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The Effect of Mechanical Force on Gene Expression of Human Bladder Smooth Muscle Cells

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The Effect Of Mechanical Force on Gene Expression of Human Bladder Smooth Muscle Cells

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Abstract

Cells are able to sense their physical surroundings through mechanotransduction. In doing so, they can translate the mechanical forces and deformations into a wide range of biochemical signals and genetic programs which can adjust their structure and extracellular environment. The urinary bladder is a physically active organ that undergoes periodic stretching as part of its normal function. In the bladder, abnormal pressure from a variety of pathologic conditions can result in forces which, over time, can alter the genetic expression of the proteins of the extracellular matrix. To determine the role that stretching or mechanical deformation may play in altering the synthetic phenotype of bladder wall cells, a series of experiments were carried out to quantify several extracellular matrix messenger ribonucleic acids (mRNAs) and their corresponding protein levels after mechanical challenge. This report first summarizes the current knowledge about regulation of cell function by mechanical forces and then presents data on the effect(s) of mechanical force on several mechanosensitive genes expressed by human bladder smooth muscle cells (BSMCs).

Using this experimental system, we demonstrated that BSMCs were acutely sensitive to mechanical deformation and showed alteration of many structural and extracellular matrix genes. This study demonstrates that BSMC extracellular matrix secretory phenotype can be altered by the mechanical deformation experienced by these cells. These data support the concept that deformation of the bladder wall affects the secretory phenotype of BSMCs and can result in an altered extracellular matrix composition.

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1. INTRODUCTION:

The purpose of this project is to define, at the molecular level, the process by which gene expression of the extracellular matrix is regulated by mechanical forces in the Human Bladder Smooth Muscle cells (BSMCs). The goal is to first localize several functionally distinct transmembrane proteins; Sarcoglycans (α, β, γ, δ and ε), cytoskeletal proteins Vimentin, and Desmin to verify their presence in the cultured BSMCs using fluorescent-labeled antibodies specific for each protein. The sarcoglycans are primarily responsible for transferring intracellular force generated by the interaction of actin and myosin while the extracellular proteins are responsible for linking the cells to the extracellular matrix. These proteins comprise several collagen matrix proteins, e.g., type I, type III and type IV collagens and an integrin-receptor (11, 64). To evaluate the functions of these proteins, we will subject the BSMCs, using a cell strain apparatus, to a level of force which mimics those experienced by the bladder wall during the filling/emptying cycles. This investigation seeks to find supporting evidence to determine if altered mechanical stimulation of bladder smooth muscle cells results in an up-regulation of type III collagen as well as additional genes which mediate the transfer of intracellular force to the extracellular environment.

1.1 Significance:

The hardening or stiffening seen in diseased tissue is typically associated with an overproduction of the abundant matrix protein collagen (11) which is the most abundant protein in the body (24). A challenge to current research is to elucidate the biochemical steps that initiate an over-accumulation of collagen and associated proteins of the

extracellular matrix. It is plausible that drugs and other interventions could be used to stop or slow the process and remove or dramatically decrease the deleterious effects of the excess matrix that mediate fibrotic disease. Since the collagen genes can be regulated and influenced by force (7), it is likely that specific steps in the control process can be targeted either by drugs or other interventions to retard its accumulation (40). Therefore, the ideal effect of such research would be to define the mechanism by which proteins of the extracellular matrix are up-regulated causing an overproduction of collagen.

Diseases such as pulmonary, liver, kidney, and bladder fibrosis, systemic sclerosis and urethral strictures are characterized by the accumulation of excess collagen and other connective tissue structural macromolecules (5). Specifically, in the bladder wall, the additional connective tissue can interfere with the normal physiologic operating pressures of the bladder which, in turn, can be transmitted in a retrograde fashion to the kidneys, ultimately compromising their function and leading to kidney failure (30). Numerous studies have documented changes in connective tissue associated with bladder dysfunction and pathology (2, 30, 63); however, the mechanism by which these changes are initiated at the cell and molecular levels is currently unknown.

1.2 Background:

Fibrosis, the excessive and persistent formation of scar tissue, is responsible for morbidity and mortality associated with organ failure in a variety of chronic diseases affecting the lung, kidney, eyes, heart, liver, bladder, and skin. Fibroproliferative diseases, including chronic pulmonary, hepatic, renal, vascular, and bladder fibrosis,

contribute to nearly half of all deaths in 2002 in the USA (34). These disorders frequently affect multiple organ systems, complicating the elucidation of their underlying pathogenesis and hindering development of effective treatments.

Many cells and tissues exist in an active mechanical environment and experience tensional, compressional, pressure-induced, and shearing deformations (61). For example, lung tissue is stretched during inspiration. Endothelial cells lining the blood vessels within the body experience shearing deformations owing to the blood flow against their luminal surfaces (44). The bladder, under normal circumstances, fills with little or no increase in pressure. When the volume capacity is reached, afferent signals from stretch receptors trigger the micturition reflex (67). The bladder then empties and a new filling process begins. In order to accomplish this function, the wall of the bladder must have mechanical properties which permit it to sustain major volume changes and strains without showing signs of irreversible changes. Although the type, duration, intensity, and frequency of these forces may be different at various anatomical sites throughout the body, cells likely perceive these forces in a similar fashion and convert them into biochemical signals which alter cell behavior. In the bladder, it is likely that the mechanical forces regulate cells by activating/deactivating different sets of genes (4).

The process by which a cell is subjected to mechanical force and converts that force into a biochemical responses is termed *mechanotransduction* (9). This process is poorly understood owing to the difficultly in reconstructing force environments to which cells are normally exposed *in vivo*; however, it is likely that cells operate optimally within a range of equilibrium force parameters. If this range were to be exaggerated or

exceeded, the cell phenotype could change and have pathological consequences resulting in hypertension, hyperplasia, altered secretion of proteins in an autocrine and/or paracrine fashion and differential responses to external stimuli and cytokines (43). Compliance (Δ volume/ Δ pressure), a measure of bladder storage function, has been shown to change in disease states and during fetal development (32). During fetal development, there is evidence of active remodeling of the bladder wall (43). Biochemical measurements of type I and type III collagens show quantitative changes which accompany physiological changes (58).

Urine storage problems arise when the bladder fails to remain relaxed when filling causing the bladder to operate at increased pressures which can be transmitted to the kidneys causing damage and ultimately kidney failure. Abnormal bladder pressure can be the result of neurogenic factors secondary to stroke, Parkinson's disease, multiple sclerosis, or spinal cord injury (28, 46, 53). Physical blockage of the bladder outlet (obstruction or BPH) can cause the same pressure-related problems. The aging process is associated with intermittent increases in bladder pressure which can result in involuntary, non-voiding contractions (bladder overactivity). Regardless of the source of a particular pathology, when the cellular components of the bladder are subjected to irregular cycles of filling and emptying, and/or if the velocity (strain rate) at which forces are applied to the bladder wall is altered as a consequence of overactivity, changes in collagen gene expression can occur (22).

Collagens are the major structural protein of the body which are encoded by a family of over 30 genes and which are responsible for its synthesis. These genes respond

to a variety of signals from both inside and outside the cell (63). Compliance changes that occur as a consequence of the overproduction of collagen are the fundamental problem behind fibrotic disorders such as pulmonary fibrosis, cirrhosis, and scleroderma (14). A thorough understanding of the role of forces in these pathologies and the biochemical mechanism underlying gene expression will guide treatment and other therapies to inhibit these diseases.

Based upon prior work and new preliminary data, it has been demonstrated that collagen gene expression can be manipulated by applying different modes of mechanical force to cells (3, 10). This information can be used to implement therapies whose goal is to ameliorate the deleterious effects caused by overproduction of collagen, i.e., structurally similar drugs such as mithramycin, mitoxantrone, daunorubicin, and bisanthracycline, which mimic and interfere with transcription factors binding to their cognate DNA elements thus preventing their activation (3). If such therapies can be further refined, they offer novel ways of preventing the harmful effects of excessive collagen deposition on the normal physiologic functions of tissues and organs. Since these drugs rely on their molecular structures to mimic the actual transcription factor, they can only be identified through an understanding of the molecular process by which gene activation occurs.

1.3 Proposal:

We will test our hypothesis that mechanical stimulation BSMCs results in increased expression of extracellular matrix genes and genes which mediate force transfer

intracellularly in BSMCs. Specifically, we will study changes in the expression of 15 genes whose encoded proteins are subdivided into 3 groups: 1); Matrix and matrix modulatory proteins, 2); Sarcolemma-associated proteins which mediate force transfer, and 3); Cytoskeletal genes which link sarcolemma associated proteins with actin and myosin intracellularly. [Expression levels of type III collagen (COL3A1), Connective Tissue Growth Factor (CTGF), Integrin β-1 (ITGB1), MMP1 and TIMP1 genes were quantified].

Matrix and Matrix Modulatory Genes

Collagen, the major structural component of the bladder wall, is a family of proteins which make up approximately 30% of the dry weight of human tissue (1). There are 26+ different types of collagen with approximately 30+ unique genes, each of which codes for a specific collagen peptide. The most abundant collagen found in connective tissue is type I collagen which is a heterotrimetric molecule composed of two α-1 polypeptides [α1(IV)] and one α-2 polypeptide [α2(IV)], each of which is the product of an individual gene; COL1A1 and COL1A2, respectively (48). The polypeptides products encoded by these unique genes associate with one another to form a rod-like triple helical molecule that has high tensile strength, is relatively inelastic, and is a major contributor to the overall mechanical properties of tissues and organs. (16).

Type III collagen is a fibrillar collagen and serves as a major structural component of the bladder wall. Type III collagen is often associated with type I collagen. Previous experiments have shown that either type III collagen is minimally regulated

during development or that there is a highly sensitive post-transcriptional response (8, 50). This suggests that there may be an additional control mechanism which regulates the levels of type III collagen in the bladder wall. Since previous studies have demonstrated that type III collagen changes during development and in pathologic states (50), it is plausible that even small fluctuations in mRNA expression could alter the rate of synthesis of type III collagen and ultimately influence the collagen fiber composition of the bladder wall. Changes at the protein level are generally preceded by alterations in the gene activity of the respective genes that encode for these proteins. Previous studies have demonstrated that alterations in type III collagen is associated with changes in the compliance properties of the bladder wall. Thus structural fibers containing type III collagen play a significant role in tissue stiffening. However, the exact mechanism by which type III collagen gene expression is up-regulated in unknown.

Collagens play a unique structural role in association with other unique elements of the bladder wall. Without the appropriate connections between the muscle cells and other components of the bladder wall extracellular matrix, the ability of the bladder to empty would be severely compromised. In normal bladders, the composition is approximately 25% type III collagen and 75% type I collagen (1). In bladders with progressive fibrotic changes, this ratio shifts to approximately 33% type III collagen and 66% type I collagen (1). Previous experiments support the idea that abnormal cycling results in progressive fibrosis of the bladder (50). This fibrosis is characterized by infiltration of some detrusor muscle bundles with increased quantities of type III collagen.

CTGF is a matrix-associated protein which, similar to transforming growth factor beta (TGF-β), can stimulate extracellular matrix production. CTGF also plays a vital role in extracellular matrix remodeling and wound healing. It is widely accepted that CTGF promotes the development of fibrosis in collaboration with transforming growth factor TGF-β (36). As well, it has been implicated in extracellular matrix remodeling, wound healing, scleroderma, and other fibrotic processes since it is capable of upregulating both matrix metalloproteases (MMPs) and their inhibitors (TIMPs). Thus, CTGF has the potential to activate both the synthesis and degradation of the extracellular matrix.

CTGF and TGF-β are ubiquitous mediators of fibrogenesis, although the precise mechanisms that underlie their concerted effect remain unclear (25). Because TGF-β is a potent inducer of CTGF, most models postulate that CTGF acts as a downstream mediator of TGF- β activity, whereas other studies support an independent, rather than sequential, relationship between the two (20).

CTGF is a multifunctional, cysteine-rich, matrix-associated, heparin binding protein that is normally expressed at low levels but dramatically enriched in virtually all fibrotic conditions. *In vitro*, CTGF mirrors some of the effects of TGF- β on skin fibroblasts, such as stimulation of extracellular matrix production, chemotaxis, proliferation, and integrin expression (20). It also regulates multiple processes that contribute to fibrogenesis, including cell proliferation, migration, adhesion, survival, and extracellular matrix production.

When an anti-CTGF antibody (FG-3019) is introduced into a system, there is consistent reduction in the fibrotic response. Thus, the anti-fibrotic activity of the

antibody, FG-3019, in this model supports a key role for CTGF in mediating fibrogenesis (41).

Integrins are a large family of membrane receptors, consisting of α-, and β-subunits, which together produce twenty-four distinct heterodimers, and play a pivotal role in the interaction of cells with the extracellular matrix (29, 42). Integrins serve as the bridge between the extracellular matrix, the intracellular signaling machinery, and the actin cytoskeleton. Such interaction regulates the organization of cells in organs and tissues during development as well as cell differentiation and proliferation (15).

Cells govern tissue shape by exerting highly regulated forces at sites of matrix adhesion, which is the integrin receptor. Cells shape tissue by pulling on neighboring cells and extracellular matrices, creating specific levels of tension. In turn, cells are finely attuned to the forces and rigidity of their surroundings. As rigidity is defined by the force per unit displacement, rigidity-sensing cells must measure both force and displacement. As the major force-bearing adhesion-receptor protein, integrins have a central role in how cells sense and respond to the mechanics of their surroundings (18). Recent studies have shown that a key aspect of mechanotransduction is the cycle by which integrins bind to the matrix at the leading cell edge, attach to the cytoskeleton, transmit mechanical force, aggregate in the plasma membrane as part of increasingly strengthened adhesion complexes, unbind and, ultimately, are recycled (17). Within this mechanical cycle, integrins themselves exhibit intramolecular conformational change that regulates their binding affinity and may also be dependent upon force.

The integrin mechanical cycle is crucial for cellular function and depends upon biophysical and biochemical changes in integrin structure post-translational modifications of integrins and associated proteins. Force likely accelerates integrin activation, both by extracellular and intracellular rearrangements, induces protein recruitment through protein stretching, and accelerates integrin clustering leading to deactivation. Dispersal of adhesion contacts is favored by the loss of tension.

Finally, we will analyze the gene expression of Matrix Metalloproteinase-1 (MMP-1) and Tissue Inhibitor of Metalloproteinases-1 (TIMP-1). These matrix-associated proteins are involved in the breakdown of the extracellular matrix.

Matrix Metalloprotease-1, or MMP-1, is an interstitial collagen protease in humans which plays a fundamental role in the re-modeling of the extracellular matrix, specifically type I, type II, and type III collagen (47). In normal physiologic processes, such as embryonic development, tissue remodeling, and reproduction, matrix metalloproteases (MMPs) are responsible for the breakdown of the extracellular matrix. MMPs are a family of twenty-four genes which are organized into six different groups based on their domain organization and substrate preference (52). These zinc-dependent endopeptidases also breakdown the extracellular matrix in disease states such as arthritis and metastasis (52). They are responsible for the degradation of a wide range of extracellular components and a small number of bioactive molecules, including laminin, fibronectin, vitronectin, elastin, and proteoglycans (47, 52).

The basic structure of the active form of MMP-1 contains an single peptide which directs MMP-1 to the secretory or plasma membrane, a domain that maintains

inactivation of the enzyme by occupying the active site, a zinc-containing catalytic domain, and a hinge region which links the catalytic domain to the hemopexin-like domain which mediated interaction with substrates and confers specificity of the enzymes (52). Activation of MMP-1 following secretion from cells depends on disruption of the prodomain region with the catalytic site, which may occur by conformation changes of proteolytic removal of the prodomain (6).

In vivo activity of MMP-1 is highly regulated. It is generally expressed at very low levels and its transcription is tightly controlled either positively or negatively by cytokines and growth factors (6). MMP-1 is first secreted as an inactive preprotein and activated only after a portion is cleaved by extracellular proteinases (38). MMP-1's activity is highly regulated by an endogenous inhibitor: Tissue Inhibitor of Metalloproteinase-1 (TIMP-1) (38). MMP genes with dysfunctional, or absent, regulation have been implicated in many diseases including arthritis, chronic ulcers, and cancer (52).

The breakdown of the extracellular matrix is systematic and highly regulated by tissue inhibitor of matrix metalloprotease (TIMP) activity (52). Four members of the TIMP family have been characterized, designated as TIMP-1, TIMP-2, TIMP-3, and TIMP-4. TIMP-1 is a natural inhibitor of matrix metalloprotease-1 (MMP-1), maintaining the balance between extracellular matrix deposition and degradation in different physiological processes (21). In addition to its role as an inhibitor of MMP-1, TIMP-1 is able to promote cell proliferation in a wide range of cell types, and may also have anti-apoptotic functions and anti- and pro-angiogenc functions (1, 33).

Normally, TIMP-1 concentrations far exceed the concentration of MMP-1 in tissue and extracellular fluids. A disturbed balance has been identified in several pathologic conditions, such as cancer, rheumatoid arthritis, and periodontitis. Accelerated growth of the extracellular matrix occurs in various pathologic processes, including inflammation, chronic degenerative diseases, and some tumors (33, 57). In these cases, TIMP-1 can slow the progression of disease and/or inhibit time growth, invasion, and metastasis due to their MMP-1 inhibitory activity (65). Thus, transcription of the TIMP-1 gene is highly regulated and inducible by cytokines and hormones, as well growth factors that influence MMP-1 expression, i.e., TGF-β and interleukins. In its soluble form, TIMP-1 is preferentially expressed in epithelial tissue, cartilage, and muscle (52). The formation of the TIMP-prometalloproteinase complex modulates the activation of the enzyme.

During mechanical stimulation of the cell, wherein the collagen production is up-regulated, we expect to see this collagenase down-regulated. Similarly, TIMP-1 is an irreversible inactivator of metalloproteinases; specifically MMP-1. If MMP-1 presence were to decline in the extracellular matrix, we believe the homeostatic process of the cell would also decrease the relative levels of TIMP-1 through a negative-feedback system.

Sarcolemma-Associated Proteins Which Mediate Force Transfer

The Sarcoglycans (α, β, γ, δ, ε and ζ) are a family of N-glycosylated transmembrane proteins characterized by a short intra-cellular domain, a single transmembrane domain, and a large extracellular domain (54). These proteins form a

complex, the sarcoglycan complex (SGC), which, with other proteins, stabilizes the muscle cell membrane and mediates transfer of force generated intracellularly to extracellular matrix components. The SGC is a component of the Dystrophin Glycoprotein Complex (DGC). A diagram of this complex is shown in Figure 1, and comprises dystrophin, dystroglycan and the sarcoglycan complex. The DGC is a membrane-spanning complex that physically links the interior cytoskeleton to the extracellular matrix in muscle (51). While dystroglycan is found in nearly all cell types, the sarcoglycan complex is exclusively found in muscle cells.

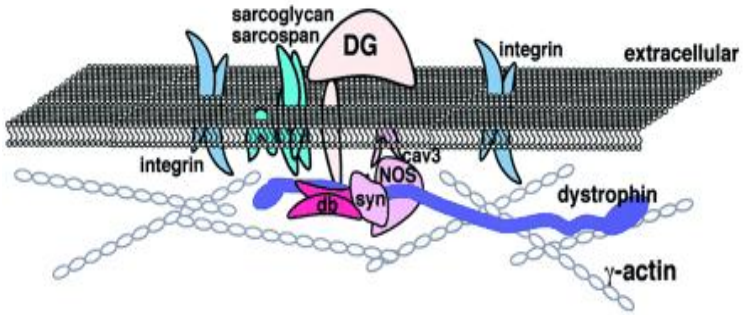

Figure 1: The dystrophin glycoprotein complex is a membrane-spanning complex that links the interior cytoskeleton to the extracellular matrix in muscle cells. It is critical for muscle integrity. The DGC is composed of Dystrophin, an elongated cytoskeletal protein that links to the cytoplasmic γ-actin and the transmembrane components of the DGC. Dystroglycan is composed of 2 subunits, α and β, each produced from the same gene. The sarcoglycan complex is composed of multiple subunits. Mutations in the genes encoding for α-, β-, γ-, and δ-sarcoglycan can lead to cardiomyopathy and muscular dystrophy in humans and mice (68).

We will determine if there are changes in the expression of the six subunits of the sarcoglycans after mechanical stimulation. The transmembrane sarcoglycans are

associated with the Dystrophin Glycoprotein Complex which is responsible for force transfer in muscle and are critical to the stability of the muscle fiber membranes as well as the linking of actin to the extracellular matrix.

Cytoskeletal Genes Which Link Sarcolemma-Associated Proteins with Actin and Myosin Intracellularly

Desmin, a type III intermediate filament protein, is muscle-specific and is found associated with the sarcomeres. Desmin filaments are mainly located at the periphery of Z-disk of striated muscles and at the dense bodies of smooth muscle cells and they have been postulated to play a critical role in the maintenance of structural and mechanical integrity of the contractile apparatus in muscle tissues (35). A 52kD protein, desmin contains three major domains: an α-helix rod, a variable non α-helix head, and a carboxy-terminal tail (35). As with all intermediate filaments, desmin is non-polar when assembled. The rod domain consists of 308 amino acids with parallel α-helical coiled dimers and three linkers to disrupt it. The rod connects to the head domain. The head domain contains 84 amino acids with many arginine, serine, and aromatic residues and is important in filament assembly and dimer-dimer interactions (23). The tail domain is responsible for integration of filaments and interaction with proteins and organelles (26).

The expression of desmin gene is regulated by a combination of different transcription control regions in muscle cells. The results from mice deficient in desmin reveal its fundamental role in cell architecture, force transmission and mitochondrial function (31). Thus, desmin also plays an important role in muscle cell architecture and structure since it connects many components of the cytoplasm (45).

Vimentin is a type III intermediate filament protein expressed in mesenchymal (stem) cells and is a major cytoskeletal component. In BSMCs it is often used as a marker of mesenchymally-derived cells or cells undergoing an epithelial-to-mesenchymal transition during both normal development and metastatic progression (26). Vimentin plays a significant role in supporting and anchoring the position of the organelles in the cytosol (26). Vimentin is attached to the nucleus, endoplasmic reticulum, and mitochondria, and is important in offering flexibility to the cell (15). Vimentin provides cells with resilience absent from the microtubule or actin filament network, when under mechanical stress *in vivo* (59). Therefore, in general, it is accepted that vimentin is the cytoskeletal component responsible for maintain cell integrity. Additionally, cells without vimentin are extremely delicate when disturbed with a micropunture (66).

Focal adhesion proteins such as paxillin serve as a point of convergence for signals resulting from the stimulation of various classes of growth factor receptor. When integrins interact with the extracellular matrix, they cluster and recruit a wide variety of intracellular proteins (1). Paxillin, along with talin and vinculin, serves as a focal adhesion proteome (51). It localizes intracellular surface sites of cell adhesion to the extracellular matrix at areas called focal adhesions while serving as a scaffold/adapter protein at the plasma membrane to coordinate interaction between adhesion and growth factor-derived signals (12). This function results in reorganization of the actin cytoskeleton at each site.

Focal adhesions form a structural link between the extracellular matrix and the actin cytoskeleton and are crucial in signal transduction as their components propagate

signals arising from the activation of integrins following their interaction with extracellular proteins, such as fibronectin, collagen, and laminin (13). Through the interactions of its multiple protein-binding modules, many of which are regulated by phosphorylation, paxillin serves as a platform for the recruitment of numerous regulatory and structural proteins that control cell adhesion, cytoskeletal reorganization, and gene expression (12, 13).

Paxillin is a 68 kDa multi-domain signal transduction adapter in specialized actin-membrane-extracellular matrix attachment sites in cultured mammalian cells *in vivo* (39). The C-terminal region of paxillin contains four LIM domains that target paxillin to focal adhesions through, presumably, direct association with the cytoplasmic tail of β-integrin. The N-terminal region of paxillin is rich in protein-protein interaction sites which are capable of binding such as tyrosine kinases, such as Src and FAK and other cytoskeletal proteins such as vinculin and actopaxin, and regulators of actin organization, such as COOL/PIX and PKL/GIT (39).

Since the cytoskeleton is intimately associated with actin and myosin and the sarcolemma, we will determine whether cytoskeletal genes that encode Desmin, Vimentin, and Paxillin proteins are also mechanically regulated by mechanical stimulation. Desmin, a type III intermediate filament specific to muscle cells, is a critical linker protein. Vimentin, another type III intermediate filament, functions to anchor the organelles of the cell and offer flexibility during periods of stress of mechanical forces *in vivo*. It is believed that vimentin is the cytoskeletal component responsible for maintaining cell integrity. Paxillin is a focal adhesion protein which localizes to

intracellular sarcolemma sites of physical linkage between cytoskeletal proteins and the extracellular matrix at areas called focal adhesions. (12).

Our rationale for studying expression of the above genes is that their altered expression would be able to functionally affect bladder physiology. Our hypothesis is that altered expression of the genes discussed above is a function of the mechanical forces experienced by BSMCs.

We will test this hypothesis by exposing BSMCs to high strain rate mechanical stretch and quantify changes in gene expression. Cells will be plated and stretched using a TensorCell machine developed by E.J. Macarak, Ph.D., which permits the application of an equibiaxial strain to cultured cells. Total RNA from stretched cells will be extracted and reverse transcribed to obtain complementary DNA (cDNA). We will perform Real-Time PCR on the cDNA sample to quantify relative levels of the mRNAs encoding the genes discussed above whose levels will be compared with identical non-stretched (control) cells. We anticipate certain genes will respond to high strain rate.

2.0 DETAILED METHODS:

2.1 TensorCell Strain:

E. J. Macarak, Ph.D. has designed and fabricated a device to impart well-characterized mechanical forces to BSMCs *in vitro*. The device is based upon a positive displacement design in which a circular plate (indenter) comes into contact with a distensible membrane upon whose upper surface cells are cultured (Figure 2).

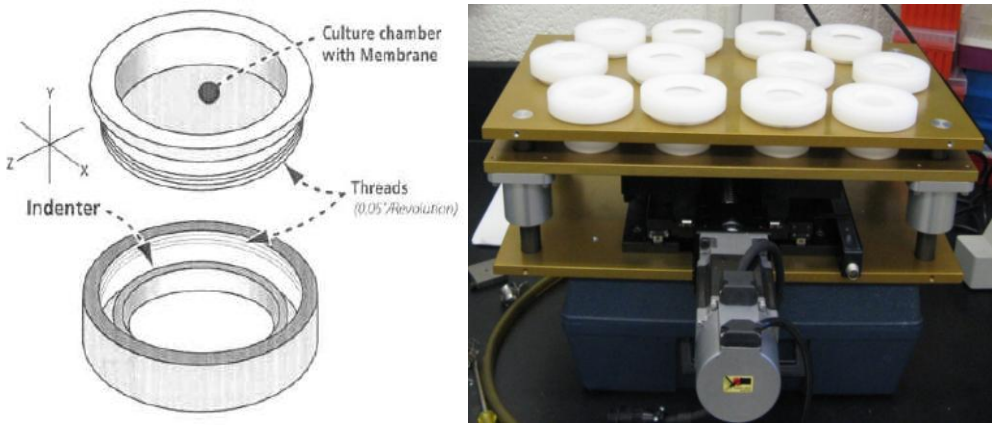

Figure 2: TensorCell culture cylinders. TensorCell culture cylinders. The upper plate contains "adapters" which accept the circular culture cylinders whose lower surfaces are the distensible membranes. Culture chambers Culture chambers lock into the adapters and are covered with a threaded cap. The distensible membranes are made of a transparent silastic material and are coated with rat tail tendon collagen (RTT). The membranes are stretched across the bottoms of the cylinders and are held in place via an elastic O-ring. Circular indenters attached to the movable plate engage the silicone membranes stretching them equibiaxially.

Deformation of a material or a cell that can be related to stresses (forces) is described as strain. As it relates to this study, cell strain can be simplistically defined as a change in a linear measurement on the surface of the cell. For example, fluorescent

beads can be attached to a cell surface and their displacement from one another measured. The change in linear distance between the beads after application of a deforming force can be measured as the "stretch ratio" $\Delta L/L$, where ΔL represents the distance between two points after force application and L represents the original length distance prior to force. In this study, we utilized 10% strain.

Strain rate is the rate at which the strain is applied to the cell or, simplistically, the rate at which the line segment between two points is stretched. A strain of 5% can be applied either over the course of 1 second or 0.1 second. In the latter example, the strain rate is 10X faster. Deformation can occur in any direction, depending on the way the force or stress is applied. Equibiaxial strain describes the stretching of the cell uniformly in all directions simultaneously; as opposed to non-equibiaxial strains, e.g., uniaxial strains. By placing a pattern of labeled dots on a distensible membrane, the relative position of the dots can be determined both before and after stretch is applied to the membrane. Selecting a trio of dots and measuring the distances between the three before and after stretch, the distances can be used to calculate the changes in length of the two principal orthogonal vectors. We have demonstrated that the magnitude of these two vectors is equivalent, meaning the strain is equibiaxial (37).

Small cylinders, across the bottom of which a distensible membrane is attached, serve as culture cylinder. Cells are plated onto the membrane and allowed to attach overnight. Once the cells have attached to the membrane, the cylinder is placed in the upper fixed plate of the apparatus and the top is screwed on, locking it in place. The whole apparatus is moved into a conventional incubator and incubated at 37°C in a

humidified atmosphere of 5% CO_2 in air. Cables from the encoder enter through a port at the front of the incubator and connect to the motor. Once the apparatus is positioned in the incubator, all strain parameters can be controlled externally via a portable computer.

A middle plate which is movable and which has indenter rings attached to it is located immediately beneath the fixed upper plate. When the indenter rings contact the underside of the elastic membranes that serve as the bottoms of the culture cylinders, the membranes are stretched causing them to expand equibiaxially as the indenter rings moves upward. The degree of expansion is linearly related to the vertical travel of the indenter and cells attached to the membrane experience a precise equibiaxial strain which is both quantifiable and reproducible.

The movement of the middle plate is via a geared "slide" whose motion is controlled by a servo motor. The rotation of the servo motor shaft is translated into a vertical movement of the middle plate by the geared slide and is controlled by software instructions that are downloaded into an encoder device that is hardwired to the motor to control the shaft's rotation. The pitch on the threads (50/1000 inch/revolution) is known, so the vertical movement of the indenter per revolution can be calculated and programmed to control the movement of the middle plate to induce the desired strain.

2.2 Membrane-Matrix Engineering:

Membranes are treated with 2-3 ml of 50 μg/ml Rat-Tail Tendon (RTT) collagen in 0.5M HAC. The collagen is permitted to dry on the membranes overnight and yields a hydrophilic membrane to which the BSMCs can adhere.

2.3 Acquisition and Growth of Bladder Smooth Muscle Cells:

Human bladder smooth muscle cells were derived from pieces of human bladder tissue normally discarded after ureteral re-implant surgery. Cells were grown in 75 ml^2 cell culture flasks in M231 medium (+ Earle's Salts, L-Glutamine, and 2.2 g/L Sodium Bicarbonate) containing 10% fetal calf serum, 25 ml of Smooth Muscle Differentiation Supplement (SMDS) and supplemented with antifungal additives of 0.25 ug/ml amphotericin B and antibacterial reagents of 100 units/ml BioWhittaker Penicillin/Streptomycin incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. SMDS is an ionically-based supplement containing fetal bovine serum and heparin. The addition of SMDS induces differentiation of normal smooth muscle cells.

2.4 Protocol for Subcultivation of Human Bladder Smooth Muscle Cells:

1. When cultures became confluent (~10^5 cells/cm^2), cells were subcultured and placed into two separate flasks to allow for continued growth and reproduction.
2. The 75 ml^2 cell culture flasks were placed in a laminar flood safety cabinet for all re-feeding and subcultivation procedures.
3. After removal of medium by suction, 10 ml of 0.05% Trypsin-EDTA is added to the flask and it is placed in the incubator at 5% CO_2 in air at 37°C for approximately 7 minutes. The Trypsin is a serine protease that cleaves cell-associated attachment proteins releasing them from the culture vessel.

4. When cells have detached from the culture flasks as observed under light microscopy (~7 minutes), the contents of the flask are suctioned off and placed into a 15 ml centrifuge tube.
5. The cells are centrifuged for 7 minutes at 1200-1400 rpm to obtain a soft pellet of BSMCs.
6. The supernatant of Trypsin-EDTA is gently removed from the test tube and the cells are resuspended in 10 ml of M231 medium (+ Earle's Salts, L-Glutamine, and 2.2g/L Sodium Bicarbonate).
7. 5 ml of the resuspended BSMCs are placed into each of two $75ml^2$ cell culture flasks and an additional 10 ml of M231 medium is added to each.
8. Both flasks are labeled and returned to the incubator at 5% CO_2 in air at 37°C until confluent.

2.5 Protein Localization/ Identification:

Human bladder smooth muscle cells were cultured and plated in separate collagen-coated plates at 5% CO_2 in air at 37°C. Cells were grown for 2-4 days or until confluent.

1. When cells were confluent, medium is suctioned out of wells and cells washed with cold PBS 3x.
2. After the final PBS rinse, cells were fixed with 4% Formaldehyde.
3. After 15 minutes fixation at room temperature, cells were washed with cold PBS 3x before adding 0.1% Triton solution to the cells.

4. After 15 minutes in room temperature, cells were washed with cold PBS 3x before adding 30 µl of the selected primary mouse antibody to the experimental slides. The antibodies used in our experiment were specific for Sarcoglycans (α, β, γ, δ and ε; 1:50) Vimentin (1:300 dilution in PBS), Desmin (1:50 dilution in PBS), and type IV collagen (1:50 dilution in PBS).
5. Cells were covered and incubated at 4°C for 1-2 days.
6. After incubation with primary antibody, cells were washed with cold PBS 3x.
7. 30 µl of the fluorescent-labeled secondary antibody was added to the slides.
8. Cells were incubated at room temperature for 1-2 hours.
9. Cells were washed with cold PBS 3x and coverslipped and sealed with a clear nail polish.
10. Slides were examined with a Zeiss microscope equipped with epifluorescence optics and a digital camera.
11. For controls, primary antibodies were omitted.

2.6 Human Bladder Smooth Muscle Plating to TensorCell Wells:

TensorCell wells were washed in detergent in a SONOGEN Ultrasound bath before being rinsed thoroughly in deionized water and sterilized in 70% ethanol for 24 hours. Wells were removed from the ethanol and dried overnight on a sterile surface in the laminar flow hood. Membranes were then coated with RTT collagen as described above.

1. Confluent cultures of cells growing in T-75 flasks were trypsinized and counted in NucleoCounter to obtain cell count per ml.

2. After centrifugation and re-suspension of the cell pellet, an equal number of cells, based upon the NucleoCounter data were added to each well. Cells plated at confluent density.
3. The wells were placed in covered petri dishes, and placed petri dishes in sealed plastic bags to prevent contamination and incubated overnight at 37°C in a humidified atmosphere of 5.0% CO_2 in air.

2.7 Application of Strain to Human Bladder Smooth Muscle Cells:

1. Confluent wells were placed on the apparatus and the program downloaded.
2. Cells were stretched at 10% strain (20 cycles/min) for 7-8 hours in TensorCell machine while incubated at 37°C in a humidified atmosphere of 5.0% CO_2 in air.
3. Control cells remain unstretched and incubated under identical conditions.

2.8 Purification of Total RNA from Human Bladder Smooth Muscle Cells:

After stretching, wells were removed and washed with clod PBS 3x. 1 ml of 0.05% Trypsin-EDTA was added to each well and it was placed in the incubator at 5% CO_2 in air at 37°C. After cells have detached from the silicone membrane, 1 ml of M231 medium was added to neutralize the trypsin/EDTA and the cells were collected by centrifugation for 7 minutes at 1200-1400 rpm in sterile tubes and counted using the NucleoCounter to obtain an accurate cell count per ml.

1. The supernatant was removed from the test tubes and Buffer RLT/ β –ME (see Table 1) was added. Cells were immediately disrupted and homogenized. Disrupted cells were pipetted into QIAGEN QIA Shredder Mini Spin Columns.

Table 1: Volumes of Buffer RLT to Tissue Disruption and Homogenization

Amount of starting material (mg)	Volume of Buffer RLT (µl)
<20	350 or 600*
20-30	600

*Use 600 µl Buffer RLT for tissue stabilized in RNA*later* RNA Stabilizing or for difficult-to-lyse tissues.

2. The lysates were centrifuged for 2 min at 14000 rpm. The filter was removed from the spin column and 1 volume of 70% ethanol was added to the cleared lysate, and mixed immediately by pipetting.
3. Samples were transferred into a QIAGEN RNeasy Mini Spin Colum and placed in a 2 ml collection tube and centrifuged for 15s at 14000 rpm. The flow-through was discarded.
4. 700 µl Buffer RW1 was added to the RNeasy Spin Column and centrifuge for 15 s at 14000 rpm to wash the Spin Column membrane after which the flow-through was discarded.
5. The column membrane was washed 3x with 500 µl of Buffer RPE followed by centrifugation for 15 s at 14000 rpm.
6. The RNeasy Spin Column was placed in a new 1.5 ml collection tube and 30-50 µl RNase-free water added directly to the Spin Column membrane. The samples

were allowed to sit at room temperature for 5 mins followed by centrifugation for 1 min at 14000 rpm to elute the RNA from the membrane.

7. The total RNA was quantified by Optical Density (OD) measurements and its quality was assessed by 2% agarose gel electrophoresis as seen in Figure 3.

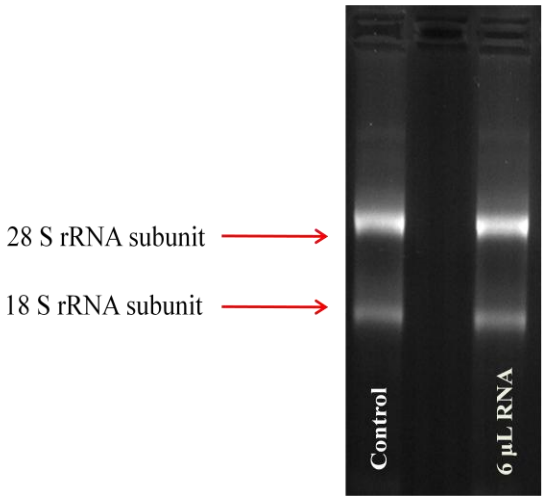

Figure 3: The 28S and 18S rRNA subunits in the control and experimental samples show that the extracted RNA was intact.

8. Collected RNA was stored at -80°C.

2.9 Reverse Transcription (RT) of total RNA from Human Bladder Smooth Muscle Cells to Compliment DNA (cDNA):

Reverse transcription of RNA-to-cDNA was accomplished via a High Capacity RNA-to-cDNA kit purchased from Applied Biosystems. Components of this kit (4387406) include 500 µl of 2X RT Buffer Mix and 50 µl of 20X RT Enzyme Mix.

1. Kit components were allowed to thaw on ice.

2. The volume of reagents needed was calculated as shown below in Table 2. Some additional reactions were included in the calculations to provide excess volume for the loss that occurs during reagent transfer.

Table 2: Volumes of kit components used for each experiment.

Component	Volume/Reaction (µl)
2X RT Buffer	10
20X RT Enzyme Mix	1
Nuclease-free H_2O	*Q.S. to 20 µl
Sample	up to 9 µl
Total per Reaction	20

*Q.S. Quantity Sufficient

3. Reagents were gently mixed and placed on ice.
4. The thermal cycler was programmed according to the manufacturer's instructions as shown below in Table 3.

Table 3: These conditions are optimized for use with the High Capacity RNA-to-cDNA Kit.

	Step 1	Step 2	Step 3
Temperature	37	95	4
Time	60 min	5 min	∞

5. Set the reaction volume to 20 µl.
6. Load the reactions into the thermal cycler and start the reaction.

2.10 Quantitative Real-Time Polymerase Chain Reaction (PCR) of cDNA:

RT-PCR analysis was performed using the Applied Biosystems 7300 PCR machine in conjunction with Sequence Detection Software (SDS) v3.1. Samples were loaded into a TaqMan 96-well plate containing a custom format (PN 4413261) of primer signatures for 14 experimental genes along with 2 control reagents. The TaqMan Gene

Expression Assays evaluate the yield of cDNA conversion through quantitative PCR analysis. Each of the plates, shown in Figure 4, contains six replicates of manufacturing control (18S rRNA) and 15 gene expression primer sets corresponding to the following genes: HPRT (control), type III collagen (COL3A1), connective tissue growth factor (CTGF), desmin (DES), vimentin (VIM), paxillin (PXN), β-1 integrins (ITGB1), matrix metalloprotease-1 (MMP1), tissue inhibitor of MMP1 (TIMP1), α-, β-, γ-, δ-, ε-, and z-sarcoglycans (SGCA, SGCB, SGCD, SGCG, SGCE, SGCZ).

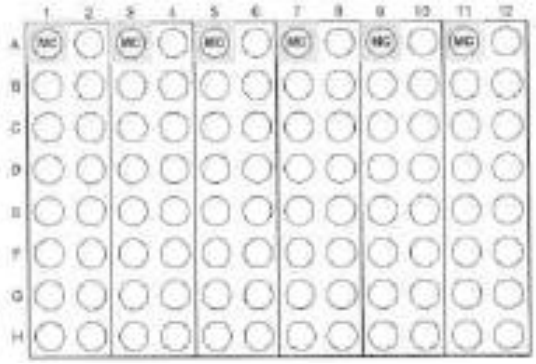

Figure 4: TaqMan 96-well custom plates containing six replicates of manufacturing control (MC) and 15 experimental assays.

Plates were loaded as described below.

1. 500 µl of Nuclease-free H_2O, 495 µl of TaqMan Master Mix, and 5 µl of RT reaction mix was combined into a 1.5 ml sterile sample test tube.
2. After mixing gently and thoroughly, 20 µl of reaction mix was aliquotted into each well of 96-well plate before plate was sealed.
3. Plate was briefly centrifuged to spin down the contents and to eliminate air bubbles.

4. The thermal cycling conditions for the plate were specified based on the manufacturer's instructions.
5. The specific detectors were loaded into the SDS software and the appropriate genes were labeled as endogenous controls.
6. When finished, data was observed and analyzed before exporting to an excel spreadsheet.
7. Once exported, the relative fold change of each gene compared to its respective control was analyzed.

For the purpose of comparison between samples, an average of every control is taken as a calibrator. Comparisons of the gene of interest mRNA levels between experimental treatments are performed by ΔΔCt analysis according to the following formulas:

Ct(gene of interest) – Ct(housekeeping gene) = ΔCt

ΔCt(experimental gene of interest) – ΔCt(calibrator gene of interest) = ΔΔCt

Assuming 100% efficiency, each cycle in an RT-PCR represents a doubling of double-stranded DNA (dsDNA). From this assumption, the fold-change in expression for each gene of interest's mRNA level versus the calibrator gene of interest's mRNA level was determined according to the following formula:

Fold Change(gene of interest) = 2^(- ΔΔCt)(gene of interest)

3.0 EXPERIMENTAL EVALUATION:

3.1 Immunolocalization of stretch-associated proteins in Human Bladder Smooth Muscle cells:

Control BSMCs were first cultured and plated in separate collagen-coated plates. Fluorescent-labeled antibodies were used to localize and identify several functional distinct proteins of study associated with the force transfer within the cell. Immunohistochemistry analysis, seen in Figures 5, 6, 7, and 8, demonstrate the presence of these proteins in our control cells.

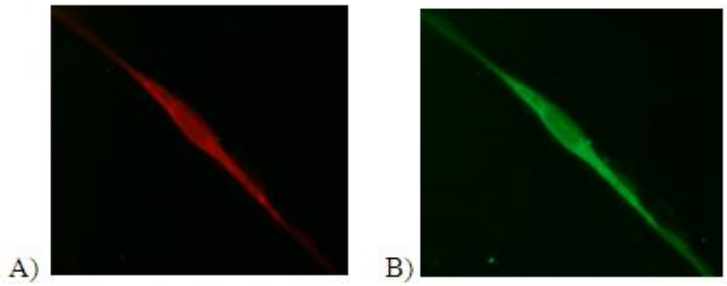

Figure 5: The immunofluorescent analysis of type [III] collagen (B; green) and β–sarcoglycans (A; red) demonstrate their co-localized positions in the cell.

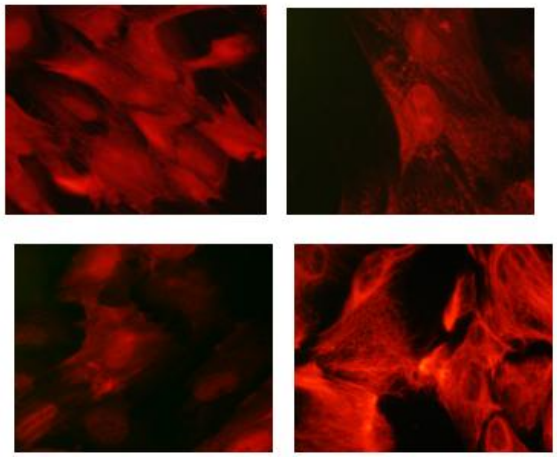

Figure 6: Immunohistochemistry characterizing the presence of (clockwise) α-sarc, β-sarc, γ-sarc, and δ-sarc in the human bladder smooth muscle cells.

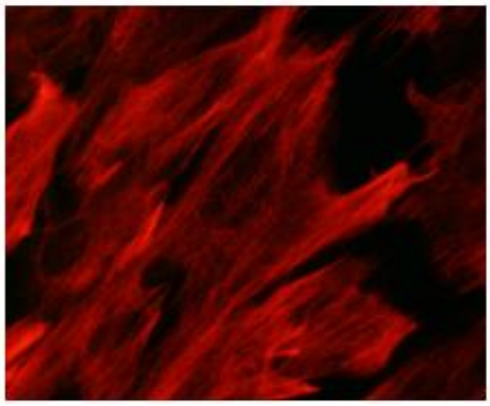

Figure 7: Immunofluorescent staining displays the location of desmin filaments in bladder cells. Desmin filaments form around the dense bodies of smooth muscle cells and play a critical role in the maintenance of structural and mechanical integrity of the contractile apparatus in muscle tissue.

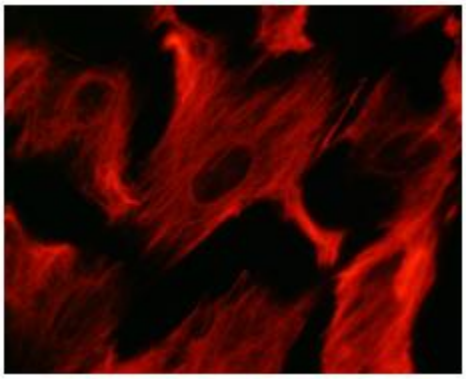

Figure 8: Immunohistochemistry used to determine the existence of vimentin in BSMCs. Vimentin filaments play a critical role in the positioning and stabilization of the muscle cell during contraction and excessive trauma to the sarcolemma.

3.2 Quantitative RT-PCR Analysis:

Analysis was carried out on 23 samples of stretched cells and 5 samples of unstretched cells. The average concentration of RNA among the stretched samples was 127.7 ng/µl, with a range of 4.8 ng/µl to 343.5 ng/µl. From these samples we reverse

transcribed the four with the highest relative concentration of RNA, between 250-350 ng/µl, and compared them against their associated controls, as seen in Figure 9.

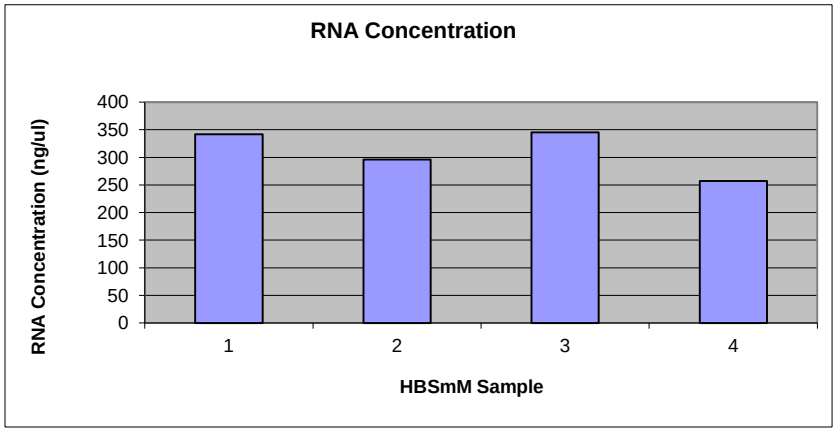

Figure 9: Four samples were selected for RT-PCR analysis based on their concentration of RNA.

After RT-PCR was performed and analyzed, the relative fold change of each gene was compared to its control. Fold change is a metric for comparing a gene's mRNA expression level between two distinct conditions. The fold change of all four samples was imported into a spreadsheet and analyzed using a paired t-test to determine the statistical significance of each. Graphical representations of the results are shown below in figures 10-23. All comparisons are between the levels of gene expression of stretched cells versus unstretched controls. Extracellular matrix genes type III collagen (COL3A1), connective tissue growth factor (CTGF) and matrix receptor β-integrin all showed increased expression. Matrix metalloprotease-1 (MMP-1) gene expression decreased as did its specific inhibitor TIMP-1. Cytoskeletal genes paxillin (PXN) and desmin (DES) both did not change significantly while a third cytoskeletal protein,

vimentin (VIM), increased its expression as a function of stretch. Within the sarcoglycan group, α, β, δ and ζ increased in expression while γ decreased and ε showed no change.

***represents statistical significance ($p < 0.05$)**

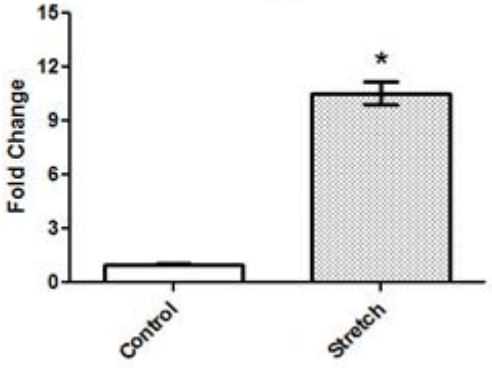

Figure 10: Quantitative PCR Analysis of α-1 [III] Collagen

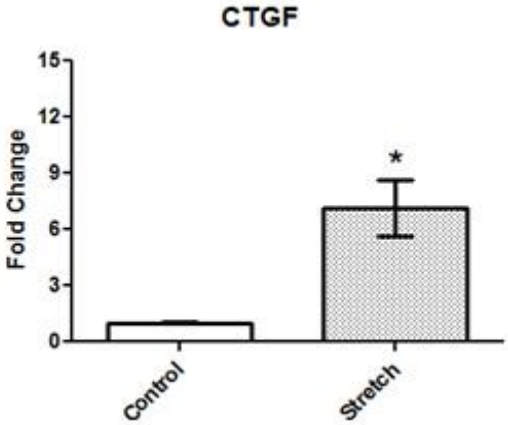

Figure 11: Quantitative PCR Analysis of Connective Tissue Growth Factor

Type III collagen and CTGF showed significant up-regulation of gene expression in the stretched sample ($p= 0.0046$ and 0.0269, respectively) when compared to their unstretched control sample.

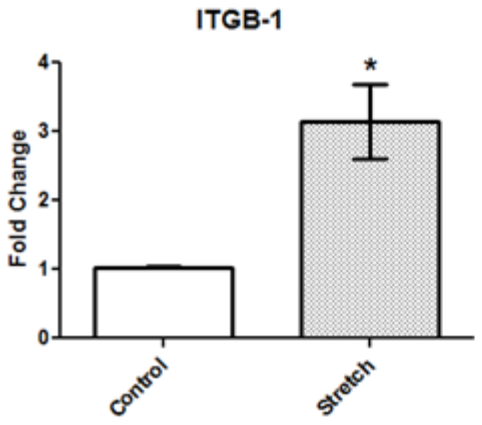

Figure 12: Quantitative PCR Analysis of Integrin β-1

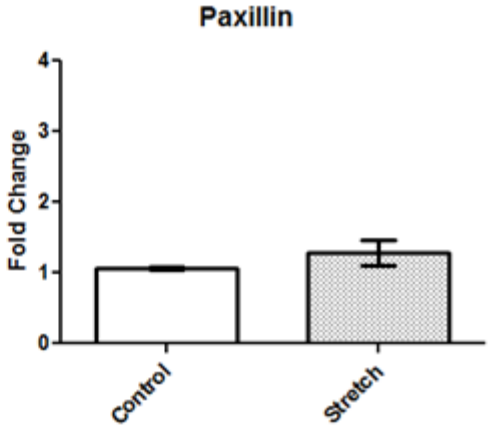

Figure 13: Quantitative PCR Analysis of Paxillin

Integrin β-1 gene expression was up-regulated to a statistically significant level after stretch application (p= 0.0305), but cytoskeletal protein paxillin did not show any significant change (p= 0.3181).

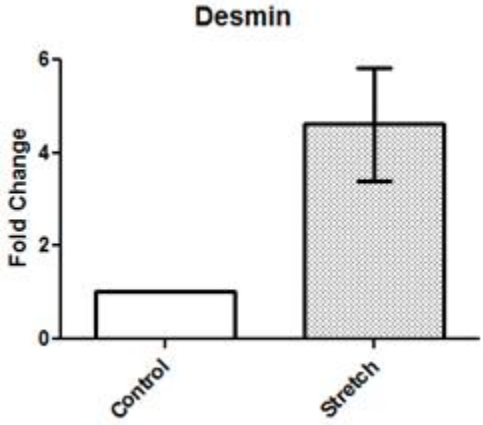

Figure 14: Quantitative PCR Analysis of Desmin

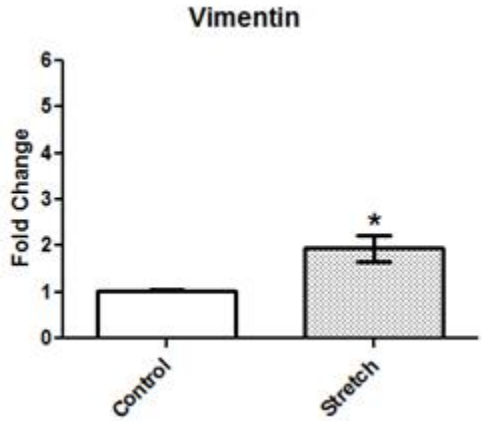

Figure 15: Quantitative PCR Analysis of Vimentin

Cytoskeletal protein desmin gene expression did not rise to a statistically significant level after the application of stretch (p= 0.0605), but vimentin showed a significant up-regulation during the same experiment (p= 0.0484).

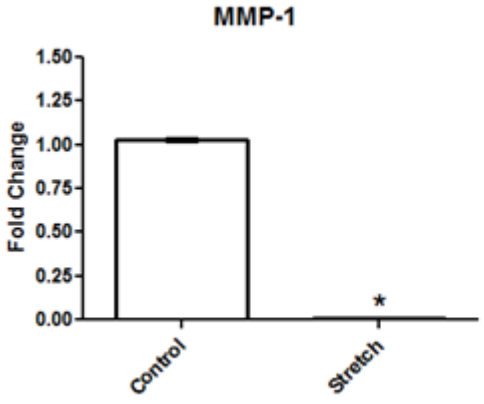

Figure 16: Quantitative PCR Analysis of Matrix Metalloprotease-1

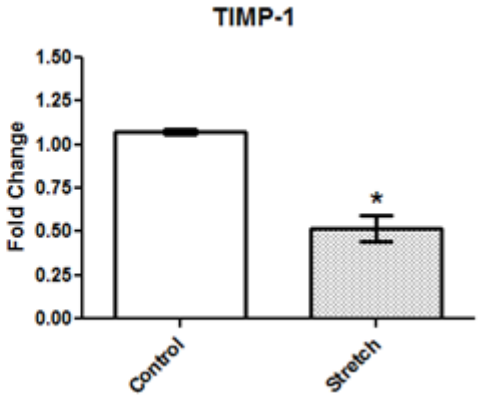

Figure 17: Quantitative PCR Analysis Tissue Inhibitor of Metalloprotease-1

Matrix-associated genes MMP-1 and TIMP-1 showed a significant change in expression after stretch. MMP-1 gene expression was effectively eliminated (p= 0.0001) while its inhibitor, TIMP-1, was also down-regulated (p= 0.0068).

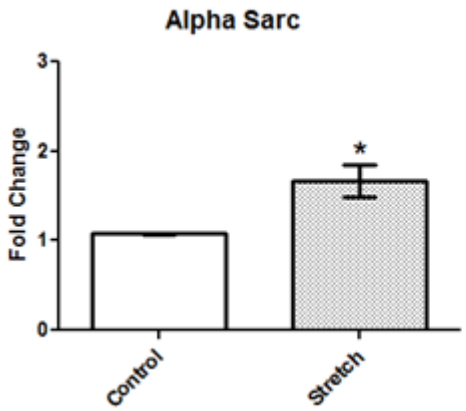

Figure 18: Quantitative PCR Analysis of α-sarcoglycans

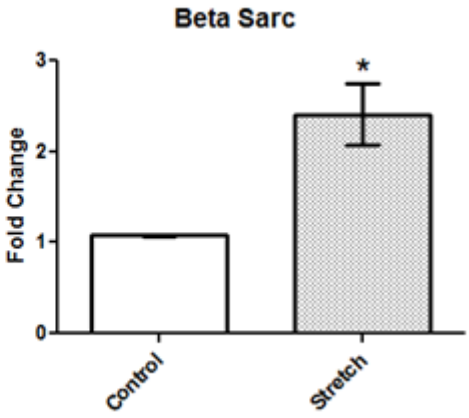

Figure 19: Quantitative PCR Analysis of β-sarcoglycans

α-, and β-sarcoglycan gene expression increased to a statistically significant level after stretch application (p= 0.0462 and p=0.0263, respectively).

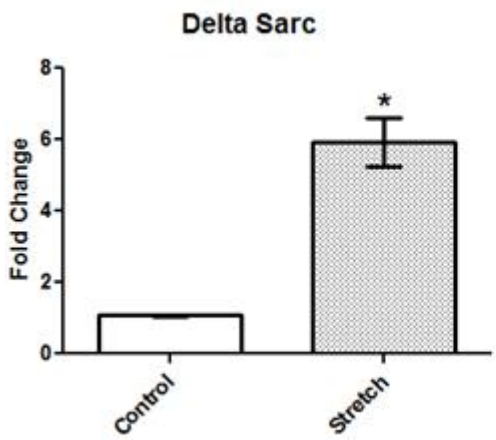

Figure 20: Quantitative PCR Analysis of δ-sarcoglycans

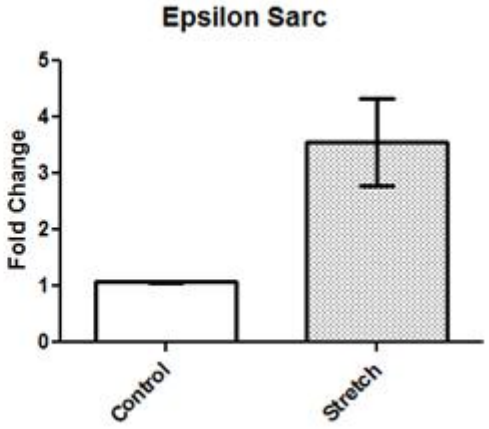

Figure 21: Quantitative PCR Analysis of ε-sarcoglycans

δ- sarcoglycan gene expression up-regulated significantly after stretch (p= 0.0053), but ε-sarcoglycan failed to show any significant alteration as a result of the stretch application (p= 0.0596).

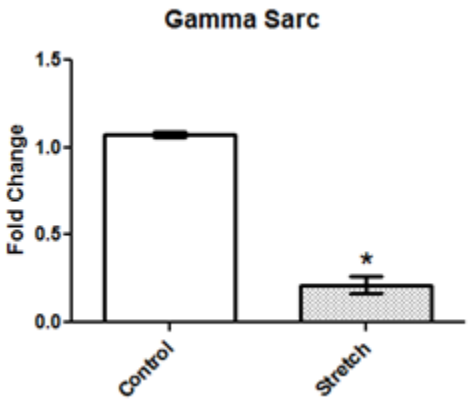

Figure 22: Quantitative PCR Analysis of γ-sarcoglycans

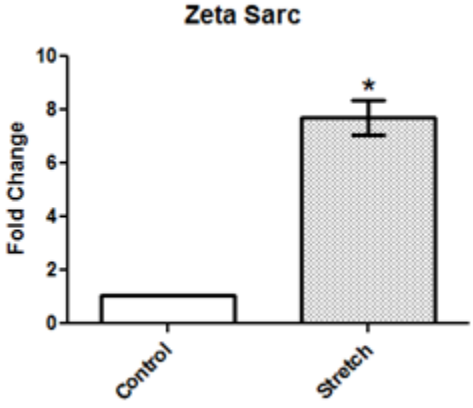

Figure 23: Quantitative PCR Analysis of ζ-sarcoglycans

γ-sarcoglycan gene expression was down-regulated significantly after stretch (p= 0.0002), in contrast to all other transmembrane sarcoglycan proteins while ζ-sarcoglycan up-regulated significantly (p= 0.0100).

3.3 Discussion:

It is known that the bladder wall undergoes profound remodeling resulting from pathologies such as spinal cord injury or obstruction, but there have been few studies examining the role of strain in the remodeling process (46, 50, 53). This research has attempted to study the effect of strain on human bladder smooth muscle cell gene expression *in vitro*, and has shown that strain is capable of inducing profound changes in a group of genes chosen for study because of their involvement in the transfer of force from within the muscle cell to the extracellular matrix. Type III collagen was chosen because it is a major structural protein found in association with the bladder smooth muscle cell *in vivo*. CTGF gene expression is involved with the control of matrix gene expression for genes like type III collagen, while MMPs and TIMPs exert control over matrix degradation. Vimentin, paxillin and desmin are all cytoskeletal proteins which have been implicated in transfer of actin-myosin-generated force in muscle cells to the extracellular matrix. The sarcoglycans are important transmembrane components of the DGC and represent physical linkages between cytoskeletal components and the extracellular matrix. Finally, β-integrin is an important matrix receptor which has been implicated in cell attachment and adhesion and, therefore, could theoretically be implicated in force transfer together with the sarcoglycans.

The strain parameters chosen mimic some of the physical conditions experienced by patients with uncontrolled and non-voiding contractions of the bladder wall. These types of contractions occur at high strain rate and occur in a variety of pathologies

including those which result as a consequence of normal aging. Our data support the view that high strain rate forces can alter gene expression in BSMCs.

In fibrotic tissues, collagen gene expression is altered. In previous studies of patients with either neurogenic or obstructive uropathies, the concentration of type III collagen increased from 25% to 33% in bladders with progressive fibrotic changes (1). Our data shows that type III collagen gene expression is up-regulated as a consequence of the application of 10% strain on BSMCs for 7 hours. Previous experiments support the concept that abnormal cycling can result in progressive fibrosis of the bladder (50). This fibrosis is characterized by infiltration of some detrusor muscle bundles with increased quantities of type III collagen. In the study presented here, we have attempted to mimic the conditions found in humans whose bladders sustain uncontrolled, intermittent, non-voiding contractions. These types of contractions can be classified as high strain contractions since they occur at high frequency over a short period of time. Normal bladder muscle tissue contracts maximally only during voiding, approximately 6 times per day. In our experiments, we have used a strain level of 10% and frequency of 20/minute to mimic high strain rate contractions. As a consequence of the force parameters used in these studies, major changes in gene expression occurred suggesting that mechanical strain plays a major role in regulating a number of genes encoding proteins involved in extracellular matrix homeostasis and force transfer within bladder smooth muscle cells.

CTGF plays a critical role in pathogenesis by promoting collagen synthesis and deposition. Similarly, CTGF appears to be regulated by mechanical force and likely

contributes to the fibrotic process found in tissues through its regulation by TGF-β. In this study, we found the concentration of CTGF mRNA to be significantly increased after exposure to mechanical force. CTGF, like collagen type III, appears to play a critical role in maintaining the steady-state level of matrix macromolecules. CTGF expression can be regulated by TGF-β (36). It is possible that TGF-β can manifest changes to the extracellular environment through its direct or indirect activation of type III collagen and CTGF.

TGF-β is a multifunctional cytokine that has been shown to induce new matrix synthesis. Intracellular signaling mediated by TGF-β has been shown to up-regulate collagen synthesis by mechanical stretch in other cell types, including smooth muscle cells (20, 25). In addition, neutralizing the TGF-β activity with anti- TGF-β antibody ablated stretch-induced up-regulation of collagen mRNA expression (36). Although we did not specifically evaluate changes in TGF- β, previous result are consistent with the view that TGF-β up-regulation by mechanical stretch stimulates gene expression of type III collagen and that up-regulation is mediated by CTGF.

Matrix metalloproteases play a critical role in the remodeling of extracellular matrix. Our findings suggest that mechanical strain does not induce MMP-1 synthesis, but rather suppresses it. This data supports the view that mechanical deformation can dramatically affect the expression of genes encoding an enzyme that plays an important role in the architecture and organization of the extracellular matrix. One of the physiological characteristics of many types of bladder uropathies is increased deposition of collagen resulting in decreased bladder compliance. If there is decreased degradation

and increased synthesis of collagen in the bladder wall, the result will be an overall increase in the level of collagen in the bladder wall. This would appear to be the case when the cells are subjected to an increased mechanical force. Overall, the data presented here show that there is an increase in gene expression of type III collagen and a decrease in expression of MMP which theoretically would result in an overall increase in type III collagen.

The enzymatic activity of MMPs is regulated at several levels, specifically by their endogenous inhibitors, TIMPs. TIMP-1 is a low, tight-binding inhibitor of MMP-1 collagenase. Thus, it is logical that the expression of MMPs and TIMPs should be inversely proportional. Our results show that as levels of MMP-1 decrease, TIMP-1 concentrations fall suggesting that TIMP expression mimics that of MMP expression.

This would suggest that it is disadvantageous to the cell to produce more TIMP-1 than is necessarily required at any time. As an irreversible inhibitor, TIMP-1's inhibition of MMP-1 occurs on a 1:1 scale. Thus, a cell would only require enough TIMP-1 to mitigate the enzymatic activity of MMP-1 and its levels should approximate those of the MMP whose activity it inhibits. Our data shows that MMP-1 activity if effectively eliminated in response to the forces applied during our experiments and that a similar change in TIMP expression also occurs perhaps via a negative-feedback system.

Integrins bind specific amino acid sequences found in extracellular matrix proteins. By virtue of its direct physical attachments to the matrix, cell surface integrins are able to respond to environmental forces thus making it a potential mechanotransducer. Our studies demonstrate that after a period of applied strain, the

bladder cells significantly up-regulated their transcription of integrin mRNA to a statistically significant level. Integrins may act, in muscle tissue, as an extra mechanism for force transfer both inside-to-outside and outside-to-inside. Integrin-based cell matrix binding may strengthen or reinforce other types of force transfer mechanisms such as those associated with the DGC. Increases in integrin expression occur rapidly and may be crucial to protect the cell from over stretching the DGC. Our laboratory has previously demonstrated an increase in adherens-based junctions in bladder smooth muscle from obstructed animals suggesting that these types of structures may help compensate for the increased force demands placed on the DGC which have been suggested to be less robust physically than integrin-based binding (68). Further studies of mechanical strain in which the time frame of applied force is varied could be used to determine if the cell increases its relative level of integrin receptor proportional to the levels of force applied.

The gene expression of several cytoskeletal proteins was also quantified. Paxillin serves as a scaffold/adapter protein at the plasma membrane. Through the interactions of its multiple protein-binding modules paxillin serves as a platform for the recruitment of numerous regulatory and structural proteins that control cell adhesion, cytoskeletal reorganization, and gene expression (12, 13). Although paxillin is an important cytoskeletal protein, it does is not appear to play a significant role in the response of BSMCs to force since there was no significant change in paxillin concentration. The same is true of muscle-specific intermediate filament protein desmin whose expression was also unchanged. This would suggest that the steady-state level of desmin mRNA is

sufficient to maintain the cells level of this protein, even during periods of increased force demands on the smooth muscle cell.

Vimentin is the only cytoskeletal protein studied whose expression increases significantly. Prior studies support the view that vimentin is especially critical in anchoring organelles to the cytoskeleton and offering flexibility to the cell during periods of mechanical stress forces and, thus, it's up-regulation may be involved with the overall process of how a cell responds structurally to an active physical environment *in vivo* (15, 66).

All sarcoglycan complex component mRNAs except ε showed altered gene expression. With the exception of γ, all sarcoglycans showed increased expression. This supports the view that the sarcoglycan transmembrane proteins function to stabilize the sarcolemma during periods of stress and/or normal transfer of intracellularly generated force to the extracellular matrix. The sarcoglycan transmembrane complex functions in connecting the muscle fiber cytoskeleton to the extracellular matrix effectively transferring actin-myosin induced force across the sarcolemma preventing damage to the fiber (54). Thus, the sarcoglycans are critical components which help the cell communicate with and monitor levels of force generated during normal cell activity. Their up-regulation is consistent with the view that a cell that is challenged mechanically must physically up-regulate those structures required to carry out force transfer. The γ-sarcoglycan subunit (SGCG) concentration decreased considerably after the cells were subjected to high strain rate. This statistically significant decrease in concentration is in contrast to the results seen among all other sarcoglycan subunits except ε which showed

no change. One possible explanation for this can be derived from research performed by Tewari, et al. (62). Their studies, involving skeletal muscle cells, found evidence to support the view that δ-sarcoglycan function is associated with cell rigidity. Mice deficient in SGCG exhibited an increased contractile response that resulted in greater shortening and widening of the sarcolemma. The researchers concluded that SGCG normally moderates contractile stress in skeletal muscle. It is possible that SGCG plays a similar role in smooth muscle and the drastic decrease in concentration is a natural response to mechanical forces to permit the sarcoglycan complex to be more flexible.

Taken together, these results suggest that BSMCs respond to stretch by up-regulating a number of genes responsible for force transfer across the sarcolemma including the sarcoglycans, β-integrin and the cytoskeletal protein vimentin. In addition, there is a very significant and substantial up-regulation of type III collagen and CTGF whose combined activities can lead to a major change in the composition of the extracellular matrix.

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