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Strain history and TGF- β 1 induce urinary bladder wall smooth muscle remodeling and elastogenesis

Rebecca L. Heise,

Cardiovascular Biomechanics Laboratory, Department of Bioengineering, Swanson School of Engineering, The McGowan Institute, School of Medicine, Pittsburgh, PA, USA

Aron Parekh,

Cardiovascular Biomechanics Laboratory, Department of Bioengineering, Swanson School of Engineering, The McGowan Institute, School of Medicine, Pittsburgh, PA, USA

Erinn M. Joyce,

Cardiovascular Biomechanics Laboratory, Department of Bioengineering, Swanson School of Engineering, The McGowan Institute, School of Medicine, Pittsburgh, PA, USA

Michael B. Chancellor, and

Department of Urology, School of Medicine, University of Pittsburgh, Pittsburgh, PA, USA

Michael S. Sacks

Cardiovascular Biomechanics Laboratory, Department of Bioengineering, Swanson School of Engineering, The McGowan Institute, School of Medicine, Pittsburgh, PA, USA

Abstract

Mechanical cues that trigger pathological remodeling in smooth muscle tissues remain largely unknown and are thought to be pivotal triggers for strain-induced remodeling. Thus, an understanding of the effects mechanical stimulation is important to elucidate underlying mechanisms of disease states and in the development of methods for smooth muscle tissue regeneration. For example, the urinary bladder wall (UBW) adaptation to spinal cord injury (SCI) includes extensive hypertrophy as well as increased collagen and elastin, all of which profoundly alter its mechanical response. In addition, the pro-fibrotic growth factor TGF- β 1 is upregulated in pathologies of other smooth muscle tissues and may contribute to pathological remodeling outcomes. In the present study, we utilized an ex vivo organ culture system to investigate the response of UBW tissue under various strain-based mechanical stimuli and exogenous TGF- β 1 to assess extracellular matrix (ECM) synthesis, mechanical responses, and bladder smooth muscle cell (BSMC) phenotype. Results indicated that a 0.5-Hz strain frequency triangular waveform stimulation at 15% strain resulted in fibrillar elastin production, collagen turnover, and a more compliant ECM. Further, this stretch regime induced changes in cell phenotype while the addition of TGF- β 1 altered this phenotype. This phenotypic shift was further confirmed by passive strip biomechanical testing, whereby the bladder groups treated with TGF- β 1 were more compliant than all other groups. TGF- β 1 increased soluble collagen production in the cultured bladders. Overall, the 0.5-Hz strain-induced remodeling caused increased compliance due to elastogenesis, similar to that seen in early SCI bladders. Thus, organ culture of bladder strips can be used as an

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John A. Swanson Endowed Chair in Bioengineering, Department of Bioengineering, McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA 15219, USA msacks@pitt.edu.

Present Address: Rebecca L. Heise Department of Biomedical Engineering, Virginia Commonwealth University, Richmond, VA, USA rlheise@vcu.edu

Present Address: Aron Parekh Department of Otolaryngology, Vanderbilt Bill Wilkerson Center, Vanderbilt University Medical Center, Nashville, TN, USA aron.parekh@vanderbilt.edu

experimental model to examine ECM remodeling and cellular phenotypic shift and potentially elucidate BSMCs ability to produce fibrillar elastin using mechanical stretch either alone or in combination with growth factors.

Keywords

Bladder wall smooth muscle; Elastogenesis; Mechanical stimulation; TGFb

1 Introduction

The urinary bladder wall (UBW) undergoes profound structural remodeling following pathologies that result in over-distension and loss of controlled voiding, such as after spinal cord injury (SCI). Specifically, the bladder smooth muscle cells (BSMC) hypertrophy, elastin content increases, and the biomechanical properties of the wall change material class, from isotropic to anisotropic (Toosi et al. 2008). Adam et al. (2004) suggested that hypertrophy (increase in bladder muscle mass) is due to sustained distension of the bladder wall. While our previous modeling efforts suggested that elastogenesis that occurs following SCI as an adaptive mechanism to increase compliance of the UBW (Wognum et al. 2009), no experimental models have been able to closely examine the importance of extracellular matrix (ECM) production as a compensatory or pathological mechanism in the injured bladder.

As a model for the physiological and pathological mechanical environment in the bladder, ex vivo culture has been used previously. Capolicchio et al. (2001) examined extracellular matrix gene response in an ex vivo model of rat bladder outlet obstruction. Collagens I, III, and XII as well as hyaluronan receptor gene expression were upregulated under bladder stretch injury. Mechanical stretch of isolated bladder smooth muscle cells also showed altered ECM gene expression, growth factors, and other cytokines (Adam et al. 2004). The mechanical environment and strain history of the bladder wall is an important, but currently not well understood, factor in the remodeling process. To date, there are no published models of the UBW under dynamic conditions.

In addition to physical stimuli, growth factors such as TGF- β 1 have been implicated in bladder pathologies since it is a well-known biochemical regulator of tissue fibrosis and remodeling. TGF- β 1 has also been implicated in various bladder pathologies in animal and patient studies resulting from diabetes (Gray et al. 2008), bladder outlet obstruction (Barendrecht et al. 2007), and interstitial cystitis and urologic cancers (Neuhaus et al. 2009). We have shown previously that TGF- β 1 gene expression is upregulated only 3 days immediately following SCI in the rat urinary bladder and continues to be upregulated out to 21 days post-SCI (Nagatomi et al. 2005; Parekh et al. 2009a,b). The TGF- β 1 expression also correlated with the expression of tropoelastin, lysyl oxidase, and IGF-1. In vitro, we have found that TGF- β 1 alters BSMC phenotype and the organization of the ECM (Parekh et al. 2009a,b). In other studies, TGF- β 1 induced hypertrophy, upregulated collagen, and inhibited proliferation of BSMCs (Barendrecht et al. 2007), as well as increasing stress fiber expression and reducing gap junctions (Neuhaus et al. 2009). TGF- β 1 modulates cellular phenotype in fibrosis (Leask and Abraham 2004) and regulates the RHAMM protein as well as CTGF in the fibrotic bladder (Capolicchio et al. 2001; Bagli et al. 1999). TGF- β 1, when administered to rabbit bladders, caused the fibroblasts within the lamina propria to differentiate into smooth muscle bundles (Roelofs et al. 1998). Based upon these previous studies from our laboratory and others, TGF- β 1 may be a key regulator of tissue fibrosis and hypertrophy in the SCI-affected bladder.

The nature of the BSMCs in the bladder wall is to remain in a contractile, non-proliferative state characterized by h-caldesmon (h-Cad) and alpha-smooth muscle actin (α -SMA) expression. However, they can become synthetic in response to injury, in tissue culture, or a pathological condition. Hypertrophic smooth muscle cells express larger amounts of α -SMA in comparison with normal cells (Bai et al. 2007), while neuropathic BSMCs are less contractile than BSMC derived from normal bladders (Lin et al. 2004). Additionally, BSMC in obstructed bladders shift to a synthetic phenotype 20 weeks after obstruction in the rat (Matsumoto et al. 2003). These changes in BSMC contractile proteins and collagen synthesis (Deveaud et al. 1998) suggest a shift to a myofibroblast phenotype akin to that of fibroblasts in healing wounds, as well as smooth muscle cells found in the cardiovascular system (Schultz et al. 2007), the kidney (Fujigaki et al. 2002), and the airway (Bai et al. 2007; Butler et al. 1987). In addition, mechanical stretch on BSMC alone can alter the BSMC phenotype by modulating cellular shape and actin reorganization (Yu et al. 2003), while TGF- β 1 can modulate the cells toward a more synthetic phenotype (Leask and Abraham 2004).

The existent data do not provide sufficient information to know the sequence and/or relationship of these remodeling events. A better quantitative understanding of how mechanical and biochemical stimuli affect BSMC phenotype and most importantly ECM production is necessary to understand BSMC pathology and tissue regeneration. Moreover, the BSMC's apparently peculiar ability to produce fibrillar elastin, both in the organ (Toosi et al. 2008) and within ECM scaffolds (Heise et al. 2009), makes such knowledge particularly interesting from both pathology and regenerative medicine perspectives. Therefore, the present study was undertaken first to determine a strain history that causes ECM deposition in the bladder wall and second to assess the role of TGF- β 1 with and without strip biaxial stretch on BSMC phenotype, ECM synthesis, and mechanical properties.

2 Methods

2.1 Tissue preparation and ex vivo organ culture

Bladders were extracted from female Sprague–Dawley rats (aged 2–3 months, 150–300 g) following euthanasia with CO₂. Bladders were opened, and strips 9–10 mm in the circumferential direction $\times$ 12–14 mm in the longitudinal direction were excised. Strips were attached on the longitudinal ends with custom-made tissue grip springs (Engelmayr et al. 2006). Use of the end springs allowed for relatively uniform distribution of stress along the specimen edge, as well as maintaining a relatively constant specimen width during deformation. The in-plane deformation gradient tensor $\mathbf{F}$ was computed by tracking four markers placed in a square on the middle of the bladder specimen. To determine any permanent deformation that occurred over the length of time in culture, measurements of the length and width of each sample were taken before and after ex vivo culture. Additionally, a strip biaxial deformation state was used, wherein the longitudinal stretch λ_L was varied while the circumferential stretch λ_C was maintained at ~ 1 . Samples were then mounted in a custom-made tension bioreactor (Fig. 1d) and stretched strip biaxially in the longitudinal direction for 7 days under sterile conditions in RPMI-1640 (Invitrogen, Carlsbad, CA) media supplemented with 10% FBS (Invitrogen, Carlsbad, CA) and 1% PenStrep (Invitrogen, Carlsbad, CA) at 37° and 5% CO₂. Bladder samples were deformed in the longitudinal direction to $\lambda_L = 1.2$ under three different stretch frequency patterns shown in Fig. 1 along with a static, free-floating control.

In diseased states such as spinal cord injury or bladder outlet obstruction, the bladder may be over-distended to 25% stretch or more. The rates of voiding also vary greatly between normal and diseased states, i.e., in the normal rat, the bladder fills over 1–1.5 h and voids

over 10 s between 18 and 24 times daily, and in the spinal cord injury rat, the bladder fills over 1 h but only voids 3–4 times daily (Streng et al. 2006). For these reasons, a “1 h” (stretched to 20% over an hour then released over 10 s) and an “8 h” (stretched to 20% over 1 h, held for 7 h, then released over 10 s) cycles were used (Fig. 1). Additionally, a rapid cyclic stretch frequency of 0.5 Hz was utilized based upon previous studies with isolated smooth muscle cells exposed to mechanical stimulation (Grote et al. 2004). We also previously showed that rapid cyclic loading caused increased ECM production in isolated BSMC grown on small intestinal submucosa (Heise et al. 2009).

2.2 TGF- β 1 experiments

Once 0.5 Hz was found to induce ECM changes, the 0.5-Hz group was used for the remainder of the studies. For TGF- β 1 experiments, 1 ng/mL TGF- β 1 was added in fresh media daily (R&D Systems, Minneapolis, MN). Freshly excised bladder strips (referred to as “Native”) and free-floating, statically cultured bladder strips (referred to as “Static”) served as controls.

2.3 Realized ex vivo tissue stretch

In order to determine the actual stretches experienced by the ex vivo bladder strips in the tension bioreactor system, a custom designed strip biaxial testing device was utilized as described previously (Stella and Sacks 2007). Briefly, the testing apparatus consisted of a small bath secured to a computer-controlled translation stage to which one end of the specimen could be mounted via a bar attached to the moving bath and the other end of the specimen mounted via a fixed bar. The bath was then moved with a linear actuator such that the bladder strip was stretched to 20% from its initial length.

2.4 Collagen and elastin assessment

Collagen and elastin concentrations were determined based on techniques adapted from Brown et al. (2000), which have previously been used to quantify ECM synthesis of ovine vascular smooth muscle cells under cyclic mechanical flexure (Engelmayr et al. 2005). Soluble collagen was extracted from tissue samples using a solution of 0.5 M acetic acid (Sigma–Aldrich, St. Louis, MO) and 1 mg/mL Pepsin A (Sigma–Aldrich, St. Louis, MO). Each sample was placed in a microcentrifuge tube and incubated in 1 ml of extraction solution overnight (~16 h) on a rocker table operating inside a refrigerator at 2–8°C. In order to determine the amounts of highly cross-linked collagen in the tissue samples, samples were denatured in a pepsin/acetic acid solution at 80°C for 1.5 h. Following denaturation, samples consisted of solubilized collagen that was measured separately from the pepsin/acetic acid digested collagen. Elastin was extracted using a hot oxalic acid treatment at 95°C for 180 min (60 min $\times$ 3). The supernatants from the oxalic acid treatments were loaded onto Centricon RC/YM-3 centrifugal filter units (Millipore, Bedford, MA) and centrifuged at 3,000 $\times$ g for an additional hour. The concentrate was then resuspended in cold (<5°C) Elastin Precipitating Reagent (UK Biocolor, County Antrim, UK). Soluble collagen from the collected media samples was precipitated with 4M NaCl. Fresh media was used as the control. Following the extraction steps, the collagen and elastin extracts were assayed according to the guidelines provided with the Sircol™ and Fastin™ assay kits, respectively (UK Biocolor, County Antrim, UK).

2.5 Matrix metalloproteinase (MMP) bulk protease activity

Since the pepsin-soluble collagen found in the tissue may be either newly formed collagen or degraded collagen, the culture media was assayed for both collagen and bulk matrix metalloproteinase activity. Protease activity was assayed from the conditioned media at days 2, 4, and 7 utilizing a similar method to Aitken et al. (2006). Net activity was assayed using

the EnzCheck collagenase/gelatinase assay kit (Invitrogen, Carlsbad, CA). DQ-gelatin fluorescein conjugate (0.1 mg/mL) was incubated in Tris buffer (50 mmol/L) with conditioned media for 2 h. The proteases then released the quenched activity of the FITC from the FITC-gelatin. The released FITC was measured on a fluorescent microplate reader at 495-nm absorption and 525-nm excitation. Collagenase produced in *Clostridium histolyticum* provided in the kit was used as a positive control. Negative controls were performed with 20 μ mol/L of 1,10-phenanthroline to inhibit the protease activity. Background from unconditioned media was subtracted from all samples. Data are reported as a summed total of activity from each day media was changed.

2.6 Mechanical testing

Mechanical testing was performed using a strip biaxial testing system as described previously (Stella and Sacks 2007). Briefly, the testing apparatus consisted of a small bath secured to a computer-controlled translation stage to which one end of the specimen could be mounted via a fixed bar. The opposite end of the specimen was attached to a flexible bending bar 316 V stainless steel, 0.229 mm diameter that extended out of the bath and was secured to a stationary mount. Through a horizontal translation of the bath, the distance between the fixed and flexible bars was increased, subjecting the specimen to prescribed elongations. The stainless steel bending bar, which had a known stiffness, was capable of achieving a load resolution of 2.91 mN/mm. With this setup, the bladder strips were mounted to the bars of the testing device using the stainless steel spring grips that were also used to stretch the sample in culture. Four graphite markers were placed in a square in the center of the specimen. Bladders were tested under three conditions: passive, inactive, and decellularized. Passive testing was performed in modified Krebs's solution at 37°C with bubbled oxygen to allow for smooth muscle tone. Inactive testing was performed after the bladders sat in a refrigerator overnight in modified Krebs's solution to inactivate the smooth muscle. Inactive testing was performed in room temperature PBS. For passive and inactive testing, the bladder strips were stretched to 1.2 or 1.3 (for more compliant groups) while the deformation of the flexible bar was monitored. For decellularized testing, the bladder strips were stretched until the tension-stretch curve moved out of the linear region, (on average a stretch of 1.35).

2.7 Bladder decellularization

Following inactive mechanical testing, bladder strips were decellularized according to a modified protocol from Nagatomi et al. in order to separate the mechanical contribution of the BSMC from the ECM (Nagatomi et al. 2008). Briefly, bladders were immersed in hypotonic Trisbuffered saline (pH 8.0; Sigma-Aldrich, St. Louis, MO) with 0.1M Phenylmethanesulfonyl fluoride (PMSF, Sigma Aldrich, St. Louis, MO) for 28 h. Second, the bladders were transferred into new solution with the Tris-buffered saline, PMSF, and the addition of 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 44 h. Finally, the bladders were transferred into new Tris solution with PMSF and 0.1% SDS (BioRad, Hercules, CA) for 28 h. At the end of the decellularization process, the bladder strips were rinsed in PBS for 30 min and then tested at room temperature in PBS.

2.8 Histological assessment

Following 7 days in culture, bladder samples were fixed in 10% neutral-buffered formalin overnight and transferred to Hank's buffered saline solution (HBSS, Invitrogen, Carlsbad, CA) for storage. Once all samples were collected, they were embedded in paraffin, cross-sectioned, and stained with elastic trichrome. Stained specimens were imaged at 400 $\times$.

2.9 Immunohistochemistry

Immunohistochemistry for elastin was performed on three representative sections from each sample group. Following paraffin embedding and sectioning, slides were deparaffinized and antigen retrieval was performed using a sodium citrate buffer (Sigma–Aldrich, St Louis, MO). Samples were then washed 3× in Tris-buffered saline (TBS, Sigma–Aldrich, St Louis, MO) and blocked with 0.3% BSA in TBS for 1 h. Then, samples were incubated with an anti-elastin primary rabbit polyclonal antibody (1:400, Abcam Inc., Cambridge, MA) for 1 h at room temperature. Following the primary antibody incubation, samples were incubated for 1 h in FITC-labeled goat polyclonal anti-rabbit IgG-H&L (1:400, Abcam Inc., Cambridge, MA). Samples were thoroughly rinsed in TBS and incubated in 300nM 4',6-diamidino-2-phenylindole dilactate (DAPI, Invitrogen, Carlsbad, CA) for 10 min. Samples were rinsed in TBS and imaged on a Leica DMRB fluorescence microscope. Negative controls for elastin antibody staining were a primary antibody delete, and positive controls were the blood vessels in the tissue.

For phenotypic staining, sections of bladder tissue from 10-day, post-surgical spinalized female Sprague–Dawley rats were obtained from a previous study (Nagatomi et al. 2005). Three bladders from each test group were fixed in 10% neutral buffered formalin overnight and then transferred to PBS until processing could be performed. Samples were paraffin embedded and sectioned en face. Three slides per sample were used for each antibody stain. Slides were deparaffinized in two changes of xylene followed by a graded ethanol rehydration. Antigen retrieval was performed using a citrate–EDTA buffer at 95°C for 30 min. All sections were blocked in 2% BSA for 1 h prior to antibody application. For the TGF- β 1 antibody staining, 5-mm sections were incubated with an anti-TGF- β 1 primary rabbit polyclonal IgG antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and visualized with a streptavidin-Cy3 goat anti-rabbit IgG secondary antibody (1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA). For the alpha-SMA staining, sections were incubated in a monoclonal anti-actin, α -Smooth Muscle–FITC antibody produced in mouse (1:250, Sigma–Aldrich, St. Louis, MO). For the h- and general CaD staining, sections were incubated in either a monoclonal anti-caldesmon antibody produced in mouse (1:100, Sigma–Aldrich, St. Louis, MO) or a monoclonal anti-caldesmon (smooth) antibody produced in mouse (1:100, Sigma–Aldrich). The sections were then incubated with a rat biotinylated adsorbed anti-mouse IgG (1:500, R&D Systems, Minneapolis, MN). Finally, the sections were rinsed in PBS and incubated with streptavidin-Cy3 (1:250, Sigma–Aldrich, St. Louis, MO). Following staining, the TGF- β 1 sections and the caldesmon sections were incubated for 10 min in 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI). Slides were then visualized using a Leica DM IRB fluorescence microscope with a 40× objective. All images were taken at the same exposure time for each group of antibody staining. Slides stained with antibody to alpha-SMA were quantified for mean fluorescence intensity using NIH Image J. Care was taken to outline smooth muscle bundles, and measurements were taken of mean fluorescent intensity per area analyzed.

2.10 Statistical analysis

All numerical data are presented as mean $\pm$ s.e.m. Statistical analyses of these data were performed using SigmaStat 3.0 software package (Chicago, IL). A one-way analysis of variance was used on all samples followed by a Tukey test for pairwise comparisons. Following these tests, p-values of less than or equal to 0.05 were considered significant.

3 Results

3.1 Realized tissue deformations

Analysis of the strains experienced under the strip biaxial organ culture revealed that the bladder strips underwent nearly pure strip biaxial stretch in the center portion of the sample. Specifically, realized stretch ratios were $\lambda_L = 1.196 \pm 0.007$ and $\lambda_C = 1.040 \pm 0.014$, with negligible shear observed. This small net increase in the circumferential direction was likely due to slight distortion artifacts in obtaining the reference state, so that a strip biaxial strain state was essentially achieved. Measurement of the bladder strips following organ culture showed that some permanent set-like effects in both the longitudinal and circumferential directions. The permanent set occurring in the longitudinal direction of the three stretched groups (0.5 Hz, 1 and 8 h) increased the length by 1.45 ± 0.65 mm, resulting in an actual realized average stretch ratio between 1.17 and 1.18 instead of the original 1.20. The circumferential direction was shortened by 2.11 ± 0.70 mm. In the static culture group, dimensions of the bladder strips decreased by 1.33 ± 0.57 mm and 1.66 ± 0.54 mm in the longitudinal and circumferential directions, respectively. This shortening most likely resulted from the unrestrained bladder strip curving back to its native shape of the UBW. Overall, the experimental system was able to subject the UBW tissue strips to a predictable and consistent strain pattern, with small effects of tissue attachment and permanent set.

3.2 Strain history-specific responses in changes in collagen and elastin production

A significantly larger amount of pepsin-soluble collagen (i.e., non-fibrillar collagen) was detected in the native tissue (actual value 2.510 ± 0.151 mg/g wet weight) compared with the 0.5-Hz and 1-h groups at 7 days (Fig. 2a); 0.5-Hz stretch caused a nearly 20-fold increase in soluble collagen found in the media compared with that of any other group (Fig. 2b). Similarly, the protease activity of the 0.5-Hz group was found to be significantly greater ($p < 0.05$) than that of any other group (Fig. 2c).

Both the immunohistochemistry and the elastin assay revealed that elastogenesis occurred in the 0.5-Hz group but not in any other test condition. Immunohistochemical staining with the antibody to elastin showed large amounts of positive staining in the 0.5-Hz group (Fig. 3b), while the only positive staining for elastin in all other groups was found only in the blood vessels (Fig. 3a arrows). Quantitatively, after 7 days in culture, elastin in the 0.5-Hz group was six-fold greater than in the native tissue (actual value native tissue: 239.267 ± 34.584 μ g (Fig. 3c). There was no change in elastin composition between the native tissue, static, 1-h, and 8-h groups (Fig. 3c). This upregulation of elastin in the cyclic group is indicative of elastogenesis and confirmed the results found by immunohistochemistry.

3.3 TGF- β 1 staining is increased in 0.5-Hz cultured bladder strips

Since we previously found an increase in TGF- β 1 in the 10-day SCI rat bladder (Parekh et al. 2009b), we examined TGF- β 1 by immunohistochemistry in native bladders, statically cultured bladders and 0.5 Hz since we also found the most extensive remodeling in this mechanical group. For comparison, we included sections from 10-day SCI rat bladders. There was abundant staining of TGF- β 1 in the SCI 10-day bladder (Fig. 4d) compared with the native bladder (Fig. 4a). Additionally, the 0.5-Hz stretched bladder (Fig. 4c) also expressed more positive staining for TGF- β 1 compared with the native bladder. Since we saw changes in ECM composition and an increase in positive staining for TGF- β 1, we then added TGF- β 1 to the cultured bladders. The two experimental groups to which TGF- β 1 was added to the media had positive staining throughout the smooth muscle bundles and blood vessels (Fig. 4e, f). The statically cultured bladders without the addition of TGF- β 1 had no positive staining for the TGF- β 1 antibody (Fig. 4b).

3.4 Impact of TGF- β 1 on collagen and elastin in the cultured bladders

Elastic trichrome staining of bladder segments revealed dense patches of elastin in the 0.5-Hz cycled bladders with more dispersed elastin seen in the 0.5 Hz+TGF- β 1 bladders (Fig. 5c, e). Cultured bladders lost the urothelial lining in all samples (Fig. 5b–e). Interestingly, the statically cultured bladders with the addition of TGF- β 1 had no clear lamina propria marked by the blue collagen staining above the smooth muscle bundles in all other groups. It appears that smooth muscle bundles made up the entirety of the cultured tissue with a larger portion of ECM separating layers of smooth muscle bundles, which may be evidence of smooth muscle migration into the lamina propria during culture (Fig. 5c).

The 0.5Hz cycling of bladder strips at 20% stretch produced significantly higher quantities of elastin compared with the native bladder and statically cultured bladders. However, the addition of 1 ng/mL of TGF- β 1 did not alter elastin protein content in either the static or cyclically stretched culture groups compared with normal culture media treated groups (Fig. 6a). There were significant differences in the amount of soluble collagen found within the bladder tissue when TGF- β 1 was added to the culture media in the 0.5-Hz stretch group (Fig. 6b). There was significantly less soluble collagen found in the tissue in the 0.5-Hz group with regular media compared with the native bladder. In the media of the cultured bladder strips, there was a significantly greater amount of collagen when TGF- β 1 was added to the culture in the static group compared with the static group with regular media (Fig. 6c). There were also large amounts of collagen found in the media of both 0.5-Hz groups.

3.5 Mechanical responses of cultured bladder tissue

The addition of carbachol for active testing only promoted a coordinated active contraction of the bladder strips in the freshly excised native bladders (data not shown). However, strip biaxial mechanical testing showed that all test groups retained passive tone compared with the inactivated group, as evidenced by decreased compliance compared with inactivated bladders (Figs. 7a, 8a–b). Static and cycled bladders with the addition of TGF- β 1 to the media exhibited greater compliance under passive and inactive testing indicated by the long toe region in the tension versus stretch curves (Fig. 8a–b) and the statistically significantly ($p < 0.05$) lower tension values at a stretch of 1.2 (Fig. 7a).

In the decellularized tissue, the static group had the largest tension values at a stretch of 1.2 that were significantly greater than all other groups ($p < 0.05$). Furthermore, the 0.5-Hz groups with and without the addition of TGF- β 1 exhibited significantly lower ($p < 0.05$) tension values at a stretch of 1.2 compared with all other groups indicating increased compliance in these decellularized bladder strips (Fig. 8c).

3.6 BMSC phenotype

En face bladder sections stained for markers of α -SMA, general CaD, and h-CaD showed that the native bladder positively expressed the contractile phenotype (Figs. 9a, 10a, g). The 10-day SCI bladders expressed significantly greater levels of α -SMA (Fig. 9g). The 10-day SCI bladders (Fig. 10d, j) also appeared to have greater CaD and h-CaD than the normal bladders (Fig. 10a–g). Additionally, the 0.5-Hz cultured bladders (Fig. 9c, g) expressed more significantly greater levels of α -SMA than the normal bladders (Fig. 9a, g). Similar results were seen in CaD and h-CaD staining (Fig. 10). The α -SMA appeared in stress fibers while the CaD and h-CaD appeared throughout the cells cytoplasm. The staining for the 0.5-Hz group appears to be equivalent to the staining in the 10-day SCI bladders for all three contractile markers.

Statically cultured bladders with and without the addition of TGF- β 1 stained positively for small amounts of α -SMA (Fig. 9b, e). The statically cultured bladder strips treated with (Fig.

10e, k) and without (Fig. 10b, h) TGF- β 1 expressed little to no CaD and h-CaD. The dynamically cultured bladders with the addition of TGF- β 1 to the media (Fig. 9f, g) expressed significantly lower staining of α -SMA than the dynamically cultured bladders with regular media (Fig. 9g–i, or native bladders (Fig. 9a, g). Similar results were seen in CaD, and h-CaD staining (Fig. 10).). These phenotypic differences show that mechanical stretch at 0.5Hz without TGF- β 1 most closely resembles the SCI bladder.

4 Discussion

4.1 Effect of mechanical stretch on ECM remodeling and BSMC phenotype

In this first study to examine the UBW SMC in a dynamic ex vivo system, we demonstrated significant ECM remodeling and in particular substantial fibrillar elastogenesis. While it is known that the bladder wall undergoes profound remodeling resulting from pathologies such as spinal cord injury or obstruction, there have been few studies examining the role of strain in the remodeling process. Unlike in vivo models of bladder pathologies, the present study has isolated the effect of strain on the UBW and has shown that strain is capable of inducing collagen turnover and biosynthesis as well as elastin production and assembly into fibrils after only 7 days in culture. Furthermore, this study has shown that the specific strain history of the UBW SMC influences the remodeling process greatly, with small changes occurring in collagen synthesis in response to physiological strain histories, yet large changes occurring in both collagen turnover and elastogenesis in response to a non-physiological strain history.

The structural role of the elastin in the cultured bladders was exhibited in mechanical testing under a decellularized state. The 0.5-Hz cyclically stretched bladders are significantly more compliant than all other groups, but not significantly different from each other. We previously showed that de novo elastin fibers play in altering UBW ECM mechanical behavior using a structural constitutive modeling approach (Wognum et al. 2009). The results in this present study corroborate our previously published model (Wognum et al. 2009) showing that elastogenesis in the cycled bladders plays a structural role in the biomechanical properties of the bladder. Additionally, the tensions at 1.2 stretch are dominated by the SMC with both passive tone and inactive tone. We have previously shown that removing the smooth muscle component and only taking into account the ECM results in larger stretch values for the same stresses (Nagatomi et al. 2005). The lamina propria has been reported to play a mechanical role at the beginning of filling and toward the end of bladder filling while the bladder wall smooth muscle governs bladder biomechanics in between Chang et al. (1999). A portion of the lamina propria may have been lost in our system since the urothelium did not survive the organ culture environment. Therefore, the changes in BSMC phenotype may be responsible for governing the biomechanical response. Culture of bladder strips at 0.5 Hz, 20% for 7 days mimicked the phenotype of the BSMC that was seen in the SCI bladders at 10 days. Furthermore, similarities between the 0.5-Hz bladders and 10-day SCI bladders were seen in mechanical testing.

Previous testing of SCI 10-day decellularized tissue under biaxial mechanical testing exhibited increased compliance compared with normal bladders (Nagatomi et al. 2004). In the present study, strip biaxial mechanical testing demonstrated increased compliance in the decellularized tissue cycled at 0.5 Hz. Although it remains to be understood exactly why the specific 0.5-Hz frequency promotes similar remodeling in both ECM composition and phenotypic change, it does appear that organ culture of bladder strips under mechanical stimulation may serve as an experimental model to examine cellular remodeling and elastogenesis.

4.2 Effect of TGF- β 1 on ECM remodeling and phenotype

The addition of TGF- β 1 to dynamic culture did not affect the elastin content within the samples. Since TGF- β 1 has been shown previously to stabilize the tropoelastin and lysyl oxidase molecules, this result was somewhat surprising (Leask and Abraham 2004). However, it is possible that since TGF- β 1 stained more positively in the 0.5-Hz culture without any additional TGF- β 1 added, the tropoelastin was already stabilized, and the elastogenic capabilities of the BSMC within the bladder strips were already maximized. The addition of TGF- β 1 to static culture did not promote any elastogenesis compared with all other groups suggesting that the stimulus for elastogenesis was mechanical stimulation at the 0.5-Hz frequency. TGF- β 1 alone may not be responsible for the early change in elastin composition in the SCI bladder.

The addition of TGF- β 1 to culture decreased bladder tone and caused more collagen to be produced in the cultured bladders. The increase in collagen in these groups may be similar to the fibrosis seen in later-stage SCI pathology (Toosi et al. 2008). In the present study, TGF- β 1 impacted collagen composition of the cultured bladders. TGF- β 1 is most commonly known as a promoter of collagen production leading to fibrosis of many organs (Howard et al. 2005). The addition of TGF- β 1 to cultures of human BSMC increases both collagen type I and III protein expression (Howard et al. 2005). Furthermore, the diseased bladder produces primarily type III collagen (Ewalt et al.). While an increase in collagen protein levels was observed in our system, mechanical properties did not necessarily follow the same pattern.

Mechanical analysis of the bladder strips with and without treatment of TGF- β 1 revealed that strips exposed to TGF- β 1 with and without mechanical stimulation were more compliant than strips cultured in regular media under passive and inactive testing. The increased compliance in the inactivated bladder strips treated with TGF- β 1 in static culture may have been due to the increase in smooth muscle bundles in the lamina propria. Similar migration and bundling of smooth muscle cells was shown in the rabbit bladder with the addition of TGF- β 1 (Roelofs et al. 1998) and in isolated BSMC seeded on collagen gel with TGF- β 1 (Parekh et al. 2009a). Phenotypic staining showed no difference between these bundles and those in the bulk of the bladder wall in the static + TGF- β 1 group. The increase in cells in this region likely altered the mechanical properties of the tissue in the inactivated state. The increased compliance in the dynamically cultured bladder strips with TGF- β 1 may have been due to the decrease in stress fibers within the tissue. Even though inactive testing does not allow for smooth muscle tone, the BSMC remains in the tissue, and the biomechanical properties of the tissue may be dependent on the cell matter that is remaining. In the decellularized bladders, TGF- β 1 treatment significantly decreased compliance in the statically cultured bladders compared with all other groups. This finding in decellularized bladders showed that the actual ECM within the bladder strips was altered with the addition of TGF- β 1 to the culture.

Under passive testing, the increased compliance for both TGF- β 1-treated groups may have been due to the decrease in BSMC contractile proteins CaD and h-CaD. The caldesmons are actin-associated proteins thought to modulate contraction by mediating phosphorylation of the myosin (Yang et al. 2008). Alterations in caldesmon proteins are key indicators of phenotypic change in bladder injury. Bladder obstruction can cause alterations in l-CaD (Burkhard et al. 2001). In the present study, exogenous TGF- β 1 added to culture decreased contractile proteins CaD and h-Cad. This result is consistent with our previously published result that TGF- β 1 added to BSMC on collagen gels decreased h-Cad expression (Parekh et al. 2009b). In addition to changes in CaD, varied changes in α -SMA expression have been observed in obstructed bladders (Matsumoto et al. 2003). In static culture, the α -SMA appeared to be unchanged with the addition of TGF- β 1. An increase in α -SMA expression

has been reported previously in static culture of isolated BSMC as well as aortic smooth muscle cells with the addition of TGF- β 1 to culture (Barendrecht et al. 2007). TGF- β 1 has also been shown previously to increase cell hypertrophy in an isolated BSMC study (Howard et al. 2005). Furthermore, the addition of TGF- β 1 to culture of BSMC increased α -SMA expression but decreased connexin gap junctions (Neuhaus et al. 2009). The present study showed that TGF- β 1 effects cellular phenotype of the BSMC within organ cultured bladder strips that is different than mechanical stimulation alone.

4.3 Relation to bladder pathologies

There is no data to date that specifically shows the phenotype of long-term SCI bladder wall SMC, so it is unclear whether the change in phenotype that was seen in the present study can be correlated with changes in the SCI bladder. However, the addition of TGF- β 1 can clearly play a role in remodeling of the bladder wall. The previous studies from our laboratory showed that TGF- β 1 expression remains upregulated out to 3-weeks post-SCI while indicators of fibrosis do not appear until later (Toosi et al. 2008). With the knowledge to date, we can speculate that the addition of TGF- β 1 to dynamic ex vivo organ culture of the bladder may mimic the SCI bladder at a later stage due to the decrease in contractile phenotype of the cells and the increase in collagen production compared with the other cycled bladders.

4.4 Limitations

Ideally, in experiments examining the effect of cellular response to a growth factor, the experiments are carried out in little to no serum to differentiate effects that may be due to the multitude of serum proteins. In the present study, fetal bovine serum (10%) was used in order to maintain cell survival in the organ culture strips. Additionally, cultures of the bladder strips were only carried out to 7 days due to a trend toward migration of the BSMC out of the tissue seen at later time points. A perfused bioreactor with oxygenated media may be the focus of future designs to examine later time points. In the present study, the 8-h cycle group was stretched to a longitudinal stretch of 1.2 in order to compare the effect of stretch frequencies on bladder wall remodeling. In the SCI bladder, the stretch level in the bladder would likely increase past 1.2 as the bladder continues to fill over the 8-h micturition cycle. Future studies will be needed with a more robust experimental system to examine the effects of larger stretch values on remodeling. Additionally, active contraction could not be measured due to the length of culture time, only passive tone could be measured. It may be possible to examine earlier time points following mechanical stimulation to determine not only when the phenotypic shift occurs but also if the phenotypic shift alters the contractility of the bladder wall. Finally, the lack of urothelium in our culture model may have also impacted our results. While the remodeling response in the bladder is thought to be governed by the smooth muscle, we cannot deny the possibility that the urothelial cells contribute to bladder wall remodeling in the disease state. This may be an interesting topic for future studies on bladder wall remodeling.

4.5 Summary

The present study highlights the complexity of mechanical stimulation and the associated biomechanical and mechanobiological consequences that occur in bladder wall remodeling. The ex vivo system described in this study may be utilized in future research to examine the TGF- β 1 signaling cascade in the bladder that likely leads to fibrosis and stiffening following SCI. For other fibrotic diseases such as scleroderma, there have been studies underway to utilize antibodies to TGF- β 1 to prevent fibrosis (Leask and Abraham 2004). With further study, the addition of TGF- β 1 antibodies to the diseased bladder may prevent stiffening of the bladder wall at early time points following SCI. Furthermore, the present study

highlighted the importance of cellular phenotype on changes collagen synthesis and overall biomechanical properties of the bladder wall. See Tables 1 and 2.

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References

- Adam R, Eaton S, Estrada C, Nimgaonkar A, Shih S, Smith L, Kohane I, Bagli D, Freeman M. Mechanical stretch is a highly selective regulator of gene expression in human bladder smooth muscle cells. *Physiol Genomics*. 2004; 20(1):36–44. [PubMed: 15467014]
- Aitken KJ, Block G, Lorenzo A, Herz D, Sabha N, Dessouki O, Fung F, Szybowska M, Craig L, Bagli DJ. Mechanotransduction of extracellular signal-regulated kinases 1 and 2 mitogen-activated protein kinase activity in smooth muscle is dependent on the extracellular matrix and regulated by matrix metalloproteinases. *Am J Pathol*. 2006; 169(2):459–470. [PubMed: 16877348]
- Bagli DJ, Joyner BD, Mahoney SR, McCulloch L. The hyaluronic acid receptor RHAMM is induced by stretch injury of rat bladder in vivo and influences smooth muscle cell contraction in vitro [corrected]. *J Urol*. 1999; 162(3 Pt 1):832–840. [PubMed: 10458391]
- Bai J, Liu XS, Xu YJ, Zhang ZX, Xie M, Ni W. Extracellular signal-regulated kinase activation in airway smooth muscle cell proliferation in chronic asthmatic rats. *Sheng Li Xue Bao: Acta physiologica Sinica*. 2007; 59(3):311–318. [PubMed: 17579786]
- Barendrecht MM, Mulders AC, van der Poel H, van den Hoff MJ, Schmidt M, Michel MC. Role of transforming growth factor beta in rat bladder smooth muscle cell proliferation. *J Pharmacol Exp Ther*. 2007; 322(1):117–122. [PubMed: 17435108]
- Brown AN, Kim BS, Alsberg E, Mooney DJ. Combining chondrocytes and smooth muscle cells to engineer hybrid soft tissue constructs. *Tissue Eng*. 2000; 6(4):297–305. [PubMed: 10992427]
- Burkhard FC, Lemack GE, Zimmern PE, Lin VK, McConnell JD. Contractile protein expression in bladder smooth muscle is a marker of phenotypic modulation after outlet obstruction in the rabbit model. *J Urol*. 2001; 165(3):963–967. [PubMed: 11176523]
- Butler GB, Adler KB, Evans JN, Morgan DW, Szarek JL. Modulation of rabbit airway smooth muscle responsiveness by respiratory epithelium. Involvement of an inhibitory metabolite of arachidonic acid. *Am Rev Respir Dis*. 1987; 135(5):1099–1104. [PubMed: 2883922]
- Capolicchio G, Aitken KJ, Gu JX, Reddy P, Bagli DJ. Extracellular matrix gene responses in a novel ex vivo model of bladder stretch injury. *J Urol*. 2001; 165(6 Pt 2):2235–2240. [PubMed: 11371952]
- Chang SL, Chung JS, Yeung MK, Howard PS, Macarak EJ. Roles of the lamina propria and the detrusor in tension transfer during bladder filling. *Scand J Urol Nephrol Suppl*. 1999; 201:38–45. [PubMed: 10573775]
- Deveaud CM, Macarak EJ, Kucich U, Ewalt DH, Abrams WR, Howard PS. Molecular analysis of collagens in bladder fibrosis. *J Urol*. 1998; 160(4):1518–1527. [PubMed: 9751406]
- Engelmayr GC Jr, Rabkin E, Sutherland FW, Schoen FJ, Mayer JE Jr, Sacks MS. The independent role of cyclic flexure in the early in vitro development of an engineered heart valve tissue. *Biomaterials*. 2005; 26(2):175–187. [PubMed: 15207464]
- Engelmayr GC Jr, Sales VL, Mayer JE Jr, Sacks MS. Cyclic flexure and laminar flow synergistically accelerate mesenchymal stem cell-mediated engineered tissue formation: implications for engineered heart valve tissues. *Biomaterials*. 2006; 27(36):6083–6095. [PubMed: 16930686]
- Ewalt DH, Constantinescu SC, Belon G, Borel JP, Snyder HM, Duckett JW, Macarak EJ. Increased collagen type III in the neurogenic non-compliant bladder. *J Urol*. 1992; 147:438a. [PubMed: 1732612]
- Fujigaki Y, Sun DF, Fujimoto T, Suzuki T, Goto T, Yonemura K, Morioka T, Yaoita E, Hishida A. Mechanisms and kinetics of Bowman's epithelial-myofibroblast transdifferentiation in the formation of glomerular crescents. *Nephron*. 2002; 92(1):203–212. [PubMed: 12187104]

- Gray MA, Wang CC, Sacks MS, Yoshimura N, Chancellor MB, Nagatomi J. Time-dependent alterations of select genes in streptozotocin-induced diabetic rat bladder. *Urology*. 2008; 71(6): 1214–1219. [PubMed: 18279932]
- Grote K, Bavendiek U, Grothausen C, Flach I, Hilfiker-Kleiner D, Drexler H, Schieffer B. Stretch-inducible expression of the angiogenic factor CCN1 in vascular smooth muscle cells is mediated by Egr-1. *J Biol Chem*. 2004; 279(53):55675–55681. [PubMed: 15492009]
- Heise RL, Ivanova J, Parekh A, Sacks MS. Generating elastin-rich small intestinal submucosa-based smooth muscle constructs utilizing exogenous growth factors and cyclic mechanical stimulation. *Tissue Eng Part A*. 2009; 15(12):3951–3960. [PubMed: 19569874]
- Howard PS, Kucich U, Coplen DE, He Y. Transforming growth factor-beta1-induced hypertrophy and matrix expression in human bladder smooth muscle cells. *Urology*. 2005; 66(6):1349–1353. [PubMed: 16360482]
- Leask A, Abraham DJ. TGF-beta signaling and the fibrotic response. *Faseb J*. 2004; 18(7):816–827. [PubMed: 15117886]
- Lin HK, Cowan R, Moore P, Zhang Y, Yang Q, Peterson JA Jr, Tomasek JJ, Kropp BP, Cheng EY. Characterization of neuropathic bladder smooth muscle cells in culture. *J Urol*. 2004; 171(3): 1348–1352. [PubMed: 14767346]
- Matsumoto S, Hanai T, Ohnishi N, Yamamoto K, Kurita T. Bladder smooth muscle cell phenotypic changes and implication of expression of contractile proteins (especially caldesmon) in rats after partial outlet obstruction. *Int J Urol*. 2003; 10(6):339–345. [PubMed: 12757606]
- Nagatomi J, DeMiguel F, Torimoto K, Chancellor MB, Getzenberg RH, Sacks MS. Early molecular-level changes in rat bladder wall tissue following spinal cord injury. *Biochem Biophys Res Commun*. 2005; 334(4):1159–1164. [PubMed: 16038877]
- Nagatomi J, Gloeckner DC, Chancellor MB, DeGroat WC, Sacks MS. Changes in the biaxial viscoelastic response of the urinary bladder following spinal cord injury. *Ann Biomed Eng*. 2004; 32(10):1409–1419. [PubMed: 15535058]
- Nagatomi J, Toosi KK, Chancellor MB, Sacks MS. Contribution of the extracellular matrix to the viscoelastic behavior of the urinary bladder wall. *Biomech Model Mechanobiol*. 2008; 7(5):395–404. [PubMed: 17690929]
- Nagatomi J, Toosi KK, Grashow JS, Chancellor MB, Sacks MS. Quantification of bladder smooth muscle orientation in normal and spinal cord injured rats. *Ann Biomed Eng*. 2005; 33(8):1078–1089. [PubMed: 16133916]
- Neuhaus J, Heinrich M, Schwalenberg T, Stolzenburg JU. TGF-beta1 inhibits Cx43 expression and formation of functional syn-cytia in cultured smooth muscle cells from human detrusor. *Eur Urol*. 2009; 55(2):491–497. [PubMed: 18406048]
- Parekh A, Long RA, Chancellor MB, Sacks MS. The effects of TGF-b1 on bladder smooth muscle cell phenotype. II. Modulation of collagen organization. *J Urol*. 2009a; 182(3):1216–1221. [PubMed: 19625051]
- Parekh A, Long RA, Chancellor MB, Sacks MS. The effects of TGF-b1 on bladder smooth muscle cell phenotype. I. Modulation of in vitro contractility. *J Urol*. 2009b; 182(3):1210–1215. [PubMed: 19625042]
- Roelofs M, Faggian L, Pampinella F, Paulon T, Franch R, Chiavegato A, Sartore S. Transforming growth factor beta1 involvement in the conversion of fibroblasts to smooth muscle cells in the rabbit bladder serosa. *Histochem J*. 1998; 30(6):393–404. [PubMed: 10192538]
- Schultz K, Murthy V, Tatro JB, Beasley D. Endogenous interleukin-1 alpha promotes a proliferative and proinflammatory phenotype in human vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol*. 2007; 292(6):H2927–H2934. [PubMed: 17293495]
- Streng T, Hedlund P, Talo A, Andersson KE, Gillespie JJ. Phasic non-micturition contractions in the bladder of the anesthetized and awake rat. *BJU Int*. 2006; 97(5):1094–1101. [PubMed: 16643498]
- Stella JA, Sacks MS. On the biaxial mechanical properties of the layers of the aortic valve leaflet. *J Biomech Eng*. 2007; 129(5):757–766. [PubMed: 17887902]

- Toosi KK, Nagatomi J, Chancellor MB, Sacks MS. The effects of long-term spinal cord injury on mechanical properties of the rat urinary bladder. *Ann Biomed Eng.* 2008; 36(9):1470–1480. [PubMed: 18622703]
- Wognum S, Schmidt DE, Sacks MS. On the mechanical role of de novo synthesized elastin in the urinary bladder wall. *J Biomech Eng.* 2009; 131(10):101018. [PubMed: 19831488]
- Yang L, He DL, Wang S, Cheng HP, Wang XY. Effect of long-term partial bladder outlet obstruction on caldesmon isoforms and their correlation with contractile function. *Acta Pharmacol Sin.* 2008; 29(5):600–605. [PubMed: 18430369]
- Yu J, Wang YF, Zhang JW. Structure of slowly adapting pulmonary stretch receptors in the lung periphery. *J Appl Physiol.* 2003; 95(1):385–393. [PubMed: 12665534]

Fig. 1. Sample waveforms for **a** two cycles of “1-h cycle”, **b** two cycles of “8-h cycle” and **c** several cycles of “0.5-Hz cycle” in the ex vivo rat bladder strips. **d** A photograph of the tension bioreactor

Fig. 2.

Collagen turnover in mechanically stretched bladders. **a** Pepsin-soluble collagen at 7 days. Data are presented as fold change compared with native tissue = 1. The 0.5-Hz and 1-h groups are significantly different $p < 0.01$ than all other groups. **b** Media assay of soluble collagen. Significantly more soluble collagen was found in the 0.5-Hz group media than in all other groups, $* p < 0.05$. **c** MMP activity of the culture media. 0.5-Hz stretch caused significantly higher MMP activity compared with other culture conditions, $* p < 0.05$. All data are presented as mean $\pm$ sem with $n = 5$ per group

Fig. 3. Non-physiological stretch caused elastin production in cultured bladders. **a–c** Immunohistochemistry with antibody to elastin and DAPI nuclei staining. **a** Overlaid image of static culture specimen, note elastin (*green*) is only seen around blood vessels (*arrows*). **b** Overlaid image of 0.5-Hz group at 7 days, note the large portion of fibrillar elastin (*green*). **c** Overlaid image of primary delete (control) and DAPI staining. **d** Elastin concentration of cultured bladders. The 0.5-Hz group was significantly greater, * $p < 0.001$, compared with all other groups. Data are presented as mean $\pm$ sem with $n = 5$ or 6 for each group

Fig. 4. TGF- β 1 antibody staining on en face bladder sections. **a** Normal native bladder, **b** Static culture 7 days, **c** 0.5-Hz culture 7 days, **d** 10-day SCI bladder, **e** Static culture + TGF- β 1 7 days, **f** 0.5-Hz culture +TGF- β 1 7 days. *Blue* represents cell nuclei. Images are reduced from 200 $\times$. *Scale bar* represents 100 μ m

Fig. 5.

Elastic trichrome staining of **a** native bladder, **b** statically cultured bladder, **c** 0.5-Hz cultured bladder **d** statically cultured bladder + TGF- β 1, **e** 0.5-Hz cultured bladder + TGF- β 1. Images are reduced from 200 $\times$. *Red/purple* are smooth muscle cells, *blue* staining is collagen, *black* staining is elastin. *Scale bar* represents 100 μ m

Fig. 6.

The effect of stretch and TGF- β 1 on elastin and collagen. **a** TGF- β 1 did not effect elastin production in 0.5-Hz bladders. * $p < 0.001$ compared with native, + $p < 0.01$ compared with native. **b** TGF- β 1 significantly increased soluble collagen in 0.5-Hz cultured bladders compared with media alone. * $p < 0.05$. **c** TGF- β 1 significantly increased soluble collagen found in the media of statically cultured bladders compared with media alone. * $p < 0.05$. Data are presented as mean $\pm$ sem with $n = 5$ –6 per group

Fig. 7.

Tension values at 1.2 stretch from strip biaxial biomechanical testing. **a** Tension values from passive testing and inactive testing. **b** Tension values following decellularization of the bladder strips. All data are presented as mean $\pm$ sem. with $n = 4$ per group. In graph A, *indicates that the TGF- β 1 groups are significantly statistically lower ($p < 0.05$) than all other groups within each testing mode. In graph B, *indicates significantly greater ($p < 0.05$) than all other test groups, and # indicates significantly lower than native and static groups but not from each other

Fig. 8. Representative tension versus stretch curves of passive testing, inactive testing, and decellularized (decell) bladder tissue

Fig. 9. Alpha-smooth muscle actin staining and quantification. **a–f** Representative images from alpha-smooth muscle actin staining from **a** Normal bladder, **b** Static, **c** 0.5 Hz, **d** 10-day SCI, **e** Static + TGF- β 1, **f** 0.5 Hz + TGF- β 1. Images are reduced from and 400 $\times$ Scale bar represents 100 μ m. **g** Mean fluorescent intensity quantification from three sections from three bladders from each group. Data are presented as mean $\pm$ standard error. * $p < 0.05$ compared with native bladder

Fig. 10.

Representative phenotypic staining of native, 10-day SCI, and cultured bladders showed that 0.5-Hz culture has similar phenotype to 10-day SCI bladders. Column 1 is general caldesmon staining, column 2 is h-caldesmon (smooth) staining, **a, g** Normal native bladder, **b, h** Static culture 7 days. **c, i.** 0.5-Hz culture 7 days. **d, j** 10-day SCI bladder, **e, k** Static culture + TGF- β 1 7 days, **f, l** 0.5-Hz culture + TGF- β 1 7 days. Images are reduced from 200 $\times$ for caldesmons *Scale bar* represents 100 μ m

Table 1

Experimental groups and *n* values

	Description	ECM assays (separate <i>n</i> for collagen and elastin)	Mechanical testing	Immuno-fluorescence
Native	Freshly isolated rat bladder	<i>n</i> = 5	<i>n</i> = 4	<i>n</i> = 3
Static	Free floating cultured bladder	<i>n</i> = 5	<i>n</i> = 4	<i>n</i> = 3
1h	Stretch to 1.2 over 1h then release, cyclic	<i>n</i> = 5	Not performed	Not performed
8h	Stretch to 1.2 over 1 h, hold at 1.2 for 7 h then release, cyclic	<i>n</i> = 5	Not performed	Not performed
0.5Hz	Cyclic stretch to 1.2 at 0.5Hz	<i>n</i> = 5	<i>n</i> = 4	<i>n</i> = 3
10 day SCI	Bladders from rats 10 days post spinal cord injury	Previous data Nagatomi et al. (2005), Nagatomi et al. (2004)	Previous data Nagatomi et al. (2005), Nagatomi et al. (2004)	<i>n</i> = 3
Static + TGF-B1	Free floating cultured bladder + TGF-β1 added to the media daily	<i>n</i> = 6	<i>n</i> = 4	<i>n</i> = 3
0.5Hz + TGF-B1	Cyclic stretch to 1.2 at 0.5Hz + TGF-β1 added to the media daily	<i>n</i> = 6	<i>n</i> = 4	<i>n</i> = 3

Table 2

Results summary

	Collagen content	Elastin content	Compliance of decellularized ECM	Compliance of intact sample	Phenotype (alpha-SMA quantification)
Static culture	No change	No change	No change	No change	No change
1 h cycle	Decreased	No change	–	–	–
8 h cycle	No change	No change	–	–	–
0.5Hz cycle	Decreased	Increased	Increased	Increased	More contractile
TGF-B1 static	No change	No change	Decreased	No change	No change
TGF-B1 0.5Hz	No change	Increased	Increased	Increased	Less contractile
SCI Bladder	Decreased Nagatomi et al. (2005)	Increased Nagatomi et al. (2004)	Increased Nagatomi et al. (2004)	Increased Nagatomi et al. (2004)	More contractile

All samples are compared with the native bladder. Groups are marked increased or decreased when values were statistically significant **p* < 0.05