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Persistent myopathy despite release of partial obstruction: *in vivo* reversal of dysfunction and transcriptional responses using rapamycin

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#, of equal importance

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1 **Nonstandard Abbreviations:**

2 PBO, partial bladder outlet obstruction, for six weeks

3 preREL or preREL+future veh, PBO rats that will be released after 6 weeks of untreated obstruction then
4 treated during release with vehicle

5 preREL+future rapa, PBO rats that will be released at 6 weeks of untreated obstruction then treated during
6 release with rapamycin

7 REL, or REL+veh, de-obstructed PBO for six weeks and treated with vehicle treatment

8 REL+rapa, de-obstructed PBO for six weeks and treated with rapamycin treatment

9 Col1a1, Collagen type Ia1*

10 Col3a1, Collagen type 3a1*

11 Eln, Elastin*

12 Mmp, matrix metalloproteinase*

13 mTOR, mammalian Target of Rapamycin*

14 SMC, smooth muscle cell

15 sham+rapa, Sham-obstructed plus rapamycin

16 sham+veh, Sham-obstructed plus vehicle

17 wk, week

18 * rat proteins are first letter capitalized, non-italicized, genes.

19 rat mRNA are expressed as first letter capitalized, all italicized.

Abstract:

Current and potential medical therapy for obstruction-induced myopathic bladder dysfunction (from benign prostatic hyperplasia or posterior urethral valves) focus on symptoms. The persistent tissue pathology and dysfunction after release of obstruction is often deemed irreversible without any systematic therapeutic approaches. As rapamycin can attenuate bladder smooth muscle hypertrophy and dysfunction during the genesis of partial obstruction *in vivo*, we tested if rapamycin could improve persistent function after release of obstruction (de-obstruction or REL). Female Sprague-Dawley rats were obstructed by suturing around the urethra and para-urethral steel rod, then removing the rod. Prior to release of obstruction (preREL), voiding parameters and residual urine volume of preREL+future rapa, preREL+future veh groups were recorded. Release of obstruction (REL) was performed by suture removal following 6 weeks of PBO. For 4 more weeks after REL (REL), animals were randomized to rapamycin (REL+rapa) or vehicle (REL+veh). PBO for 6 weeks were used as positive controls. In shams, the urethra was exposed, but no suture tied. Voiding parameters and residual urine volume were measured prior to sacrifice of sham and REL+veh or REL+rapa, and PBO. Rapamycin efficacy was tested by pair-wise comparison of changes in individual voiding data from preREL+future veh or preREL+future rapa vs. REL+veh or REL+rapa, respectively, as well as by comparisons of REL+veh to REL+rapa groups. Bladders were measured and utilized using a high-throughput QPCR array, and histopathology. Bladder/body mass ratios with PBO increased significantly and remained higher in the release phase in REL+veh animals. REL+rapa vs. veh improved residual volumes and micturition fractions toward sham levels. Three genes encoding extracellular proteins, BMP2, SOD3 and IGFBP7, correlated with functional improvement by Pearson's correlations. The promoters of these genes showed enrichment for several motifs including circadian E-boxes. While obstruction and REL augmented CLOCK and NPAS2 expression above sham levels, rapamycin treatment during release significantly blocked their expression. This experimental design of pharmaco-intervention during the de-obstruction phase revealed a novel pathway dysregulated during the clinically relevant treatment phase of obstructive bladder myopathy.

Key Words: rapamycin, bladder obstruction, core clock genes, smooth muscle, drug therapy.

1 **Introduction**

2 Obstructive bladder myopathy is highly prevalent, affecting >50% of men over 50 years of age at some point in
3 their lives(1-3), yet only a narrow list of pharmacologic therapies are presently available. PBO is prototypically
4 either anatomic (e.g. Posterior Urethral Valves (PUV), Benign Prostatic Hyperplasia (BPH)) or neurogenic (e.g.
5 spinal cord injury and myelomeningocele). Drug therapy often begins in lieu of, preceding or following
6 surgical ablation of an obstructing lesion, in order to supplement intermittent catheterization, relax the bladder
7 and prevent upper tract dysfunction. Novel treatments that go beyond treating symptoms of overactivity and
8 target pathways that address the structural and phenotypic changes in bladder smooth muscle are needed to
9 replace current anti-muscarinic and beta-adrenergic drugs which focus on lower urinary tract symptoms
10 (LUTS). However, effective experimental modeling of treatment that targetting the therapeutic stages, after
11 surgery concomitant with catheterization, is needed for development of appropriate patient-centred therapies.

12 Previously, we identified a novel ability of the mTOR inhibitor rapamycin that reduced bladder overcapacity,
13 smooth muscle hypertrophy and improved pathologic residual urine volumes when given during the genesis of
14 obstruction(4). The mechanism of action *in vivo* involved prevention of both gross and smooth muscle cell
15 (SMC) hypertrophy as well as modulation of matrix gene dysregulation(4-14). Rapamycin inhibits obstruction-
16 and stretch-induced signaling downstream of mTOR (mammalian target of rapamycin). In smooth muscle,
17 rapamycin is known to work through several mechanisms, including S6K and elongation initiation factor 4E
18 (EIF4E), which affect SMC marker dysregulation(4, 15-17), as we and others observed *in vitro*(16, 18-20).

19 However, functional improvements from therapeutic treatment during ongoing obstruction differ from those
20 after removal of obstruction (REL). Clinical treatments focus on mitigating the source of out-flow obstruction,
21 However, removal of the anatomical PBO itself does not completely reverse the pathology and dysfunction, in a
22 large proportion of BPH and PUV patients, even months or years following REL. The failure to resolve after
23 REL is seen at multiple levels (matrix, neural input, and smooth muscle cellular phenotype) and leads to
24 ongoing sequelae such as increased residual volumes and altered activity(5, 15, 17, 21-31).

25 To treat REL bladders, experimental approaches will have to move beyond treating early and ensuing responses
26 to PBO(32-34). Indeed, the clinically relevant period available to treat and study this condition is during the
27 post-release phase(35), once the anatomic obstruction itself has been detected and mitigated. While several
28 reports have described the effect of REL(24, 31, 36-40), the response to targeted pharmacotherapy in the REL
29 period has not systematically combined molecular and functional analyses to find relevant candidates. While
30 some molecular studies have looked at early genome-wide responses after a 10 day REL(5), this timepoint may
31 still be a part of the healing phase post-release. Later timepoints may be reveal recalcitrant and unique responses
32 of the REL bladder.

In the present study, we developed a clinically translatable approach to integrate functional and molecular responses following release of organ obstruction (REL) in the presence of pharmacotherapy, again using rapamycin to establish proof-of-principle. We tested physiology before and after the release of obstruction to measure differences in each animal between PBO bladder dysfunction and change in function during REL +/- treatment. Assessing 87 candidate genes and their associations with changes in function during treatment allowed us to identify a functionally important group of genes, IGFBP7, BMP2 and SOD3, along with a regulatory pathway of these genes. By testing function and gene expression in a clinically and therapeutically relevant model after release of obstruction, we uncovered novel dysregulated targets, e.g. circadian transcripts, differing from the reiterative types of anti-muscarinic or adrenergic drugs, to move towards achieving structural and biological repair to aid in functional recovery of the de-obstructed bladder.

Materials and Methods

Partial bladder outlet obstruction (PBO): PBO was performed as in our previous work(4). Briefly, in female Sprague-Dawley rats weighing 250 to 300 g(4), the proximal urethra was exposed and a 4-0 silk suture ligated around both a 0.9 mm diameter metal rod and the proximal urethra. Subsequently, the rod was removed leaving a standardized partial constriction of the urethra, and the abdomen was closed. As in the Figure 1 schematic, a positive control group of PBO alone for 6 weeks was created to isolate bladder tissue for comparison to the release (REL) groups. Sham obstructions were performed by exposing the bladder, passing the suture behind the bladder neck without leaving the suture in place, and reclosing the abdomen.

Release (REL) of partial bladder outlet obstruction: Two days prior to release surgery, animals were placed in metabolic cages for micturition studies (Schematic in Fig.1, and Section below). Under anaesthesia, the urethra was catheterized with a 22-gauge angio-catheter to collect residual volumes (RV). An elevated RV is a functional indicator of obstructive disease. At the time of release, obstructed animals with RV's in the sham range (0.2 mL or less) were not considered obstructed, and removed from the study. Release from obstruction (REL) was performed by exposing the bladder, removing the silk suture and reclosing the abdomen. REL animals were followed for 6 weeks after de-obstruction. Total experimental duration for REL and 12w-sham groups was 12 weeks. Animals in REL and sham groups were randomized to treatment with rapamycin (rapa) or vehicle. After 12 weeks, both 12w-sham and REL rats were placed in metabolic cages for 12-hours to record voiding. REL Residual volumes were measured under anaesthetic. Bladders were harvested for RNA and frozen sectioning, with bladder mass and body mass recorded. The 12w-sham groups were included as controls for comparison to REL groups. Total sample numbers for each group were as follows: PBO=8, REL+veh=22, REL+rapa =18, 12wsham=17, 12wsham+rapa=13. From the released groups, there were 3 rats removed: 1 mortality in the REL group, and 2 euthanized for signs consistent with UTI each from a REL and a REL+rapa group. Harvested bladders were randomized to different analyses, including HT-QPCR, immunofluorescence and histology.

Micturition measurements: Prior to release of the obstructing suture, each animal was randomized to one of two groups for **post-release** treatment: preREL+future rapa or preREL+future veh. Each group had voiding parameters recorded after 6 weeks of PBO (Schematic in Fig. 1, Supplemental Figure S1) **prior to** release of obstruction. **After** release, animals from each group were treated with either rapamycin or vehicle 6 weeks and had voiding parameters recorded. The data from the post-release groups are labeled as REL+veh or REL+rapa to differentiate them from each animal's pre-release/pre-treatment recording. Using non-invasive functional assays, we followed each animal as their own voiding control prior to release and after the treatment period. We recorded micturition patterns for 10 hours during the sleep (0800 to 1800 hours, rat night) cycle at two time points: (1) 1-2 days prior to release of obstruction (preREL), and in 6 week obstructed controls (PBO); and (2) again after 6 weeks of de-obstruction for REL and at 12-weeks for the 12w-sham groups. Each rat was placed in a metabolic cage which funneled urine to a cup placed on a weigh scale attached to LoggerPro software(4). Recording of urine output began after one hour of adaptation to the metabolic cage environment. Micturition frequency was defined as the number of voids in the measurement time period. 'Small voids' were defined as less than 0.25g. An increased frequency of small voids (over controls) was used as a surrogate for new onset bladder hyperactivity. Other voiding parameters are described in Sidler et al, 2017(41) and Supplemental Fig.S1. These voiding data prior to obstruction release were utilized to identify outliers using principle component analysis (Supplemental Fig.S1B). Voiding data immediately prior to surgical obstruction release (preREL) and 6 weeks after surgical release (postREL) were compared using pairwise t-tests. Each postREL data point from REL animals was compared to its own preREL voiding control. Student's two-tailed t-tests were used to compare sham, PBO, and REL groups. REL bladders were examined for their level of obstruction; pre-release residual volumes within two standard deviations of the mean sham RV (≤ 0.23 mL) were removed from the analysis. Conversely, successful obstruction was defined by a preREL RV $>2SD$ greater than the mean RV of sham residual volumes. Harvesting of bladder samples was randomized by group, and tended to occur between 0700 and 1100 hours.

Rapamycin treatment: Initial experiments with rapamycin in DMSO dissolved directly into saline during obstruction demonstrated large skin lesions at the injection site and poor survival in both sham and obstructed groups receiving the drug. Crucial aspects of rapamycin dissolution comprised making a stock of rapamycin in DMSO plus 5% Tween80, which is then dissolved further into saline. This resulted in better solubility and improved toleration of the drug alongside improvements in hypertrophy of the bladder(4). Both the 12wsham and REL rats were randomized to treatment with rapamycin 1 mg/kg/day or vehicle, and injected subcutaneously for 6 weeks(4). A strong decrease in S6 phosphorylation was confirmed by immunofluorescent staining during rapamycin treatment *in vivo* (Representative micrographs, Supplemental Fig. S2).

Histology of Bladder Sections: 7 μ M-thick equatorial sections were stained with Haemotoxylin and Eosin and modified elastic-Masson's Trichrome dyes. Slides were scanned using Aperio software. Wall thickness of

scanned sections of the entire bladder wall was measured on Aperio software, and compared using student's t-test on 'R'. Further details provided in Supplemental materials.

Optical polarimetry of collagen fibril structure: Cryosections (7 μ M) from equatorial regions of the bladder were sectioned, fixed in formalin and stained with PicroSirius Red. Photomicrographs were taken using a Zeiss camera and cross polarizing filters as in previous work(4). The colour of the light released from the sections was noted as the fibril thickness varies with the colour from red (thick) >orange >yellow >green (thin). Quantitation of red vs. green colour intensity of photomicrographs was performed on ImageJ using mean red or green fluorescence in detrusor regions from 3 slides for each sample, and 3-7 samples per group.

RNA extraction and Custom PCR Array: Samples were placed in RNAlater (Life Technologies) then later snap frozen and stored at -80°C. Samples were homogenized in TRIzol Reagent and extracted before(16). RNA was dissolved in DNase/RNase free water and quantified by nanodrop. RNA Integrity Number (RIN) was tested using a Bioanalyzer (The Center for Applied Genomics at the Hospital for Sick Children). Only samples with RIN > 8 were processed further. We designed a custom high throughput QPCR (HT-QPCR) array similar to the array published in Sidler *et al*, 2018. Candidate genes (71) were selected through curation of the literature for genes related to bladder SMC differentiation, mechanical strain and bladder obstruction (Table S1). This custom-ordered PCR array (RT² Profiler PCR Array, Qiagen, Hilden, Germany) including 5 reference genes and built-in quality controls was standardized and highly quantitative. For each bladder sample, 800 ng of RNA was used for cDNA synthesis with the RT² First Strand Kit (Qiagen). Sample cDNAs, the cDNA blank and water blank were mixed with RT² SYBR Green ROX qPCR Mastermix (Qiagen) and dispensed into custom QPCR plates for cycling under the manufacturer's suggested conditions. An 'R' package, 'HTqPCR', was used to identify by ddc(t) normalization and limma, the most significantly altered genes between groups(42). Promoters of genes with significantly altered expression were compared to those without altered expression utilizing DIRE, a program that analyses transcription factor binding sites, (TFBS) (43). The significantly changed rapamycin-responsive genes were compared to the background set of unaltered genes from the HTqPCR gene set in DIRE (Table S1). QPCR primers for cDNA of reference genes B2M, Ubc, Sdha, Rpl13, Rpl32 and Hprt were previously described(44). Pre-designed primers for target genes Igfbp7, Bmp2, Dec1+2, Clock, Npas2, Per1+2, Cry1+2, Nr1d1, Bmal and Sod3 were purchased from IDT.

Bladder smooth muscle cell cultures: Human bladder SMC (Sciencell, Carlsbad, California, USA) were cultured in SMC medium (Sciencell), plus additives including serum for maintenance, and passaged using with Trypsin-EDTA as before(45). SMC were plated at 0.5 or 1X10⁵ cells/mL onto 6 well tissue culture treated plates or permanox-treated chamber slides, incubated overnight and switched into EMEM (Wisent, St-Bruno, Quebec) plus 0.5% FCS. SMC were treated with vehicle (DMSO, Sigma-Aldrich) or 10 μ M SR9009 (Focus Biomolecules, Plymouth Meeting, Pennsylvania, USA). SMC were harvested for RNA and QPCR(16, 46) or protein lysates. For immunofluorescence, SMC chamber slides were stained as in immunofluorescence section

below previously(45). Quiescent cells were pretreated with SR9009, rapamycin (15ng/mL) or vehicle one hour prior to stretch, e.g. mechanical strain for 6 hours then a further relaxation for the remaining 12 hours, as previously described(47). For controls, vehicle was added to non-stretched cells. For stretch relax experiments, serum-starved cells were stretched for 6 hours at 5% elongation on BioFlex collagen I plates(16, 47), then relaxed for 14 hours. For circadian cycling, serum-starved cells were induced to start circadian cycling with Dexamethasone (100 nM) treatment for 2 hours with a subsequent washout period in normal starvation media.

Immunofluorescence: Harvested bladder tissue was briefly rinsed in 9% sucrose, placed in OCT then frozen on dry ice. Cryosections (7 micron-thick) were fixed in 4% paraformaldehyde for 20 minutes, washed, treated with 0.2% Triton-X 100, washed and blocked in 10% donkey serum, 1 hour. Primary antibodies for calponin (mouse-anti-calponin 1:200, Sigma-Aldrich, St. Louis, Missouri, USA), alpha-smooth muscle myosin heavy chain (rabbit-anti-Myh11, 1:200, Sigma-Aldrich), phospho-S6, phospho-4EBP (both Cell Signaling) or Dec1 (rabbit, 1:400, Neolabs) were incubated at 1:200, 4°C overnight (45). After washing, secondary donkey anti-mouse-Cy3 or donkey anti-rabbit-Alexa 647 conjugated Affini Pure F(ab)² fragments (Jackson Immunolabs, West Grove, PA, USA) were incubated for one hour, counterstained with Hoechst 33342 (Thermo Fisher Scientific, Waltham, Massachusetts, USA), then washed in PBS. Slides were mounted in Dako fluorescence mounting medium. We acquired confocal micrographs using an Olympus IX81 microscope equipped with a Hamamatsu camera, and spectral borealis lasers attached to Volocity software (ver. 6.2, Perkin Elmer, Waltham, MA, USA). Volocity software was utilized to assess intensity of calponin, myosin and Dec1 staining. To understand the contribution of SMC hypertrophy to gross bladder wall hypertrophy in REL and PBO bladders, we measured cross-section areas of myosin-positive (Green) cells containing circular nuclei. Volocity was used to evaluate hypertrophy of cell areas (um²) in transnuclear cross-sections of myocytes (4, 48).

Overexpression of mTOR pathway proteins: Aliquots of 5X10⁵ SMC (grown as above) were re-suspended in 100 uL of room temperature Nucleofector™ Solution (Amaxa SMC electroporation kit, Lonza, Morristown, NJ, USA) and combined with 1ug of plasmid DNA for each treatment groups (vehicle, pcDNA3-FLAG-RHEB-N153T, pcDNA3-FLAG-RHEB (gift from Fuyuhiko Tamanoi, Addgene plasmids #19996 and 19997(49)) or active pRK7-HA-S6K1-E389D3E (gift from John Blenis, Addgene plasmid # 8993 (50)). Electroporation proceeded with the P-024 protocol in the Nucleofector™ 2b device. Pre-warmed culture media was added to the cuvette, and cells aliquoted into 6-well plates for kept at 37°C / 5 % CO₂, for 12-20 hours. Cells were lysed for either RNA using Trizol as above, or for protein in RIPA buffer. Protein was quantified by BioRad protein assay. 25µg of sample were diluted into Laemmli buffer and loaded into and electrophoresed on a 7.5% polyacrylamide gel, which was then transferred to supported nitrocellulose for 16 hours at 12 Volts. Western blotting of lysates was performed using antibodies that targeted phosphorylated-S6, Serine-2448 phosphorylated-mTOR, total mTOR, GAPDH (all from Cell Signaling), DEC1, DEC2(Proteintech), CLOCK (Abcam), as well as Flag (Sigma) and HA(Covance)-specific antibodies.

Statistics: Pair-wise t-tests and Pearson's correlation analysis of functional parameters were performed for pre- and post-release functional data. Analysis of variance (ANOVA) with post-hoc Student-Newman-Keuls t-tests were performed on all data, including sham animals treated with vehicle or rapamycin, with $p < 0.05$. SMC size analysis: ANOVA was performed for the REL+rapa or +veh, sham+rapa or +veh, and PBO groups with post hoc Student-Newman-Keuls t-tests, with $p < 0.05$ considered significant. The high throughput expression data was analyzed using 'HT-qPCR', 'limma' and 'car' package on 'R' with Bioconductor package(42). Only samples that passed the array's built-in quality checks were included in the analysis. The data was quantified using $\Delta\Delta c(t)$ and limma normalization methods, leaving 62 genes plus 5 reference genes for further analysis. Surgery and treatment groups were normalized to 12-week sham (Sham). Due to the extremity of the physiologic responses to Obstruction alone (PBO), we considered that these PBO data points might overwhelm smaller, but still significant de-obstruction patterns. To find relevant de-obstruction responses to therapy, we identified Pearson's correlations between expression and function in treated and untreated de-obstructed samples, i.e. REL+veh and REL+rapa. Pearson's correlations were plotted using 'ggcorrplot' on 'R'.

Circadian rhythm induction and analysis: Bladder smooth muscle cells, which had been pre-starved to induce quiescence, were treated with dexamethasone (100 nM) for 1 hour. RNA from replicates ($n=4$) were isolated in Trizol as above, every 4 hours, and the cDNA examined by QPCR for expression of circadian genes and the three genes of interest using the R package 'rain' or 'cosinor' and Pearson's correlations.

Study approval: The Animal Use Protocol for the following experiments was approved by Hospital for Sick Children's Animal Care Committee. The protocol followed policies established in the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals.

Results

Rapamycin treatment of de-obstruction improves bladder function: We confirmed the activity of rapamycin by immunostaining cryosection for S6 activity (Supplemental Figure S2). After 6 weeks of partial bladder obstruction, physiologic parameters were significantly altered in the obstructed animals in both pre-REL+future veh and pre-REL+future rapa groups vs. sham (Right vs left panels, Fig.2, Supplemental Figs. S3 vs. S4). Importantly, the pre-REL+future veh or pre-REL+future rapa groups were not significantly different (Right panels, Fig.2A,B). After 6 weeks of de-obstruction (REL), residual volumes and micturition fractions did not return to sham levels (Fig. 2A,B, $*p < 0.05$), consistent with ongoing obstructive disease. Rapamycin significantly improved residual urine volume and micturition fraction towards sham levels in postREL+rapa animals (Fig. 2A,B, $*p < 0.05$). Some functional parameters inherently recovered in the 6 weeks following release of obstruction, to sham levels equally with or without therapy (Supplemental Fig.S4). The mean and maximum voided volumes were no different between pre-REL+future veh and pre-REL+future rapa bladders.

The pre-REL mean and maximum voided volumes returned to sham levels in both REL+vehicle and REL+rapamycin ($p<0.05$, [Supplemental Figs. S3 vs. S4](#)). The number of voids in preREL+veh was lower than REL or sham, but the number of voids was increased in REL vs. sham and unaffected by rapamycin ([Supplemental Figs.S3B,S4A](#)).

Gross bladder hypertrophy persists after release of bladder obstruction: PBO significantly increased bladder mass as well as bladder to body mass ratio (Fig. 3A,B, both >5-fold vs. sham, $p<0.05$). Both REL+rapa and REL+veh had lower bladder mass and bladder/body mass ratios than obstruction ($p<0.05$). However, REL+veh bladder mass and mass ratios were 1.7- and 2.3-fold higher than sham levels, respectively ($p<0.05$; Figs. 3A, B). The starting body mass of the animals varied from 250 to 300 g, but body mass gain during the experiment varied with surgical intervention and treatment (Fig. 3C). In PBO, REL+rapa or sham+rapa animals, total body mass did not change significantly from nadir levels.

SMC hypertrophy and ECM alterations during Release of obstruction (REL): PBO cross-sections demonstrated consistently higher SMC hypertrophy compared to all groups (Fig. **3D,E**, $p<0.05$, [Supplemental Figure S5](#)). REL+veh SMC hypertrophy was 2.5-fold less than PBO ($p<0.01$), but still 30% higher than shams, ($p<0.05$). Rapamycin treatment did not significantly mitigate the cross-sectional SMC hypertrophy, although a trend towards decreasing hypertrophy in REL+rapa compared to REL+veh was noted (83 vs 96 μm^2). No difference between REL+rapa and 12wsham (83 vs. 74 μm^2 , Fig. 3) was seen. Gross wall thickness in H&E sections of both REL groups was increased compared to sham or PBO, $p<0.05$ (**Fig.4**). In terms of matrix of the detrusor wall, elastin fibres were more prominent in the rapamycin treated REL samples (Fig. **5A**). Optical polarimetry of REL samples showed a decrease in collagen fibrils, whereas rapamycin partially reversed this (Fig. **5B**).

Gene expression during the release phase after obstruction is significantly responsive to pharmacotherapy.

To understand mechanisms of rapamycin effects on physiology, we assessed candidate gene mRNA levels using a custom high throughput QPCR (HT-QPCR). 6 genes were significantly dysregulated during obstruction (See Table 1). One persistently dysregulated gene after de-obstruction compared to sham, KCNB2, is the subject of another study (Jiang *et al*, Submitted). 8 genes were altered significantly by treatment during release: 3 downregulated, and 5 upregulated (Table 1). Treatment also significantly downregulated REL+rapa levels vs. REL+veh. Of ECM genes, Col3a1 was differentially expressed during PBO and REL (Table1), and MMP2 showed a difference in expression with REL and sham by one-tailed t-test. Pearson's correlations were performed to detect associations between bladder physiology and all HTQCPR genes ([Supplemental Fig.S6](#)). There were many genes associating with each other, but few correlated with function. Pearson's correlations of the significantly changed genes in both REL+ veh and REL+rapa groups from Table I, helped to identify rapamycin-responsive genes whose expression correlates with improved physiology (Fig. 6A). Three genes, IGFBP7, BMP2 and SOD3, inversely correlated with improvements in micturition fraction, and 1 gene,

1 ATP2A2, correlated with residual volume. We further confirmed in a larger group of samples that these three
2 genes, BMP2, SOD3 and IGFBP7, were significantly upregulated by PBO and REL+veh vs. sham (Fig. 6B,C,
3 D).

4 ***Exogenous BMP2 and SOD3 directly affect smooth muscle phenotype:*** Exogenous IGFBP7 did not appear to
5 alter the intensity of the early SMC marker ACTA2 (Fig.6E), though cell number appeared to increase. In
6 contrast, exogenous BMP2 increased ACTA2, whereas exogenous SOD3 decreased ACTA2 intensity.

7 ***Transcriptional regulation of E-box activators during obstruction, release and rapamycin treatment:*** TFBS
8 analysis on revealed an enrichment in Igfbp7, Bmp2 and Sod3 promoters for E-box motifs that bind Arntl/Bmal,
9 Dec1 and other clock genes (Table 2). As circadian activators and repressors regulate smooth muscle
10 function(51-59) but have unknown roles in bladder obstructive disease, we assessed the transcriptional
11 regulation of core circadian genes in our obstruction and de-obstruction models. Obstruction increased
12 expression of Clock, Npas2, Dec1, and Dec2, which regulate circadian E-boxes (Fig. 7, Supplemental Figure
13 S7). Arntl, Cry1, Per1 and Per2 did not show consistent upregulation with obstruction (Fig. 7). Nr1d1 was
14 decreased during obstruction, $p<0.05$. After release (REL), Clock, Cry1, Dec1, Dec2, Npas2, showed reduced
15 expression. In contrast, Nr1d1 expression levels during REL were higher than obstruction, and no different from
16 sham levels. Interestingly, Cry2 expression was increased in both REL (by 34-fold) and obstruction (12.5-fold),
17 $p<0.05$ using logarithmic ddc(t) values for the t-test. Dec1 and Clock protein expression was upregulated in the
18 nucleus of bladder SMC, but not as dramatically as their corresponding mRNA expression (Fig. 7,
19 Supplemental Fig. S7A). Rapamycin treatment during release significantly reduced the expression of Clock,
20 Cry1, Cry2, Per1, Npas2 and Nr1d1 mRNAs $p<0.05$, REL vs. REL+rapa. Similarly, Per2 expression in
21 REL+rapa was significantly lower than obstructed bladders. In contrast, Arntl was not reduced by rapamycin.

22 ***BMP2, SOD3 and IGFBP7 correlated with particular clock genes expression in vitro:*** In vitro,
23 dexamethasone induction of circadian cycling in bladder SMC resulted in changes of expression of the three
24 target genes (IGFBP7, SOD3, BMP2) compared to vehicle control at discrete timepoints (**Fig. 8A-J**). The three
25 target genes demonstrated similar patterns of expression to several circadian genes, including NPAS2 and
26 CLOCK (**Fig. 8A-E**). Dexamethasone induced differential patterns of expression of CLOCK, NPAS2, NR1D1,
27 and others consistent with circadian patterns. NR1D1 and IGFBP7 and SOD3 showed similar patterns of
28 expression that were highly correlated with NPAS2 and CLOCK expression, $R^2<0.53$ to 0.71 . In contrast, the
29 pattern of BMP2 expression was more similar to PER1+2, or Cry1 expression, with a correlation of $R^2<0.86$,
30 0.76 , 0.75 , respectively. Dexamethasone also upregulated the protein of CLOCK in bladder SMC, which was
31 mimicked by stretch plus relaxation at the same final timepoint (**Fig.8K**). In contrast, 4 hours of stretch without
32 the relaxation period failed to show any upregulation of CLOCK compared to no stretch (data not shown).
33 Coordinate with the upregulation of CLOCK was a downregulation of DEC2 protein in both stretch/relax and
34 Dexamethasone treatment.

Disruption of circadian signaling antagonizes obstruction-induced gene expression and increases SMC marker expression: To further examine the dependency of IGFBP7, SOD3 and BMP2 gene expression on circadian rhythm (Fig. 9A-C), we treated human bladder SMC *in vitro* with the Nr1d1 agonist SR9009(60). After 18 hours, SR9009 reduced expression of BMP2 and IGFBP7 by 20 and 41%, respectively (Fig. 9B) in resting cells. In SMC that were stretched for 6 hours then left to relax for another 12 hours, SOD3 was strikingly increased by stretch (Fig. 9A), and stretch-induced SOD3 was significantly inhibited by SR9009 ($p<0.05$). A similar pattern was seen with several core clock genes, CRY2, CRY1, PER1, NPAS2 and CLOCK (Fig.9C-G). These genes showed stretch-responsive upregulation that was inhibited by SR9009. In the same genes, rapamycin showed strong trends towards inhibition of the gene expression. In NPAS2 and CLOCK, basal expression was also downregulated by SR9009 (Fig. 9C,D). Expression of two genes, BMAL and NR1D1 (Fig.9H, I), however, was repressed during stretch, and recovered by SR9009. Interestingly, SR9009 or rapamycin were able to increase expression of the SMC marker calponin in SMC plated on the mitogenic substrate of denatured collagen (Fig.9J).

Overexpression of RHEB, upstream of mTOR, leads to increased CLOCK expression (Fig.10). The lysates from RHEB but not S6K overexpressing cells, showed a significant increase in CLOCK protein (Fig. 10A). Although both FLAG-RHEB and constitutively active HA-S6K increased the phosphorylation of S6, vs. GAPDH (Fig.10B), the S6K clone failed to increase CLOCK expression at the 20-hour timepoint. Immunoprecipitation did not find a clear association between CLOCK protein and either RHEB or S6K (data not shown), suggesting that indirect effects lead to the increase in CLOCK in the cell lysates. We also examined mRNA expression in RHEB overexpressing SMC at an earlier timepoint of 12 hours. RHEB overexpression increased in DEC2 and BMAL mRNA, yet decreased CLOCK, PER1 and PER2 mRNA (line indicates $p<0.05$, Fig.10C) revealing a reciprocal relationship between CLOCK and DEC2 expression in bladder SMC, as in Fig. 8K.

Discussion

Physiology and statistical method for assessing treatment efficacy:

This study was designed to test the potential to modulate functional and mechanistic bladder pathology after release of bladder obstruction. After de-obstruction or release of obstruction (REL), the bladder partially recovers but also shows persistent pathophysiological changes that remain unresponsive to treatment, both clinically and experimentally. By monitoring pre- and post-release functional endpoints of obstruction, directly assess treatment efficacy in the post-obstruction milieu by internally comparing results in individual animals acting as their own controls. A high degree of variability in both the obstruction and de-obstruction phase require filtering prior to de-obstruction for a consistent set of animals, which can be done using non-invasive

functional testing and principle component analysis (PCA), as we have shown. The variability in the release response may be due to the variability of the initiating obstruction, the de-obstruction variability or the length of time after release (6 weeks). This enabled the detection of beneficial effects of rapamycin on the release phase. Rapamycin was used as a prototype for pharmaco-manipulation due to its reported non-cholinergic modulation of disease development during the onset of obstruction. We measured both voiding function and residual urine volumes in all animals immediately prior to release (pre-release; preREL) and also six weeks after release (postREL) of the partial obstruction. Further analysis of the bladder tissues harvested after the total 12 weeks allowed us to examine concordance between the functional data and bladder structure, hypertrophy, and gene expression. Both micturition fraction, the number of small voids and residual volumes were persistently altered in REL, remaining significantly above sham but below corresponding preREL values in respectively matched animals. These observations are consistent with patient and experimental literature(23, 61), attesting to the clinical relevance of this novel study of the post-obstruction release phase of this disease state. Importantly, rapamycin resolved some persistent changes in post-release bladder function, reducing the residual volumes and improving micturition frequency (Fig.2). This is consistent with the beneficial effect of rapamycin on ongoing bladder obstruction, where it also improved residual urines and bladder capacity(4). Neither existing nor new medical treatments for REL bladder dysfunction have been systematically tested with functional data obtained before and after the REL phase. Indeed the responses to therapy may be unique during this phase of the disease, and need to be addressed separately from obstruction alone.

Persistence of hypertrophy:

During obstruction, rapamycin treatment reduces bladder mass and smooth muscle hypertrophy(4), yet approximately 50% of 6 wk obstruction-induced tissue hypertrophy was resistant to rapamycin treatment suggesting additional mechanisms remain to be identified. Following de-obstruction in the present study, tissue and SMC hypertrophy persisted (Fig. 3), similar to that reported by Gabella and Uvelius(62), in which SMC remained twice their normal size even 8 weeks after de-obstruction. Rapamycin-resistant hypertrophy during both PBO and REL may be dependent on other growth pathways of bladder smooth muscle, such as NFATc/calcineurin, STAT3, IL1 β , PDGF, FGF2 and TGF β (9, 12, 63-70). Consequently, to understand persistent hypertrophy in future it may be of interest to further delve into the gene expression patterns and transcriptional pathways induced despite rapamycin treatment, which correlate with the mTOR-independent gross hypertrophy. Nevertheless, while reducing hypertrophy remains an empiric therapeutic goal, it is not clear if mitigating ongoing gross hypertrophy postREL is of additional functional benefit. In this particular setting of de-obstruction, it does not appear that a direct ratio exists between functional benefit and hypertrophic reduction. A tipping point may exist where augmented mass leads to functional deficits.

While hypertrophy of bladder SMC during de-obstruction appears resistant to rapamycin treatment, luminal size was altered with rapamycin treatment. Luminal volume and related residual volume are dependent upon the

ability of the bladder to empty, and consequently also matrix stiffness and contractile function. Wall thickness can increase through multiple mechanisms including both matrix parameters and cellular contractile phenotypes of the detrusor. The non-significant increase in wall thickness with rapamycin treatment was concordant with a decrease in luminal area of the whole bladder cross-sections and residual volumes (Fig. 2A, residual volume in REL+veh vs. REL+rapa, $p<0.04$, and Fig. 4). The slightly increased wall thickness but decreased luminal volumes is concordant with the lack of reduction in overall absolute bladder mass or bladder-to-body mass ratio with rapamycin treatment during release (REL+rapa vs. REL+veh Fig. 4A,B).

Rapamycin partially restores function:

Despite the persistence of gross bladder hypertrophy in this de-obstruction model, rapamycin treatment during REL was able to partially improve the function. Part of the return to function may relate to changes in matrix proteins. ECM gene expression and MMP expression is altered during *in vitro* bladder SMC stretch(16, 71), *ex vivo* distension alone and bladder obstruction(4, 72). Indeed, ECM expression changes during the release phase present a pattern distinct from sham or obstruction. In another study, obstruction decreased the ECM master regulator TGF β , whereas 10 days release of obstruction increased it(73). As we looked at 6 weeks of de-obstruction, not 10 days, differences are expected. However, our goal was not to study the acute response to de-obstruction, but lasting changes persisting after de-obstruction, which did not appear to include TGF β . Despite this, it is important to note that the mTOR pathway is considered a crucial translational regulator of TGF β , even in the absence of transcriptional changes. In future, we may examine the protein levels of TGF β with and without rapamycin treatment.

In drug-eluting stents, rapamycin enhances deposition of normal elastin fibrils(74). During obstruction, rapamycin helps to maintain or restore elastin fibrils, while elastin mRNA expression is decreased by rapamycin treatment(4). Obstruction induces collagen production in the endomysium surrounding cells, whereas rapamycin-treatment concentrated collagen in the epimysium and interstitial spaces. Similarly, in REL tissues, the fibrillar matrix appears to be undergoing extensive remodelling which is improved by rapamycin (Fig.4). As elastin networks can be degraded by MMPs and elastases, which are induced during obstruction(4, 75, 76) this pathology could persist into the REL phase, where rapamycin would exert a beneficial effect on elastin through inhibition of elastin degradation. In previous obstruction-related studies, functional improvements due to treatment of partial obstruction with rapamycin segregated with normalization of expression levels for many genes including the structural genes collagens, elastin and MMP7, which acts on elastin(4). However, MMP inhibition using doxycycline after relief of 3 week partial obstruction lead to detrimental remodelling and worsening function, including increased mean voided volumes and voiding frequencies(77). Unlike Doxycycline, which affects only activity of MMPs, rapamycin alters expression of both MMPs and ECM structural genes(4), which could explain the differential effects on bladder function. As a group, 2 ECM genes were significantly reduced in expression altered during REL, with, e.g. Col3a1 above PBO levels and Mmp2

below sham levels (Table 2). Also, rapamycin downregulated Mmp2 (using 1-tailed t-test) compared to vehicle alone (data not shown). As the matrix is regulated not just by expression, but also by activity of the MMPs and other ECM enzymes, it is possible that the activity of MMPs is altered through rapamycin effects on the circadian proteins, mTOR pathway or the three genes, which could be teased out most readily *in vitro*. Together, changes in gene expression of these genes that affect degradation, production and cross-linking could exert a cumulative or synergistic effect on the collagen network.

Indeed, the biochemical integrity of collagen networks (which depends upon the balance of production, fragmentation of fibrils and cross-linking of fibrils) has not been previously examined during de-obstruction, although collagen protein accumulation has been correlated with loss of function in a rabbit model of released obstruction(78). Using Optical polarimetry which quantifies fibril arrangement of intact fibrils, we found that rapamycin appears to improve fibril structure during de-obstruction (Fig.5B). Collagen fibril formation is dependent upon production and modifications by prolyl hydroxylases, LOX, MMPs, free radicals, and small collagens, such as COL12A1 (for Review of ECM in bladder(76)). Without structural collagen integrity and the attendant buttressing that the intact matrix scaffold provides, the mechanical forces required during de-obstruction may increase, perhaps altering the ability of the muscle to form efficient contractile bundles.

Circadian clock gene expression in stretch and obstruction:

Using the limma statistical method, we found a restricted number of differentially expressed genes during rapamycin treatment, and only three genes of these were associated with functional improvements during release plus rapamycin treatment: BMP2, IGFBP7 and SOD3. This low number of differentially expressed genes may be due to inherent physiologic variability in the obstruction and subsequent de-obstruction responses of the bladder, but also due to the low number of initial candidates. However, QPCR for these genes using a larger dataset, showed that these three target genes increase during obstruction, persist in REL and are profoundly inhibited by rapamycin. All 3 encode extracellular proteins, and have known effects on mesenchymal and smooth muscle differentiation(79-84). Indeed, we saw that they have diverging effects on SMC number and SMC phenotype (Fig. 6E).

Importantly, we found that the promoters of these genes are associated with motifs bound by the transcription factor Dec1 or Dec2, which bind to a class B E-box element, similar to binding sites of other BHLH and circadian rhythm proteins. The increase in BMP2, IGFBP7 and SOD3 during obstruction, and their reduction in response to rapamycin was concordant with the expression patterns of several transcriptional activators of the circadian E-boxes, including core clock (circadian) genes Cry2, Clock and Npas2.

Bladder smooth muscle is known to have peripheral circadian rhythm(55), which can be mechanically induced *ex vivo*. However the effect of obstruction, its release and the mTOR pathway on core clock gene expression in the bladder has not been previously studied. It was clearly shown here that bladder

obstruction and release dysregulates core clock gene expression, with the exception of ARNTL. Furthermore, rapamycin has a severe dampening effect on the core clock genes (Figs.7 and 9). Interestingly, we also found that our target genes follow either one circadian pattern or another either peaking earlier or later. This may be due to co-regulation by E-boxes coordinate with binding at sites for vitamin D receptor (DBPE) and retinoic acid receptor (RORE). Interestingly, one core clock gene was downregulated during obstruction and stretch: NR1D1, a repressor of gene transcription (Fig.7 and 9H). We found that the NR1D1 agonist, SR9009, was able to alter expression of target genes and circadian genes either basally or during a stretch/release program. This was even seen in the gene Cry2, which showed a very high persistent upregulation in REL bladders. This is suggestive of a role for NR1D1 repressor function in regulation of upstream effectors of circadian genes during obstruction and perhaps during REL as well. The in vitro overexpression studies suggest that the normal regulation of circadian networks may be disrupted by hyperactivation of the mTOR pathway. As stretch plus relaxation in bladder SMC was also shown to alter circadian gene expression, the question remains whether stretch is acting through mTOR and which arm of the mTOR pathway is required. The impact of NR1D1 agonism on SMC phenotype was comparable to that of rapamycin, as they both lead to an increase in the differentiation marker calponin in cultured cells. Given the role of NR1D1 on SMC differentiation, it would be of interest to examine, if NR1D1 and other circadian regulators affect the activity of the obstructed bladder during sleep (day-time for rats) vs. during wakefulness (night for rats), and how it may benefit experimental obstruction and contractile function. Our findings are also corroborated by data in human studies. The expression of key clock genes and their proteins found in rodent bladders(55, 57, 85-87) are also shown in human bladder tissues (88). Furthermore, circadian dysregulation may also cause bladder pathologies in humans including bladder cancer, prostate cancer, LUTS, nocturia and overactive bladder (89-92). Specifically, the circadian rhythm of androgen in humans, which is key to the development of benign prostatic hyperplasia also exists and is under the control of clock genes in human studies(93). The mechanism whereby clock genes control BMP2, IGFBP7 and SOD3 in bladder obstruction, discovered here, may have important implications in the pathogenesis and treatment of prostatic hypertrophy and bladder outflow obstruction.

Summary:

The question of whether or not persistent bladder pathology post-release from obstruction can be altered by a mechanistically relevant pharmaco-therapy was addressed by analysing functional and mechanistic changes pre- and post- release from PBO. This approach provides a foundation for evaluating functional, histologic and molecular changes through collection of micturition and residual volumes both immediately prior to obstruction relief (preREL) and 6 weeks after reversal of obstruction (REL) (see Model in Fig. 11). While used as a clinically available pharmacologic prototype, rapamycin at this dosage also suppresses weight gain in this

study and observed previously. Thus, more specific inhibitors targeting specific arms of the mTOR pathway as well as further dose-finding studies are needed to reduce off target effects. Using this novel clinically relevant framework, the effect of pharmacologic interventions on postREL function and expression should reveal approaches with more potential for clinical translation than those revealed during evolving obstruction alone. The present study demonstrates that treatment specifically during the release phase from obstruction provides multiple opportunities for further study and novel manipulation of biologic response and recovery of normal gene expression and tissue function. Indeed, this strategy has revealed a new post-obstruction transcriptional mechanism, circadian pathways, which have increased our understanding of post-release biology and future avenues for therapeutic research. This approach demonstrates for the first time that post-obstruction histopathology and dysfunction formerly believed to be immutable displays a unique molecular biology, and is physiologically malleable.

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TABLES

TABLE 1: Expression analysis by limma of HT-QPCR gene expression changes in REL, REL+RAPA, PBO and sham. Ddct=delta-delta c(t) changes.

TABLE 2: Transcription factor binding site (TFBS) analysis. Top five distant and proximal transcription factor (TF) binding sites during rapamycin- vs. vehicle+Release of obstruction from DIRE. DEC= STRA13 , LYF1 = ikaros or IKZF1, AP2ALPHA = TFAP2A, RPBJK = Intracellular Notch binding factor. Other factors are listed in supplemental data. DEC TFBS associated with E-box binding circadian BHLH transcription factors: DEC1 (STRA13), DEC2 (HES1), CLOCK/NPAS2 with BMAL.

Figure Legends:

Figure 1: Animal model schematic. Functional endpoints were collected after 6 weeks of partial bladder outlet obstruction (preREL+future veh or preREL+future rapa) or obstruction plus release of obstruction during another six weeks (REL+veh or REL+rapa), or 12week sham+veh or 12sham+rapa. Micturition readings

collected using metabolic cages included 12hr total excreted urine volume, micturition fraction, mean voided volume, and functional bladder capacity and maximal single volume voided. Residual volumes were collected by 22 gauge angio-catheter. Micturition readings collected at 6 weeks were recorded in untreated preREL animals (preREL+future veh or preREL+future rapa), which were later treated after release with vehicle or rapamycin (REL+ veh or REL+rapa, see dark arrows) for another 6 weeks. Differences between samples in preREL+future veh or preREL+future rapamycin were compared with REL+veh or REL+rapamycin, respectively, to ascertain improvements during the 6-week REL treatment protocol. These readings were also compared with 12-week sham-obstructed animals treated with vehicle or rapamycin in the last 6 weeks. Endpoints collected from harvested bladders after partial outlet obstruction (PBO) for 6 weeks, release of PBO for another 6 weeks (REL), or sham obstruction for 12 weeks total (sham) included bladder mass, immunofluorescent staining, histology and RNA expression analysis.

Figure 2: Rapamycin improves bladder function after release of obstruction. **A:** Residual volumes return to normal levels in rapamycin-treated animals. **B:** Micturition fraction post-release (REL) increased to normal levels with rapamycin vs. vehicle. Pre-release residual volumes (in right panel A) and micturition fractions (right panel B), measured 1-2 days prior to the 6 week release timepoint, did not show significant differences between groups randomized for future post-release treatment with either vehicle or rapamycin (future veh or future rapamycin). Sample numbers are described in Figure 1: pre-release (preREL+veh) and post-release (REL+veh) vehicle=22, preREL+rapa and REL+rapa=18, sham+veh=17, sham+rapa=13. * $P < 0.05$, REL vs. sham controls or REL+rapa treatment.

Figure 3: Gross hypertrophy occurs during bladder obstruction and persists after release of obstruction. Bladder to body mass ratios (A) and Bladder mass (B) remained above normal after release of obstruction with or without rapamycin treatment. Bladders mass from sham release (sham), released (REL), and obstructed rats (PBO) were significantly different, with a >1.7-fold difference between sham (black bars) and both REL groups (white bars, $p < 0.02$, dashed lines indicate difference). (C) Body mass was significantly lower when rapamycin was given to either sham or de-obstruction. Sample numbers consisted of PBO=8, REL=22, REL+rapa=18, sham+veh=17, sham+rapa=13. (D) Expression of myosin is decreased during obstruction and release compared to sham vehicle levels. Representative images were taken from $n=6$ samples per group. Immunofluorescent staining of smooth muscle myosin heavy chain (Green) was used to mark smooth muscle bundles in the detrusor in the images in left panels. In REL+veh or +rapa and obstruction (PBO), myosin staining revealed a mean decreased intensity in muscle bundles. SMC hypertrophy was noted in the REL+veh or +rapa SMC. Marked hypertrophy is seen in SMC at six weeks of PBO. Representative images of negative controls (primary antibody absent, with secondary included) are shown in right panels. Myosin intensities decreased with obstruction and release of obstruction (REL) with either vehicle or rapamycin treatment ($p < 0.05$). Original magnification 200X for all photos. Scale bar is indicated. **E,** Morphometry for SMC and immunofluorescent staining reveals persistence of hypertrophy with release of obstruction (REL). Cross-sectional SMC hypertrophy of cells containing circular nuclei (indicating the middle of the cell) revealed an increase in hypertrophy of SMC during obstructions (PBO). Moreover, REL+veh or REL+rapa SMC to show continued hypertrophy by cross-section compared to sham. *, $p < 0.05$, ** $p < 0.01$.

Figure 4: Release of obstruction leads to increased equatorial wall thickness. Haemotoxylin and Eosin staining reveals that bladders released post-obstruction show improvement in lumen size and wall architecture compared to 6 week obstruction and sham bladders. The lumen of the bladders (green "L") appears less expanded with rapamycin treatment. All slide sections scanned (Aperio software) and imaged at same magnification (4X original). Representative images of sections were taken from the following groups: A= sham+veh, B=sham+rapa, C= REL+veh, D= REL+rapa, E and F= PBO (6 week obstruction), showing samples with extreme dilatation of the luminal area.

Figure 5: ECM alterations after Release of obstruction (REL). **A.** Modified Elastin Masson's Trichrome Stain stained sections taken at 400X original magnification. Shorter elastin fibrils are more abundant in partially obstructed (PBO), de-obstructed (REL) bladder sections. Longer elastin fibrils are more prominent in the bladders of rapamycin treated animals. Modified Masson's Elastin Trichrome Stain. Elastin Fibrils=dark blue (yellow arrows); Collagen= bright green-blue. All photos 400X original magnification. **B.** Optical polarimetry of Picrosirius Red stained bladders reveals that release (REL) of obstruction is associated with loss of collagen

fibrils, which is reversed by rapamycin. To assess collagen fibril structure in the bladder, polarized light was used to induce fluorescence of the collagen networks of PicroSirius Red stained sections. Red and orange fibrils are thicker collagen fibrils (quantitated in left graph) that are more common in the sham+veh, sham+rapa treated samples, and less apparent in vehicle-treated REL samples ($p=0.005$). Rapamycin in REL bladders demonstrated an increase in red, orange and yellow collagen fibrils compared to either REL+veh or sham bladders ($p<0.05$).

Figure 6: Differential gene expression and correlations with function reveals three genes with effects on SMC phenotype. A: Heatmap of Pearson's correlations of gene expression and physiology distinguish a few discrete genes correlating with Physiologic changes. From Table 1, the significantly altered genes between REL+RAPA and REL groups were compared with micturition measurements by Pearson's correlations. Using data from rapamycin- vs. vehicle-treated animals, significant correlations with physiology were seen in the bladder for ATP2A2, Bmp2, Igfbp7 and Sod3 (black arrows). **B-D: Bmp2, Igfbp7 and Sod3 gene expression patterns were significantly altered during obstruction, de-obstruction and rapamycin treatment.** We confirmed the changes in expression of HT-QPCR genes altered by rapamycin during the release phase by QPCR. All three genes were upregulated during obstruction. SOD3 and BMP2 were significantly downregulated by rapamycin (REL+RAPA) vs. release of obstruction (REL). QPCR and functional analysis were compared using the larger dataset, revealing correlations with pathophysiologic parameters. **E: Bmp2, and Sod3 induce changes in bladder SMC phenotype.** IGFBP7 appeared to increase cell proliferation, which BMP2 and Sod3 altered ACTA2 expression (up or down, respectively). Exogenous BMP2 (10 ug.mL), IGFBP7 and SOD3 (10 uM) were added to bladder SMC for 48 hours, then stained for alpha-smooth muscle actin (ACTA2), counterstained with Hoechst, and analysed on Volocity. *, $p<0.05$; **, $p<0.01$.

Figure 7: Dysregulation of core clock genes during obstruction and after release of obstruction in the bladder. Clock, Npas2, Per1+2, Cry1+2, Arntl and Dec1+2 were analysed by QPCR for alterations in gene expression during obstruction and after release of obstruction. Cry2, Clock, Dec1+2 and Npas2 showed persistent upregulation, which was improved with rapamycin treatment. Nr1d1 was downregulated with obstruction, but returned toward normal with release. Many core clock genes returned towards basal levels with release of obstruction.

Figure 8: Bmp2, Igfbp7 and Sod3 are expressed in a circadian pattern and 20 hours of circadian cycling induces clock expression similar to stretch. (A-J) Quiescent human bladder SMC were examined for core clock gene expression after the standard treatment for induction of circadian cycling: dexamethasone treatment for 1 hour, followed by a washout and timecourse. QPCR of the core clock genes and BMP2, SOD3 and IGFBP7 revealed that several of these genes had similar expression patterns over the time course. (K) A representative blot from 1 of 3 samples shows that 4 hours stretch plus 16 hours relaxation is similar to circadian cycling by dexamethasone. Both induce upregulation of clock protein by western blotting, coordinate with a reduction of DEC2 protein. *, $p<0.05$, **, $p<0.01$.

Figure 9: Inhibition of mTOR (rapamycin) and circadian modulation (SR9009) alter transcription of circadian genes and Bmp2, Igfbp7 and Sod3 during Stretch/relaxation of bladder SMC. Bladder SMC stretched or unstretched for 4 hours and released 16 hours were treated with SR9009. Genes were assayed by QPCR and analyzed using $\Delta\Delta c(t)$ against reference genes. (A) Stretch-induced alterations in SOD3 gene expression were disrupted by the RevErba agonist, SR9009, a highly selective RevErba agonist, and rapamycin. BMP2 and IGFBP7 expression were reduced by SR9009 (B). (A, C-I) Stretch plus release upregulated SOD3, PER1, CRY1+2, NPAS2 and CLOCK. NR1D1 and BMAL were repressed by stretch. Interestingly, although NR1D1 is downregulated, SR9009 agonist activity inhibited downregulation of NR1D1 and BMAL. Rapamycin had similar but less significant effects than SR9009, demonstrating trends in reduction. SR9009 downregulated stretch-induced SOD3, PER1, CRY1+2, NPAS2 and CLOCK (A, C-I) as well as basal Clock and Npas2 expression (C, D). (J) SMC marker expression (calponin) was restored by SR9009 and rapamycin treatment in SMC cultured on denatured collagen. *, $p<0.05$ and **, $p<0.005$, by 2-tailed t-test.

Figure 10: Overexpression of RHEB and activated S6K increases S6 phosphorylation, but only RHEB shows increased CLOCK expression at 20 hours, yet decreased mRNA expression at 12 hours. Cell lysates or RNA were isolated from SMC electroporated with FLAG-RHEB and HA-S6K-active mutant and cultured

for 12 or 20 hours. Lysates were probed by Western and show that RHEB, but not active-S6K increases (A) CLOCK expression. However, both clones show an increase in overall activity of the S6K pathway, as S6 phosphorylation is increased in both mutants (B). In Supplemental Fig.S8, mTOR phosphorylation was increased by RHEB overexpression. After 12 hours however, the mRNA of DEC2 and BMAL expression are increased in parallel with decreased CLOCK, PER1+2 (C). Other core clock genes were studied by QPCR, but did not show significant changes in expression at this timepoint (data not shown). Lines, $p < 0.05$.

Figure 11: Schematic model of alterations in during obstruction and release of obstruction. Bladder distension as a result of outlet obstruction leads to increased stretch, leading to ongoing alterations in matrix and circadian rhythm disruption, which alters expression of the three genes described in this paper, BMP2, IGFBP7 and SOD3, and altering SMC phenotype.

Supplemental DATA: HTQPCR data will be uploaded to NCBI GEO.

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- Contributed new reagents or analytic tools: MS contributed new analytic tools for micturition analysis (published in Bladder, 2018)
- Analyzed data: KJA JXJ ASch MS
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Table 1: HT-QPCR limma analysis of gene expression changes in REL, REL+RAPA, PBO and sham**REL+RAPA vs. REL**

<u>genes</u>	<u>t.test</u>	<u>p value</u>	<u>adjusted p value</u>	<u>ddCt</u>	<u>fold-change</u>
IGFBP7	-22.23	0.000070	0.004720	9.23	0.0017
SOD3	-4.59	0.01	0.19	1.22	0.43
KCNB2	4.31	0.01	0.19	-0.65	1.57
BMP2	-5.20	0.02	0.27	3.11	0.12
VEGFA	3.75	0.02	0.27	-0.96	1.95
FKBP5	3.34	0.02	0.27	-1.94	3.84
NFKB2	3.37	0.03	0.27	-1.49	2.82
HIF1A	2.77	0.04	0.34	-0.63	1.55

sham vs. PBO

<u>genes</u>	<u>t.test</u>	<u>p value</u>	<u>adjusted p value</u>	<u>ddCt</u>	<u>fold-change</u>
NOS3	-5.41	0.01	0.49	0.96	0.51
FKBP5	-7.22	0.02	0.49	1.10	0.47
DES	3.57	0.03	0.49	-2.52	5.74
TPM1	5.18	0.04	0.49	-2.04	4.10
RYR2	4.78	0.04	0.49	-1.47	2.76
KCNB2	-3.84	0.05	0.49	0.63	0.65

REL vs. sham

<u>genes</u>	<u>t.test</u>	<u>p value</u>	<u>adjusted p value</u>	<u>ddCt</u>	<u>fold-change</u>
KCNB2	-4.93	0.01	0.70	0.93	0.52
MMP2	-2.83	0.08	0.98	1.16	0.45

REL vs. PBO

<u>genes</u>	<u>t.test</u>	<u>p.value</u>	<u>adjusted p value</u>	<u>ddCt</u>	<u>fold-change</u>
CPT1A	-7.95	0.0028	0.18	1.95	0.26
KCNK1	-5.24	0.01	0.31	2.42	0.19
KCNB2	-4.22	0.02	0.31	0.80	0.57
SOD3	3.76	0.02	0.31	-1.12	2.17
TPM1	3.35	0.03	0.31	-1.71	3.27
MECP2	-3.73	0.03	0.31	1.59	0.33
CCND1	3.18	0.03	0.31	-1.07	2.09
HIF1A	-3.47	0.04	0.31	0.88	0.54
CCND3	-2.89	0.04	0.31	1.11	0.46
COL3A1	2.94	0.05	0.31	-1.75	3.37

TABLE 2: Transcription factor binding site (TFBS) analysis. Top five distant and proximal transcription factor (TF) binding sites during rapamycin- vs. vehicle+Release of obstruction from DIRE. DEC= STRA13 , LYF1 = ikaros or IKZF1, AP2ALPHA = TFAP2A, RBPJK = Intracellular Notch binding factor. Other factors are listed in supplemental data. DEC TFBS associated with E-box binding circadian BHLH transcription factors: DEC1 (STRA13), DEC2 (HES1), CLOCK/NPAS2/BMAL.

<u>Transcription Factor</u>	<u>Occurrence</u>	<u>Importance</u>
DEC	25	2.1836
LYF1	50	1.5352
AP2ALPA	50	0.9125
RBPJK	50	0.8750
HNF4	50	0.8750

Figure 1

A

B

Figure 2

Figure 2

A

B

Figure 3**A** **B** **C** **Figure 3****D** **E**

Figure 4

Figure 4

Figure 5

Figure 5

A

B

Figure 6

Figure 6

A

C

B

D

E

Figure 7

Figure 7

Figure 8

Figure 8

Figure 9

Figure 9

Figure 10

A

20 hours

B

Figure 10

C

12 hours

Figure 11

Supplemental Table 1: Target Genes for HT-QPCR

<u>Gene Symbol</u>	<u>Gene Name</u>
ACTB	actin, beta
ADRBK1	adrenergic, beta, receptor kinase 1
ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2
BMP2	bone morphogenetic protein 2
CAT	catalase
CCND1	cyclin D1
CCND3	cyclin D3
CD44	CD44 antigen (homing function and Indian blood group system)
CEBPD	CCAAT/enhancer binding protein (C/EBP), delta
CGRRF1	cell growth regulator with ring finger domain 1
CHRNA5	cholinergic receptor, nicotinic, alpha 5
CIP1a	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
COL12A1	collagen, type XII, alpha 1
COL1A1	collagen, type I, alpha 1
COL3A1	collagen, type III, alpha 1
CPT1A	carnitine palmitoyltransferase 1A (liver)
CTGF	connective tissue growth factor
CYR61	cysteine-rich, angiogenic inducer, 61
DBP	D site of albumin promoter (albumin D-box) binding protein
DES	desmin
DUSP1	dual specificity phosphatase 1
EDNRA	endothelin receptor type A
ELN	elastin
FGF2	fibroblast growth factor 2 (basic)
FGF9	fibroblast growth factor 9 (glia-activating factor)
FKBP5	FK506 binding protein 5
FN1	fibronectin 1
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog
GEM	GTP binding protein overexpressed in skeletal muscle
GYS1	glycogen synthase 1 (muscle)
HBEGF	heparin-binding EGF-like growth factor
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
IGF1	insulin-like growth factor 1 (somatomedin C)
IGFBP7	insulin-like growth factor binding protein 7
JUN	jun oncogene
KCNB2	potassium voltage-gated channel, Shab-related subfamily, member 2
KCNK1	potassium channel, subfamily K, member 1
KCNMA1	potassium large conductance calcium-activated channel, subfamily M, alpha member 1
LOX	lysyl oxidase
MECP2	methyl CpG binding protein 2 (Rett syndrome)
MEF2D	myocyte enhancer factor 2D
MMP2	matrix metalloproteinase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)
MT3	metallothionein 3
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)
NOS3	nitric oxide synthase 3 (endothelial cell)
PCNA	proliferating cell nuclear antigen
PDGFA	platelet-derived growth factor alpha polypeptide
RAC1	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)
RIMS1	regulating synaptic membrane exocytosis 1
RYR2	ryanodine receptor 2 (cardiac)
SOD3	superoxide dismutase 3, extracellular
SRF	serum response factor (c-fos serum response element-binding transcription factor)
TGFB	transforming growth factor, beta 1
THRB	thyroid hormone receptor, beta (erythroblastic leukemia viral (v-erb-a) oncogene homolog 2, avian)
TLK1	tousled-like kinase 2
TPM1	tropomyosin1
TRAF6	TNF receptor-associated factor 6
TRAM1	translocation associated membrane protein 1
TSC22D3	TSC22 domain family, member 3; GRAM domain containing 4
VEGF	vascular endothelial growth factor A
VIM	vimentin

S2 Table: Full Table of promoter analysis by Distal Regulatory Elements of co-regulated genes (DIRE).

Transcription Factor	Occurrence	Importance
DEC	25	2.1836
LYF1	50	1.5352
AP2ALPA	50	0.9125
RBPJK	50	0.8750
HNF4	50	0.8750
ER	25	0.8594
HNF6	25	0.7961
E2F1DP1	25	0.7961
ERR1	25	0.7961
AP2GAMMA	50	0.7438
PXR	25	0.6695
OLF1	25	0.6680
AP1FJ	25	0.6268
TTF1	25	0.6063
WHN	25	0.5828
AHR	50	0.5750
CREB	50	0.5117
P300	25	0.5031
ROAZ	25	0.4563
MYOD	25	0.4376
CACCC-BINDING FACTOR	25	0.4375
AP2	50	0.4250
TBX5	25	0.4094
TF111	25	0.3719
BACH2	25	0.3695
ZF5	50	0.3500
PEBP	25	0.3156
AHRHIF	25	0.3063
KROX	50	0.2844
NMYC	25	0.2781
MINI19	25	0.2500
HIC1	50	0.2375
LPOLYA	25	0.2371
T3R	25	0.2371
NFE2	25	0.2313
OSF2	25	0.1797
HIF1	25	0.1781
ZNF219	25	0.1750
CBF	25	0.1688
TATA	25	0.1286

Supplemental Figure S1:A: Schematic of functional voiding analyses as in Sidler et al, 2018(44).The maximum volume of the bladder is defined as Maximum Bladder Capacity (MBC) and the subsequent Micturition is referred to as Maximum Voiced Volume. Residual Volume in our case was determined by needle aspiration. Any random volume voided is called Voiced Volume (or micturition volume, MV). Mean or Maximum Voiding efficiency or Micturition Fraction is the ratio of mean or maximum MV over MBC, respectively. Principle component analysis of physiology enabled identification of outliers from the analysis of the pre-release data from animals: B-E. All pre-release data should be highly consistent prior to randomization of animals for future analysis of a treatment regime. Individual rats in particular in purple circle diverged from the other experimental animals in different PCA dimensions. F. Additional PCA analysis of the physiology parameters showed the relationships between different pathophysiologic characteristics, with mean and maximum voided volumes clustering loosely together with micturition fraction (voiding efficiency), but separately from the number of voids (large or small) or residual volumes, suggesting that there are three groups of physiologic parameters regulated separately.

Supplemental Figure S2: mTOR activity represented by phospho-S6 immunofluorescence was increased during obstruction and REL *in vivo* and decreased by rapamycin treatment. Nuclear localization was apparent in the highly stained sections, with notable staining in between bundles, perhaps suggesting that high degree of mTOR signaling led to support of de-differentiation SMC or myofibroblasts. Rapamycin reduced the mTOR activation. Scale bar = 23 microns.

Supplemental Figure S3: Pre-release functional parameters of obstruction animals were highly similar. Mean and Maximum voided volumes, and voiding frequencies of the Sham and REL groups 1 day prior to the time of pre-release.

Supplemental Figure S4: Post-release bladder function. While the number of voids (hyperactivity) was persistently upregulated (A), many functional parameters 6 weeks post-release returned almost completely to sham levels (B) or completely (C-F). Maximum voided volumes (D), mean voided volumes (B) all showed improvements toward sham levels between pre-release (preREL) and post-release. Number of voids was significantly different with REL (*, $p < 0.05$), but did not improve significantly with rapamycin treatment.

Supplemental Figure S5: Haemotoxylin and Eosin staining reveals that bladders released post-obstruction an show differences in hypertrophy and bundle architecture (yellow arrows) compared to 6 week obstruction (green arrows) and sham bladders (black arrows). Lumenal size (green “L”) appears to improve with rapamycin treatment. Slide sections were scanned and visualized with Aperio software and imaged at same magnification (4X original). Representative images of sections were taken from the following groups: A= sham plus vehicle, B=sham plus rapamycin, C= Release plus vehicle, D= Release plus rapamycin, E and F= 6 week obstruction, showing samples with varying thickness of the wall.

Supplemental Figure S6: Pearson’s correlations of bladder functional and expression patterns in sham, released and released plus rapamycin samples. Obstruction data was omitted, as the magnitude of some of the responses would mask the relationships in REL+veh vs REL+rapamycin samples. The expression patterns of many genes clustered together, but few showed correlations with physiology (see black boxes).

Supplemental Figure S7: Clock and Dec1 expression are enhanced in nuclei of released bladders, but are also present in the cytoplasm of all bladders. (A) Clock protein was localized clearly to the nuclei of muscle in PBO bladders, while REL showed less nuclear staining. Released bladders treated with rapamycin (REL+Rapa) showed less intense Clock than REL+Veh, while Sham + rapamycin (Rapa) demonstrated an unexpected increased Clock intensity. Scale bar = 12 microns. (B) The repressor Dec1 was apparent in both sham and REL bladders, but without any significant changes in average intensity between groups (data not shown). In contrast, the intensity of Dec1 in the smooth muscle compartment of only REL bladders as compared to the sham was significantly increased ($p<0.05$), although mean Dec1 intensity in all cell types of the bladder did not differ between REL nor sham bladders. Rapamycin treatment during release (REL+rapa) significantly decreased nuclear Dec1 protein compared to REL+vehicle ($p<0.05$).

Supplemental Figure S8: mTOR activity was increased by FLAG-RHEB overexpression, but not by FLAG-RHEB-N153T constitutively-active mutant. (x indicates a lane in which transfer did not occur efficiently in the upper molecular weight range due to an air bubble in the upper molecular weight area of the lane. After electroporation, overexpression of RHEB (wild-type) was sufficient to increase mTOR phosphorylation relative to the reference protein, GAPDH, in SMC.