclin D, and proliferation in vivo consistent with the pathway delineated by our in vitro studies.

Materials and Methods

Cells

Rat MLL, murine CT-26 and human SW620, Caco-2, MCF-7 and PC3 cancer cells were cultured by American Type Culture Collection (ATCC, Rockville, MD) recommendations. Cells were studied on collagen I-precoated plates.

Pressure regulation

Pressure was manipulated for 24 hours utilizing an airtight box with inlet and outlet valves for gas and manometry, as previously (Downey et al., 2008).

Proliferation

MTT absorbance was assayed per ATCC protocol. Briefly, we exposed 5,000 cells/well in 96 well plates to increased or ambient pressure, added MTT reagents, and quantitated 570nm absorbance. Control and pressure-treated cells were also manually

counted in 20 random fields of 6 well plates (Downey et al., 2008) with similar results.

Inhibitors

Extracellular and intracellular Ca^{2+} were chelated with 1mM EGTA and 5μM BAPTA-AM, respectively (EMD Chemicals, Gibbstown, NJ). 10μM lanthanum chloride (EMD Chemicals) blocked non-specific divalent cation channels and 5μM SFK96365 (Tocris Bioscience, Bristol, UK) blocked receptor-mediated calcium-entry (Merritt et al., 1990). Gadolinium chloride (Sigma-Aldrich, St. Louis, MO) inhibited stretch-activated ion channels (Yang and Sachs, 1989). 5μM nimodipine [77] blocked L-type Ca^{2+} channels and 1μM NNC 55-0396 blocked T-type channels (Tocris) (Huang et al., 2004). NiCl2 (Sigma-Aldrich) blocked T-type channel subtypes, with 20μM blocking Cav3.2 and 200μM blocking Cav3.1 and Cav3.3 (J. H. Lee et al., 1999). 100nM calphostin-C blocked PKC globally, 6nM GO6976 blocked PKC-α and PKC-β, 15nM 3-(1-(3-Imidazol-1-yl propyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione blocked PKC-β alone, and 10nM PKC-ε translocation inhibitor peptide (EMD chemicals) blocked PKC-ε. 10mM IKK-2 inhibitor [5-(p-fluorophenyl)-2-ureido]-thiophene-3- carboxamide, 40nM IKK-3 inhibitor [5-(5,6-dimethoxybenzinidazol-1-yl)-3-(2- methanesulfonyl-benzyloxy)-thiophene-2-carbonitrile], and a 90nM IKK inhibitor N(6-chloro-9H-β-carbolin-8-yl)-nicotinamide, that blocks IkB phosphorylation, were used per manufacturer's protocol separately and in combination (EMD Chemicals). 30μM NSC23766 inhibited rac1 (EMD Chemicals). 65nM PP2 (EMD Chemicals) was used for Src family inhibition, and Akt was inhibited using 1μM Akt inhibitor IV (EMD Chemicals). 12μM SN50 (EMD Chemicals) blocked NF-kB p50 nuclear localization. An SN50 inactive analog that does

not affect NF-kB nuclear translocation was used as a control. 25μM NF-kB Serine 276 inhibitory peptide (Imgenex, San Diego, CA) acts as a p65 decoy through phosphorylation at that site. Cells were treated with the inhibitory peptide or an inactive control. 1μM TCH-021, a novel imidazoline, inhibits NF-kB gene transcription by modulating IkB degradation and subsequently inhibiting DNA binding (Kahlon et al., 2009; Peddibhotla and Tepe, 2004; Sharma et al., 2004). All inhibitors were diluted in sterile PBS, DMSO, or water and used for 24 hours unless stated.

Small interfering RNA

Cav3.1, Cav3.3, PKC-ß, PKC-α, and NF-kB proteins were reduced using at least siRNA specific to each protein with similar results (Cell Signaling, Beverly, MA), oligofectamine, and Plus reagent (Invitrogen, Carlsbad, CA) by manufacturer's protocol, using non-targeting controls in parallel (Dharmacon, Lafayette, CO). These experiments were performed 48 hours after transfection.

Intracellular calcium visualization

Cells cultured at 70% confluence on glass coverslips (CS-22/40, Warner Instruments, Hamden, CT) for 24 hours were either treated with siRNA 24hr before plating or with PKC-ß inhibitor 30 minutes before visualization. Coverslips were then incubated in the dark for 30 min with fluorescent calcium-sensitive dye, X-Rhod I AM (X-1420, Invitrogen), prepared in Ringer's lactate, placed into the RC-30 chamber (Warner Instruments), and subjected to ambient or 15mmHg increased pressure. Pressure was manipulated by raising the reservoir of Ringer's lactate connected to the RC-30

chamber to either the level of the chamber or 21cm above it. Cells were visualized by fluorescent confocal microscopy.

Immunoprecipitation and western blotting

Cells were lysed, and protein was quantitated and resolved by SDS-PAGE before western blotting on nitrocellulose (GE Healthcare, Little Chalfont, Buckinghamshire, UK) as previously (Downey et al., 2008). Coimmunoprecipitation studies were performed using 400μg of protein with appropriate antibody and agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) as described (Downey et al., 2008). Rabbit polyclonal antibodies to pIKKα/β (ser180/181), NF-kB p50 (Cell Signaling), total IkB (Sigma-Aldrich), Histone H1 (Santa Cruz) and NF-kB p65 (Millipore, Billerica, MA), and appropriate anti-rabbit secondary antibodies were used. Mouse monoclonal antibodies to cyclin D1, pIkB (ser 32/36, Cell Signaling), and actin (Sigma-Aldrich) were also used, with a horseradish peroxidase conjugated anti-mouse secondary antibody. Protein was visualized by ECL-Plus (GE Healthcare) and quantitated by Kodak Phosphoimager (Perkin Elmer, Boston, MA) within the linear range of exposure.

Nuclear fractionation

Nuclear fractions were obtained using the Qproteome nuclear subfractionation kit (Qiagen, Frederick, MD).

NF-kB p50 and p65 transcription factor activity

We evaluated NF-kB p50 and p65 DNA binding activity 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).

NF-kB activation

Cellular NF-kB activation was assayed using a luciferase-based NF-kB lentiviral reporter assay (Qiagen). 5,000 cells/well were plated in 96 well plates for 24 hours and lentiviral particles were introduced with SureENTRY transduction reagent per manufacturer's protocol (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).

Flow cytometry

S-phase fraction was measured in previously serum-starved cells exposed to ambient or increased pressure for 24 hours as previously (Walsh et al., 2004).

TUNEL staining

Apoptosis was evaluated by TUNEL staining (Roche Applied Science, Indianapolis, IN) per manufacturer's protocol. Apoptotic cells were counted on each slide, and control and pressure-treated cells compared.

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°C 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). An observer 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. Discernible 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

Differences between two groups were analyzed using Student’s t-test, and

differences in immunostaining intensities by chi-squared test. Statistical significance was set at $P<0.05$.

Results

Increased extracellular pressure stimulates proliferation in colon, breast, and prostate cancer cells in vitro

SW620, Caco-2, and CT-26 colon; MCF-7 breast; and MLL 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 pressures, with greatest responses at 40-60mmHg increased pressure (n=6;$p<0.05$, Figure A.1). Parallel

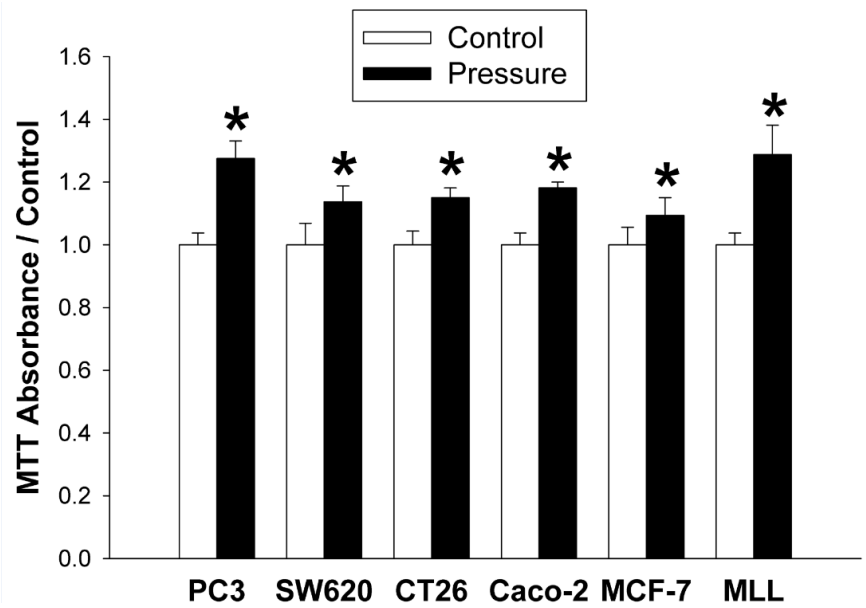


Figure A.1. Extracellular pressure stimulates proliferation
PC3 prostate cancer, SW620, CT-26, and Caco-2 colon cancer; MCF-7 breast cancer; and MLL prostate cancer cells were incubated at either ambient pressure or 40mmHg increased pressure for 24 hours and quantified by MTT assay. (*$p<0.05$ vs. paired controls)

manual cell counting confirmed increased cell numbers after exposure to 40 mmHg increased pressure vs. ambient (n=4;p<0.05, not shown). Subsequent studies used even lower pressures to conservatively stay within the pathophysiologically relevant range.

Ca^{2+} influx required for pressure-stimulated proliferation in colon adenocarcinoma

The extracellular calcium chelator EGTA (1mM) and intracellular chelator BAPTA-AM (5μM) each abolished the mitogenic effect of pressure, demonstrating the necessity of calcium

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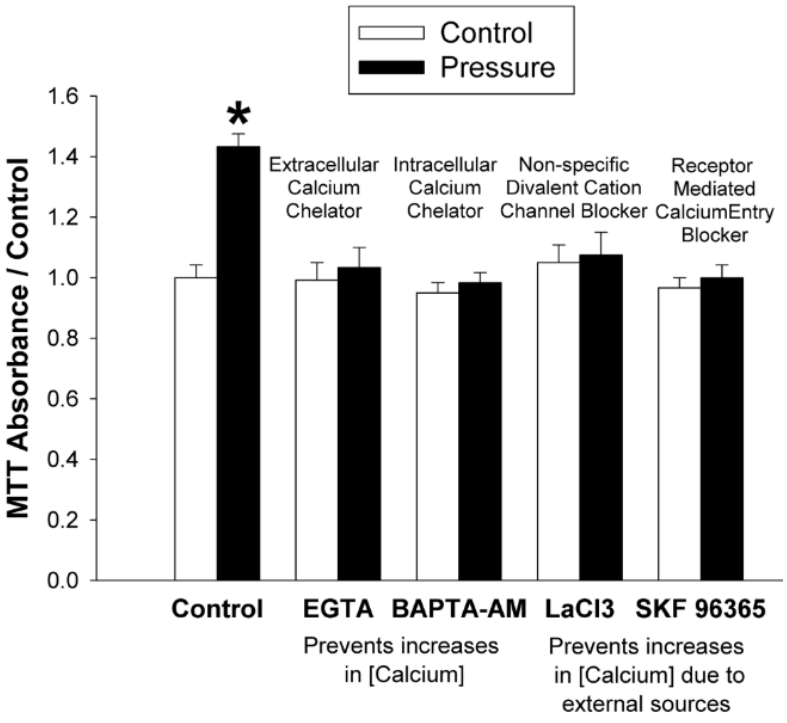

Figure A.2. Extracellular pressure induces an influx of calcium that is required for pressure-stimulated proliferation
(2A) SW620 cells were treated with either an extracellular calcium chelator (EGTA 1mM), an intracellular chelator (BAPTA-AM 5μM), a non-specific divalent cation channel

Figure A.2 (cont'd)

blocker (lanthanum chloride 10μM), or a blocker of receptor-mediated calcium-entry (SFK96365 5μM) during 24 hour exposure to ambient (open bars) or 15mmHg increased extracellular pressure (shaded bars). Cells quantified by MTT assay. (*p<0.05 vs. paired controls) (2B) X-rhod- 1, a fluorescent calcium-sensitive dye, was added to SW620 cells 30 minutes before visualization by fluorescent confocal microscopy. The study began at ambient pressure which was increased transiently by 15mmHg at arrow 1 and returned to baseline ambient pressure at arrow 2.

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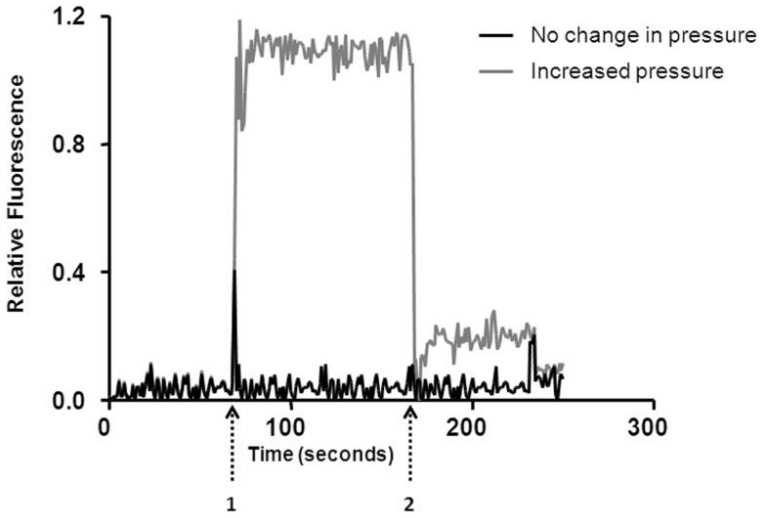

for the effect (n=8;p<0.05 Figure A.2A). The non-specific divalent cation channel blocker lanthanum chloride (10μM) and SFK96365 (5μM), a blocker of receptor-mediated calcium-entry, differentiated between intracellular calcium increases due to receptor mediated and/or voltage-gated channels from those due to release from internal stores,. Like the chelators, each inhibitor of Ca^{2+} entry inhibited pressure-driven proliferation (n=8;p<0.05 Figure A.2A). Observing real-time calcium flow under confocal microscopy with the calcium-sensitive dye, X-rhod- 1, we observed a distinct increase in $[Ca^{2+}]i$ following a 15mmHg increase in hydrostatic pressure (n=8;p<0.05 Figure A.2B).

Pressure-induced Ca^{2+} influx and proliferation dependent on T-type Ca^{2+} channel Cav3.3

The proliferation assay was repeated in the presence of channel-specific inhibitors. Gadolinium chloride, which inhibits stretch-activated calcium-channels, did not block the pressure effect at 100μM, a concentration ten times higher than the reported IC50 (Yang and Sachs, 1989) (n=8;p<0.05, not shown).

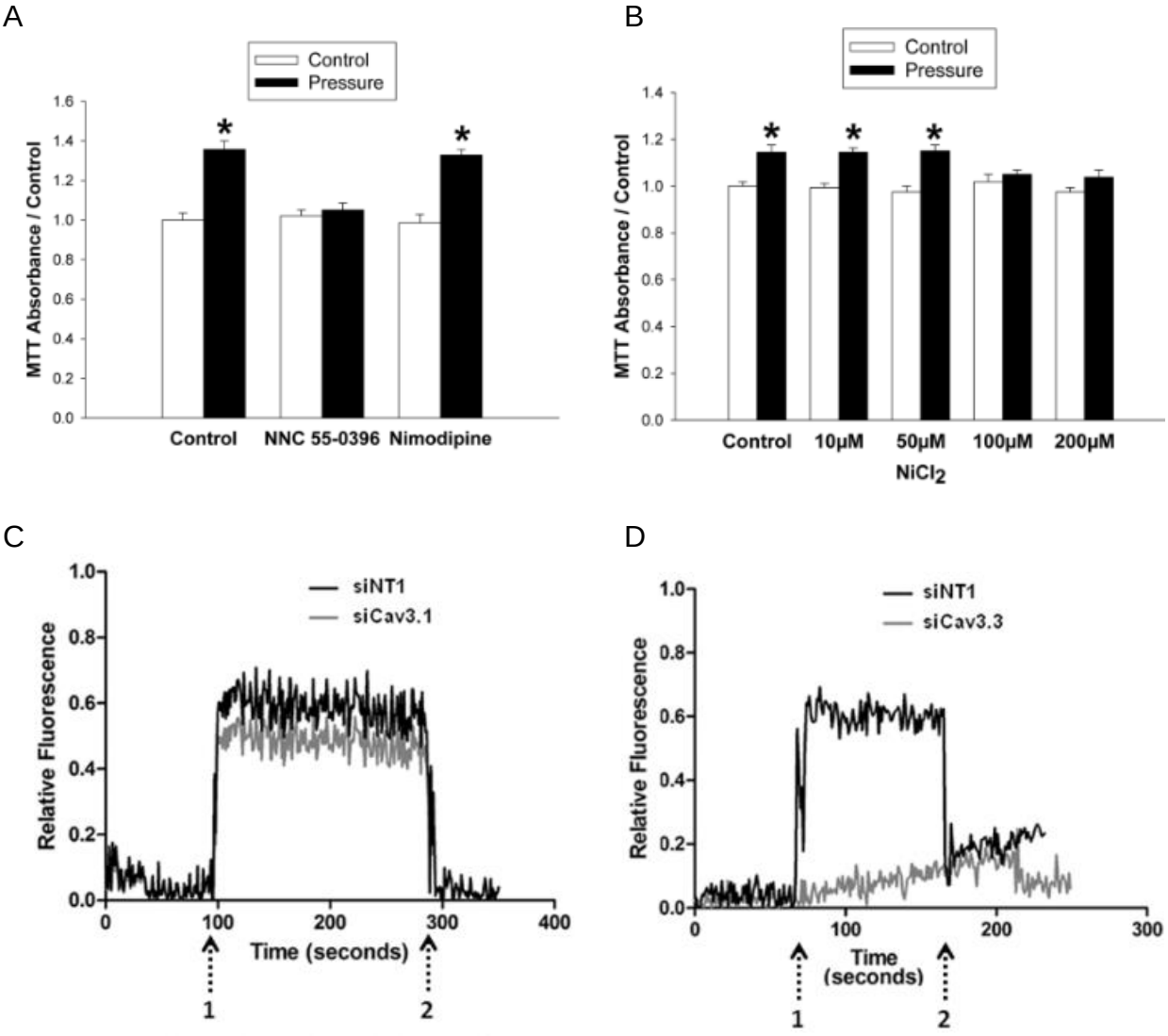

Figure A.3. T-type Ca^{2+} channel Cav3.3 is necessary for pressure-induced Ca^{2+} influx and proliferation

(A.3A) SW620 cells treated with T-type channel blocker (NNC 55-0396 5μM) or L-type

Figure A.3 (cont'd)

Ca^{2+} blocker (nimodipine 1μM) were subjected to ambient pressure (open bars) or 15mmHg increased extracellular pressure (filled bars) and MTT assay was performed at 24 hours. (A.3B) SW620 cells were treated with varying concentrations of nickel chloride to inhibit either Cav3.2 (IC_{50} 20μM) or Cav 3.1 and 3.3 (IC_{50} 200μM) prior to pressurization to 15mmHg. Both experiments show a loss of pressure-induced proliferation with blockade of T-type calcium channels Cav3.1 and 3.3. (A.3C) Cav3.1 or Cav3.3 was reduced in SW620 cells via siRNA. 48 hours after transfection, Ca^{2+} was visualized within the cells by fluorescent confocal microscopy. The study began at ambient pressure which was increased transiently by 15mmHg at arrow 1 and returned to baseline ambient pressure at arrow 2. (A.3D) The bar graph shows the area under the curve of the cumulative fluorescence seen in the Cav 3.1 and 3.3 siRNA transfected cells vs. non-targeting controls. (A.3E) SW620 cells were treated with siRNA specific to Cav3.1, 3.2, or 3.3, or with a non-targeting control for 48 hours before 24 hours of incubation under ambient (open bars) or 15mmHg increased pressure (shaded bars). Cells were then quantified using an MTT reagent. (*p<0.05 vs paired controls)

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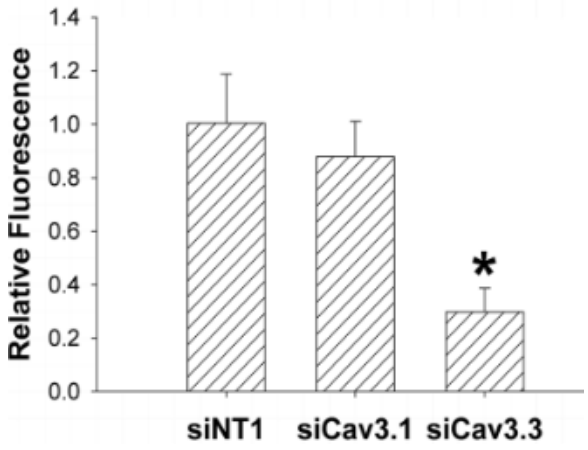

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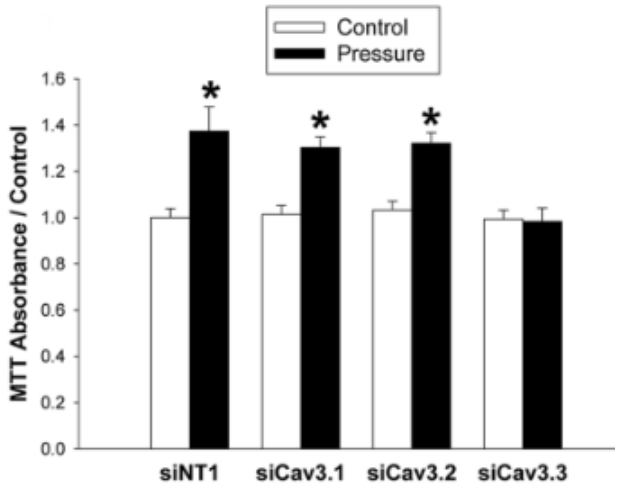

5μM nimodipine (IC50 = 3μM), a L-type Ca^{2+} blocker, was also unable to block the mitogenic effect of pressure, whereas 1μM NNC 55-0396 (IC50 = 7μM), the T-type channel blocker, negated the stimulation of proliferation by pressure (n=8;p<0.05 Figure A.3A). Nickel chloride only blocked proliferation at concentrations of 100μM or higher, suggesting that Cav3.1 or Cav3.3 might be the channel responsible, rather than Cav3.2

which is inhibited at 20μM (J. H. Lee et al., 1999) (n=8;p<0.05 Figure A.3B). Ca^{2+} visualization with confocal microscopy after treatment with channel-specific siRNA revealed that the pressure-driven influx of calcium persisted after ≈50% Cav3.1 reduction but was abolished by ≈50% Cav3.3 reduction (n=8;p<0.05, Figure A.3C, D). Pressure induced proliferation despite ≈50% siRNA reduction of Cav3.1, or Cav3.2 but not after ≈50% Cav3.3 reduction (n=12;p<0.05, Figure A.3E).

Pressure-induced Ca^{2+} influx activates PKC-β to stimulate proliferation

The reliance of the pressure signal on $[Ca^{2+}]i$ increases led us to compare the effects of inhibiting Ca^{2+} dependent PKC-α and PKC-β with that of Ca^{2+}-independent

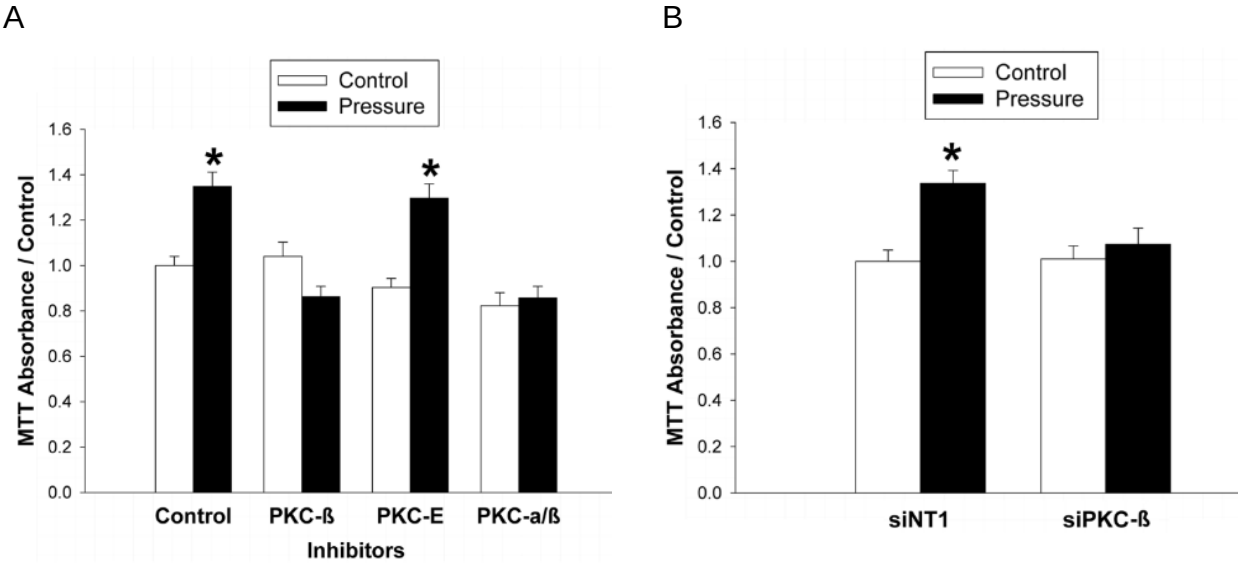

Figure A.4. PKC-β is activated by extracellular pressure
(A.4A) SW620 cells were treated with a PKC-α /PKC-β inhibitor (GO6976 6nM), a PKC-β specific inhibitor (3-(1-(3-Imidazol-1-ylpropyl)-1H-indol-3-yl)-4-a nilino-1H-pyrrole-2,5-dione 15nM), or a PKC-ε specific inhibitor (PKC-ε translocation inhibitor peptide10nM) immediately before incubation at ambient (open bars) or 40mmHg increased (shaded

Figure A.4 (cont'd)

bars) pressure for 24 hours before MTT assay. (A.4B) Knockdown of PKC-β was achieved by 48 hours of siRNA transfection before 24 hours of pressurization at ambient or 15mmHg increased pressure and MTT assay. (A.4C) Knockdown of PKC-α was achieved by 48 hours of siRNA transfection before 24 hours of pressurization at ambient or 15mmHg increased pressure and MTT assay. (A.4D) SW620 cells were transfected for 48 hours with siRNA against Cav3.3 or with a non-targeting control before being exposed to 15mmHg pressure. PKC-β levels in the membrane fraction were measured after 24 hours. (A.4E) SW620 cells treated with PKC-β inhibitor (5nM) immediately prior to Ca^{2+} visualization by fluorescent confocal microscopy. The study began at ambient pressure which was increased transiently by 15mmHg at arrow 1 and returned to baseline ambient pressure at arrow 2. (A.4F) The bar graph shows the area under the curve of the cumulative fluorescence in the DMSO and PKC-β inhibitor treated groups. (*p<0.05 vs paired controls)

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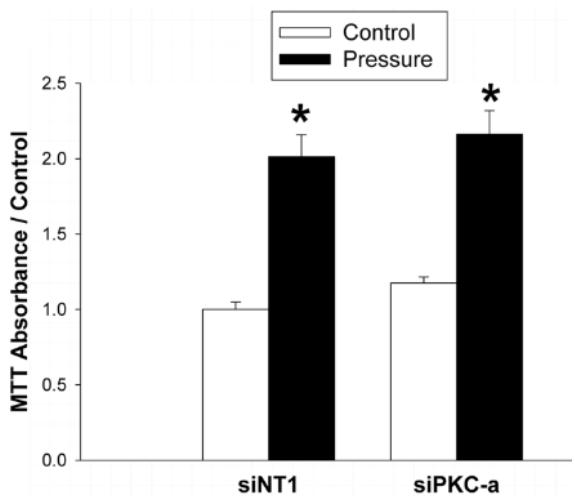

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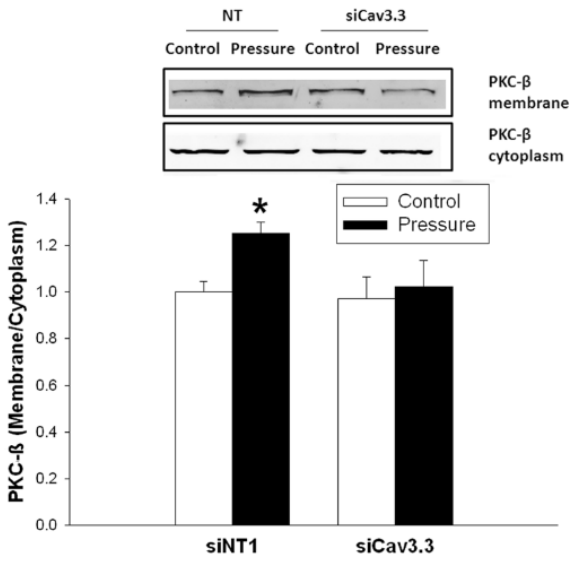

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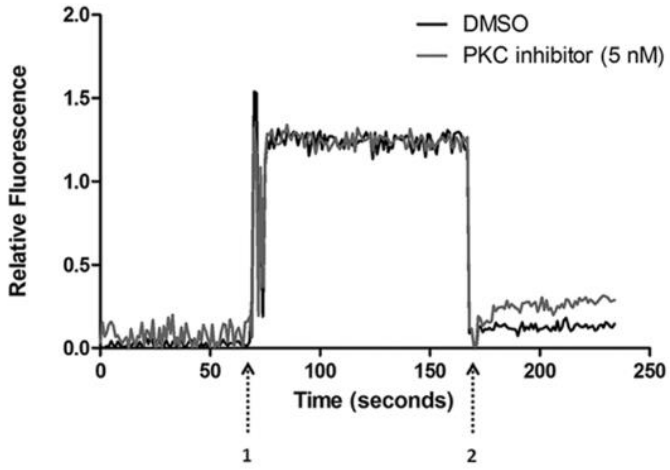

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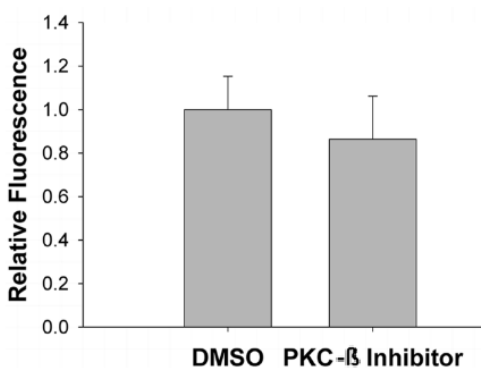

PKC-ε (Braz et al., 2002). Inhibiting either PKC-α/β together or PKC-β alone abolished the effect of pressure on proliferation to suggest that PKC-β is a necessary component of the pathway (n=9;p<0.05, Figure A.4A). Conversely, inhibition of Ca^{2+} independent PKC-ε failed to do so. Similarly, siRNA knockdown of PKC-β, but not PKC-α, abolished pressure-stimulated proliferation (n=7;p<0.05, Figure A.4B) (n=12;p<0.05, Figure A.4C). 15mmHg pressure increased PKC-β levels in the membrane fraction, and this was blocked by siRNA to Cav3.3 (n=6;p<0.05, Figure A.4D). Alternatively, PKC-β inhibition failed to attenuate the pressure-induced calcium, consistent with PKC-β being downstream of the Cav3.3-induced calcium flux in the pressure pathway (n=6;p<0.05, Figure A.4E).

Cav3.3 required for pressure-activation cascade of IKK, IkB, and NF-kB

NF-kB is sequestered within the cytosol by the binding of its inhibitor IkB. When

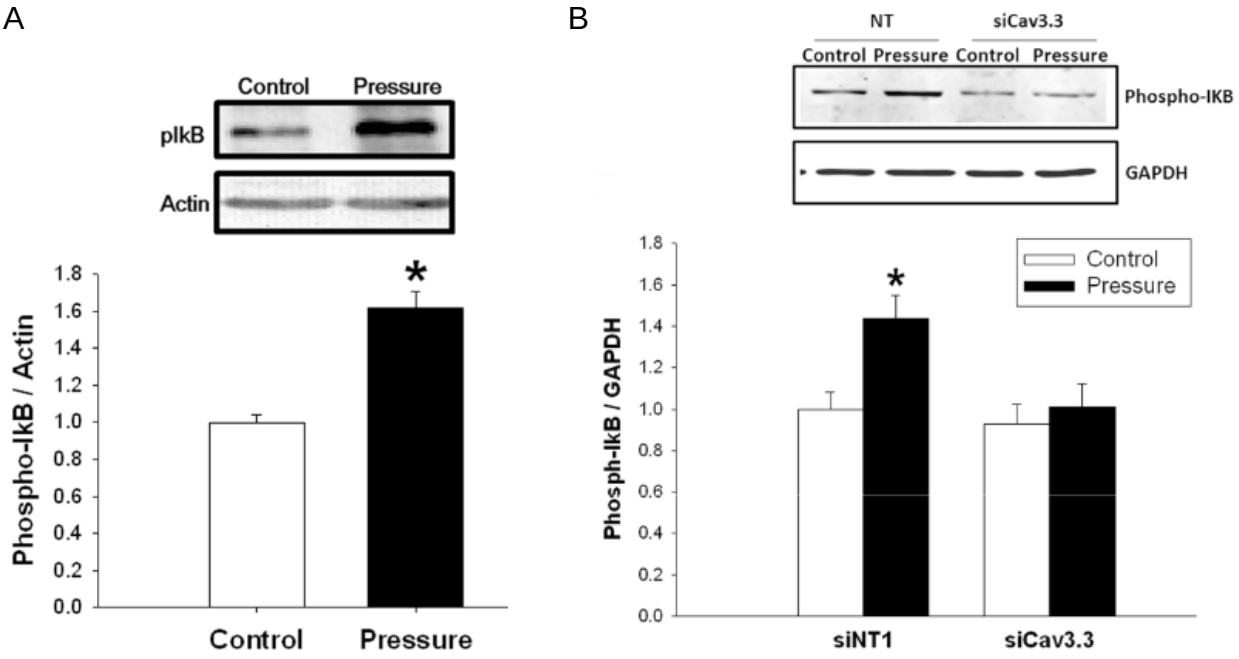

Figure A.5. Pressure activates the IKK-IkB-NF-kB signaling cascade in a Cav3.3-dependent manner

(A.5A) SW620 cells were exposed to 40mmHg for 24 hours before Western blotting with phospho-IkB antibodies. Densitometric results were normalized to actin. (A.5B) SW620 cells were transfected with siRNA targeting Cav3.3 or with a non-targeting control siRNA for 48 hours and then incubated under at ambient or 15mmHg increased pressure for 24 hours. Western blots with phospho-IkB antibodies were analyzed and normalized to GAPDH. (A.5C) Lysate from SW620 cells incubated at ambient or 40mmHg increased pressure for 24 hours was immunoprecipitated with antiNF-kB antibodies and the resulting immunoprecipitates were immunoblotted with IkB antibodies to identify IkB associated with NF-kB. (A.5D) Nuclear fractions from SW620 cells that had been incubated at ambient or 40mmHg increased pressure for 24 hours were immunoblotted with antibodies against the p65 and p50 subunits of NF-kB. Histone H1 served as a loading control. (A.5E) Lysate from SW620 cells transfected with siRNA targeting Cav3.3 or with a non-targeting control for 48 hours and then incubated at ambient or 15mmHg increased pressure for 24 hours was then used to quantify NF-kB p65 and p50 subunit activity by ELISA. (A.5F) SW620 cells were treated with NF-kB lentiviral reporter particles expressing firefly luciferase and incubated under ambient or 40mmHg increased pressure for 24 hours in the presence of an IKK-2 inhibitor ([5-(p-fluorophenyl)-2-ureido]-thiophene-3- carboxamide, 10mM), an IKK-3 inhibitor ([5-(5,6-dimethoxybenzinidazol-1-yl)-3-(2- methanesulfonyl-benzyloxy)-thiophene-2-carbonitrile] 40nM), or an IKK inhibitor that blocks IkB phosphorylation (N(6-chloro-9H-β-carbolin-8-yl)-nicotinamide 90nM). (* $p<0.05$ vs paired controls)

Figure A.5 (cont'd)

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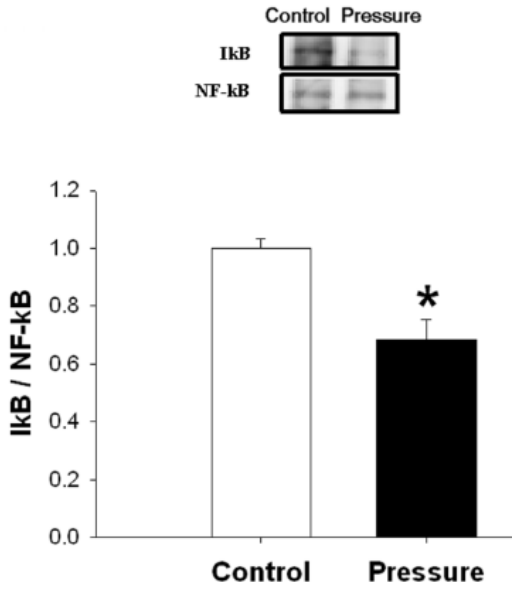

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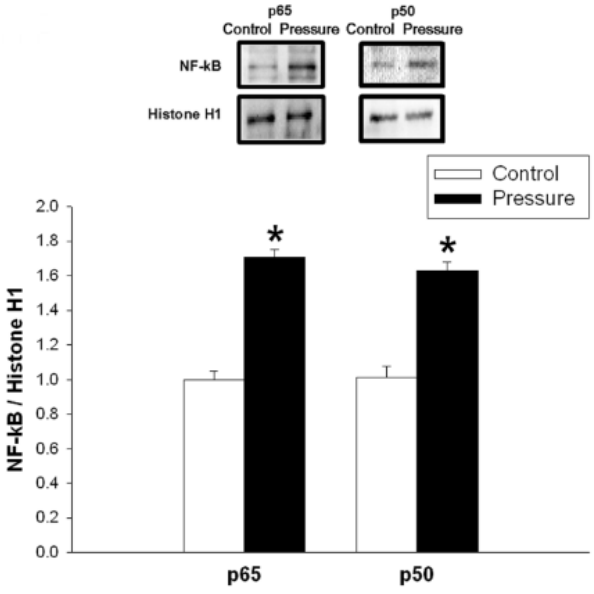

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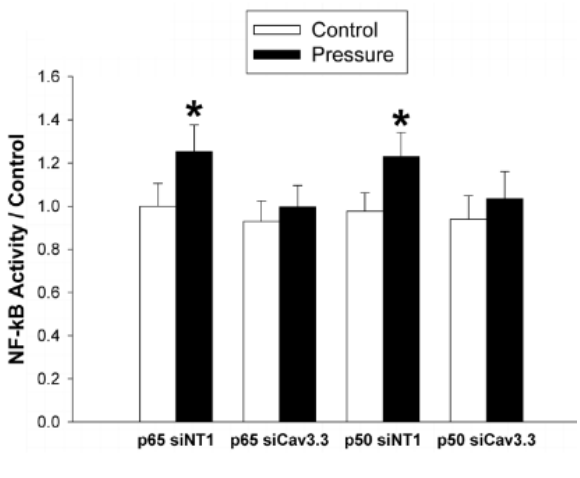

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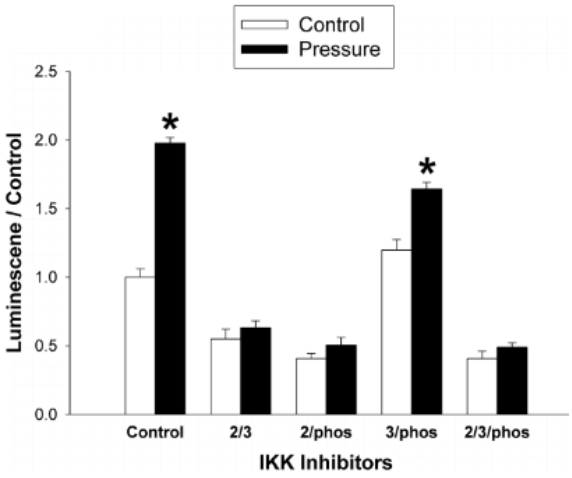

IkB is phosphorylated by the kinase IKK, it releases NF-kB, exposing the NF-kB nuclear localization signal (C. H. Lee et al., 2007; Yamamoto and Gaynor, 2001). IKK

phosphorylation was increased 27±4% in pressure-treated cells relative to ambient pressure control cells (n=8;p<0.05, not shown). IkB phosphorylation also increased 61±8% in pressure-treated cells (n=8;p<0.05, Figure A.5A), but not after siRNA reduction of Cav3.3 (n=7;p<0.05, Figure A.5B). Pressure decreased NF-kB-IkB association 31±4% (n=5;p<0.05, Figure A.5C). NF-kB translocation was confirmed via measuring p50 and p65 subunit levels in nuclear fractions, which increased 58±6% and 67±8% respectively in pressure-treated versus ambient pressure cells (n=6;p<0.05, Figure A.5D). ELISA based measurements of NF-kB p50 and p65 transcription factor activity showed increases of 50±4% and 48±6% respectively in response to pressure that were reversed with siCav3.3 treatment (n=6;p<0.05, Figure A.5E). To assess the effect of IKK on pressure-stimulated NF-kB activation we treated SW620 cells with NF-kB lentiviral reporter particles expressing firefly luciferase. We incubated them under ambient or 40mmHg increased pressure for 24 hours in the presence of 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 (n=12;p<0.05, not shown), but the combination of the IKK-2 and IKK-3, the IKK-2 and global IKK, or the three inhibitors used together each abolished pressure activation of NF-kB (n=12;p<0.05, Figure A.5F).

Pressure activates NF-kB through PKC-β

Pressure increased NF-kB activation 94±7% (n=12;p<0.05, Figure A.6A). The NF-kB nuclear decoy and the NF-kB inhibitors SN50 and TCH 021 each prevented pressure-associated NF-kB activation (n=6;p<0.05, Figure A.6A). FAK and Akt facilitate the effects of extracellular pressure on integrin-mediated adhesion in colon cancer cells

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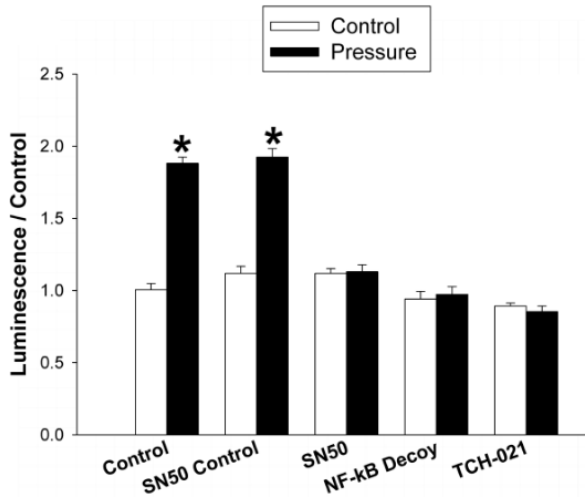

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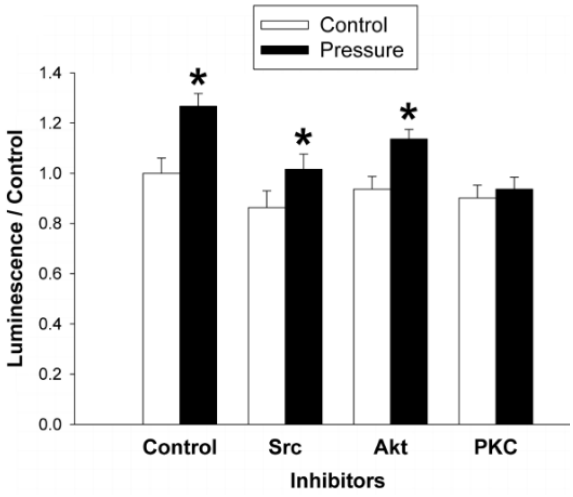

Figure A.6. Pressure activates NF-kB in a PKC-β dependent manner
(A.6A) SW620 NF-kB activation was assayed using a luciferase-based NF-kB lentiviral reporter assay. After transduction, the cells were incubated under ambient or 40mmHg increased pressure for 24 hours in the presence of NF-kB inhibitor SN50 (12μM), or its control peptide, NF-kB nuclear decoy (25μM), or NF-kB inhibitor TCH 021 (1μM). (A.6B) NF-kB activation was also measured in cells treated with an inhibitor to Src (PP2 65nM), Akt (Akt inhibitor IV 1μM), or PKC (calphostin 100nM) before undergoing 24 hours of ambient or 40mmHg pressure exposure. (A.6C) The experiment was repeated using the global PKC inhibitor as well as inhibitors specific for PKC-β (3-(1-(3-Imidazol-1-yl propyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione 15nM) and PKC-ε (PKC-ε translocation inhibitor peptide 10nM). (A.6D) SW620 cells were transfected for 48 hours with siRNA against Cav3.3, PKC-β, or with a non-targeting control before being exposed to ambient or 15mmHg pressure. NF-kB activation was measured via luminescence from the luciferase reporter gene. (*$p<0.05$ vs paired controls)

Figure A.6 (cont'd)

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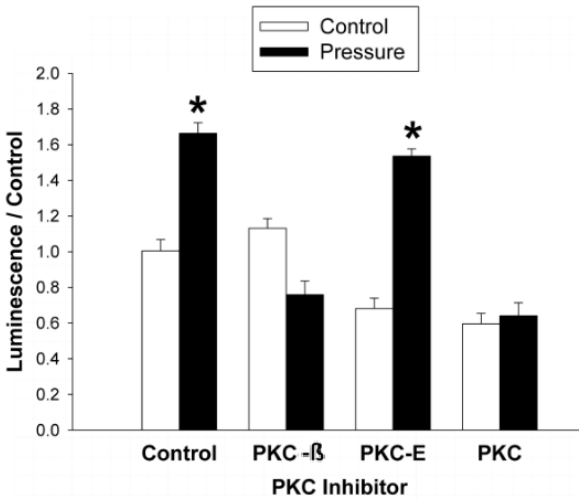

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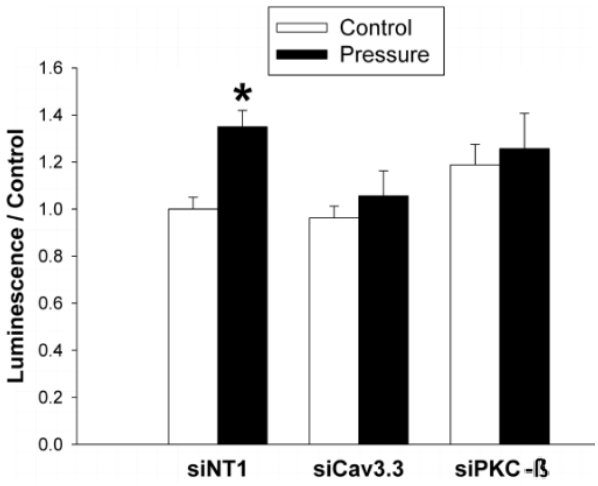


(Basson, 2008), and FAK also stimulates proliferation in response to another physical force, cyclic deformation (Li et al., 2001). However, neither FAK nor Akt inhibition prevented pressure activation of NF-kB ($n=9;p<0.05$, Figure A.6B). Conversely, the global PKC inhibitor calphostin C, the PKC-α/β inhibitor Go6976, and a pure PKC-β inhibitor all abolished the effect of pressure on NF-kB activation, while PKC-ε inhibition did not ($n=9;p<0.05$, Figure A.6C). Consistent with the chemical inhibitor data, siRNA knockdown of PKC-β reversed the effect ($n=6;p<0.05$, Figure A.6D). The pressure-driven increase in NF-kB activation was also abolished by siRNA to Cav3.3 ($n=7;p<0.05$, Figure A.6D).

Pressure stimulates proliferation through NF-kB

To connect NF-kB to the pressure proliferation effect, we attempted to block the increase using NF-kB inhibitors as well as an inhibitor of another mitogen signal, rac1 for comparison. The rac1 inhibitor NSC23766 did not prevent increased MTT fluorescent intensity in pressure-treated cells, but the NF-kB inhibitor SN50, a NF-kB nuclear decoy, and a novel imidazoline inhibitor, TCH-021, abolished the effect ($n=4;p<0.05$, Figure A.7A). Flow cytometry showed S-phase fraction increases of 22±6% in cells exposed to increased pressure ($n=3;p<0.01$, Figure A.7B) but not in those treated with inhibitors as well ($n=4;p<0.05$, Figure A.7B). TUNEL staining demonstrated that pressure did not alter cell apoptosis ($n=3;p<0.05$, not shown). A

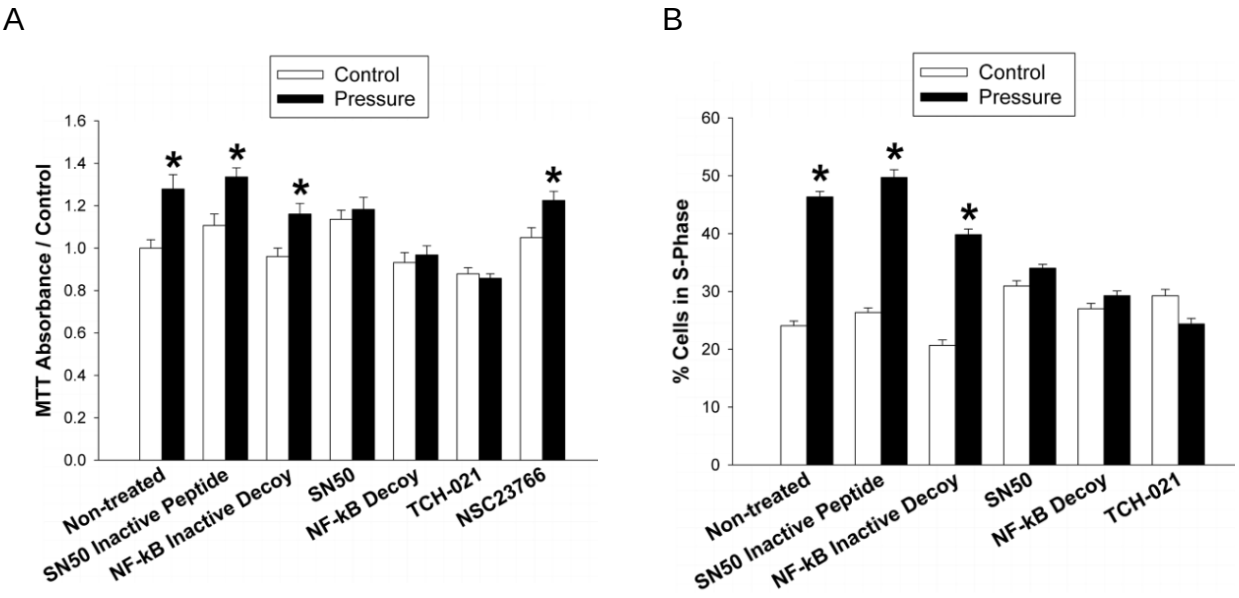
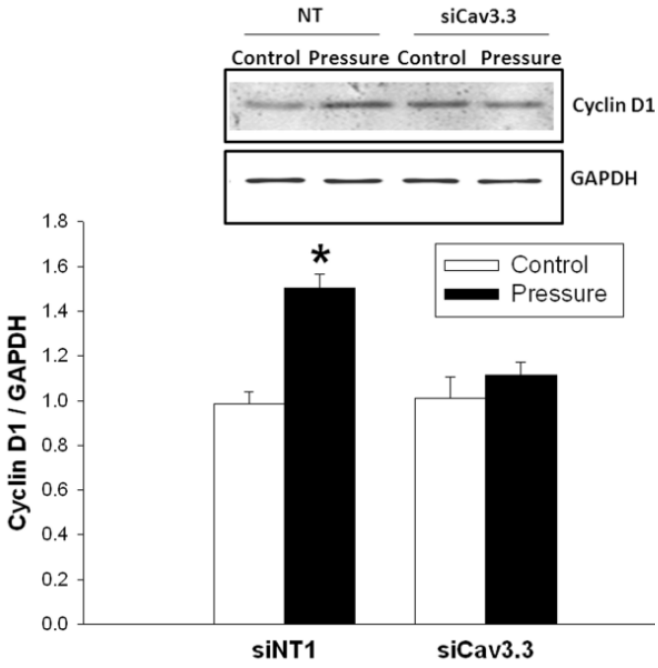


Figure A.7. Pressure induces proliferation through Cav3.3 and NF-kB
(A.7A) SW620 cells were incubated under ambient or 40mmHg increased pressure for 24 hours in the presence of an NF-kB inhibitor SN50 (12μM) or its control peptide, an NF-kB nuclear decoy (25μM) or its inactive control, an NF-kB inhibitor TCH 021 (1μM), or a rac1 inhibitor (NSC23766 30μM) before MTT assay. (A.7B) S-phase fraction was measured in previously serum-starved SW620 cells via flow cytometry. Before

Figure A.6 (cont'd)

measurement, cells were incubated under ambient or 40mmHg increased pressure for 24 hours in the presence of an NF-kB inhibitor SN50 (12μM) or its control peptide, an NF-kB nuclear decoy (25μM) or its inactive control, an NF-kB inhibitor TCH 021 (1μM). (A.7C) SW620 cells were transfected with siRNA targeting Cav3.3 or with a non-targeting control for 48hr and exposed to ambient or 15mmHg for 24hr before being lysed and immunoblotted with cyclin D1 antibodies and normalized to GAPDH. (*p<0.05 vs paired controls)

C



measurable product of the mitogenic effect of NF-kB is the accumulation of the cell cycle regulator Cyclin D1, which increased in pressure-treated cells that did not undergo siRNA Cav3.3 treatment (n=6;p<0.05, Figure A.7C).

NF-kB, IkB and cyclin D1 staining increases in the center of human tumors

Since extracellular interstitial pressures are higher in the center of large solid human tumors than at their peripheries (Boucher et al., 1990; Sarntinoranont et al.,

2003), 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 per hpf in the central regions of the tumors relative to their peripheries (n=15;p<0.05, Figure A.8D). Parallel studies demonstrated 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. (n=27;p<0.05, Figure A.8A-C). Assessment of immunoreactivity on a scale from 0 (negative staining)

A

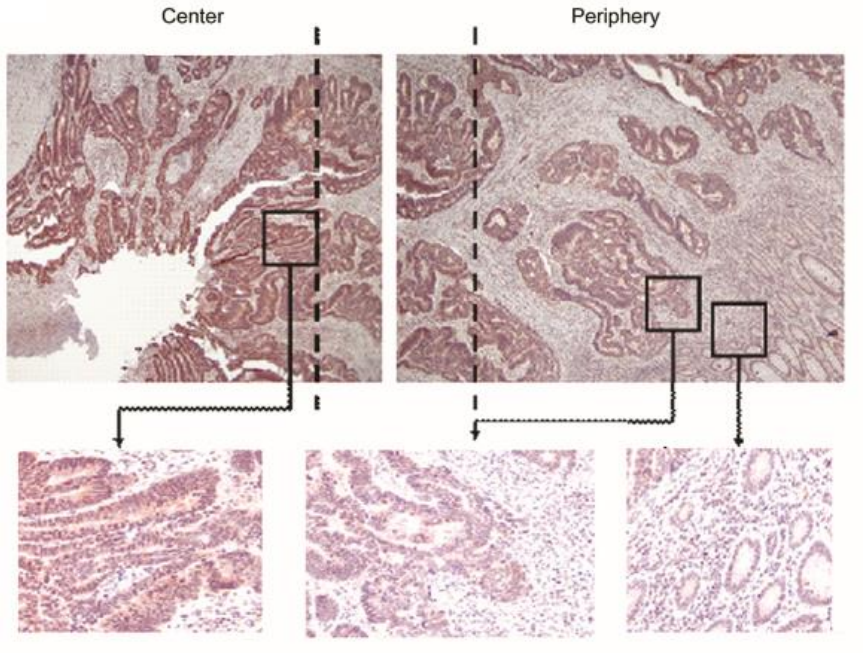
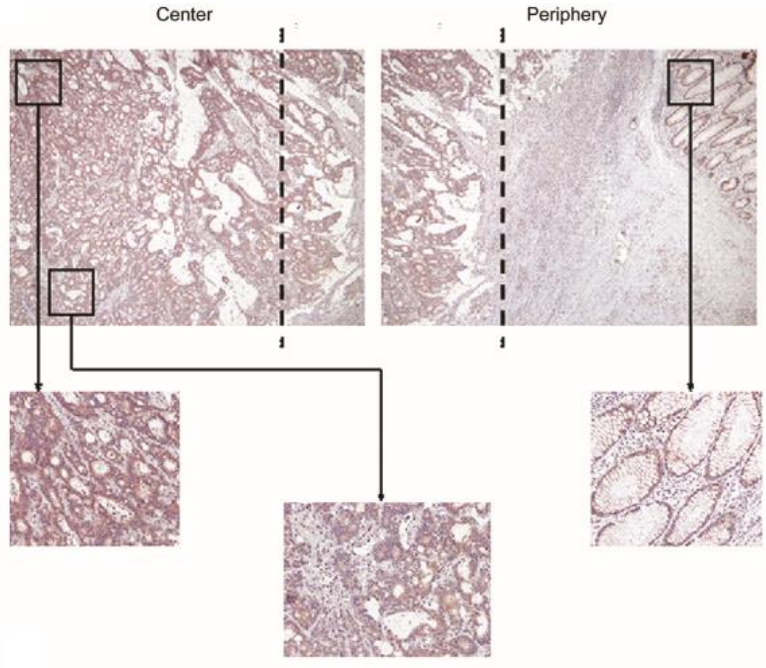


Figure A.8. Immunohistochemical staining of colorectal carcinomas
Staining of colorectal carcinomas was representative of that observed in colorectal, head and neck and lung tumors. To obtain a full view of the sample, a panel of overlapping photographs were taken and placed together. Dashed lines represent zones of overlap within the image. Overall panel images were taken at 4X magnification and higher power images within each zone at 20X. NF-kB (A.8A), phospho-IkB (A.8B), and cyclin D1 (A.8C) immunoreactivity and mitotic index (A.8D) are increased in tumor

Figure A.7 (cont'd)

centers vs. peripheries. Non-malignant tissue showed minimal staining and few mitotic figures.

B



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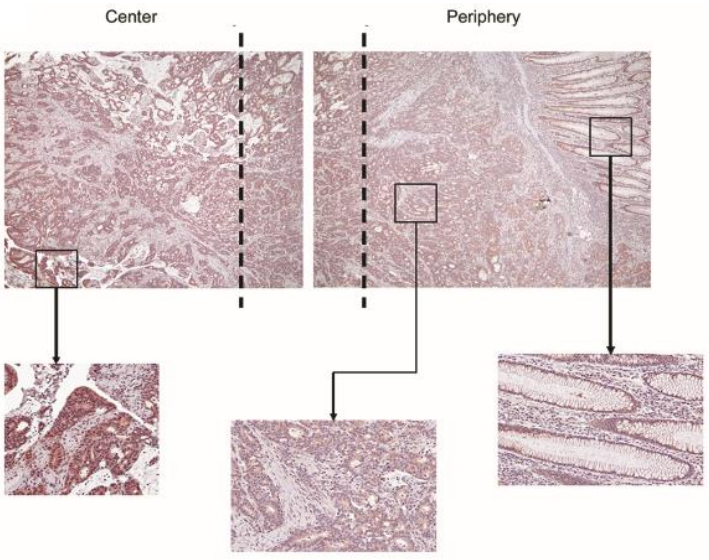

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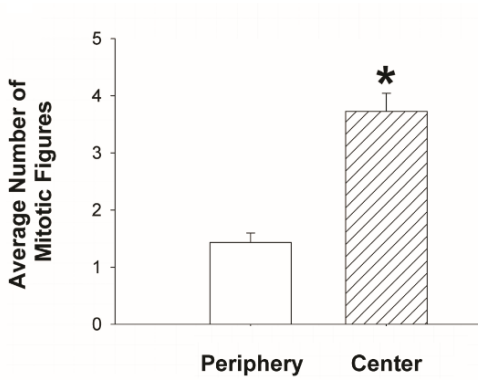

Table 2: Immunohistochemical Scores
Table 2: Immunohistochemical staining of colorectal carcinomas, representative of staining observed in colorectal, head and neck and lung tumors. Mean immunohistochemical NF-kB, phospho-IkB, and cyclin D1 intensity in tumor centers vs. peripheries vs. adjacent non-malignant tissue, scored by a blinded observer.

		Average Intensity (Number of zones counted)			
Antibody	Cases	Non-Malignant	Periphery	Center	P-value
NF-kB	19	0.8 (95)	2.2 (95)	3.6 (95)	< 0.01
Phospho-IkB	23	0.6 (115)	2.0 (115)	3.4 (115)	< 0.001
Cyclin D1	21	0.9 (105)	2.4 (105)	3.6 (105)	< 0.01

to 4 (high staining), specimens immunostained for NF-kB were scored in 95 zones from 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 2). 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).

A.4 Discussion

Malignant cells within tumors are exposed to increased extracellular pressure as the tumors grow (Gutmann et al., 1992; Less et al., 1992; Raju et al., 2008). This study suggests that such increased extracellular pressure can stimulate tumor cell proliferation by activating the T-type Ca^{2+} channel Cav3.3. The consequent calcium

influx activates a PKC β-dependent pathway. Stepwise phosphorylation of IKK and IkB permits NF-kB activation and ultimately results in tumor cell proliferation. Although physical forces have been reported to be mitogenic for other cell types, these results are interesting because they implicate a calcium channel that is not thought stretch-activated as a primary mechanoreceptor. In addition, they suggest a specificity of activity of the PKC-ß isoform in this regard. Finally, since the pressures studied here are consistent with those reported in large human tumors (Boucher et al., 1990; Gutmann et al., 1992; Less et al., 1992; Raju et al., 2008) and since we observed gradients of proliferation and related signaling between the periphery and center of large human tumors that parallel previous observations of similar gradients in interstitial pressures, these results suggest the possibility that cancer cells in rapidly growing tumors are exposed to such pressures in vivo, causing more rapid proliferation that further increases pressure in a vicious positive feedback cycle that may potentiate cancer growth.

Existing models of pressure signaling are split between two theories of mechanoperception. Intrinsic mechanosensitivity relies on tension along the lipid membrane to drive protein subunit recruitment or realignment to affect ion channel conductance (Hamill and McBride, 1994; Martinac et al., 1990; Opsahl and Webb, 1994). Extrinsic mechanosensitivity depends on cytoskeletal or extracellular elements to relay mechanical stress to cellular enzymes (Guharay and Sachs, 1984; Hamill and McBride, 1992; Ingber, 1997). Early mechanosensitive channels were discovered under negative pressures and characterized as intrinsic stretch-activated channels that

opened with lateral membrane tension but were insensitive to the perpendicular forces exerted by hydrostatic pressure (Sokabe et al., 1991). Because the lipid bilayer is volumetrically incompressible under physiological conditions, it is poorly suited to conduct the forces generated by tumor growth (Hamill and Martinac, 2001). Therefore, the pressure-proliferation pathway might be hypothesized to depend on the cytoskeleton, consistent with the effects of pressure on cell migration and adhesion (Kovalenko et al., ; Thamilselvan and Basson, 2004). However, phalloidin, which discourages actin depolymerization (Cooper, 1987), attenuates the effect of pressure on adhesion but not proliferation (Basson, 2008). While actin depolymerization occurs within minutes during adhesion (Wang et al., 1993) and is necessary for pressure-stimulated adhesion by suspended cells (Thamilselvan and Basson, 2004), cytoskeletal rearrangement seems less likely to be required for pressure to stimulate proliferation in adherent cells.

Indeed, the pathway delineated here by which pressure stimulates proliferation differs markedly from that by which similar pressures stimulate adhesion to matrix substrates in non-adherent cells. The latter depends upon cytoskeletal mechanosensing, PI3K, Src, FAK, Akt and rac1 activation (Basson, 2008; Downey et al., 2008). However, we have previously reported that the mitogenic effects of pressure are independent of Src, PI-3-kinase and actin depolymerization (Basson, 2008; Walsh et al., 2004) and show here they are also independent of FAK, AKT and rac1. This may reflect differences in the intracellular kinome associated with the effects of adhesion itself. Internal pre-stress naturally remodels the cytoskeletons of suspended cells into a

spherical shape, changing the kinetic properties of attached enzymes (Ingber, 1997). Adherent cells, however, maintain a state of isometric tension, balanced between the inward pulling forces of the contractile cytoskeleton and the outward pulling forces of the extracellular matrix and adjacent cells, and thus experience a different kinomic landscape (Ingber, 1997). Cells experiencing such tensegrity forces are more responsive to mitogens (Ingber, 2008) and sites of high mechanical stress produce higher cell growth rates in monolayer models (Nelson et al., 2005). The proliferative effect of pressure may be another example of a mitogen that is better perceived by the cytoarchitecture of adherent cells.

While one type of mechanical stimulus can effect several different changes in a cell, different mechanical forces may conversely stimulate the same cellular process albeit through different pathways. Repetitive deformation stimulates proliferation in human Caco-2 colon cancer cells and non-malignant IEC-6 enterocytes through a cytoskeleton-dependent pathway involving Src, rac1, FAK and Erk (Chaturvedi et al., 2007b; Chaturvedi et al., 2007a; Li et al., 2001), none of which are required for the mitogenic effects of pressure (Kovalenko et al.). Stretch-activated ion channels have also been implicated in mechanotransduction and proliferation (Hamill and Martinac, 2001; M. Liu et al., 1994). These channels activate under negative pressure and are inhibited by gadolinium chloride, which stunts the lipid bilayer's ability to transmit lateral tension (Ermakov et al., 2001; Tanaka et al., 2002). However, gadolinium failed to block pressure-induced proliferation, which echoes the Cav3.3 stretch insensitivity reported by Morris (Calabrese et al., 2002). We believe this pressure-activated Cav3.3-PKC-β-NF-

kB pathway to be novel among reported mechanisms for mechanically-stimulated proliferation.

Our studies identify, for the first time, a non-stretch activated calcium channel, the T-type calcium channel Cav3.3, that responds to increased pressure and stimulates proliferation. Most mechanosensitive ion channels are intrinsically mechanosensitive and exhibit stretch-activation under negative pressures, as is the case for L-type calcium channels (Kraichely and Farrugia, 2007). There are reports of calcium permeable channels that respond to positive pressure in rat endocardial endothelium, but even these are stretch-activated (Kohler et al., 1998). We found that the mitogenic effects of positive pressure required an influx of extracellular calcium, and despite the lack of reports of T-type Ca^{2+} channel mechanoperception, in our hands Cav3.3 indeed reacted to positive pressures of 15mmHg to induce calcium influx. Cav3.3 is mostly found in neuronal cells and not known to be over-expressed in tumors (Lu et al., 2008). Cav3.1 and Cav3.2, on the other hand, are over-expressed in breast cancer, human retinoblastoma and rat glioma cells (Bertolesi et al., 2002). However, neither the L-type channels nor Cav3.1/Cav3.2 seemed to be required for pressure-induced proliferation. Although T-type Cav channels have previously been studied in the context of neural function, these findings suggest a possible role for Cav3.3 and calcium signaling in pressure-mediated tumor growth.

Several PKC isoforms have been implicated in signaling responses to various mechanical forces, but most of these isoforms are calcium-independent, and no

mechanisms to date have implicated the PKC-ß isoform. The calcium-independent isozymes PKC-δ, ε, θ and η are activated by diacylglycerol alone, whereas the isozymes PKC-α, βI, βII and γ also require calcium (Mochly-Rosen et al.). We previously reported that repetitive deformation of Caco-2 cells induces activation and membrane translocation of both PKC-α and PKC-ζ (Han et al., 1998), while Cheng observed similar results in endothelial cells and further noted that repetitive deformation failed to potentiate PKC-ß membrane translocation (Cheng et al., 2001). The mechanical force of shear stress activated Erk1/2 in endothelial cells via PKC-ε, but not PKC-α or PKC-ζ (Traub et al., 1997), and IL-11 expression was stimulated in murine primary osteoblasts via PKC-δ (Kido et al.). Interestingly, pressure stimulates integrin-mediated adhesion independently of PKC in suspended Caco-2 and SW620 cells (Thamilselvan and Basson, 2004). However, the same 15 mmHg pressure applied to adherent colon cancer cells stimulated both PKC-dependent proliferation and PKC-α membrane translocation (Walsh et al., 2004). In our current study, consistent with the calcium-dependent effect of pressure on proliferation, specific inhibition of calcium-dependent PKC-ß prevented the mitogenic effects of pressure.

Pressure activated NF-kB and its upstream regulators IkB and IKK in a PKCß-dependent manner. Our results are consistent with previous observations that PKCß plays a role in NF-kB activation (Sommer et al., 2005), although the opposite effect has also been reported in human umbilical vein endothelial cells. Both hyperphosphatemia and hypophosphatemia increase PKCßII but decrease activated NF-kB (Peng et al.). Traditionally, IKK and IkB phosphorylation release NF-kB to translocate the nucleus

where it is mitogenic (Dolcet et al., 2005). However, NF-kB can also cause cell death through interactions with anti-apoptotic factors like FLIP and TRAF1/2 (Dolcet et al., 2005), and is associated with cell apoptosis in colon cancer xenografts (Stark et al., 2007). IKK-2 ablation similarly causes proliferation in myocytes (Mourkioti et al., 2006), although IKK-2 is required for TNF-α -induced proliferation in human mesenchymal stem cells (Bocker et al., 2008). Thus, it was not obvious that activation of the NF-kB pathway would stimulate cancer cell proliferation in response to pressure before these studies. We blocked the pressure-induced increase in NF-kB activity with siCav3.3 to demonstrate its downstream position relative to Ca^{2+}, and then with various NF-kB inhibitors to demonstrate the specificity of the effect. That the inhibitors of IKK-2, IKK-3 and IKK phosphorylation of IkB could only prevent pressure activation of NF-kB when used in combination suggests pathway redundancy and confirms IKK relevance. The overall effect of pressure on the conventional NF-kB pathway was a conserved activation that increased cell number through proliferation not decreased apoptosis.

The magnitudes of pressure used in our studies resemble those experienced by large human tumors in vivo. The reported range of average internal pressures for large tumors is 4-50mmHg (Gutmann et al., 1992; Less et al., 1992; Raju et al., 2008). In vivo measurements from 219 cervical cancer patients delineated a mean tumor interstitial fluid pressure of 18mmHg (Milosevic et al.). A similar study in colorectal carcinoma tumors averaged 21mmHg pressure (Less et al., 1992). Our studies were conducted at both 40mmHg, to elicit the maximal proliferative effect, and at 15mmHg, to simulate the relevant pathophysiology. The significance of elevated pressure was validated in

sections of large human tumors by the gradient of phospho-NF-kB, IkB, and cyclin D1 immunoreactivity from the high pressure center towards the lower pressure periphery until nearly disappearing in the adjacent normal tissue. NF-kB immunoreactivity of the magnitude observed correlates with poor prognosis (Ismail et al., 2004). These observations in human tumors further suggest a link between pressure-mediated NF-kB activation and increased proliferation.

Pathophysiologically relevant increases in pressure may stimulate colon, breast and prostate cancer cell proliferation by a Cav3.3-dependent mechanism involving activation of PKC-ß and the IKK complex, NF-kB p50 and p65 nuclear localization and activation, and increased cyclin D1 expression. This is consistent with the increased NF-kB, IkB and cyclin D1 immunoreactivity and increased proliferation that we observed within the higher pressure centers of human tumors. While the mechanism by which Cav3.3 senses pressure awaits further study, these results suggest that the increases in extracellular pressure generated by tumor growth against a stiff surrounding stroma may stimulate proliferation within the tumor, eliciting an unfortunate 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.

REFERENCES

REFERENCES

Basson, Marc D. 2008. “An Intracellular Signal Pathway That Regulates Cancer Cell Adhesion in Response to Extracellular Forces.” *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-07-2992.

Basson, Marc D., Cheng Fang Yu, Oliver Herden-Kirchoff, Marc Ellermeier, Matthew A. Sanders, Ronald C. Merrell, and Bauer E. Sumpio. 2000. “Effects of Increased Ambient Pressure on Colon Cancer Cell Adhesion.” *Journal of Cellular Biochemistry*. https://doi.org/10.1093/brain/114.3.1297.

Basson, Marc D., B. Zeng, and S. Wang. 2017. “The C-Terminal Region of the Focal Adhesion Kinase F1 Domain Binds Akt1 and Inhibits Pressure-Induced Cell Adhesion.” *Journal of Physiology and Pharmacology*.

Basson, Marc D., Bixi Zeng, Christina Downey, Madhu P. Sirivelu, and Jetze J. Tepe. 2015. “Increased Extracellular Pressure Stimulates Tumor Proliferation by a Mechanosensitive Calcium Channel and PKC-β.” *Molecular Oncology*. https://doi.org/10.1016/j.molonc.2014.10.008.

Bonjer, H. Jacob, Wim C J Hop, Heidi Nelson, Daniel J. Sargent, Antonio M. Lacy, Antoni Castells, Pierre J. Guillou, et al. 2007. “Laparoscopically Assisted vs Open Colectomy for Colon Cancer: A Meta-Analysis.” *Archives of Surgery*. https://doi.org/10.1001/archsurg.142.3.298.

Buckley, M J, A J Banes, L G Levin, B E Sumpio, M Sato, R Jordan, J Gilbert, G W Link, and R Tran Son Tay. 1988. “Osteoblasts Increase Their Rate of Division and Align in Response to Cyclic, Mechanical Tension in Vitro.” *Bone & Mineral*.

Butler, Thomas P., and Pietro M. Gullino. 1975. “Quantitation of Cell Shedding Into Efferent Blood of Mammary Adenocarcinoma.” *Cancer Research*.

Ceccarelli, Derek F.J., Kyu Song Hyun, Florence Poy, Michael D. Schaller, and Michael J. Eck. 2006. “Crystal Structure of the FERM Domain of Focal Adhesion Kinase.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M509188200.

Chaturvedi, Lakshmi S., H. Michael Marsh, Xun Shang, Yi Zheng, and Marc D. Basson. 2007. “Repetitive Deformation Activates Focal Adhesion Kinase and ERK Mitogenic Signals in Human Caco-2 Intestinal Epithelial Cells through Src and Rac1.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M605817200.

Chaturvedi, Lakshmi S, Harold M Marsh, and Marc D Basson. 2007. “Src and Focal Adhesion Kinase Mediate Mechanical Strain-Induced Proliferation and ERK1/2

Phosphorylation in Human H441 Pulmonary Epithelial Cells." *American Journal of Physiology-Cell Physiology*. https://doi.org/10.1152/ajpcell.00529.2006.

Chen, William S., Pei Zhang Xu, Kathrin Gottlob, Mei Ling Chen, Karen Sokol, Tanya Shiyanova, Igor Roninson, et al. 2001. "Growth Retardation and Increased Apoptosis in Mice with Homozygous Disruption of the Akt1 Gene." *Genes and Development*. https://doi.org/10.1101/gad.913901.

Cheng, Zhaokang, Laura A. DiMichele, Mauricio Rojas, Cyrus Vaziri, Christopher P. Mack, and Joan M. Taylor. 2014. "Focal Adhesion Kinase Antagonizes Doxorubicin Cardiotoxicity via P21Cip1." *Journal of Molecular and Cellular Cardiology*. https://doi.org/10.1016/j.yjmcc.2013.12.002.

Cho, H., J. Mu, J. K. Kim, J. L. Thorvaldsen, Q. Chu, E. B. Crenshaw, K. H. Kaestner, M. S. Bartolomei, G. I. Shulman, and M. J. Birnbaum. 2001. "Insulin Resistance and a Diabetes Mellitus-like Syndrome in Mice Lacking the Protein Kinase Akt2 (PKBβ)." *Science*. https://doi.org/10.1126/science.292.5522.1728.

Conway, William C., Jochem Van Der Voort Van Zyp, Vijayalakshmi Thamilselvan, Mary F. Walsh, David L. Crowe, and Marc D. Basson. 2006. "Paxillin Modulates Squamous Cancer Cell Adhesion and Is Important in Pressure-Augmented Adhesion." *Journal of Cellular Biochemistry*. https://doi.org/10.1002/jcb.20819.

Craig, D H, C P Gayer, K L Schaubert, Y Wei, J Li, Y Laouar, and M D Basson. 2009. "Increased Extracellular Pressure Enhances Cancer Cell Integrin-Binding Affinity through Phosphorylation of Beta1-Integrin at Threonine 788/789." *American Journal of Physiology. Cell Physiology*. https://doi.org/10.1152/ajpcell.00355.2008.

Craig, David H., Cheri R. Owen, William C. Conway, Mary F. Walsh, Christina Downey, and Marc D. Basson. 2008. "Colchicine Inhibits Pressure-Induced Tumor Cell Implantation within Surgical Wounds and Enhances Tumor-Free Survival in Mice." *Journal of Clinical Investigation*. https://doi.org/10.1172/JCI34279.

Craig, David H., Hiroe Shiratsuchi, and Marc D. Basson. 2009. "Increased Extracellular Pressure Provides a Novel Adjuvant Stimulus for Enhancement of Conventional Dendritic Cell Maturation Strategies." *Biochemical and Biophysical Research Communications*. https://doi.org/10.1016/j.bbrc.2009.07.010.

Craig, David H, Christina Downey, and Marc D Basson. 2008. "SiRNA-Mediated Reduction of α-Actinin-1 Inhibits Pressure-Induced Murine Tumor Cell Wound Implantation and Enhances Tumor-Free Survival." *Neoplasia*. https://doi.org/10.1593/neo.07945.

Craig, David H, Beatrice Haimovich, and Marc D Basson. 2007. "⎵-Actinin-1 Phosphorylation Modulates Pressure-Induced Colon Cancer Cell Adhesion through

Regulation of Focal Adhesion Kinase-Src Interaction." *Am J Physiol Cell Physiol*. https://doi.org/10.1152/ajpcell.00118.2007.

Craig, Lisa, Paul C. Sanschagrin, Annett Rozek, Steve Lackie, Leslie A. Kuhn, and Jamie K. Scott. 1998. "The Role of Structure in Antibody Cross-Reactivity between Peptides and Folded Proteins." *Journal of Molecular Biology*. https://doi.org/10.1006/jmbi.1998.1907.

Datta, Sandeep Robert, Henryk Dudek, Tao Xu, Shane Masters, Fu Haian, Yukiko Gotoh, and Michael E. Greenberg. 1997. "Akt Phosphorylation of BAD Couples Survival Signals to the Cell- Intrinsic Death Machinery." *Cell*. https://doi.org/10.1016/S0092-8674(00)80405-5.

Downey, Christina, Kamal Alwan, Vijayalakshmi Thamilselvan, Liyue Zhang, Yan Jiang, Arun K. Rishi, and Marc D. Basson. 2006. "Pressure Stimulates Breast Cancer Cell Adhesion Independently of Cell Cycle and Apoptosis Regulatory Protein (CARP)-1 Regulation of Focal Adhesion Kinase." *American Journal of Surgery*. https://doi.org/10.1016/j.amjsurg.2006.08.006.

Dregelid, E., and E. Svendsen. 1988. "Endothelial Cell Injury in Human Saphenous Veins after Manipulation and Tweezer Grasping." *Journal of Cardiovascular Surgery*.

Fidler, Isaiah J. 1970. "Metastasis: Quantitative Analysis of Distribution and Fate of Tumor Emboli Labeled with 125l-5-Lodo-2'-Deoxyuridine." *Journal of the National Cancer Institute*. https://doi.org/10.1093/jnci/45.4.773.

Fosgerau, Keld, and Torsten Hoffmann. 2015. "Peptide Therapeutics: Current Status and Future Directions." *Drug Discovery Today*. https://doi.org/10.1016/j.drudis.2014.10.003.

Fuller, Jonathan C., Nicholas J. Burgoyne, and Richard M. Jackson. 2009. "Predicting Druggable Binding Sites at the Protein-Protein Interface." *Drug Discovery Today*. https://doi.org/10.1016/j.drudis.2008.10.009.

Gayer, Christopher P., Lakshmi S. Chaturvedi, Shouye Wang, David H. Craig, Thomas Flanigan, and Marc D. Basson. 2009. "Strain-Induced Proliferation Requires the Phosphatidylinositol 3-Kinase/AKT/Glycogen Synthase Kinase Pathway." *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M804576200.

Giantonio, Bruce J., Christine Derry, Cecilia McAleer, Joseph J. McPhillips, and Peter J. O'Dwyer. 2004. "Phase I and Pharmacokinetic Study of the Cytotoxic Ether Lipid Ilmofosine Administered by Weekly Two-Hour Infusion in Patients with Advanced Solid Tumors." *Clinical Cancer Research*. https://doi.org/10.1158/1078-0432.CCR-0837-02.

Gupta, Gaorav P., and Joan Massagué. 2006. “Cancer Metastasis: Building a Framework.” *Cell*. https://doi.org/10.1016/j.cell.2006.11.001.

Han, Okhee, Guang Di Li, and Marc D Basson. 1998. “Strain Induces Caco-2 Intestinal Epithelial Proliferation and Differentiation via PKC and Tyrosine Kinase Signals.” *American Journal of Physiology*.

Hanahan, Douglas, Weinberg, Robert. 2000. “Hallmarks of Cancer.” *Cell*. https://doi.org/10.1007/s00262-010-0968-0.

Hartman, Z. R., M. D. Schaller, and Y. M. Agazie. 2013. “The Tyrosine Phosphatase SHP2 Regulates Focal Adhesion Kinase to Promote EGF-Induced Lamellipodia Persistence and Cell Migration.” *Molecular Cancer Research*. https://doi.org/10.1158/1541-7786.MCR-12-0578.

Ingber, D. E. 2006. “Cellular Mechanotransduction: Putting All the Pieces Together Again.” *The FASEB Journal*. https://doi.org/10.1096/fj.05-5424rev.

Ingber, Donald E., Ning Wang, and Dimitrije Stamenović. 2014. “Tensegrity, Cellular Biophysics, and the Mechanics of Living Systems.” *Reports on Progress in Physics*. https://doi.org/10.1088/0034-4885/77/4/046603.

Irwin, John J., and Brian K. Shoichet. 2005. “ZINC - A Free Database of Commercially Available Compounds for Virtual Screening.” *Journal of Chemical Information and Modeling*. https://doi.org/10.1021/ci049714+.

Jain, Rakesh K., Yves Boucher, and Norman Wolmark. 1992. “Interstitial Hypertension in Human Breast and Colorectal Tumors.” *Cancer Research*.

Kao, Y. C., J. R. Jheng, H. J. Pan, W. Y. Liao, C. H. Lee, and P. L. Kuo. 2017. “Elevated Hydrostatic Pressure Enhances the Motility and Enlarges the Size of the Lung Cancer Cells through Aquaporin Upregulation Mediated by Caveolin-1 and ERK1/2 Signaling.” *Oncogene*. https://doi.org/10.1038/onc.2016.255.

Ko, Hyo Rim, Il Sun Kwon, Inwoo Hwang, Eun Ju Jin, Joo Ho Shin, Angela M. Brennan-Minnella, Raymond Swanson, Sung Woo Cho, Kyung Hoon Lee, and Jee Yin Ahn. 2016. “Akt1-Inhibitor of DNA Binding2 Is Essential for Growth Cone Formation and Axon Growth and Promotes Central Nervous System Axon Regeneration.” *ELife*. https://doi.org/10.7554/eLife.20799.

Koch, Moritz, Peter Kienle, Ulf Hinz, Dalibor Antolovic, Jan Schmidt, Christian Herfarth, Magnus Von Knebel Doeberitz, and Jürgen Weitz. 2005. “Detection of Hematogenous Tumor Cell Dissemination Predicts Tumor Relapse in Patients Undergoing Surgical Resection of Colorectal Liver Metastases.” *Annals of Surgery*. https://doi.org/10.1097/01.sla.0000151795.15068.27.

Krieger, Elmar, Tom Darden, Sander B. Nabuurs, Alexei Finkelstein, and Gert Vriend. 2004. “Making Optimal Use of Empirical Energy Functions: Force-Field Parameterization in Crystal Space.” *Proteins: Structure, Function and Genetics*. https://doi.org/10.1002/prot.20251.

Labbé, Céline M., Guillaume Laconde, Mélaine A. Kuenemann, Bruno O. Villoutreix, and Olivier Sperandio. 2013. “IPPI-DB: A Manually Curated and Interactive Database of Small Non-Peptide Inhibitors of Protein-Protein Interactions.” *Drug Discovery Today*. https://doi.org/10.1016/j.drudis.2013.05.003.

Lamore, Sarah D., Ernst Ahlberg, Scott Boyer, Michelle L. Lamb, Maria P. Hortigon-Vinagre, Victor Rodriguez, Godfrey L. Smith, et al. 2017. “Deconvoluting Kinase Inhibitor Induced Cardiotoxicity.” *Toxicological Sciences*. https://doi.org/10.1093/toxsci/kfx082.

Le Devedec, SE.,Geverts B, de Bont H, Yan K, Verbeek FJ, Houtsmuller AB, and van der Water B. 2018. “The Residence Time of Focal Adhesion Kinase (FAK) and Paxillin at Focal Adhesions in Renal Epithelial Cells is Determined by Adhesion Size, Strength, and Life Cycle Status.” *J Cell Sci*.

Leunig, Michael, Rakesh K. Jain, Alwin E. Goetz, Konrad Messmer, and Ernst Kastenbauer. 1992. “Interstitial Hypertension in Head and Neck Tumors in Patients: Correlation with Tumor Size.” *Cancer Research*.

Li, Wei, Aydin Duzgun, Bauer E. Sumpio, and Marc D. Basson. 2001. “Integrin and FAK-Mediated MAPK Activation Is Required for Cyclic Strain Mitogenic Effects in Caco-2 Cells.” *Am J Physiol Gastrointest Liver Physiol*.

Li, Weiqun, Jiachang Zhang, Lawrence Flechner, Teresa Hyun, Alan Yam, Thomas F. Franke, and Jacalyn H. Pierce. 1999. “Protein Kinase C-α Overexpression Stimulates Akt Activity and Suppresses Apoptosis Induced by Interleukin 3 Withdrawal.” *Oncogene*. https://doi.org/10.1038/sj.onc.1203065.

Liu, T.-J., T. LaFortune, T. Honda, O. Ohmori, S. Hatakeyama, T. Meyer, D. Jackson, J. de Groot, and W.K. A. Yung. 2007. “Inhibition of Both Focal Adhesion Kinase and Insulin-like Growth Factor-I Receptor Kinase Suppresses Glioma Proliferation in Vitro and in Vivo.” *Molecular Cancer Therapeutics*. https://doi.org/10.1158/1535-7163.MCT-06-0476.

Liu, Weiguo, David A. Bloom, William G. Cance, Elena V. Kurenova, Vita M. Golubovskaya, and Steven N. Hochwald. 2008. “FAK and IGF-IR Interact to Provide Survival Signals in Human Pancreatic Adenocarcinoma Cells.” *Carcinogenesis*. https://doi.org/10.1093/carcin/bgn026.

London, Nir, Barak Raveh, and Ora Schueler-Furman. 2013. “Druggable Protein-Protein Interactions - from Hot Spots to Hot Segments.” *Current Opinion in Chemical Biology*. https://doi.org/10.1016/j.cbpa.2013.10.011.

Luzzi, Keith J., Ian C. MacDonald, Eric E. Schmidt, Nancy Kerkvliet, Vincent L. Morris, Ann F. Chambers, and Alan C. Groom. 1998. “Multistep Nature of Metastatic Inefficiency: Dormancy of Solitary Cells after Successful Extravasation and Limited Survival of Early Micrometastases.” *American Journal of Pathology*. https://doi.org/10.1016/S0002-9440(10)65628-3.

Madara, Karen, John E. Janik, John W. Smith, and Dan L. Longo. 1993. “Interstitial Pressure of Subcutaneous Nodules in Melanoma and Lymphoma Patients: Changes during Treatment1.” *Cancer Research*.

Molladavoodi, Sara, Matthew Robichaud, David Wulff, and Maud Gorbet. 2017. “Corneal Epithelial Cells Exposed to Shear Stress Show Altered Cytoskeleton and Migratory Behaviour.” *PLoS ONE*. https://doi.org/10.1371/journal.pone.0178981.

Moran-Jones, Kim, Laura M. Brown, and Goli Samimi. 2015. “INC280, an Orally Available Small Molecule Inhibitor of c-MET, Reduces Migration and Adhesion in Ovarian Cancer Cell Models.” *Scientific Reports*. https://doi.org/10.1038/srep11749.

Motro, René. 2003. *Tensegrity: Structural Systems for the Future*. *Tensegrity: Structural Systems for the Future*. https://doi.org/10.1016/B978-1-903996-37-9.X5028-8.

Nishizaki, Takashi, Takashi Matsumata, Takashi Kanematsu, Chikao Yasunaga, and Keizo Sugimachi. 1990. “Surgical Manipulation of VX2 Carcinoma in the Rabbit Liver Evokes Enhancement of Metastasis.” *Journal of Surgical Research*. https://doi.org/10.1016/0022-4804(90)90116-J.

Oki, Yasuhiro, Michelle Fanale, Jorge Romaguera, Luis Fayad, Nathan Fowler, Amanda Copeland, Felipe Samaniego, et al. 2015. “Phase II Study of an AKT Inhibitor MK2206 in Patients with Relapsed or Refractory Lymphoma.” *British Journal of Haematology*. https://doi.org/10.1111/bjh.13603.

Oltersdorf, Tilman, Steven W. Elmore, Alexander R. Shoemaker, Robert C. Armstrong, David J. Augeri, Barbara A. Belli, Milan Bruncko, et al. 2005. “An Inhibitor of Bcl-2 Family Proteins Induces Regression of Solid Tumours.” *Nature*. https://doi.org/10.1038/nature03579.

Parsons, J. T. 2003. “Focal Adhesion Kinase: The First Ten Years.” *Journal of Cell Science*. https://doi.org/10.1242/jcs.00373.

Patel, Hitesh, Nadia Le Marer, Richard Q. Wharton, Zulfiqar A.J. Khan, Ruth Araia, Clare Glover, Michael M. Henry, and Timothy G. Allen-Mersh. 2002. “Clearance of

Circulating Tumor Cells after Excision of Primary Colorectal Cancer." *Annals of Surgery*. https://doi.org/10.1097/00000658-200202000-00010.

Perry, Brandon C., Shouye Wang, and Marc D. Basson. 2010. "Extracellular Pressure Stimulates Adhesion of Sarcoma Cells via Activation of Focal Adhesion Kinase and Akt." *American Journal of Surgery*. https://doi.org/10.1016/j.amjsurg.2010.07.013.

Sadoshima, Junichi, and Seigo Izumo. 1997. "THE CELLULAR AND MOLECULAR RESPONSE OF CARDIAC MYOCYTES TO MECHANICAL STRESS." *Annual Review of Physiology*. https://doi.org/10.1146/annurev.physiol.59.1.551.

Shapovalov, Maxim V., and Roland L. Dunbrack. 2011. "A Smoothed Backbone-Dependent Rotamer Library for Proteins Derived from Adaptive Kernel Density Estimates and Regressions." *Structure*. https://doi.org/10.1016/j.str.2011.03.019.

Shiratsuchi, Hiroe, and Marc D. Basson. 2007. "Akt2, but Not Akt1 or Akt3 Mediates Pressure-Stimulated Serum-Opsonized Latex Bead Phagocytosis through Activating MTOR and P70 S6 Kinase." *Journal of Cellular Biochemistry*. https://doi.org/10.1002/jcb.21295.

Shiratsuchi, Hiroe, and Marc D Basson. 2004. "Extracellular Pressure Stimulates Macrophage Phagocytosis by Inhibiting a Pathway Involving FAK and ERK." *American Journal of Physiology. Cell Physiology*. https://doi.org/10.1152/ajpcell.00553.2003.

Souers, Andrew J., Joel D. Leverson, Erwin R. Boghaert, Scott L. Ackler, Nathaniel D. Catron, Jun Chen, Brian D. Dayton, et al. 2013. "ABT-199, a Potent and Selective BCL-2 Inhibitor, Achieves Antitumor Activity While Sparing Platelets." *Nature Medicine*. https://doi.org/10.1038/nm.3048.

Tamura, Masahito, Jianguo Gu, Erik H J Danen, Takahisa Takino, Shingo Miyamoto, and Kenneth M. Yamada. 1999. "PTEN Interactions with Focal Adhesion Kinase and Suppression of the Extracellular Matrix-Dependent Phosphatidylinositol 3-Kinase/Akt Cell Survival Pathway." *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.274.29.20693.

Thamilselvan, Vijayalakshmi, and Marc D. Basson. 2004. "Pressure Activates Colon Cancer Cell Adhesion by Inside-Out Focal Adhesion Complex and Actin Cytoskeletal Signaling." *Gastroenterology*. https://doi.org/10.1053/j.gastro.2003.10.078.

Thamilselvan, Vijayalakshmi, David H Craig, and Marc D Basson. 2007. "FAK Association with Multiple Signal Proteins Mediates Pressure-Induced Colon Cancer Cell Adhesion via a Src-Dependent PI3K/Akt Pathway." *The FASEB Journal : Research Communication*. https://doi.org/10.1096/fj.06-6545com.

Thomas, J W, M A Cooley, J M Broome, R Salgia, J D Griffin, C R Lombardo, and M D Schaller. 1999. “The Role of Focal Adhesion Kinase Binding in the Regulation of Tyrosine Phosphorylation of Paxillin.” *The Journal of Biological Chemistry*. https://doi.org/10.1074/JBC.274.51.36684.

Thubrikar, Mano J., and Francis Robicsek. 1995. “Pressure-Induced Arterial Wall Stress and Atherosclerosis.” *The Annals of Thoracic Surgery*. https://doi.org/10.1016/0003-4975(94)01037-D.

Turnbull, R. B. 1970. “Cancer of the Colon. The Five- and Ten-Year Survival Rates Following Resection Utilizing the Isolation Technique.” *Annals of the Royal College of Surgeons of England*. https://doi.org/10.5833/jjgs.9.539.

Vassilev, Lyubomir T., Binh T. Vu, Bradford Graves, Daisy Carvajal, Frank Podlaski, Zoran Filipovic, Norman Kong, et al. 2004. “In Vivo Activation of the P53 Pathway by Small-Molecule Antagonists of MDM2.” *Science*. https://doi.org/10.1126/science.1092472.

Walsh, Mary F., Vijayalakshmi Thamilselvan, Rainer Grotelueschen, Lulu Farhana, and Marc D. Basson. 2003. “Absence of Adhesion Triggers Differential FAK and SAPKp38 Signals in SW620 Human Colon Cancer Cells That May Inhibit Adhesiveness and Lead to Cell Death.” *Cellular Physiology and Biochemistry*. https://doi.org/10.1159/000071864.

Wang, S., and M. D. Basson. 2011. “Akt Directly Regulates Focal Adhesion Kinase through Association and Serine Phosphorylation: Implication for Pressure-Induced Colon Cancer Metastasis.” *AJP: Cell Physiology*. https://doi.org/10.1152/ajpcell.00377.2010.

Wang, Shouye, and Marc D Basson. 2011. “Protein Kinase B/AKT And Focal Adhesion Kinase: Two Close Signaling Partners in Cancer.” *Anti-Cancer Agents in Medicinal Chemistry*. https://doi.org/10.2174/187152011797927661.

Wen, Li, Tanveer A. Javed, Dean Yimlamai, Amitava Mukherjee, Xiangwei Xiao, and Sohail Z. Husain. 2018. “Transient High Pressure in Pancreatic Ducts Promotes Inflammation and Alters Tight Junctions via Calcineurin Signaling in Mice.” *Gastroenterology*. https://doi.org/10.1053/j.gastro.2018.06.036.

Wolfenson, Haguy, Bo Yang, and Michael P Sheetz. 2018. “Steps in Mechanotransduction Pathways That Control Cell Morphology.” *Annual Review of Physiology* 81 (1): annurev-physiol-021317-121245. https://doi.org/10.1146/annurev-physiol-021317-121245.

Wu, Meng Yao, Rong Rui Liang, Kai Chen, Meng Shen, Ya Li Tian, Dao Ming Li, Wei Ming Duan, et al. 2015. “FH535 Inhibited Metastasis and Growth of Pancreatic Cancer Cells.” *OncoTargets and Therapy*. https://doi.org/10.2147/OTT.S82718.

Yamaguchi, Kazuya, Yukihiro Takagi, Shinichirou Aoki, Manabu Futamura, and Shigetoyo Saji. 2000. “Significant Detection of Circulating Cancer Cells in the Blood by Reverse Transcriptase-Polymerase Chain Reaction during Colorectal Cancer Resection.” *Annals of Surgery*. https://doi.org/10.1097/00000658-200007000-00009.

Zanotelli, Matthew R., and Cynthia A. Reinhart-King. 2018. “Mechanical Forces in Tumor Angiogenesis.” In *Advances in Experimental Medicine and Biology*, 1092:91–112. https://doi.org/10.1007/978-3-319-95294-9_6.