The Effect of Hydrostatic Pressure on Bladder Smooth Muscle Cell Function

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THE EFFECT OF SUSTAINED HYDROSTATIC PRESSURE ON BLADDER SMOOTH MUSCLE CELL FUNCTION

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

by
Margaret Rebecca Drumm
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Accepted by:
Dr. Jiro Nagatomi, Committee Chair
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Dr. Anand Ramamurthi
ABSTRACT

Previous research has demonstrated that bladder smooth muscle cells (BSMC) respond to various forms of mechanical stimuli, including stretch and hydrostatic pressure, by increases of cell proliferation, activation of intracellular signaling pathways, and alteration of contractile and synthetic marker protein expression. These cellular/molecular level changes are all indicative of a BSMC phenotypic shift that can negatively impact the bladder function at the tissue and organ level. The objective of the present study is to test a hypothesis that bladder SMCs shift their phenotype from contractile to synthetic in response to elevated hydrostatic pressure. Rat bladder SMC cultures were exposed to 7.5 cm H₂O of hydrostatic pressure in custom-made columns up to 48 hours. Following exposure to pressure, the SMCs were fixed, stained, and imaged using fluorescence microscopy. Image analyses revealed that, compared to the control, SMCs exposed to hydrostatic pressure for 4 hours exhibited a more spread morphology, which was quantitatively confirmed when examining the aspect ratio of the cell population. Moreover, cell density of BSMCs exposed to hydrostatic pressure exhibited an increase after 24 and 48 hours when compared to their respective controls. Additionally, total proteins collected from these cells were analyzed using the Western blotting technique to quantify extracellular signal-regulated kinase (ERK½) activation as well as phenotype marker proteins, alpha-smooth muscle actin (α-SMA) and SM-22 in SMCs. Compared to control (0 minutes), the expression of activated ERK ½ was up to two-fold when these cells were exposed to hydrostatic pressure (7.5 cm H₂O) for up to 180 minutes. In contrast, α-SMA and SM-22 expression was similar in the control and cells exposed to hydrostatic pressure for 48 hours. While the proliferative and morphological responses suggest a possible ERK ½ mediated phenotypic shift from a contractile phenotype toward a synthetic phenotype under mechanical stimulus, the expression of contractile proteins did not corroborate the other aspects of SMC phenotypic modulation in the time
points examined in this study (48 hours). These results suggest that contractile protein expression in response to mechanical stimulus could possibly be mediated by ERK ½ at earlier or later time points not detected in this study, or by a different signaling pathway altogether. Future study recommendations include, but are not limited to, exploring the expression of contractile proteins at various time points and their relationship with ERK ½ activation.
DEDICATION

This work is dedicated to my friends and family who have supported me through my undergraduate and graduate education. To my parents, thank you for providing me with the opportunity to attend Clemson University and for the multitude of encouragement and advice throughout my life. To Daniel Widener, thank you for your motivation and support for the past two years. With every obstacle I faced, you were there to listen and help in any way you could. My research and graduate experience would not have been the same without the support of my loving family and friends.
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CHAPTER 1

INTRODUCTION

The urinary bladder is a low pressure vessel that constantly experiences mechanical forces due to its functions of urine storage and micturition. Alterations in structure and composition of the urinary bladder due to cellular responses to mechanical force can subsequently affect bladder compliance and, thus, its function. Mechanotransduction is the process by which cells interpret mechanical stimuli and respond through biochemical signals to regulate their function.\(^1\) The mechanotransduction of sustained hydrostatic pressure by bladder smooth muscle cells and their role in bladder tissue compliance will be assessed in this research.

1.1 Bladder Anatomy and Physiology

The urinary bladder is a hollow, distensible, muscular organ located within the pelvic cavity (Figure 1). The main functions of the urinary bladder are to store and voluntarily void urine that is produced in the kidneys.\(^1,2\) This spherical organ expands as it fills with urine, causing the shape to alter. When empty, the bladder has many inner folds,\(^2\) which smoothen and flatten out as the bladder fills and expands to more than fifteen times its contracted size.\(^1,3\)

The urinary bladder experiences considerable mechanical forces such as hydrostatic pressure and stretch during filling and voiding cycles. To maintain a physiological pressure level (0-10 cm H\(_2\)O),\(^4-6\) the
bladder wall distends and is therefore a highly compliant tissue. The bladder is able to recover from repeated distension due to elastin content in the bladder tissue.\textsuperscript{1} Elastin is thought to create a recoiling effect for collagen after repetitive filling and voiding cycles.\textsuperscript{1, 7} The bladder wall is able to withstand levels of hydrostatic pressure created by a capacity of 400-500 mL under normal conditions, thereby protecting the kidneys and upper urinary tract from damage.\textsuperscript{1}

1.1.1 \textbf{Tissue Layers}

The bladder wall consists of three tissue layers functioning together to expand and contract cyclically as the bladder fills with and voids urine: the urothelium, the lamina propria, and the detrusor muscle\textsuperscript{1} (Figure 2). A network of nerves in connective tissue can be found in all layers of the bladder, particularly in the urothelium and detrusor layers.\textsuperscript{1, 8} The external surface of the bladder is covered with a dense, fibrous layer of fine collagen fibrils, called the adventitia.\textsuperscript{1, 2}
Figure 1: Physiology of the bladder. Image taken from http://academic.kellogg.cc.mi.us/herbrandsonc/bio201_McKinley/f27-9a_urinary_bladder_c.jpg
1.1.1.1 Urothelial Layer

The urothelium consists of several layers of epithelial cells, similar to those lining the ureters and upper portion of the urethra. The urothelium is composed of three layers: a basal layer attached to the basement membrane, an intermediate layer, and a superficial layer containing umbrella cells. The superficial layer is in direct contact with the urine and acts as a protective barrier to prevent constituents of urine from passing into the tissue and bloodstream. The barrier role of the superficial layer is dependent on tight junctions between the umbrella cells. These tight junctions reduce the movement of water, ions, solutes, and macromolecules between cells into underlying tissue. Conditions such as interstitial cystitis or bladder dysfunction due to spinal cord injury can cause conditions that alter the urothelial barrier and can compromise the underlying tissue and muscle layers causing bladder dysfunction.

1.1.1.2 Lamina Propria

The lamina propria in humans is approximately 1.3 mm thick and functions to maintain the structural shape of the bladder wall. This layer is comprised of several sublayers: superficial lamina propria, lamina muscularis mucosa, submucosa, and deeper lamina propria. The superficial lamina propria contains the capillary network of the bladder surrounded by a dense layer of randomly oriented collagen fibers. The lamina muscularis mucosa is a thin layer of muscle cells between the superficial lamina propria and the submucosa, a thin layer of collagen. The deeper lamina propria is a thick layer of collagen Type I (>300 μm in humans) that makes up the majority of the lamina propria. These sublayers of the lamina propria create a connective tissue matrix that contains crucial cells responsible for maintaining the matrix during bladder development and remodeling.
1.1.1.3 Detrusor Layer

The detrusor layer of the human bladder wall is approximately three-fourths of the total bladder wall thickness and is responsible for the mechanical function of the bladder, contraction and relaxation, during and in between urine voiding.\textsuperscript{1,2} The detrusor layer is comprised of smooth muscle cells (SMC) surrounded by the extracellular matrix (ECM) including collagen fibrils and elastic fibrils; its interstitium contains blood vessels, and intrinsic nerves.\textsuperscript{15} The SMCs are arranged within collagen sheaths to form muscle fiber bundles (50-150 µm in diameter and 20-50 µm apart) that provide the bladder contraction capabilities to aid in voiding.\textsuperscript{1} Altered or impaired bladder function is either due to the effects of neurogenic disorders, obstruction, or dysfunction within the detrusor or urothelial layers.\textsuperscript{15}
Figure 2: Histology cross section of a porcine urinary bladder, stained with Van Geison’s stain. Bar-250µm¹
1.1.2 Physiology of the Bladder: Neural Control of Micturition

The micturition process involves coordination through a complex neural system of the smooth muscles of the bladder and urethra, and of the striated muscles of the sphincter and pelvic floor. As the bladder fills, the sphincter muscles are contracted around the urethra to prevent leaking. Initiation of the micturition process causes the sphincter muscles to relax while the detrusor muscle of the bladder simultaneously contracts causing urine release. This simultaneous coupling of sphincter relaxation and detrusor contraction is obtained through neural control.

The desire to void urine originates from the stretching of the bladder wall. Sensory nerve cells collect information in the bladder wall and transmit signals to the spinal cord. Studies have shown most sensory afferents of the bladder and urethra originate in the thoracolumbar region and travel through the pelvic nerve, although some signals may be transmitted along the hypogastric nerve.
1.2 Bladder Smooth Muscle Cells

Smooth muscle is located in the wall of various hollow organs, such as the bladder and vasculature, throughout the body representing an estimated 2% of human body weight. An estimated 30-60 grams of smooth muscle exists in bladder tissue. SMCs are involved in various functions that maintain homeostasis in the body including control of blood pressure and bladder contraction.

1.2.1 Smooth Muscle Cell Morphology and Structural Organization

SMCs are long, spindle-shaped, mono-nucleated cells (Figure 3). The widest portion of a smooth muscle cell ranges from 2-4 µm in width and the length can be up to 1000 µm in visceral organs, such as the bladder, but is significantly shorter in length in vasculature. Smooth muscle structure is dependent on myofibrils oriented in three-dimensional direction. The myofibrils are the contractile apparatus composed of actin and myosin. A structure called a dense body is located at the terminal site of myofibrils in the sarcoplasm. The dense membrane is a heterophilic cell adhesion structure in the plasma membrane mediated by the extracellular matrix. Intermediate filaments connect the dense body to the dense membrane causing a three-dimensional contraction.
Figure 3: Smooth Muscle Cell Morphology. BSMCs stained with rhodamine-phalloidin for actin filaments with a DAPI overlay to detect nuclei. 100x magnification.
1.2.2 **Smooth Muscle Cell Contractile Mechanism**

The main function of smooth muscle is contraction through an actin-myosin interaction (Figure 4). Calcium plays a key role in regulating this actin-myosin interaction, thus controlling contraction.\(^\text{19, 21}\) Contraction occurs when the intracellular calcium ion concentration increases to a level greater than 10\(^{-6}\) M. Calcium ions bind to the regulatory protein, calmodulin, associated with the enzyme myosin light chain kinase (MLCK).\(^\text{19, 21}\) MLCK is activated by binding to this Ca\(^{2+}\)/calmodulin complex, then catalyzing the phosphorylation of regulatory light chain of myosin.\(^\text{21-23}\) Phosphorylation of myosin causes actin-activated Mg-ATPase activity to increase.\(^\text{21}\) Simultaneously, the Ca\(^{2+}\)/calmodulin complex binds to caldesmon, releasing its inhibitory effect by inducing actin-myosin interaction.\(^\text{19}\) This series of events causes smooth muscle contraction (Figure 4).\(^\text{19, 21}\)

Relaxation occurs when intracellular calcium-ion levels deplete to a concentration below 10\(^{-6}\) M.\(^\text{19}\) In this situation, MLCK is dephosphorylated by myosin light chain phosphatase (MLCP).\(^\text{19}\) Simultaneously, caldesmon binds to the actin/tropomyosin complex inhibiting the actin-myosin interaction, thus causing cell relaxation (Figure 4).\(^\text{19}\)
Figure 4: Calcium-ion dependent mechanism for SMC contraction.\textsuperscript{19}

CaM=Calmodulin; CaD= caldesmon; A= actin filament; TM= tropomyosin; MLCK= myosin light chain kinase; p-M= phosphorylated myosin; MLCP= myosin light chain phosphatase; M= dephosphorylated myosin
1.2.3 Phenotypic Modulation of Smooth Muscle Cells

SMCs \textit{in vivo} have the potential to undergo phenotypical changes in response to an altered environment such as stress or injury.\textsuperscript{3, 24} SMC phenotypic modulation is involved in the onset of vascular diseases such as atherosclerosis\textsuperscript{19, 25, 26} and hypertension.\textsuperscript{19} This modulation is also hypothesized to occur in bladder smooth muscle cells (BSMCs) under certain pathological conditions where the BSMCs are exposed to abnormal mechanical environments.\textsuperscript{3, 27} In vascular research, SMCs have been shown to exhibit the ability to transition from a contractile (differentiated) to a synthetic (dedifferentiated) state, or vice versa.\textsuperscript{19, 25, 27, 28} This phenotypic modulation of SMCs toward a more synthetic phenotype is associated with morphological change, decreased expression of smooth muscle-specific proteins,\textsuperscript{25, 27, 28} an increase in cell proliferation and migration,\textsuperscript{19, 27-29} and decreased tissue compliance due to extracellular matrix (ECM) remodeling.\textsuperscript{3, 28, 30}

1.2.3.1 Phenotypic Modulation Effects on SMC Morphology

Contractile and synthetic phenotypes represent the two extreme ends of the SMC phenotypic spectrum with infinite varying intermediates existing in between. A contractile SMC \textit{in vitro} exhibits an elongated, spindle-like morphology seen in normal, physiological environments (Figure 3). SMCs showing a synthetic phenotype exhibit a more spread-out, rounded morphology \textit{in vitro}.\textsuperscript{27, 31}
1.2.3.2 Phenotypic Modulation Effects on Smooth Muscle Marker Protein Expression

Changes in the expression of several contractile proteins are closely associated with smooth muscle cell phenotypic modulation.\textsuperscript{32} Fifty percent of total proteins in SMCs are contractile proteins,\textsuperscript{19} which include \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA),\textsuperscript{19, 28, 33} smooth muscle myosin heavy chain (isoforms SM1 and SM2),\textsuperscript{19, 28, 33} \(\beta\)-tropomyosin,\textsuperscript{19, 33} caldesmon,\textsuperscript{19, 33} SM22,\textsuperscript{19, 33} calponin,\textsuperscript{19, 28, 33} desmin,\textsuperscript{28} and \(\alpha\) integrin.\textsuperscript{19, 33} Both \(\alpha\)-SMA and smooth muscle myosin heavy chain are proteins involved in the contractile apparatus of a SMC, while SM22 is an actin-associated protein involved in the regulation of contraction.\textsuperscript{27, 31} SMCs of varying phenotype express different levels of marker proteins rather than different proteins altogether,\textsuperscript{27, 31} thus contractile protein quantification is a common way to characterize SMC phenotype.

1.2.3.3 Phenotypic Modulation Effects on Increased SMC Proliferation

An increase in cell proliferation occurs when SMC modulate toward a more synthetic phenotype, rather than a contractile phenotype.\textsuperscript{27, 31} Increased SMC proliferation, hyperplasia, in the detrusor muscle layer causes the bladder wall to become thicker and stiffer and therefore decreasing compliance.\textsuperscript{30, 34} An increase in cell proliferation has been shown to result from activation of both the extracellular signal regulated kinase (ERK \(\frac{1}{2}\)) pathway\textsuperscript{29, 35, 36} and through the phosphoinositide-3-kinase (PI3K)/Akt intracellular pathway.\textsuperscript{34, 37}

The ERK intracellular pathway is one signaling pathway in a family of mitogen-activated protein kinases (MAPK) that also includes c-Jun N-terminal kinase (JNK) and p38 stress-activated protein kinase 2 (p38SAPK2) (Figure 5). The ERK \(\frac{1}{2}\) family of MAPK converts extracellular stimuli, such as stretch,\textsuperscript{22, 36} adhesion,\textsuperscript{38} and fluid flow,\textsuperscript{39} into signals that control gene expression, cell proliferation,\textsuperscript{36} and dedifferentiation in numerous types of cells.\textsuperscript{35}
Akt is an intracellular signaling molecule that mediates cellular functions such as cell survival, cell-cycle progression, and proliferation.\textsuperscript{34, 37} The activation of Akt occurs through various stimuli such as growth factors,\textsuperscript{34} activation of G protein coupled receptors (GPCRs),\textsuperscript{40} and possibly by increased intracellular concentration of Ca\textsuperscript{2+} ions.\textsuperscript{37, 41}

1.2.3.4 Extracellular Matrix Remodeling

Phenotypic modulation in BSMCs also leads to ECM remodeling characterized by fibrosis\textsuperscript{3} and decreased tissue compliance.\textsuperscript{30} Bladder compliance is primarily influenced by the amount of the extracellular matrix proteins within the wall tissue, which is determined by the balance between proteolytic enzymes such as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs).\textsuperscript{30} Typically, an increase in the ECM of SMCs representing the synthetic phenotype is associated with the down-regulation of MMP-1 and MMP-2\textsuperscript{3, 42} and an up-regulation of TIMP-1 levels.\textsuperscript{30} ECM remodeling associated with a SMC phenotypic shift can be monitored through quantifying Type I collagen deposition\textsuperscript{3, 43, 44} and levels of MMPs and TIMPs.
Figure 5: Intracellular-signaling cascades in bladder SMC.\textsuperscript{22}
1.3 Effects of Mechanical Stimuli on Bladder Cell Function

1.3.1 In Vivo Observations of the Bladder Under Physiological Loading

The urinary bladder experiences physiological mechanical loading in the form of tensile loads within the tissue due to distension and compressive loads perpendicular to the bladder surface caused hydrostatic pressure. These forms of mechanical loading are necessary for the growth and development of the urinary bladder. The mechanical stimulation that occurs during normal bladder filling and emptying induces cellular communication through signaling, also referred to as mechanotransduction.

For proper development and maintenance of structure and function, the bladder must undergo mechanical stress. Under normal physiological conditions of humans, the bladder is exposed to cyclic variations of hydrostatic pressure ranging from 0 to 10 cm H$_2$O due to filling and emptying. This cyclic hydrostatic pressure may be necessary for proper bladder development and growth. In fetal bladder development, urine storage begins at 16 weeks of gestation while the completion of muscle formation occurs at 21 weeks, suggesting that bladder mechanics during urine storage may stimulate normal bladder development.

1.3.1.1 Effects of Urine Diversion on the Bladder

The response of the bladder tissue to defunctionalization (diversion) and then refunctionalization (undiversion) is of interest in SCI patients and in clinical settings such as surgical procedures where the filling and emptying cycle of the bladder is interrupted. Machado et al reported the response of rabbit bladders (10-12 weeks old) to defunctionalization by hemisecting the bladder from dome to trigone into a
functioning and nonfunctioning chamber, and then refunctionalizing the bladder after 3 months by reattachment of the bladder tissue. Three months post refunctionalization, the animals were sacrificed and the urinary bladders were harvested. The defunctionalized hemibladders were reported to have a lower weight, capacity and compliance compared to the refunctionalized and control bladders; however the defunctionalized bladders showed progressive recovery of both capacity and compliance with time. The contractile response and tissue to muscle ratio of the bladder were also reported as abnormal in the defunctionalized bladders, but recovered also with time after refunctionalization. The results reported by Machado et al suggest that urinary bladders have an ability to recover after diversion is restored. This conclusion is in agreement with Chun et al, who investigated the intravesical capacities, compliance, and contractility in defunctionalized canine bladders. Chun et al reported significantly decreased bladder capacities 1, 3, and 6 months after diversion as well as a weight and contractility decrease, all of which returned to normal levels in bladder that were undiverted.

These studies by Machado et al and Chun et al report data for bladder diversion that occurred for only 3 and 6 months respectively. In a clinical study by Jayanthi et al, patients who underwent urinary bladder diversion for a 7 year period showed that complete urinary diversion leads to significantly decreased bladder function, most of which is not recovered when diversion is corrected. Likewise, Lipski et al reported that defunctionalized bladders developed much more slowly than those bladders that underwent a cyclic filling and emptying regimen. Diversion studies suggest that mechanical stimuli are necessary in the growth and development of bladder tissue, but if diversion occurs for only minimal time recovery of bladder properties and function can occur.
1.3.2 *In Vivo Observations of the Bladder Under Pathological Loading*

Although appropriate mechanical stimuli are needed for growth and development of the urinary bladder, the abnormal mechanical environment under various pathological conditions may jeopardize the integrity of the bladder tissue. These abnormal conditions can be a result from voiding dysfunctions due to SCI, such as areflexia (flaccid bladder), hyperreflexia (overactive bladder), and detrusor-sphincter dyssnergia (loss of coordination between the detrusor muscle and external sphincter) or obstruction due to benign prostate hypertrophy.\(^{37, 50}\) These complications cause alterations at the cellular and molecular levels such as hypertrophy, hyperplasia, and changes in the ECM leading to changes in bladder structure, biomechanics, and compliance.\(^{1, 27, 37}\)

1.3.2.1 Hypertrophy and Hyperplasia

When the bladder’s intravesical pressure exceeds a homeostatic level (\(>10\ \text{cm H}_2\text{O}\)) the tissue responds at a cellular level to resist over-distention and prevent damage to the upper urinary tract.\(^{37, 51}\) Bladder responses to increased levels of pressure include hyperplasia (an increase in cell number) and/or hypertrophy (an increase in cell size) in the detrusor muscle layer causing bladder wall to become thicker and stiffer compared to a bladder under normal internal pressure.\(^{34}\) While the initial stimulus may be mechanical stress, growth factors and cytokines have been shown to mediate tissue remodeling.\(^{34}\)
1.3.2.2 Fibrosis and Decreased Bladder Compliance

Bladder fibrosis is a common complication of bladder injury or disease where scar tissue forms. The formation of scar tissue has been related to a phenotypic shift of SMCs towards a more synthetic phenotype, which leads to excessive production of collagen. Since collagen is significantly stiffer compared to SMCs, fibrosis results in reduction of tissue compliance and the bladder is unable to adequately perform its normal bladder functions, such as expanding to its fullest potential.

1.3.3 In Vitro Observations of the Bladder

1.3.3.1 Cyclic Stretch

BSMCs in vitro exhibit hypertrophy, hyperplasia, and increased stretch-regulated gene expression compared to the control when exposed to cyclic stretch. A common method of exposing SMCs to cyclic stretch is through a commercially available flexible membrane system, such as Flexcell®. This process allows for cells to be cultured on a membrane and exposed to conditions similar to those in vivo by subjecting cells to mechanical stretch with desired specifications. By applying controlled vacuum under the cell cultured flexible silicone membrane continuously or at cyclic intervals, the membrane as well as the cultured cells are subjected to stretch up to 20% elongation.

Studies to determine a link between cyclic stretch stimulation and response of BSMCs have identified several stretch-regulated genes (insulin-like growth factor-1, heparin-binding EGF-like growth factor, nerve growth factor, COX-2, and the cysteine-rich protein Cyr61) as possible mediators of mechanical stimuli. BSMCs in vitro exhibited a concomitant increase of heparin-binding EGF-like growth factor and COX-2 expression under cyclic stretch (20% maximum) at 0.1 Hz for up to 48 hours.
Likewise, Tamura et al. observed a five to nine-fold increase in Cyr61 expression in BSMCs *in vitro* subjected to cyclic stretch (a magnitude of 2.5% to 7.5%) after 30 min and 1 hour of testing. In addition to gene expression, stretch stimulation in BSMCs has been shown to activate MAPK and PI3K/Akt pathways to upregulate DNA synthesis and gene expression. Kushida et al compared the activation of JNK, p38, and ERK 1 in BSMCs subjected to either cyclic (10 cycles per minute) or sustained stretch. The activation of JNK increased 11-fold within 10 minutes of mechanical stretch. Activation of p38 was present (4 fold) but with less intensity as JNK activation. ERK 1 showed no activation in BSMCs however in response to cyclic stretch. These results provide evidence that mechanotransduction through signaling cascades and cell to cell communication can modulate cell proliferation and homeostasis.

Several intra-cellular signaling pathways have been examined to explore the relationship between the response of stretch-regulated genes in BSMCs under cyclic stretch to tissue remodeling at the cellular level such as proliferation, hypertrophy, and hyperplasia. Adams et al reported significant phosphorylation of Akt within 1 to 3 minutes and increased phosphorylation of p38 in BSMC with peak activation at 5 to 10 minutes of cyclic stretch stimulation using Flex Cell®. In response to the same level of stretch, BSMC exhibited 3 to 4-fold increase in DNA synthesis, which was abolished in the presence of PI3K inhibitors, LY294002 and wortmannin, and by the p38 pathway inhibitor, SB203580. Together, the authors concluded that activation of PI3K/Akt and p38 SAPK2 pathways by cyclic stretch promote cell proliferation of BSMCs *in vitro*.

Growth regulatory HB-EGF signaling via activation of its receptor EGFR has also been linked to BSMC response to stretch and pressure. Estrada et al demonstrated that rat bladders subjected to 40 cm H$_2$O pressure *in vivo* exhibited an increase in DNA synthesis after 18 and 24 hours of sustained distension, which was abrogated in the presence of an EGFR inhibitor, ZD1839. In addition, BSMCs
subjected to cyclic stretch (20% elongation, 0.1 Hz) in vitro for 24 hours exhibited increased DNA synthesis, which was also abrogated in the presence of inhibitor ZD1839. Using similar models, Aitken et al investigated the relationship between mechanical stretch and ECM remodeling through MAPK signaling pathways. The authors observed elevated ERK ½ activation in whole bladders, predominantly in the detrusor layer of the bladder wall, ex vivo after 24 hours of bladder distension (40 cm H₂O pressure), as well as distension followed by relaxation, or emptying of the bladder. The effects of mechanical strain on ECM remodeling and proliferation were explored ex vivo through MMP activity in distended intact bladders. In situ zymography revealed areas where FITC-gelatin was proteolyzed by MMPs in response to 24 hours of distension (40 cm H₂O pressure) in both the detrusor and urothelial layers of the bladder wall. In addition, gelatinase activity was increased in the condition medium of bladders distended for 15 minutes. When the bladder was incubated with doxycycline, an inhibitor of MMP activity, prior to distension significantly reduced stretch-induced gelatinase activity in the condition medium. Additionally, BSMC proliferation was shown to increase in bladders distended (40 H₂O cm pressure) for 15 minutes and 24 hours when compared to undistended bladders.

The effect of cyclic mechanical stretch, both transient and continuous, on ERK ½ activation in BSMCs in vitro was also explored using Flex Cell®. Previous research shows that exposure of BSMCs cultured on collagen type I to cyclic strain resulted in increased activation of MAPK cascades and enhanced transcription of MMP-1. Transient cyclic stretch (5 minutes stretch plus 55 minutes relaxation) of BSMCs induced ERK ½ activation only during the time period of stretch exposure. Continuous stretch (60 minutes of cyclic stretch), however, led to increased ERK ½ activation compared to its controls for time periods up to 1 hour. To better explore the gelatinase activity reported in the in vivo studies, BSMCs seeded on collagen matrices pre-incubated in condition medium from whole distended bladders showed significant proliferation. This proliferation however was abolished when the MMP inhibitor GM-6001 was supplemented to the condition medium. These in vivo and in vitro
findings lead the authors to conclude that MMPs not only alter the ECM but also induce BSMC ERK $\frac{1}{2}$ signaling in response to distension, suggesting a pathway in stretch-induced proliferation. These studies (Adams, Estrada, and Aitken et al) report increased cell proliferation and activation of PI3K/Akt and MAPK, more specifically ERK $\frac{1}{2}$, signaling pathways in BSMCs in response to cyclic stretch both in vivo and in vitro, supporting the current study’s focus to investigate the relationship between hydrostatic pressure in vitro and ERK $\frac{1}{2}$ activation in BSMCs.

1.3.3.2 Sustained Tension in 2D and 3D culture

To determine the role of signal transducers and activators of transcription 3 (STAT3), in mitogen and stretch-induced BSMC proliferation, Halachmi et al subjected BSMCs to sustained tension in vitro using FlexCell® and whole mice bladders to overdistention. STAT3 phosphorylation was reported to increase in mice bladders during 30 minutes of ex vivo distention. BSMCs stretched statically on both collagen and carboxyl matrices in vitro exhibited a significant increase in phosphorylated STAT3 after 60 minutes of sustained stretch, with an increase in BSMC proliferation occurring in cells seeded on collagen matrices. Moreover, STAT3 phosphorylation increased in BSMCs after treatment with mitogens EGF and PDGF. Both mitogenic and sustained stretch induced proliferation of BSMCs was significantly inhibited in the presence of a JAK2/STAT inhibitor, AG490, confirming the involvement of JNK2/STAT3 in stretch stimulated cell proliferation. Halachmi et al concluded that STAT3 signaling is activated by ex vivo conditions of 40 cm H$_2$O pressure in mouse bladders closely associated with hypertrophy, and JAK2/STAT3 mediates BSMC proliferation in response to mitogens and sustained stretch in vitro.

To mimic an in vivo environment where BSMCs are surrounded by ECM, BSMCs were exposed to uni-axial sustained tension in a 3-D collagen environment. Roby, et al. demonstrated that BSMCs in
3D collagen culture exposed to sustained tension for 7 days show decreased expression of phenotypic marker proteins, α-SMA and SM22, compared to 2-D cultures of SMCs on cell culture polystyrene. BSMCs under sustained tension however showed greater levels of α-SMA expression when compared to those exposed to no tension after 48 hours. Additionally, BSMCs exposed to sustained tension were reported to exhibit a spindle-like morphology and a greater aspect ratio when compared to free-floating cultures. Roby at al concluded that sustained tension may be an important stimulus in maintaining the contractile phenotype of BSMCs in vitro. Sustained tension in 2D exhibits a similar response of BSMCs to 2D cyclic stretch through an activation of intracellular signaling pathways and increased cell proliferation. BSMCs were reported to respond differently to 3D sustained tension by altered expression of contractile proteins and cell morphology.

1.3.3.3 Hydrostatic Pressure

Containment of fluid within hollow organs such as the bladder generates higher hydrostatic pressure inside compared to the abdominal cavity. As a result, the SMCs that exist within the bladder wall tissue are subjected to a pressure gradient between the internal and external pressures. A seminal study by Haberstroh et al exposed BSMCs to normal physiological ranges of hydrostatic pressure (4, 6, and 8.5 cm H$_2$O) for up to 7 days and observed a significant increase in cell proliferation after 5 days. Additional studies by the same group demonstrated that conditioned supernatant medium from BSMCs subjected to 8.5 cm H$_2$O pressure for 5 days exhibited mitogenic activity when compared to the controls. BSMCs maintained under the conditioned medium from BSMCs subjected to hydrostatic pressure exhibited a significant increase in cell proliferation, which was then abrogated in the presence of HB-EGF inhibitor, CRM197. These results suggest a BSMC proliferative response to elevated hydrostatic pressure is mediated, at least in part, through the release of HB-EGF by BSMC. Although these authors conclude
that physiological-level hydrostatic pressure is an important mechanical stimulus for detrusor development, the results of these and other studies suggest that excessive levels or prolonged exposure could lead to hyperplasia or other cellular responses that are damaging to the bladder tissue.

Backhaus et al reported that, while collagen type I and III mRNA levels in BSMC lysates showed no change after exposure to sustained hydrostatic pressure (either 20 or 40 cm H\textsubscript{2}O), decreased levels of MMP-1 (35\% and 44\%) was seen in the supernatant of BSMCs after exposure to hydrostatic pressure of 20 and 40 cm H\textsubscript{2}O after 24 hours, and decreased levels of MMP-2 and MMP-9 activity were seen after 7 hours.\textsuperscript{30} This study also reported an up-regulation of TIMP-1 in the supernatant of BSMCs following exposure to hydrostatic pressure (40 cm H\textsubscript{2}O) for 3, 7, and 24 hours.\textsuperscript{30} The coupled findings of decreased MMP-1 activity and increased TIMP-1 activity suggest that such molecular-level changes within the ECM may be the cause of decreased tissue compliance often found in high pressure bladders.\textsuperscript{30} More recently, Stover et al reported that application of cyclic pressure (an amplitude of 40 cm H\textsubscript{2}O at a frequency of 0.1 Hz) on BSMCs \textit{in vitro} led to PI3K/Akt pathway-dependent increase in cell proliferation.\textsuperscript{37} While several previous studies report increased Akt phosphorylation in SMCs in response to cyclic stretch is triggered by growth factor release (PDGF-BB,\textsuperscript{34} IGF,\textsuperscript{66} or HB-EGF\textsuperscript{62}), which then effects downstream events such as cell proliferation,\textsuperscript{37} this was the first study that demonstrated activation of an intracellular signaling pathway in BSMC by hydrostatic pressure. Potential mechanisms for the activation of Akt in BSMCs include growth factor release,\textsuperscript{34} activation of G protein coupled receptors (GPCRs),\textsuperscript{40} and increased intracellular Ca\textsuperscript{2+} ion concentrations.\textsuperscript{37, 67} In the study by Stover et al, Akt activation in response to cyclic pressure is hypothesized to be triggered by pressure-sensitive ion channels that allow for an increase in Ca\textsuperscript{2+} concentration.\textsuperscript{37} These studies provide growing evidence that BSMC is sensitive to changes in hydrostatic pressure and that increased cell proliferation and changes in the ECM may represent a possible phenotypic shift from contractile to synthetic. Further investigation, however, is necessary to elucidate the link between the mechanical stimulus and cellular/molecular events.
CHAPTER 2

RESEARCH RATIONALE

The majority of patients with diabetes (23.6 million people in U.S.), 68 spinal cord injuries (SCI), and other chronic disorders suffer from various forms of bladder dysfunction such as detrusor areflexia, often known as flaccid bladder, and detrusor hyperreflexia, or overactive bladder. 1, 37, 50 Another common urological condition in SCI patients is detrusor sphincter dyssynergia, which is a discoordination between the detrusor muscle of the bladder and external sphincter contractions around the urethra. 1, 37, 50 These urological complications are in most cases not life threatening but impair their quality of life and place burden upon the patient. Moreover, these conditions can lead to an abnormal mechanical environment in the bladder, which can then lead to cellular and molecular changes such as SMC hyperplasia or hypertrophy, and remodeling of the ECM. 27, 37 These cellular changes can result in tissue damage such as bladder wall thickening and fibrosis, 3, 30 thus decreased compliance. 1, 30, 37 These alterations in the urinary bladder tissue are the clinical motivation to find the relationship between BSMC stimulation and phenotypic changes that cause bladder dysfunction.

Previous literature suggests that BSMCs are sensitive to hydrostatic pressure (4 to 40 cm H$_2$O) exhibited through increased proliferation, 6, 37 and ECM protein changes, 30 which are characteristics of phenotypic shifts. However, the links between mechanical stimulus and downstream effects such as phenotypic shift are still unclear. This project aimed to determine potential mechanisms by which BSMCs sense pressure to convert this mechanical stimulus into biological events that are pertinent to phenotype expression. To explore these mechanisms, my research plan consisted of the following four aims:
**Aim 1: To Quantify Cell Morphology and Proliferation of BSMCs in Response to Hydrostatic Pressure**

SMC phenotypic shift from contractile to synthetic phenotype often results in a shift from a more elongated morphology to a more rounded morphology.\textsuperscript{27, 31} The cell morphology and proliferation of BSMCs exposed to sustained hydrostatic pressure (7.5 cm H\textsubscript{2}O) for 4 and 48 hours, respectively, were analyzed through aspect ratio and cell density calculations.

**Aim 2: To Quantify Contractile-Marker Protein Expression by BSMCs in Response to Hydrostatic Pressure**

The expressions of these phenotypic marker proteins in BSMCs can indicate a possible phenotypic shift toward a more synthetic phenotype when expression is decreased.\textsuperscript{27, 28} The expression of contractile marker proteins, \(\alpha\)-smooth muscle actin and SM22, were quantified in BSMCs after sustained hydrostatic pressure (7.5 cm H\textsubscript{2}O) for 48 hours.

**Aim 3: To Determine the Effects of Hydrostatic Pressure on ERK \(\frac{1}{2}\) Intracellular Signaling Pathway Activation in BSMCs**

An increase in phosphorylated ERK \(\frac{1}{2}\), or activated ERK \(\frac{1}{2}\), has been linked with cell proliferation\textsuperscript{29, 35, 36} in previous literature which is a characteristic of a BSMC synthetic phenotype.\textsuperscript{27, 31} The activation of ERK \(\frac{1}{2}\) signaling pathway in BSMCs was quantified using western blotting after hydrostatic pressure exposure up to 3 hours.
Aim 4: To Determine the Involvement of ERK $\frac{1}{2}$ in Downstream Events Related to BSMC Phenotypic Shift

To potentially link downstream effects of hydrostatic pressure stimulus on BSMCs such as expression of contractile-marker proteins and morphological changes, a pharmacological inhibitor, U0126, was applied to BSMCs before hydrostatic pressure exposure (7.5 cm H$_2$O).
CHAPTER 3

MATERIALS AND METHODS

3.1 Cells

3.1.1 Cell Isolation, Culture, and Characterization

BSMCs were isolated from female Sprague-Dawley rats (250-350 grams) using the following techniques. Post anesthetization, bladders were harvested, cut open along the urachus, and pinned down on a silicone rubber coated petridish. The urothelium was removed from the smooth muscle layers using sterile scissors and forceps under a dissection microscope. After the urothelial and periphery tissue were removed and the remaining tissue was placed in a small beaker containing 10 ml sterile RPMI medium 1640 (Invitrogen: Carlesbad, CA) supplemented with 0.1% collagenase (Sigma Aldrich: St. Louis, MO) and 0.5% Trypsin EDTA (Invitrogen). The bladder was finely diced in this beaker using sterile scissors and then incubated at 37°C with gentle stirring for 30 minutes. The collagenase digested tissue was then filtered through a 100µm cell strainer (BD Biosciences: Bedford, MA) into a sterile 15 cc microcentrifuge tube (VWR: West Chester, PA). The filtrate was combined with 10 ml of fresh RPMI medium 1640 supplemented with 10% fetal bovine serum (FBS; HyClone: Logan, UT) and 1% penicillin-streptomycin (P/S; Invitrogen) and centrifuged using Centrifuge 5810 R (Eppendorf:Westbury, NY) at 1200 rpm for 3 minutes. The supernatant was discarded and the cell pellet was resuspended in the culture medium previously described. The cells were cultured in T-75 tissue culture polystyrene flasks (VWR) under
standard cell culture conditions (i.e. 37°C humidified 5% CO₂/95% air environment) with medium change every 3-4 days.

When necessary, the BSMCs were passaged by first rinsing with sterile phosphate buffered saline (PBS; Invitrogen) 3 times, and then incubating the cells with 2 ml of 0.5% Trypsin EDTA (Invitrogen) at 37°C for 5 minutes. The suspended cells were transferred to a sterile 15 cc tube along with culture medium in a 1:1 ratio to Trypsin EDTA (Invitrogen). Remaining cells were rinsed from the flask by adding 1 ml of PBS (Invitrogen) and then combined with the first cell suspension. The cells were centrifuged at 1500 rpm for 5 minutes, and following removal of the supernatant, were resuspended in RPMI medium 1640 supplemented with 10% FBS (HyClone) and 1% Penicillin-streptomycin (Invitrogen). Using a hemacytometer (Hausser Scientific: Horsham, PA) and Trypan Blue dye (MP Biomedicals, Inc: Solon, Ohio) a cell count was performed by counting the viable cells in four quadrants then calculating the total cell density. The cells were then split per desired density and seeded on polystyrene T-75 flasks.
3.2 Sustained Hydrostatic Pressure Experiments

3.2.1 Cell Substrate: Glass Coverslips

Glass coverslips (18 mm in diameter, product no. 48382-041: VWR) were cleaned by soaking in acetone (Fisher Scientific) for 10 minutes followed by 10 minutes of sonication, and then rinsed with distilled H₂O. The coverslips then soaked in 70% ethanol (Ricca Chemical Company: Arlington, TX) for 10 minutes followed by 10 minutes of sonication and rinsing in distilled H₂O. The coverslips were then autoclaved at 134°C for 30 minutes then dried in an oven at 80°C overnight.

3.2.2 Hydrostatic Pressure Columns

Hydrostatic pressure columns were prepared by sawing off the conical end of 50 cc conical tubes (VWR) with a band saw leaving both ends open. Caps that correspond with the 50 cc tubes were kept in duplicate to serve as a top (cover) and bottom (well for coverslips) to the pressure column (Figure 6). The columns and caps were sterilized by ethylene oxide exposure (Anprolene: Haw River, NC) for 24 hours.
Figure 6: Custom-made hydrostatic pressure columns.
3.2.3 **Cell Seeding**

BSMCs were seeded at a pre-determined density (10,000 or 200,000 cells per coverslip for morphological or molecular analysis respectively) on sterile glass coverslips (as described in section 3.2.1 Glass Coverslips) in RPMI medium 1640 supplemented with 10% FBS (HyClone) and 1% Penicillin-streptomycin (Invitrogen). The cells were seeded in a volume of 200 µl on a coverslip within a small dish and incubated for 4 hours to allow for attachment of cells. After attachment, 2 ml of medium were added to each dish containing an individual coverslip. After 24 hours, the medium were replaced with a low serum media consisting of RPMI medium 1640 supplemented with 1% FBS (HyClone) and 1% penicillin-streptomycin (Invitrogen). Incubate for 24 hours before exposing cells to sustained hydrostatic pressure.

3.2.4 **Exposure of Bladder Smooth Muscle Cells to Sustained Hydrostatic Pressure**

Prior to hydrostatic pressure exposure, media were changed with fresh low serum media. Seeded glass coverslips were attached to a sterile cap of a 50 cc tube with sterile vacuum grease (Dow Corning Corporation: Midland, MI). The inverted sterile column (described in 3.2.2 Hydrostatic Pressure Columns) was then screwed onto the cap and filled slowly with low serum medium to a pre-marked line that subjected cells to 7.5 cm H₂O pressure. A second cap was used to cover the top, and these cells were exposed to the sustained hydrostatic pressure for 30 minutes up to 48 hours. The cells maintained under atmospheric pressure (normal culture medium height = 0.3 cm) for the duration of experiments were used as the control.
3.2.5 **Exposure of Bladder Smooth Muscle Cells to MEK ½ Inhibitor**

In specified experiments, prior to pressure experiments, BSMCs were incubated in a 10µM solution of MEK ½ inhibitor (U0126; Cell Signaling Technology: Danvers, MA) in RPMI medium 1640 (Invitrogen) supplemented with 1% FBS (HyClone) and 1% Penicillin-streptomycin (Invitrogen) for two hours. The cells that were incubated with low serum RPMI medium (Invitrogen) supplemented with 1% FBS (HyClone) and 1% Penicillin-streptomycin (Invitrogen) served as the control.

3.2.6 **Analysis of Bladder Smooth Muscle Cell Responses to Sustained Pressure**

3.2.6.1 **Cell Morphology and Proliferation**

At the end of each prescribed time period, the seeded coverslips were removed from the columns. The cells were rinsed in 1x PBS (Invitrogen) and fixed in freshly prepared 2% paraformaldehyde (Sigma-Aldrich) at room temperature for 15 minutes. The excess aldehyde was quenched by adding 0.1 M glycine (Sigma-Aldrich) for 5 minutes. The fixative reagent was removed and cells were permeabilized in 0.1% TritonX-100 (Sigma-Aldrich) for 1 minute. The cells were then incubated with rhodamine-phalloidin (Invitrogen/Molecular Probes) diluted 1:100 in PBS (Invitrogen) for 1 hour to stain f-actin. The cells were then rinsed 3 times with PBS (Invitrogen), 5 minutes each rinse. After rinsing, the cells were then incubated with 300nM DAPI (Invitrogen) for 5 minutes to stain for cell nuclei and rinsed briefly. To keep from drying out, 1 ml of PBS (Invitrogen) was added to each dish and then imaged using fluorescence microscopy. An inverted microscope (Nikon Eclipse TE2000-S; Nikon Instruments: Melville, NY) and digital camera (MicroPublisher 3.3 RTV; Q Imaging: Surrey, BC Canada) at a magnification of 10X and using an objective of 10X were used to image the BSMCs.
3.2.6.1.1  **Image Analysis**

For each coverslip, 8-12 images of BSMCs were taken in each quadrant. The cell morphology was analyzed using Image Pro Plus 5.0 software (Media Cybernetics, Inc: Bethesda, MD) and the aspect ratio (major axis: minor axis) of 16 randomly selected cells in each image. The cell proliferation was quantified by counting all stained cell nuclei in each image (0.577 mm$^2$) and averaging. The data were reported as the number of cells per mm$^2$.

3.2.6.2  **Intra-Cellular Signaling Pathway and Phenotypic Marker Protein Activity**

3.2.6.2.1  **Cell Protein Extraction and Quantification**

After the hydrostatic pressure exposure for the prescribed time periods (30, 90, and 180 minutes for signaling molecules and 24 and 48 hours for phenotypic marker proteins), cell lysates were analyzed. For this purpose, cells were first rinsed with ice cold PBS (Invitrogen) 3 times and removed from the coverslips by scraping in PBS (Invitrogen). The cells were centrifuged at 300x g for 7 minutes at 4°C and following removal of the supernatant the cell pellet was resuspended in 150 µl of Complete Cell Extraction Buffer (CCEB). CCEB was prepared from 5 ml of Cell Extraction Buffer (CEB; Invitrogen), 250 µl of protease inhibitor cocktail (PIC; Sigma Aldrich), and 17 µl of 0.3 M stock solution of Phenylmethylsulfonyl fluoride (PMSF; Sigma Aldrich). The cell suspension in CCEB was then incubated on ice with periodic vortexing for 30 minutes. The lysates were clarified with centrifugation at 14,000 rpm at 4°C for 10 minutes and prepared for protein quantification or stored at -80°C.
The protein concentration in each sample was quantified using a protein assay kit based on the Bradford Method\textsuperscript{69} and following the manufacturer’s instructions (Bio-Rad: Hercules, CA). In each well of a transparent 96-well flat bottom plate (Corning Incorporated: Corning, NY), 200 µl of dye reagent (Bio-Rad) was added along with 10 µl of either a BSA standard (Sigma-Aldrich) or a 10x dilution of each sample. The absorbance was measured at a 595 nm wavelength using a GENios Plate Reader and Magellan Software (Tecan: Research Triangle Park, NC). A standard curve was created at each time the assay was performed. Using the linear regression and absorbance values, the concentration of each sample was determined.

3.2.6.2.2  \textit{SDS PAGE}

A polyacrylamide gel [separation 12%: 3.5 ml dH\textsubscript{2}O, 2.5 ml 1.5 M Tris/HCl (pH 8.8) 100 µl 10% SDS (Bio-Rad), 4.0 mL Acryl/Bis (Bio-Rad), 50 µl 10% APS (Sigma-Aldrich), 5 µl TEMED (Bio-Rad), stacking 4%: 6.1 ml dH\textsubscript{2}O, 2.5 ml 0.5 M Tris/HCl (pH 6.8), 100 µl 10% SDS (Bio-Rad), 1.3 ml Acryl/Bis (Bio-Rad), 50 µl 10% APS, and 10 µl TEMED (Bio-Rad)] was cast using a Mini-PROTEAN 3 System (Bio-Rad) with a pair of glass plates with a 1.5mm spacer plates.

Samples (5-20 µg protein) were mixed with a 2X sample buffer (1% sodium dodecyl sulfate (SDS; Bio-Rad), 10% Glycerol (Sigma-Aldrich), 50mM Dithiothreitol (DTT; Bio-Rad), 0.12 mM Trs/HCl at a pH of 7.1 (Bio-Rad), distilled water (dH\textsubscript{2}O), and a small pinch of Bromophenol Blue (Bio-Rad)) at a 1:1 dilution and heated at 95°C to allow for the protein to denature for 5 minutes. After cooling, 40 µl (20 µl protein) of the protein-buffer mixture and 10 µl of molecular weight ladder (Precision Plus Protein Unstained Standard; Bio-Rad) were loaded and the gels were subjected to a constant voltage in 10% 1x Tris/Glycine/SDS Buffer (Bio-Rad) at 200V for 1 hour.
Separated protein bands on the acrylamide gels were transferred to polyvinylidene difluoride (PVDF; Bio-Rad) membranes using a wet blot method. The gels and membranes were first incubated in Transfer Buffer [10% 10x Tris/Glycine Buffer (Bio-Rad), 20% methanol (Sigma-Aldrich) in dH$_2$O] to equilibrate for 30 minutes. The gels and membranes were then loaded into the transferring cartridge of the Bio-Rad kit along with an ice pack and subjected to a constant voltage of 100 V for 1 hour with gentle stirring of the buffer. The membranes were stained with Ponceau S [0.5% Ponceau S (MP Biomedical), 1.0% Acetic Acid (Fisher Scientific) in dH$_2$O] for 1 minute to ensure that all protein bands transferred.

### 3.2.6.2.3 Western Blotting

Western blot techniques were used to determine the relative amounts of the proteins of interest (Figure 7) present in each band. The PVDF membranes containing the separated protein bands (as described in section 3.2.6.2.2 SDS PAGE) were incubated in either a 10% nonfat dry milk (Bio-Rad) or 5% bovine serum albumin (BSA; Sigma Aldrich) blocking solution in 0.01% Tween/PBS at room temperature for 1 hour to block non-specific binding sites for proteins. The membranes are then rinsed with a Tween/PBS rinse [0.2% Tween-20 (Bio-Rad) in PBS (Invitrogen)] for 5 minutes 3 times. The membranes were then incubated with primary antibody for the protein of interest at an appropriate dilution (Figure 7) in either a 5% BSA/Tween/PBS solution or 2% nonfat dry milk/Tween/PBS solution overnight at 4°C. The membranes were then rinsed 3 times for 15 minutes each in the Tween/PBS rinse before incubating with secondary antibodies at dilution of 1:8000 in the presence of 1 µl Streptactin-HRP (Bio-Rad), in either 5% BSA/Tween/PBS solution or 2% nonfat dry milk/Tween/PBS solution at room temperature for 1 hour. The membranes were then rinsed 3 times for 15 minutes each in Tween/PBS rinse before incubating in a chemiluminescence reagent for 10 minutes to tag the secondary antibodies following the manufacturers’ instructions (Immun-Star HRP Substrate Kit: Bio-Rad).
<table>
<thead>
<tr>
<th>Protein of Interest</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho ERK ½</td>
<td>Rabbit polyclonal to ERK1 + ERK2 (phosphoERK) (ab16869; Abcam: Cambridge, MA)</td>
<td>Goat Anti-rabbit IgG-HRP (sc-2004; Santa Cruz Biotech: Santa Cruz, CA)</td>
</tr>
<tr>
<td></td>
<td>(1:1000)</td>
<td>(1:8000)</td>
</tr>
<tr>
<td>Total ERK ½</td>
<td>Mouse monoclonal to ERK1 + ERK2 (ab36991; Abcam) (1:1000)</td>
<td>Goat Anti-mouse IgG-HRP (sc-2005; Santa Cruz Biotech) (1:8000)</td>
</tr>
<tr>
<td>Alpha-smooth muscle actin</td>
<td>Mouse Monoclonal Anti-αSMC Actin (a2547; Sigma Aldrich) (1:500)</td>
<td>Goat Anti-mouse IgG-HRP (sc-2005; Santa Cruz Biotech) (1:8000)</td>
</tr>
<tr>
<td>SM-22</td>
<td>Goat polyclonal to SM22 alpha (ab10135; Abcam) (1:1000)</td>
<td>Bovine anti-goat IgG-HRP (sc-2378: Santa Cruz Biotech) (1:8000)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAPDH (FL-335) (sc-25778; Santa Cruz Biotech) (1:200)</td>
<td>Goat Anti-rabbit IgG-HRP (sc-2004; Santa Cruz Biotech) (1:8000)</td>
</tr>
</tbody>
</table>

Figure 7: Corresponding Primary and Secondary Antibodies and Dilutions.
3.2.6.2.4 **Membrane Stripping for Re-Probing**

Prior to re-probing with a different primary antibody, the PVDF membranes were stripped following a method adapted from Abcam’s mild stripping procedure. The membranes were incubated in a mild stripping buffer (0.2M Glycine (Sigma-Aldrich), 3.5 mM SDS (Sigma-Aldrich), 1% Tween-20 (Acros Organics: Morris Plains, NJ) in dH2O at pH 2.2) for 10 minutes. Following this incubation, the membranes were rinsed twice with 10X PBS (Invitrogen) for 10 minutes each, and then rinsed twice in Tween/PBS rinse solution for 5 minutes each. The membranes were then reblocked in either 5% BSA blocking solution or 10% nonfat dry milk blocking solution (as described in 3.2.6.2.3 Western Blots) at room temperature for 1 hour. The PVDF membranes were then incubated with the next appropriate primary antibody and secondary antibody sequence (Figure 7).

3.2.6.2.5 **Image Analysis**

Images of the chemiluminescence labeled membranes were taken using FluorChemSP (Alpha Innotech: San Leandro, CA). The intensity of each protein band was analyzed to detect changes in protein expression using spot densitometry using AlphaEase FC software (Alpha Innotech: San Leandro, CA). The quantified protein expression of phosphorylated ERK ½ was normalized to by dividing by the quantified protein expression of total ERK ½ to determine the amount of ERK ½ activated after hydrostatic pressure exposure. Also, the intensity of alpha-smooth muscle actin and SM-22 protein expressions were normalized by dividing by the intensity of GAPDH expression present after hydrostatic pressure exposure.
3.3 **Statistical Analysis**

All experiments were run in duplicate and were repeated at a minimum of three separate times. Numerical data were analyzed using Analysis of Variance (ANOVA) and Tukey’s Test in SigmaPlot software (Systat Software: Chicago, IL); values of $p < 0.05$ were considered significant.
CHAPTER 4

RESULTS

4.1 BSMC Morphology and Proliferation in Response to Hydrostatic Pressure

4.1.1 BSMC Morphological Response to Hydrostatic Pressure in the Presence of a MEK ½ Inhibitor

The morphological response of BSMCs to hydrostatic pressure was qualitatively reported through representative images (Figure 8). To quantitatively analyze the morphological changes in response to sustained hydrostatic pressure, histograms were created to visualize trends (Figure 9). When compared to the control (in the absence of hydrostatic pressure and MEK ½ inhibitor), BSMCs that were exposed to sustained hydrostatic pressure for 4 hours in the absence of an inhibitor exhibited an increase in number of cells within the population with an aspect ratio between 1.0 and 2.0 and a decrease in cells with an aspect ratio of 4.0 or higher occurred with the application of hydrostatic pressure (Figure 9-A and C). This trend represents a greater number of BSMCs with a rounded morphology and less BSMCs with an elongated morphology in cultures exposed to sustained hydrostatic pressure in vitro.

To further analyze the morphological response of BSMCs to sustained hydrostatic pressure, box plots were created to observe the aspect ratio percentiles along with statistical observations such as the mean and median aspect ratios (Figure 10). The mean and median aspect ratios are greater in controlled
conditions when compared to conditions of sustained hydrostatic pressure. Moreover, the 95% percentile under controlled conditions contained BSMCs with a much greater aspect ratio range suggesting BSMCs with a more elongated morphology which is reduced under sustained hydrostatic pressure conditions.

When compared to the control, BSMCs subjected to sustained hydrostatic pressure in the presence of U0126, a MEK $\frac{1}{2}$ inhibitor, exhibited a slight decrease in the percentage of elongated morphology cells and increase in rounded morphology cells. This trend was similar to that of the cells exposed to pressure in the absence of the MEK $\frac{1}{2}$ inhibitor.

### 4.1.2 BSMC Proliferative Response to Hydrostatic Pressure

The proliferative response of BSMCs to sustained hydrostatic pressure (7.5 cm H$_2$O) for 48 hours was quantified through cell density (cells/mm$^2$) measurements (Figure 11). The cell density of BSMCs subjected to hydrostatic pressure for 24 hours was similar to the no pressure control at the same time point. However, the cell density in BSMCs subjected to hydrostatic pressure for 48 hours exhibited a significant increase (p< 0.05 n=3) when compared to the control after 48 hours (Figure 11 B).
Figure 8: Cell morphology of rat BSMC exposed to sustained hydrostatic pressure (7.5 cm H₂O) for 4 hours. At the end of experiments BSMCs were fixed and stained with rhodamine phalloidin and DAPI for actin filaments and nuclei, respectively. A) Cells maintained under control (no pressure) conditions in the absence of MEK ½ inhibitor, U0126, B) Cells maintained under control (no pressure) conditions in the presence of MEK ½ inhibitor, C) Cells exposed to pressure in the absence of MEK ½ inhibitor, D) Cells exposed to pressure in the presence of MEK ½ inhibitor. The arrows indicate examples of major and minor axis measurements used to calculate the aspect ratios. 100 x magnification.
Figure 9: Histograms of aspect ratios of BSMC exposed to sustained hydrostatic pressure for 4 hours. (A) BSMCs under controlled conditions of minimal hydrostatic pressure and an absence of MEK ½ inhibitor, U0126. (B) BSMCs under minimal hydrostatic pressure after exposure of a MEK ½ inhibitor. (C) BSMCs after sustained hydrostatic pressure for 4 hours without exposure to a MEK ½ inhibitor. (D) BSMCs after sustained hydrostatic pressure for 4 hours with a prior exposure to a MEK ½ inhibitor. Data are mean ± SD; analyzed using t-test; *p<0.05; n=3 experiments; 384 cells per group per experiment.
Figure 10: Aspect ratio of BSMCs exposed to sustained hydrostatic pressure for 4 hours. The boxes represent the aspect ratios of the lower quartile and higher quartile of the cell population. The dots represent the aspect ratio in the 5th and 95th percentile of the cell population. The mean and median aspect ratio of the cell population is also indicated. (--) BSMCs under controlled conditions of minimal hydrostatic pressure and an absence of MEK \( \frac{1}{2} \) inhibitor. (+) BSMCs under minimal hydrostatic pressure after exposure of a MEK \( \frac{1}{2} \) inhibitor. (++) BSMCs after sustained hydrostatic pressure for 4 hours without exposure to a MEK \( \frac{1}{2} \) inhibitor. (++) BSMCs after sustained hydrostatic pressure for 4 hours with a prior exposure to a MEK \( \frac{1}{2} \) inhibitor. Data are percentile of cell population; range of 832-1024 cell population plotted.
Figure 11: Proliferative response of BSMCs exposed to sustained hydrostatic pressure for 24 and 48 hours. At the end of experiments, cells were fixed and stained with DAPI for nuclei, which were then counted using ImagePro software. Data are mean ± SD; analyzed using ANOVA and paired t-test; *p<0.05 when compared to the control; n=3 experiments; 16 random fields per sample, 2 samples per group.
4.2 Activation of ERK ½ Intracellular Signaling Pathway

The phosphorylation, or activation, of ERK ½ intracellular signaling pathway in SMCs has been linked to mechanical stimuli such as stretch\textsuperscript{22,26,36} in previous studies. In this study, the activation of ERK ½ in BSMCs exposed to sustained hydrostatic pressure for up to 3 hours was quantified through western blotting and spot densitometry of chemiluminescence imaging (Figure 12). When compared to the control, BSMCs exposed to sustained hydrostatic pressure exhibited up to a 2-fold increase in the activation of ERK ½ at 30, 90, and 180 minutes. In 3 of the 4 experiments conducted (samples 1-3), the peak activation of ERK ½ occurred at the 180 minutes time point, but in one other experiment (sample 4) ERK ½ activation occurred earlier and peaked at 90 minutes (Figure 12). The mean increase in activation of ERK ½ in samples 1-3 in response to sustained hydrostatic pressure for 180 minutes is significantly (p< 0.05 n=3) greater (1.6-fold) than that of the control (baseline, at 0 minute) and at 30 minutes (Figure 13).

4.3 Expression of Contractile-Marker Proteins α-SMA and SM22

BSMCs pre-treated with MEK ½ inhibitor, U0126, were exposed to hydrostatic pressure (7.5 cm H\textsubscript{2}O) for 48 hours and the expression of contractile proteins α-SMA and SM22 were quantified. When compared to the control (no pressure in the absence of U0126), BSMC expression of α-SMA and SM-22 was similar in all other groups (no pressure in the presence of U0126, and cells exposed to sustained hydrostatic pressure both in the absence and presence of U0126) at 48 hours (Figure 14).
Figure 12: Activation of ERK ½ in rat BSMCs after sustained hydrostatic pressure for 4 hours. Spot densitometry was used to quantify the ratio of phosphorylated ERK ½ expression and total ERK ½ expression. The fold increase was calculated by dividing the ratio of phosphorylated ERK ½ and total ERK ½ expression at a specified time point with that of the control. (A) Chemiluminescence images for ERK ½ activation; (B) ERK ½ activation in BSMCs after sustained hydrostatic pressure from 4 independent experiments. The peak activation occurred at 180 minutes for 3 of the 4 experiments.
Figure 13: Average ERK ½ activation from 3 of 4 pressure experiments. Data are mean ± SD; analyzed using ANOVA; *p<0.05 when compared to the control; **p<0.05 when compared to the 30 minute group; n=3.
Figure 14: Expression of contractile proteins, α-SMA and SM22, in rat BSMCs after exposure to sustained hydrostatic pressure for 48 hours. Spot densitometry was used to quantify contractile protein expression and normalized to GAPDH protein expression. (A) Representative western blot spot densitometry for α-SMA and SM22 protein expression; (B) Expression of contractile-marker proteins α-SMA and (C) SM22 after exposure to hydrostatic pressure and in the presence of a MEK ½ inhibitor. Data are mean ± SD; analyzed using ANOVA; n=4.
CHAPTER 5

DISCUSSION

This study investigated the effect of sustained hydrostatic pressure on BSMCs \textit{in vitro}. Hydrostatic pressure is one of many mechanical stimuli that result from filling and voiding cycles of the bladder. Since a number of previous \textit{in vivo} studies demonstrated that urinary diversion (removal of pressure load) leads to bladder atrophy\textsuperscript{46-49} while excessive pressure (due to obstruction) led to hypertrophy,\textsuperscript{34} it was hypothesized that pressure is an important stimulus for the health of the bladder. For this reason, using a custom setup, rat BSMCs were exposed to 7.5 cm H\textsubscript{2}O hydrostatic pressure for various time periods and their response was examined. The level of hydrostatic pressure used in this study was chosen since it is within the physiological range (< 10 cm H\textsubscript{2}O)\textsuperscript{4-6} to which bladder tissue is exposed, but when compared to our control (typical cell culture conditions: 0.3 cm H\textsubscript{2}O), represents elevated hydrostatic pressure. A similar experimental setup was used in previously published studies to expose BSMCs\textsuperscript{6, 65} and vascular endothelial cells\textsuperscript{71} to hydrostatic pressure; this design allowed for application of hydrostatic pressure to cultured cells with minimal variation between samples, and no change in pH, but lower pO\textsubscript{2}, and slightly elevated pCO\textsubscript{2} values in collected medium exposed to up to 15 cm H\textsubscript{2}O hydrostatic head after 7 hours when compared to the control (3 mm H\textsubscript{2}O).\textsuperscript{71} While the changes in pO\textsubscript{2} and pCO\textsubscript{2} on long term may have a slight effect on cellular responses such as proliferation, Acevedco et al reported that the changes observed were not severe enough to alter cellular responses.\textsuperscript{71} In the present study, the longest experimental period was 2 days, and since gas pressure changes occur via diffusion, it was determined that any changes observed were due to hydrostatic pressure.
In the present study, the morphological and proliferative responses as well as contractile protein expression and ERK ½ activity of BSMCs subjected to hydrostatic pressure were examined since these events characterize SMC phenotype. The time points were chosen based on previous data from our lab. For example, a time-course experiment demonstrated a notable change in cell morphology after 4 hours of sustained hydrostatic pressure exposure (unpublished). Likewise, previous studies in our lab demonstrated a change in cell proliferation and in α-SMA expression in BSMCs at 24 and 48 hours at the earliest, respectively, thus leading to the time points chosen for this study.

5.1 Morphological and Proliferative Response of BSMCs to Hydrostatic Pressure

Both morphological changes and increased cell proliferation have been reported to be induced by various sources of mechanical stimuli that bladder tissue is routinely subjected to during micturition, such as hydrostatic pressure and cyclic stretch. These cellular alterations are characteristics of a phenotypic modulation toward a synthetic phenotype and are associated with hypertrophy and hyperplasia altering bladder tissue structure and integrity. In the present study, the morphological and proliferative responses to hydrostatic pressure (7.5 cm H₂O) were evaluated through cell aspect ratio and cell density calculations, respectively. A BSMC exhibiting a more rounded, spread-out morphology compared to an elongated, spindle-like morphology has been suggested to be indicative of a phenotypic shift toward a more synthetic phenotype. Likewise, BSMCs with increased proliferative rates compared to their control are characteristic of a shift toward this synthetic phenotype. In vitro as well as in vivo, BSMCs
can represent either phenotype with a vast continuum of intermediates at any given time. This morphological study analyzes any apparent trends of a phenotypic shift with the understanding that all cells in culture do not express of the same phenotype at a given time.

The results of the present study demonstrate that BSMCs subjected to sustained hydrostatic pressure for 4 hours showed a population shift in morphology from elongated to more rounded morphology. This was evidenced by more cells (48%) exhibiting an aspect ratio of 1.0- 2.0 and fewer cells (6.5%) exhibiting an aspect ratio of greater than 4.0, following exposure to sustained hydrostatic pressure exposure (7.5 cm H$_2$O), when compared to the control (40%, 11%). While these are the first observations of BSMC morphological response to pressure, others have investigated and reported changes in SMC morphology in response to mechanical stimuli. For example, Roby et al reported that BSMCs within 3D collagen constructs exposed to sustained tension for 48 hours exhibited significantly higher mean and median aspect ratios, suggesting a more contractile phenotype, than that of the control.$^{27}$ Qu et al reported that rat vascular SMC exhibited a change in morphology from a more spread-out and stellate state to a spindle-like morphology in response to cyclic strains (10%) at 0.5, 1, and 2 Hz for 24 hours when compared to a static control.$^{26}$ This change in morphology was abolished in the presence of SB 202190, an inhibitor of the p38 signaling pathway, but not when ERK ½ or Akt were inhibited by PD 98059 and Wortmannin.$^{26}$ Similarly, in the present study, there was an observed change in cell morphology in response to hydrostatic pressure, even in the presence of MEK ½ inhibitor, U0126, when compared to the control; suggesting the inhibition of ERK ½ does not affect changes in cell morphology. These findings suggest a morphological response, characteristic of a phenotypic shift, of SMCs to various sources of mechanical stimuli including hydrostatic pressure, sustained tension, and cyclic stretch, possibly mediated through p38 signaling pathway.
In addition to the cell morphology change, the results of the present study provide evidence that exposure of BSMCs to sustained hydrostatic pressure (7.5 cm H$_2$O) for 48 hours led to a significant increase in cell proliferation when compared to the control (Figure 11). While these results are in agreement with Haberstroh et al., our data showed a BSMC proliferative response to occur more rapidly (within 48 hours) when compared; these authors reported a significant increase in lamb BSMC density following exposure to sustained hydrostatic pressure (4, 6, and 8.5 cm H$_2$O) for 5 and 7 days. The difference in time of proliferative response between these studies may be due to the original cell density prior to experimentation, 982 cells/cm$^2$ versus 3900 cells/cm$^2$ in the present study. A greater original cell density would create more cell to cell contact and communication which could alter rates of cell response to mechanical stimuli. Other possible explanations for the different time course in proliferative response of BSMCs to hydrostatic pressure include species, rat versus sheep, as well as the serum concentration in which the cells are subjected to hydrostatic pressure. Haberstroh et al used a similar mechanical set up as the present study, but with culture media containing 10% FBS, where in the present study 1% FBS was used. Using medium containing only 1% FBS ensures that the BSMCs are all at the G0 phase in the cell cycle of growth at the onset of pressure application. In addition, this precaution measure was taken so that the proliferative response of the BSMCs is due solely to the hydrostatic pressure stimulus and not masked by the serum-induced cell proliferation, which may explain the different outcomes between these two studies.

Morphological and proliferative responses of SMCs to mechanical stimuli may be mediated, at least in part, by activation of the members of Rac-MAPK signaling pathways. For example, Katsumi et al investigated the response of vascular SMC cell morphology mediated by Rac activation in response to strain. This group reported more rounded, polarized cell morphology when SMCs are subjected to tension possibly mediated through Rac down regulation. The rounded cell morphology observed occurred through lamellipodia inhibition, where this dense meshwork of actin fibers retracted over the
cell surface, known as ruffling, as opposed to extending outward from the cell elongating the morphology. The relationship between SMC morphology activated by mechanical stimuli and signaling pathways is being investigated in various ways by other research groups as well as ours. Moreover, Stover et al reported that BSMCs in vitro subjected to cyclic pressure (amplitude of 40 cm H$_2$O maximum) at a frequency of 0.1 Hz for 24 hours also exhibited an increase in cell proliferation compared to static controls. The PI3K/Akt pathway was determined to be a potential signaling pathway that mediated the cyclic mechanical stimulus and observed increase in cell proliferation. Aitken et al also reported a significant increase (800%) in BSMC proliferation in response to cyclic stretch followed by a relaxation period (4 hrs stretch + 20 hrs relaxation and 16 hrs stretch + 8 hrs relaxation) when compared to 24 hours of continuous stretch and a static control. The stretch-induced cell proliferation was inhibited (2-fold) in the presence of ERK½ inhibitor, PD98059, suggesting involvement of ERK activation in this event. Although there are a number of signaling pathways that can potentially mediate SMC response to hydrostatic pressure, the present study chose to focus on the ERK½ pathway.

### 5.2 BSMC Signal Transduction Pathway in Response to Hydrostatic Pressure

In the present study, BSMCs subjected to sustained hydrostatic pressure in vitro for 3 hours exhibited up to a 2-fold increase in activated ERK½ expression (Figure 12). Three of the four samples (samples 1-3) exhibited a peak in activation of ERK½ after 180 minutes of sustained hydrostatic pressure, while one (sample 4) exhibited this peak activation after 90 minutes (Figure 12). This variation
in peak activation in response to hydrostatic pressure is possibly due to the cell batch and passage number differences. Increasing the numbers of trials and examination at additional time points will probably allow determination of more detailed time course. However, the results to date certainly demonstrate that BSMCs exhibit ERK ½ activation in response to sustained hydrostatic pressure. The ERK ½ family of MAPK convert extracellular stimuli into signals that control gene expression, cell proliferation, and dedifferentiation in numerous types of cells. Aitken et al report a 5-fold and 3-fold increase in phosphorlyated ERK ½ expression in vitro in response to statically stretched BSMCs (5 % elongation) and cyclically stretched BSMCs (5% elongation) for 60 minutes, respectively. The source of mechanical stimuli, cyclic stretch versus hydrostatic pressure, could be rationale for the more rapid response and greater magnitude of activation of ERK ½ (within 60 minutes) when compared to the current study (180 minutes).

ERK ½ activation in response to hydrostatic pressure may potentially be mediated by integrins and cell adhesion to the ECM. Integrins have been shown to activate, through cell surface receptor interactions on various ECM proteins, signaling pathways such as focal adhesion kinase (FAK), c-src, and the Ras/ERK pathway. FAK and c-Src have been shown to be important regulators of cellular responses to mechanical stimuli, such as shear stress. Integrins aggregate and cluster in the focal adhesion sites to transduce mechanical stress into chemical signals that trigger the accumulation of FAK and Src family PTKs leading to the activation of MAPKs, such as ERK ½. This integrin-mediated ERK ½ activation is suggested to occur through the following process: (1) integrin-ECM interactions induce phosphorylation of FAK on Tyr397 which promotes the binding of Src family protein tyrosine kinase (PTKs) to FAK; (2) c-Src further phosphorylates FAK on Tyr925 to create an Src homology 2 (SH2)-domain-binding site for the Grb2 adaptor protein, (3) thus potentially leading to the phosphorylation, or activation, of ERK ½ through the Grb-2-Sos-Ras pathway. In vascular research, Morla et al concluded that SMC proliferation and phenotype are strongly controlled by FAK mediated integrin signaling when
culturing VSMCs on fibronectin and laminin surfaces. However, the exact relationship between FAK activation and ERK ½ activation is still controversial. Previous studies report that ERK ½ activation is a downstream effect of FAK activation, while other systems report that ERK ½ activation can occur in the absence of FAK activation, but in the presence of growth factors or the Shc protein. To advance our understanding of the mechanism underlying the ERK ½ activation in BSMCs subjected to hydrostatic pressure, investigating integrin-mediated FAK may be necessary.

5.3 Contractile-Marker Protein Expression in BSMCs Exposed to Hydrostatic Pressure

Results of the present study indicated that expression of contractile proteins, α-SMA and SM22, was similar in the BSMCs exposed to sustained hydrostatic pressure for 48 hours when compared to the control. Expression of these contractile marker proteins has been previously used as an index of a phenotypic shift seen in SMCs in addition to the morphological and proliferative changes, while the exact relationship between cell proliferation and contractile-marker protein expression is still partially understood. Several studies have demonstrated that exposure of SMCs to platelet-derived growth factor (PDGF) can cause decreased levels of α-SMA expression through activation of the ERK ½ intracellular signaling pathway. Activation of the ERK pathway has also been shown to cause an increase in cell proliferation, but it has been suggested that the downstream pathways leading to changes in cell proliferation and α-SMA expression are separate. Stegemann et al reported an increase in cell proliferation of vascular SMC but no change in α-SMA expression when cultured in the presence of
PDGF (5.0 ng/ml) for 6 days in a 2D monolayer on collagen Type I coated (2.0 mg/ml) polystyrene culture flasks. When vascular SMC were cultured in 3D collagen Type I gels (2.0 mg/ml), exposure to PDGF (5.0 ng/ml) for 6 days caused both an increase in cell proliferation and a decrease in α-SMA expression. These findings suggest that cell proliferation and α-SMA expression of SMCs are not governed by a single pathway, but may also be regulated by the environment in which they are cultured (2D versus 3D). Additionally, Qu et al reported that the inhibition of ERK ½ and Akt by PD 98059 and wortmanin, respectively, did not abolish the increased expression of contractile proteins, α-SMA, calponin, and SM22, nor cell morphology change in vascular SMCs subjected to cyclic strain (10% elongation), for 24 hours. These results provide further evidence that there may not be a discernable correlation between mechanically induced ERK ½ activation and contractile protein expression or cell morphology. However, Zeidan et al reported somewhat contradicting results from the present and these previous studies (Stegemann and Qu et al); contractile protein expression increased and inhibition of ERK ½ with PD-98059 resulted in decreased expression of α-SMA and SM22 in strips of rat portal veins subjected to sustained stretch (0.6 g) for 3 days. While these results suggest a direct link between mechanical activation of ERK ½ and subsequent up regulation of contractile marker proteins, unlike our study, this study used whole tissue that would contain a variety of cell types. Therefore, the observed response to mechanical loading could come from other cells besides SMCs (e.g. endothelial cells, fibroblasts) or a combination of these cells. To fully understand if a link between ERK ½ activation by mechanical stimuli and contractile protein expression in SMCs exists, further investigation is necessary. Contractile protein expression in BSMCs in response to sustained hydrostatic pressure should be analyzed at both mRNA and protein-levels, for additional time periods other than 48 hours, using more effective approaches to inhibit ERK ½.
In the present study, BSMCs exposed to sustained hydrostatic pressure exhibited morphological and proliferative responses that indicate a phenotypic shift. Moreover, the activation of ERK ½ intracellular signaling pathway occurred within 180 minutes of hydrostatic pressure exposure (7.5 cm H$_2$O). The expression of contractile proteins, however, did not agree with the other morphological and proliferative characteristics of SMC phenotypic modulation within the time point examined in this study (48 hours). These in vitro results suggest that the tissue-level changes such as hypertrophy in pathological bladders may result from SMC phenotypic change triggered by elevated pressure and mediated by ERK ½ activation. Future study recommendations include:

- To explore the expression of contractile proteins in BSMCs subjected to hydrostatic pressure for multiple time points and their relationship with ERK ½ activation.
  - Rationale: The expression of contractile proteins should be observed under a more physiological time course and under inhibition of ERK ½ to determine the relationship between this cell signaling pathway and protein expression in BSMCs exposed to hydrostatic pressure.

- To investigate the proliferative response of BSMCs subjected to hydrostatic pressure under inhibition of ERK ½
  - Rationale: The proliferative response in the presence of an ERK ½ inhibitor of BSMCs subjected to hydrostatic pressure should confirm the relationship between this signaling pathway and downstream events such as cell proliferation and morphological changes.
• To investigate the p38 signaling pathway as a possible mediator of BSMCs response to sustained hydrostatic pressure.
  
  o *Rationale:* A previous study reported that the p38 (another MAPK) pathway mediates downstream responses (morphological, proliferative, and contractile protein expression) of vascular SMCs subjected to cyclic stretch.\(^2^6\) This pathway could also be activated through hydrostatic pressure exposure in BSMCs leading to the downstream morphological and proliferative responses reported in the present study.

• To investigate collagen synthesis in BSMCs subjected to sustained hydrostatic pressure.
  
  o *Rationale:* Excess collagen synthesis is a characteristic of a synthetic phenotype and leads to fibrosis in bladder tissue.\(^3\) Investigating collagen synthesis of BSMCs subjected to hydrostatic pressure would provide further evidence of a phenotypic shift related to cellular and molecular changes within the bladder tissue.

• To explore the response of BSMCs to multiple levels of sustained hydrostatic pressure.
  
  o *Rationale:* In the present study, we report the effect of only one (physiological) level of hydrostatic pressure on BSMCs. Multiple levels of hydrostatic pressure that range between a physiological level representative of an empty bladder (4 cm H\(_2\)O) and pathological levels (20 and 40 cm H\(_2\)O) would provide more insight on how the BSMCs responses lead to cellular and molecular changes within the bladder tissue.

• To explore the response of BSMCs from different animal species other than rat.
  
  o *Rationale:* While rats are commonly used in studies of urological complications, other species of BSMCs could possibly respond to hydrostatic pressure differently than rat BSMCs. By using BSMCs from a higher order of species, we can determine a more accurate hypothesis about the response of human BSMCs to hydrostatic pressure.
To explore the response of BSMCs to ex vivo bladder distension and in vivo animal testing.

- **Rationale:** In vivo, BSMCs are not directly subjected to hydrostatic pressure due to the urothelium and lamina propria tissue layers. Experiments that explored ex vivo bladder distension and in vivo animal testing could more accurately explore the physiological effect of hydrostatic pressure on BSMCs.

These study recommendations will provide further insight into how hydrostatic pressure regulates BSMC functions that can lead to cellular and molecular changes within the bladder tissue. The results will then guide the design of the prevention and treatment measures for bladder hypertrophy and/or hyperplasia often seen under pathological conditions such as spinal cord injury and diabetes.
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