BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

DNA Methylation Reduces the Yes-Associated Protein 1/WW Domain Containing Transcription Regulator 1 Pathway and Prevents Pathologic Remodeling during Bladder Obstruction by Limiting Expression of *BDNF*

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Chronic bladder obstruction and bladder smooth muscle cell (SMC) stretch provide fibrotic and mechanical environments that can lead to epigenetic change. Therefore, we examined the role of DNA methylation in bladder pathology and transcriptional control. Sprague-Dawley female rats underwent partial bladder obstruction by ligation of a silk suture around the proximal urethra next to a 0.9-mm steel rod. Sham operation comprised passing the suture around the urethra. After 2 weeks, rats were randomized to normal saline or DNA methyltransferase inhibitor, 5-aza-2-deoxycytidine (DAC) at 1 mg/kg, three times/week intraperitoneally. After 6 weeks, bladders were weighed and divided for histology and RNA analysis by high-throughput real-time quantitative PCR arrays. DAC treatment during obstruction *in vivo* profoundly augmented brain-derived neurotrophic factor (BDNF) expression compared with the obstruction with vehicle group, which was statistically correlated with pathophysiologic parameters. BDNF, cysteine rich angiogenic inducer 61 (CYR61), and connective tissue growth factor (CTGF) expression clustered tightly together using Pearson's correlation analysis. Their promoters were associated with the TEA domain family member 1 (TEAD1) and Yes-associated protein 1/WW domain containing transcription regulator 1 pathways. Interestingly, DAC treatment increased BDNF expression in bladder SMCs ($P < 0.0002$). Stretch-induced BDNF was inhibited by the YAP/WWTR1 inhibitor verteporfin. Verteporfin improved the SMC phenotype (proliferative markers and SMC marker expression), in part by reducing BDNF. Expression of BDNF is limited by DNA methylation and associated with pathophysiologic changes during partial bladder outlet obstruction and SMC phenotypic change *in vitro*. (*Am J Pathol* 2018, 188: 2177–2194; <https://doi.org/10.1016/j.ajpath.2018.06.024>)

Partial bladder outlet obstruction (PBO), occurring in diseases such as prostate hyperplasia and urethral valves, is a widespread cause of urinary dysfunction, patient discomfort, and overactive bladder. It was responsible for \$65.9 billion in US 2007 health care costs.¹ Although removing the obstruction partially improves the functional and pathologic sequelae, ongoing symptoms remain in

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many patients. Epigenetic changes, such as DNA methylation, play an important role in both adaptive and recovery processes, including fibrosis and proliferation.^{2,3} Epigenetic mechanisms may be critical components of the response to obstructive stimuli (matrix, hypoxia, inflammatory, and mechanical stimuli). Because DNA methylation may be crucial to the maladaptive or adaptive responses to these stimuli, a hypomethylating agent was used in a rodent model of PBO to evaluate the role of DNA methylation in bladder remodeling and gene expression during obstruction. This invites investigation of potential epigenetic mechanisms in bladder tissue homeostasis and repair.

During aging, global loss of DNA methylation occurs across the genome. The aging bladder is commonly clinically associated with dysfunctional changes that include the gradual onset of irritative and obstructive bladder symptoms. However, it is not known if DNA methylation may also contribute to the clinical pathology of the aging bladder. *In vitro*, DNA methylation changes dynamically in response to obstructive stimuli in bladder smooth muscle cells alongside proliferation.³ In this study, we investigated the effect of DNA methylation during obstruction of nonaging rats to isolate DNA methylation from other age-related pathologies. Functional bladder parameters were assessed using metabolic cages and associated gene expression during obstruction in nonaging rats while inhibiting DNA methylation. By studying candidate gene expression, the most profound and significant changes in gene expression among all groups were identified as brain-derived neurotrophic factor (BDNF) and connective tissue growth factor (CTGF). Using correlation analysis, novel and discrete changes in gene expression in *Bdnf* and *CTGF*, relevant to obstructive bladder dysfunction, were identified. It was further determined that BDNF, cysteine rich angiogenic inducer 61 (CYR61), and CTGF share transcriptional regulation through the YAP/WWTR1 pathway. BDNF's massive increase in transcript level and localization around smooth muscle bundles led us to investigate its role more clearly. BDNF can induce highly contrasting effects during bladder obstruction,^{4,5} including overactivity, but also restoration of neuronal function. The functional role of BDNF on bladder smooth muscle phenotype has previously not been examined in detail, despite potential implications for regulation of contractile machinery. We examined the role of DNA methylation and YAP/WWTR1 in gene expression and their coordinate effects on bladder smooth muscle cell (BSMC) pathophysiology.

Materials and Methods

Partial Bladder Outlet Obstruction

Partial bladder outlet obstruction was performed as described,⁶ under a protocol approved by the Animal Care Committee at The Hospital for Sick Children Research Institute, following

policies established in the Canadian Council on Animal Care *Guide to the Care and Use of Experimental Animals*. In this design, 200 to 250 g Sprague-Dawley female rats ($n = 20$; Charles River Laboratories, Wilmington, MA) underwent PBO by tying a silk suture around the proximal urethra and a 0.9-mm steel rod; the rod was then removed, leaving the suture behind to form the partial obstruction. Sham operation was performed without tying the suture ($n = 12$).⁶ Pain medication was given for the first 24 hours and whenever symptoms of pain were evident.

Decitabine Treatment

Hypomethylating activity of 5-aza-2'-deoxycytidine (or decitabine; DAC; LC Laboratories, Woburn, MA) treatment was tested by examining Line-1 methylation in the spleens of rats during a separate dose-finding experiment (Supplemental Figure S1). After 2 weeks of obstruction, half of each group was treated with the DNA methyltransferase inhibitor, DAC, at 1 mg/kg, three times/week intraperitoneally for 4 weeks (Supplemental Figure S2). Control animals received normal saline in the same course and route as the treatment.⁶

Micturition Measurements

Micturition patterns were recorded at 6 weeks 1 to 2 days before sacrifice, using previously described methods.^{7–9} Voiding patterns and volumes (Supplemental Figure S3)⁸ were recorded on a computer using Logger Pro software version 3.9 (Vernier Software & Technology, Beaverton, OR). Residual volumes were measured in anesthetized rats at the time of sacrifice.⁶

SMC Culture and Mechanical Stretch

Human BSMCs (Sciencell, Carlsbad, CA) were maintained in BSMC media including growth supplements, fetal bovine serum, and antibiotic/antimycotic (Sciencell). Cells were plated onto collagen I-coated BioFlex plates (Flexcell International, Burlington, NC) at 4×10^4 cells/mL in a 1:1 mix of BSMC medium plus Eagle's minimal essential medium (EMEM) (Wisent, St. Bruno, QC, Canada) plus antibiotic/antimycotic (Wisent). After 24 to 48 hours, cells were serum starved for 1 day in EMEM, then treated in EMEM plus either vehicle (dimethyl sulfoxide), verapamil (VP; 0.1 μ mol/L; MilliporeSigma, Burlington, MA), or decitabine (DAC; 2 μ mol/L), 1 hour. Cells were mechanically strained to 5% static elongation on the Flexcell 3000 (Flexcell International) for 6, 8, or 24 hours, using a ramping pattern, as previously described.^{10,11} For exogenous BDNF, Trk inhibitor GNF 5837 (both from Alamone Labs, Jerusalem, Israel), and verapamil treatments, human BSMCs were plated on denatured collagen-coated glass coverslips,¹² and serum starved overnight before treatments. BDNF was added to serum-starved cells at 0, 2.5, 5,

or 10 ng/mL in serum-free EMEM. VP or vehicle (dimethyl sulfoxide at same volume) was added to BSMCs. An initial dosage finding study on human BSMCs showed that VP was not cytotoxic at this level, because it did not decrease cell counts or morphology in unstretched cells (data not shown). Neonatal Sprague-Dawley rat bladder domes were minced and digested using collagenase type IV (Elastin Products Company, Owensville, MO). Digests were washed and cells were preplated to remove fibroblasts. Passaging cells with 0.25 mmol/L trypsin removed contaminating urothelial cells. Cells were plated at passage 3, as late passage primary rat SMC loose differentiation markers. Rat neonatal SMCs were serum starved and stretched in a static ramping pattern for 8 hours, as previously described.¹⁰

Collagen Gel Contraction Assay

Collagen gels were assembled using neutralized type I collagen (2.4 mg/mL; Elastin Products Company, Owensville, MO) and plating into 24-well plates. Serum-starved cells that had been pretreated with vehicle or 10 ng/mL BDNF for 2 days were plated at 5×10^3 cells/mL onto collagen gels in EMEM. After 2 hours to allow for cell attachment, cells were then treated with vehicle, 10 ng/mL BDNF, or BDNF + GNF 5837 (10 μ mol/L), then gels were released from the sidewall of the 24-well plate for another 2, 4, and 18 hours.

RNA Isolation, Custom HT-qPCR Array, and Quantitative RT-PCR

Bladder tissues were harvested, treated in RNAlater at 4°C for 48 hours, dried on sterile gauze, and stored at –80°C until RNA isolation. Tissue samples were crushed under liquid nitrogen, then homogenized at 4°C in Trizol (Thermo Fisher, Waltham, MA) using a refrigerated Bullet Blender (Next Advance, Troy, NY) with steel 0.3 to 1.5 mmol/L pellets for 3 minutes at setting 8. After the steel pellets settled by gravity, the supernatant was processed for RNA isolation.⁶ Samples from cell culture experiments were isolated by adding Trizol to wells and proceeding as previously described.¹¹ Each RNA sample from groups PBO, PBO + DAC, and sham ($n = 4$) was reverse transcribed using Quant-it (Qiagen, Hilden, Germany) and assessed by high-throughput real-time quantitative PCR (HT-qPCR) with a custom array using built-in quality checks. The 85 candidate genes were selected through *in silico* curation of literature on bladder obstruction and bladder smooth muscle dedifferentiation (Supplemental Table S1). The HTqPCR package on R was used to identify statistical significance of the custom array by limma and *t*-test.¹³ Five reference genes, *Rpl13a*, *B2M*, *Hprt1*, *Ldha*, and *Rplp1*, plus β -actin were assessed for optimal normalization on Excel (Microsoft Corp., Redmond, WA) and then the $\Delta\Delta$ Ct method. Confirmation of significant HT-qPCR results was performed on a larger set of RNA samples by real-time

quantitative PCR of cDNA produced by reverse transcription with Superscript III and Oligo d(t) (Thermo Fisher) and PCR amplification with iQ SYBR Green Supermix (BioRad, Hercules, CA).^{3,14} Primers are defined in Table 1. Total Ntrk2 expression of neonatal rat BSMC obstructed and sham bladders was assayed using rat Ntrk2 primers from Qiagen. Analysis of variance and post hoc *t*-tests were performed to identify significant changes in gene expression.

Transcription Factor Binding Site Analysis and Pathway Analysis

To predict transcription factors and pathways of importance for the clustered genes of *BDNF*, *CYR61*, and *CTGF*, the promoters of these genes were analyzed using the web-based programs, TOPGO and OPOSSUM 3.0 (<http://opossum.cisreg.ca>, last accessed October 31, 2017).

Western Blotting of BSMCs

Protein extracts from mechanical strain and *in vivo* obstruction experiments were isolated in radio-immunoprecipitation assay buffer plus protease inhibitor cocktail (Roche, Basel, Switzerland), at 4°C. They were quantitated using the Pierce Bradford Protein quantification kit (Thermo Fisher). Protein samples (10 μ g) were electrophoresed on 10% or gradient polyacrylamide gels (BioRad) and transferred to nitrocellulose membranes (Amersham, GE Healthcare, Little Chalfont, UK).¹¹ Membranes were blocked in skim milk and bovine serum albumin, then probed using antibodies against smooth muscle myosin (rabbit; MilliporeSigma), calponin (mouse; MilliporeSigma), and reference proteins total actin (rabbit; MilliporeSigma) and glyceraldehyde-3-phosphate dehydrogenase (rabbit; Cell Signaling Technology, part of NEB, Ipswich, MA), with secondary anti-rabbit or anti-mouse horseradish peroxidase conjugates (Cell Signaling Technology), as appropriate. Autoradiographs were developed using electrochemiluminescence (Amersham/GE Healthcare). Normalization and quantification were performed on ImageJ version 1.51 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>) by analysis of band densities from equivalent band areas on blots for reference proteins compared with target proteins.

Enzyme-Linked Immunosorbent Assay for Tissue BDNF

Bladder tissues were washed in phosphate-buffered saline (PBS) and frozen as for RNA isolation. Protein was isolated and quantitated as for Western blotting in radio-immunoprecipitation assay buffer. Protein lysates (10 μ g) and standards were added to the Chemikine BDNF sandwich enzyme-linked immunosorbent assay kit immunoassay plates, following the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Table 1 qPCR Oligonucleotide Sequences

Target	Forward	Reverse	Amplicon size, bp
Human <i>BDNF</i> promoter gDNA (<i>ChIP</i> primer in gene desert)	5'-AGCCCAACAACCTTCCCTTT-3'	5'-CGGGGCTGTTAACTCACATT-3'	162
Human <i>BDNF</i> promoter gDNA (<i>ChIP</i> primer)	5'-AGATCACAGAGCCTGCCAGT-3'	5'-AATTTGTGTGAGGGGTCTCG-3'	177
Human <i>BDNF</i> promoter gDNA at <i>EGR1</i> site (<i>ChIP</i> primer)	5'-AGCCTTTCGGGTCTCATTT-3'	5'-AGGTCCAGGGACTCCAAGTT-3'	196
Human <i>BDNF</i> cDNA	5'-TAACGGCGGCAGACAAAAAGA-3'	5'-GAAGTATTGCTTCAGTTGGCCT-3'	101
Human and rat <i>CTGF</i> cDNA	5'-AAGACCTGTGGGATGGGC-3'	5'-TGGTGCAGCCAGAAAGCTC-3'	194
Human <i>CYR61</i> cDNA	5'-CGAGGTGGAGTTGACGAGAAA-3'	5'-CTTTGAGCACTGGGACCATGA-3'	159
Human <i>YAP</i> cDNA	5'-ACCCTCGTTTTTGCCATGAAC-3'	5'-TGTGCTGGGATTGATATTCGGTA-3'	221
Human <i>TAZ/WWTR1</i> cDNA	5'-CCATCACTAATAATAGCTCAGATC-3'	5'-GTGATTACAGCCAGGTTAGAAAAG-3'	338
Human β -actin cDNA	5'-CGGCTACAGCTTCACCACCA-3'	5'-CGGGCAGCTCGTAGCTCTTC-3'	140
Human <i>B2M</i> cDNA	5'-AGGCTATCCAGCGTACTCCA-3'	5'-TCATCCAATCCAAATGCGGC-3'	348
Human <i>GAPDH</i> cDNA	5'-GTCAGTGGTGGACCTGACCT-3'	5'-TGCTGTAGCCAAATTCGTG-3'	245
Human <i>HPRT</i> cDNA	5'-GGCGAACCTCTCGGCTTT-3'	5'-CATCACTAATCACGACGCCA-3'	159
Human <i>SDHA</i> cDNA	5'-ACTGTTGCAGCACAGCTAGAA-3'	5'-TTATGCGATGGATGGACCG-3'	283
Human <i>UBC</i> cDNA	5'-TTAGGACGGGACTTGGGTGA-3'	5'-TCACGAAGATCTGCATTGTCAAG-3'	240
Rat total (pan) <i>Bdnf</i> cDNA	5'-TGGGGAGCTGAGCGTGTGTGA-3'	5'-TGTGACCGTCCC GCCAGACAT-3'	90
Rat <i>CYR61</i> cDNA	5'-CTTCCTGTCTTTGGCACGGA-3'	5'-AACTCGTGTGGAGATGCCAG-3'	134
Rat <i>Tead1</i> cDNA	5'-TTTGTGCAGCAGGCCTACCCCATC-3'	5'-GGCGAAGCTTGGTTGTGCCAATGGA-3'	129
Rat <i>Tead4</i> cDNA	5'-ACAGTGACCCCTACCTCGAA-3'	5'-GGTCTGCCCAGAACTTCACA-3'	135
Rat <i>Wwtr1</i> cDNA	5'-GAGGAAGGGCTCGCTTTTGT-3'	5'-ATGTTGACCTCGGGACTTTGG-3'	88
Rat <i>Yap</i> cDNA	5'-AATATCAATCCAGCACAGCA-3'	5'-CATTCTGAGTCCCTCCATCC-3'	109
Rat <i>Ntrk2</i> cDNA (<i>TrkB T1</i>)	5'-GAGCATCTCTCGGTCTATGC-3'	5'-CCCATCCAGGGGATCTTAT-3'	146
Rat <i>Ntrk2</i> cDNA (total)	Qiagen	Qiagen	71
Rat β -actin cDNA	5'-GGTCGTACCACTGGCATTGTG-3'	5'-GCTCGGTGAGGATCTTCATGAG-3'	150
Rat <i>Gapdh</i> cDNA	5'-GATCGTGGGAGGGCTAATGA-3'	5'-GAGCTCTGGGATGACTTTGC-3'	165
Rat <i>Hprt</i> cDNA	5'-AGGCCAGACTTTGTTGGATT-3'	5'-GCTTTTCCACTTTTCGCTGAT-3'	118
Rat <i>Rpl32</i> cDNA	5'-CATCTGTTTTGCGGCATCA-3'	5'-CACCCTGTTGTGCGATGCCTC-3'	152

ChIP, chromatin immunoprecipitation; *EGR*, early growth response; gDNA, genomic DNA; qPCR, real-time quantitative PCR.

Immunofluorescence

Cells or 7 $\mu\text{mol/L}$ equatorial bladder cryosections were fixed in paraformaldehyde and washed twice in PBS. Antigen exposure was performed by treatment with 0.2% Triton X-100. Blocking was performed in normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA). Then, staining was performed with the smooth muscle myosin heavy chain rabbit antibody (Medical Group/Alfa Aesar, Tewksbury, MA; or MilliporeSigma), calponin (MilliporeSigma), collagen type I (MilliporeSigma), *BDNF* (rabbit host; Abclonal, Woburn, MA), *YAP*, mouse host *YAP* (Santa Cruz Biotechnology, Dallas, TX), rabbit host *YAP/TAZ* and phosphorylated *YAP* (Cell Signaling, a part of NEB), *WWTR1* [mouse host (R&D Systems) or rabbit host (Novus Biologicals, Littleton, CO)], and secondary donkey anti-rabbit-Farred Fab fragments or donkey anti-mouse-Cy3 Fab (Jackson ImmunoResearch Laboratories). Nuclei were stained with Hoechst. Slides were mounted with Dako fluorescence mounting medium (Agilent, Santa Clara,

CA). Immunofluorescence was captured using an Olympus IX81 microscope (Olympus Corp., Tokyo, Japan) equipped with a Hamamatsu camera (Hamamatsu Photonics K.K., Hamamatsu, Japan) and spectral borealis lasers attached to Volocity software version 6.2 (Perkin Elmer, Waltham, MA). To test for myocyte hypertrophy, trans-nuclear cross sections of myocytes¹⁵ were measured on Volocity. Intensity of immunofluorescence signals in muscle bundles was evaluated by normalizing the sum of the intensity for each smooth muscle bundle region to the number of cells in each bundle. Immunofluorescence staining for nerves was performed with the $\beta 3$ tubulin rabbit antibody (MilliporeSigma). Additional processing for both $\beta 3$ tubulin and *YAP* antibodies included pre-clearing treatment with ice-cold methanol for 10 minutes, antigen exposure with 0.3% Triton X-100, blocking in 10% bovine serum albumin (MilliporeSigma) and 10% normal donkey serum (Jackson ImmunoResearch Laboratories), for 30 minutes, and washing steps in 0.1% Triton X-100 in PBS.

Bladder Histology and Wall Thickness

Equatorial bladder cryosections (7 μm thick) were stained with PicroSirius Red or hematoxylin and eosin. Sections were scanned and imaged using the Aperio system to ascertain the wall thickness at equatorial regions of the bladder, and degree of collagen accumulation by in muscle and nonmuscle tissue compartments.⁶ Data were included in Pearson's correlations.

Chromatin Immunoprecipitation PCR for YAP/WWTR1 Association with *BDNF* Genomic Regions during Stretch

Chromatin was isolated from human bladder smooth muscle cells, which had been treated with VP or vehicle during stretch or no stretch for 6 hours. Initially, it was found that nuclei could not be efficiently isolated from fixed cells by any kind of homogenization at multiple settings. To maximize isolation of chromatin, it was found that isolation of nuclei was optimized by first washing cells in ice-cold PBS with protease inhibitors, then solubilizing unfixed cell membranes by incubating with 1% Triton X-100 in PBS for exactly 5 minutes on ice. Nuclei were pelleted by centrifugation at 4°C for 5 minutes at 3000 $\times$ g. The pelleted nuclei were gently washed at 4°C and fixed in 1% paraformaldehyde in PBS for 10 minutes, after which fixation was immediately blocked with glycine/PBS. Nuclei were washed three times in PBS and placed at -80°C until further processing. Nuclear pellets were sonicated on ice to attain optimal DNA fragmentation (400-bp fragment size). Debris was pelleted at 3000 $\times$ g, 3 minutes. Input DNA was isolated from the resulting chromatin in the supernatant by reversing cross-links, phenol extraction, and ethanol precipitation. The chromatin was immunoprecipitated as in the High-Sensitivity ChIP kit (Active Motif, Carlsbad, CA), using the YAP/WWTR1 or species control IgG antibody for immunoprecipitation. Cross-links were reversed with heat and NaCl, then DNA isolated by phenol chloroform extraction. PCR primers were designed around the promoter of *BDNF* (Table 1).

Statistical Analysis

Physiology data were analyzed using Welch's *t*-test, when variances were not equal, and *t*-test, with $P < 0.05$ considered significant. All *t*-tests were performed as two-tailed, except where indicated in the text. Correlation analysis was performed by Pearson's correlation (using the stats package Hmisc on R) to identify parameters from physiological and expression data that correlate and cluster together. Expression data were analyzed initially using limma on R, with an interquartile range of 0.9, using the HTqPCR package.¹³ Correlation analysis of expression and physiology data were analyzed in both linear and logarithmic formats [-delta c(t)] to facilitate comparisons.¹³ Correlations with $P < 0.05$ and $R^2 > 0.40$ were selected as

potential candidates and plotted graphically. In addition, R , R^2 , and P values for correlations were plotted using R ggcorrplot packages to identify genes whose expression patterns were associated with physiological parameters. Post hoc Student's tests, Welch's *t*-tests, and analysis of variance of gene expression data were performed. For all tests, P values were considered significant if $P < 0.05$.

Results

Inhibiting DNA Methylation during Partial Bladder Outlet Obstruction Exacerbates Gross Bladder Hypertrophy, SMC Hypertrophy, and Loss of Smooth Muscle Differentiation Markers

PBO alone (without inhibitor) caused a significant increase in mean bladder mass ($P < 0.005$) and bladder/body mass ratio ($P < 0.005$) compared with sham animals (Figure 1, A and B). DAC-treated PBO animals showed further elevations in mean bladder mass ($P < 0.05$) and bladder/body mass ratio ($P < 0.05$, one-tailed *t*-test) versus PBO alone (Figure 1, A and B). Body mass at time of surgery varied from 250 to 300 g, and body mass was not significantly altered (Supplemental Figure S4, A–F and H). More important, DAC treatment had only minimal effects on final mass of sham bladders. Transnuclear cross-sectional SMC area was increased significantly during PBO, compared with sham ($P < 0.01$) (Figure 1, C and D). Consistent with bladder mass, the increase in obstruction-induced SMC area was significantly exacerbated by DAC treatment ($P < 0.05$) (Figure 1D). In contrast to increased SMC size, obstruction led to a twofold decrease in smooth muscle myosin marker fluorescence within SMC bundles ($P < 0.05$) (Figure 1, C and E). SM myosin signal was further dampened by PBO + DAC treatment versus sham (Figure 1, C and E).

Inhibiting DNA Methylation during Partial Bladder Outlet Obstruction Exacerbates Obstruction-Induced Bladder Dysfunction

PBO decreased maximal micturition fraction ($P < 0.05$) (Figure 1F), which is a surrogate for efficiency (completeness) of voiding.⁸ DAC treatment during PBO specifically decreased maximum and mean micturition fractions by greater than twofold versus PBO alone ($P < 0.01$) (Figure 1, F and G, respectively). Residual volume and maximal bladder capacity increased significantly in both PBO and PBO-DAC groups compared with sham ($P < 0.01$) (Figure 1, H and I, respectively). Moreover, DAC treatment of PBO versus vehicle treatment of PBO significantly increased residual volume (6.88-fold, $P < 0.05$) (Figure 1H) and bladder capacity (2.75-fold, $P < 0.05$, by one-tailed *t*-test) (Figure 1I). DAC did not affect capacity or residual volume in sham-operated rats (Figure 1, H and I). Nonsignificant changes between PBO and PBO-DAC were observed in maximum voided volumes, total voided

Figure 1 DNA methylation protects against smooth muscle hypertrophy and loss of function during partial bladder obstruction. **A** and **B**: Inhibition of DNA methylation by 1 mg/mL of 5-aza-2-deoxycytidine (or decitabine; DAC) leads to worsening of the physiological response to obstruction, including bladder mass (**A**) and bladder/body mass ratios (**B**). **C**: Immunofluorescence staining of smooth muscle myosin heavy chain (green) was used to mark smooth muscle bundles in the detrusor. Collagen type I was also detected (red). Negative control images (primary antibodies absent, with secondaries included) shown for each represented sample. Representative images were taken. **D**: Cross-sectional smooth muscle cell (SMC) hypertrophy of cells containing circular nuclei (indicating the middle of the cell) has higher hypertrophy by cross section compared with sham. **E**: Myosin intensity normalized to areas of SMC show a decrease in myosin content by immunofluorescence detection. **F** and **G**: Contractile dysfunction during obstruction is restrained by DNA methylation. **G–I**: Changes in maximum (**I**) and mean (**G**) micturition fraction, and bladder capacity and residual urine volumes (**H**) during partial bladder outlet obstruction are restrained by DNA methylation. **H** and **I**: Partial bladder outlet obstruction (PBO) increases residual volumes (**H**) and maximum bladder capacity (**I**), above sham or sham + DAC groups, whereas decitabine treatment of PBO animals (PBO + DAC) further elevates volumes in both residual volumes and maximum bladder capacity. Sample numbers are described in the animal model schematic ([Supplemental Figure S2](#)). $n = 6$ samples for sham or sham+DAC (**C**); $n = 10$ PBO or PBO+DAC (**C**). $*P < 0.05$, $**P < 0.01$. Scale bars: 52 μm (white bars); 34 μm (black bars). Original magnification, $\times 200$ (**C**).

volumes, and wall thickness ([Supplemental Figure S4](#)). Other physiological parameters, such as frequency of voids or small voids, and mean voided volumes ([Supplemental Figure S4](#)) showed only trends toward reversal of PBO effects by DAC treatment. The mean voided volume of PBO

alone showed a trend toward increasing 1.5-fold higher than PBO-DAC ($P < 0.10$) ([Supplemental Figure S4G](#)). By Pearson's correlations, neither bladder kinetics nor hyperactivity (ratio of number of small voids/number of total voids and number of small voids) correlated with gross or

Table 2 Significant Changes in Gene Expression Comparing Sham, Obstruction (PBO), and PBO + DAC Treatment by High-Throughput qPCR

Gene	P value	$\Delta\Delta\text{Ct}$	FC
Sham versus PBO			
<i>Atp2a2</i>	2.50×10^{-2}	2.554	0.17
<i>Bdnf</i>	1.13×10^{-2}	-3.396	10.53
<i>Ccnd1</i>	3.67×10^{-2}	2.579	0.17
<i>Dbp</i>	1.21×10^{-2}	2.569	0.17
<i>Dnmt1</i>	2.82×10^{-2}	3.119	0.12
<i>Dnmt3b</i>	1.26×10^{-2}	4.4315	0.05
<i>Galr2</i>	3.52×10^{-2}	2.6415	0.16
<i>Gem</i>	2.18×10^{-2}	2.549	0.17
<i>Kcnb2</i>	1.98×10^{-2}	2.839	0.14
<i>Kcnk1</i>	2.99×10^{-2}	2.684	0.16
<i>Kcnma1</i>	2.24×10^{-2}	3.4765	0.09
<i>Chrna10</i>	3.36×10^{-2}	2.339	0.20
<i>RIMS1</i>	3.24×10^{-2}	2.2515	0.21
<i>Thrb</i>	4.23×10^{-3}	3.574	0.08
<i>Tsc22d3</i>	1.95×10^{-2}	3.5665	0.08
<i>Ctgf</i>	4.55×10^{-2}	-2.206	4.61
<i>Chrna5</i>	4.26×10^{-2}	3.944	0.06
<i>Mecp2</i>	4.35×10^{-2}	2.444	0.18
Sham versus PBO + DAC			
<i>Bdnf</i>	5.04×10^{-5}	-6.33	80.45
<i>Ctgf</i>	2.20×10^{-3}	-3.66	12.64
<i>Dnmt3b</i>	2.98×10^{-2}	3.7775	0.07
<i>Kcnb2</i>	3.50×10^{-2}	2.5325	0.17
<i>Thrb</i>	4.34×10^{-2}	2.3775	0.19
PBO versus PBO + DAC			
<i>Adra1d</i>	2.11×10^{-2}	-3.0165	8.09
<i>Bdnf</i>	2.55×10^{-2}	-2.934	7.64

The only gene significantly altered in mRNA expression among all three comparisons was *Bdnf*. *Ctgf* was significantly different in two comparisons (sham-PBO and sham-PBO + DAC). $P < 0.05$ was considered significant (t-tests by limma).

CTGF, connective tissue growth factor; DAC, 5-aza-2'-deoxycytidine (or decitabine); FC, fold change; PBO, partial bladder outlet obstruction; qPCR, real-time quantitative PCR.

SMC hypertrophy (Supplemental Figure S5 and Supplemental Table S2). Rather, correlations were observed between hyperactivity, bladder capacity, and residual volumes. More important, the maximum micturition fraction correlated inversely with residual volume, bladder capacity, and gross hypertrophy.

BDNF, CYR61, and CTGF Expression Levels Correlate with Bladder Pathophysiology

mRNA expression of multiple candidate genes was examined by custom HT-qPCR array in sham, PBO, and PBO + DAC groups. PBO caused a variety of changes in candidate gene expression, with *Bdnf* appearing as the most significantly increased mRNA among all comparisons ($P < 0.05$) (Table 2). Principal component analysis revealed that each group formed separate clusters (Figure 2A). The greatest divergence between treatment groups was seen in principle

component 2 (Table 3), differentiating the PBO + DAC groups from the PBO and sham groups. Two genes contributed greatly to the weight of principle component 2: *Bdnf* and *Ctgf* (Table 3).

Pearson's correlation analysis of the entire data set of HT-qPCR genes alongside the physiological parameters revealed that expression of three genes, *Bdnf*, *Ccn1*, and *Ctgf*, cosegregated (Supplemental Figure S6). *Ctgf* highly correlated with *Bdnf* ($P < 10^{-6}$), and strongly correlated with maximum micturition fraction and SMC area (Pearson's correlations, $R^2 > 0.4$, $P < 0.05$) (Figure 2B). *Bdnf* expression was coordinately linked to alterations in SMC hypertrophy, maximum bladder capacity, mean and maximum micturition fraction, and residual volume (Pearson's correlations, $R^2 > 0.4$, $P < 0.01$) (Figure 2B). Moreover, correlations between *Bdnf* and gross muscle hypertrophy, bladder mass or bladder/body mass ratio, were significant ($R^2 > 0.25$, $P < 0.01$) (Supplemental Figure S7, A and B). In addition, bladder dysfunction showed a trend in correlating with two extracellular matrix genes, *Lox* and *Fnl* ($R^2 > 0.4$, $P \leq 0.20$) (Supplemental Figure S6). *Fnl*, a known YAP/WWTR1 target, was up-regulated in both PBO and PBO + DAC ($P < 0.05$) (Supplemental Figure S6), whereas *Lox* showed a trend in up-regulation only with PBO + DAC treatment. *Fnl* also contributed to the principle component 2, with the third highest eigenvalue (Table 3). In contrast to *Bdnf*, *Cyr61* + *Ctgf*, *Lox*, and *Fnl*, most of the candidate genes tended to be down-regulated in the PBO + DAC group versus PBO vehicle (Supplemental Figure S8). The *Bdnf* regulator methyl CpG binding protein 2 (MECP2) and alpha-1A adrenergic receptor (*Adra1d*) were up-regulated during obstruction but did not show any significant change with DAC treatment (Supplemental Figure S9). Although *Bdnf*, *Cyr61*, and *Ctgf* expression are well known to increase during obstructive myopathy in the bladder, this report shows their coregulation and cosegregation with physiological bladder dysfunction.

To confirm the dysregulation of the two most significantly altered genes, *Bdnf* and *Ctgf*, conventional real-time quantitative PCR was performed on cDNA from a larger set of samples. *Bdnf* and *Ctgf* were up-regulated 11.3- and 2.4-fold, in PBO alone versus sham, respectively ($P < 0.05$) (Figure 2, C and D). *Ctgf* and *Bdnf* expression levels were further amplified during PBO + DAC versus PBO in the confirmation data set ($P < 0.05$) (Figure 2, C and D). *Ctgf* mRNA was increased 27-fold in PBO + DAC versus sham ($P < 0.05$). *Bdnf* mRNA showed the highest obstruction-induced up-regulation with decitabine treatment, 100-fold above sham ($P < 0.05$, using $2^{-\Delta\Delta\text{Ct}}$ linear values; and $P < 0.0001$, using ΔCt values). In sham plus DAC, these genes did not significantly change compared with sham plus vehicle. Using the larger confirmation data set, *Bdnf* expression correlated inversely with voiding efficiency (residual volume, micturition fraction, bladder capacity, $R^2 > 0.4$, $P < 0.005$) (Supplemental Figure S7, C–E). By enzyme-linked immunosorbent assay, BDNF protein levels from PBO or PBO + DAC increased above sham

Table 3 Coordinates of PCs 1 and 2 of the 20 Most Significantly Changed Genes in the 3 Groups [Obstruction, Obstruction Plus 5-Aza-2-Deoxycytidine (or Decitabine), and Sham]

Gene	PC1	PC2
Ctgf	0.023	0.305
Bdnf	0.024	0.304
<i>Adra1d</i>	0.139	0.076
<i>RIMS1</i>	0.131	−0.129
Fn1	0.121	0.180
<i>Gys1</i>	0.152	0.008
<i>Socs3</i>	0.135	0.123
<i>Des</i>	0.148	0.033
<i>Ryr2</i>	0.132	0.055
<i>Galr2</i>	0.149	−0.026
<i>Ccnd1</i>	0.156	−0.020
<i>Chrna5</i>	0.126	−0.067
<i>Traf6</i>	0.150	−0.037
<i>Gem</i>	0.150	−0.083
Dbp	0.129	− 0.177
Mmp7	0.013	0.163
Cyr61	0.086	0.173
Mmp9	0.002	0.241
<i>Srf</i>	0.153	−0.046
<i>Atp2a2</i>	0.148	−0.099

Genes with PC analysis values > 0.160 are in bold, including *Bdnf*, *Ctgf*, and *Cyr61*. Data is displayed in [Figure 2](#).

PC, principal component.

($P < 0.05$) ([Figure 2E](#)). Likewise, BDNF from PBO + DAC was also 60% higher than PBO ($P < 0.01$). Similarly, immunofluorescence revealed that BDNF was up-regulated in PBO and PBO-DAC groups, particularly around smooth muscle cells and bundles identified by smooth muscle actin ([Figure 2F](#)).

DNA Methylation Regulates *Bdnf* and *Ctgf* Expression in Cultured BSMCs

Because DNA methylation has been reported to correlate with *Bdnf* expression during rat partial obstruction, the response of BDNF or CTGF transcription to inhibition of DNA methylation in human BSMCs was determined. *In vitro* treatment of resting BSMCs with decitabine for 24 hours increased baseline BDNF and CTGF expression ($P < 0.01$) ([Figure 3](#), A and B). DNA methylation of the *BDNF* locus was also explored using pyrosequencing of genomic DNA from *in vivo* obstruction samples. Only three sites of 17 analyzed demonstrated significant alterations in methylation by one-tailed *t*-test ($P < 0.05$), between sham, PBO, and PBO + DAC groups (data not shown). However, the DAC-induced changes were small, 0.3% to 0.9% changes in cytosine-guanosine dinucleotide (CpG) methylation, suggesting that regulation of these genes by DNA methylation may be occurring downstream of other epigenetic activity.

Stretch-Induced Gene Expression of BDNF, *CYR61*, and CTGF mRNA Requires YAP/WWTR1 Activity

Because a 0.9% change in DNA methylation of the *Bdnf* promoter is unlikely to lead to the observed 8.9-fold decitabine-induced up-regulation of *Bdnf* in PBO rats, the pathways that may be involved in up-regulation of the three clustered genes, *BDNF*, *CYR61*, and *CTGF*, were analyzed *in silico*. The YAP/WWTR1 pathway was identified along with the transcription factor binding site transcriptional enhancer activator domain transcription factor 1 (TEAD1) in TOPGO and OPOSSUM 3.0 ([Supplemental Tables S3](#) and [S4](#)). Other pathways were identified for these three genes, including macrophage integrin signaling, extracellular matrix protein and proteoglycans, hypertrophy, mitogen-activated protein kinase signaling, RhoA signaling, transforming growth factor- β , neurotrophic signaling, and Wnt signaling. Within the highly conserved human and mouse promoters of *BDNF*, *CYR61*, and *CTGF*, OPOSSUM 3.0 identified many motifs that were common to these three genes compared with the background set of genes. Particularly, TEAD1 consensus binding regions were identifiable in *CYR61* + *CTGF*, *LOX*, and the large *BDNF* gene locus ([Supplemental Table S4](#)). The TEAD1 transcription factor binding site is previously unexplored in BSMCs and bladder obstruction. Other transcription factor binding site identified included sites previously explored by other researchers, including NF- κ B, CCAAT-enhancer-binding proteins, and nuclear factor of activated T cells. TEADs bind DNA with cofactors YAP/WWTR1, which comprise a crucial part of the Hippo growth regulatory pathways.

Because YAP/WWTR1 localization to the nucleus is needed for function, nuclear YAP/WWTR1 expression and localization were evaluated during mechanical bladder strain injury, an *in vitro* functional surrogate for bladder obstruction.^{10,11} Using an antibody that reacts to both YAP and WWTR1, YAP and WWTR1 localization to the nucleus was significantly increased in mechanically strained human BSMCs at 6 hours (3.3-fold, $P < 0.05$) ([Figure 3](#), C and D). Time points earlier than 6 hours failed to show a significant increase in localization.

We then tested if transcription responses to 8 hours of mechanical strain affected BDNF and CTGF mRNA in human BSMCs. This time point was selected to allow for transcription of target genes after nuclear localization of YAP/WWTR1 at 6 hours. Mechanical strain significantly induced mRNA of BDNF (1.5-fold, $P < 0.05$) ([Figure 3E](#)) and CTGF (2.4-fold, $P < 0.005$) ([Figure 3F](#)). To examine YAP/WWTR1 dependency of BDNF and CTGF expression, the YAP/WWTR1 inhibitor VP was used to treat BSMCs during mechanical strain. VP reduced strain-induced and basal CTGF mRNA expression ($P < 0.001$) ([Figure 3F](#)) and strain-induced mRNA expression of BDNF (1.9-fold, $P < 0.01$) ([Figure 3E](#)), supporting a role for YAP/WWTR1 signaling in BDNF and CTGF expression *in vitro*. In contrast to this, the SMC differentiation marker, MYH11, was increased by VP ($P < 0.01$) ([Figure 3G](#)) during both stretch and no stretch.

Figure 3 Brain-derived neurotrophic factor (BDNF) and CTGF mRNAs are repressed by DNA methylation but increased by mechanical strain. **A** and **B**: Expression (mRNA) of BDNF (**A**) and CTGF (**B**) is significantly constrained by DNA methylation, as loss of DNA methylation by 2 $\mu\text{mol/L}$ 5-aza-2-deoxycytidine (or decitabine; DAC) treatment increases BDNF and CTGF mRNA expression, 3.5- and 2.4-fold, respectively. Human bladder smooth muscle cells (BSMCs) seeded onto type I collagen BioFlex plates were treated for 1 hour with vehicle or verteporfin (VP). **C–K**: Cells were then stretched on the Flexcell 3000 system for 8 hours (**C–G**), 6 hours (**H** and **K**), or up to 24 hours (**I** and **J**), using a ramping program of increasing mechanical strain to 5% elongation. **C** and **D**: Immunocytochemistry for YAP/WWTR1 shows increased nuclear localization after mechanical strain in bladder smooth muscle. **E–G**: Cells were analyzed by real-time quantitative RT-PCR for *Bdnf*, *Ctgf*, and *Myh11*. Discrete mechanical stimulation of BSMCs *in vitro* significantly increases mRNA of *Bdnf* 1.5-fold and of CTGF 2.4-fold. In stretched cells, *Bdnf* and CTGF are reduced by VP 1.9- and 11.6-fold, respectively. **G**: MYH11 shows the opposite pattern. **H**: Chromatin Immunoprecipitation/PCR was performed on human BSMCs after 7 hours of mechanical strain, compared with no stretch and stretch + VP. YAP binding to the *BDNF* promoter increases with mechanical strain and is blocked by verteporfin, an inhibitor that sequesters YAP in the cytoplasm. **I** and **J**: Levels of YAP and WWTR1 mRNA in human bladder smooth muscle cells are also dependent on DNA methylation as WWTR1 increases 1.8-fold with decitabine treatment. **K**: Expression of WWTR1 increases during bladder smooth muscle stretch, as examined by Western blotting. $n = 4$ (**H**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; † $P < 0.05$ versus all other time points. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Min, minutes; Str, stretch.

Mechanical Strain Increases YAP/WWTR1 Binding to the *Bdnf* Locus in BSMCs

To test for a YAP/WWTR1 association at the *BDNF* promoter, chromatin immunoprecipitation/PCR was performed. Binding of YAP/WWTR1 to the region before

the second promoter increased during mechanical strain (33-fold increase, $P < 0.05$, by one-tailed *t*-test) (Figure 3H). This binding was dependent on YAP/WWTR1, because their inhibitor, VP, greatly reduced stretch-induced binding at this locus (5.3-fold reduction, $P < 0.05$, one tailed).

Figure 4 *In vivo* expression of YAP and WWTR1 is repressed by DNA methylation. **A** and **B**: *In vivo*, transcription of WWTR1 during partial bladder outlet obstruction (PBO) 5-aza-2-deoxycytidine (or decitabine; DAC) treatment significantly increases (**A**), whereas YAP only shows nonsignificant trends in mRNA fluctuations (**B**). **C**: Western blotting for YAP and WWTR1 demonstrates an increase in total bladder levels of YAP and WWTR1. **D**: Immunofluorescence staining of representative cryosections (7 μ m thick) with a YAP/WWTR1-specific antibody reveals that YAP/WWTR1 appear to be increased during obstruction and localized to the nucleus more during decitabine treatment in bladder smooth muscle (identified by smooth muscle myosin). **Left panels**: Intensity of nuclear compared to cytoplasmic YAP/WWTR1 immunofluorescent signal was quantified on Volocity software version 6.2. **Right panels**: Phosphorylated YAP signal decreases in the smooth muscle compartment. **D: Insets**: White arrows: nuclear-localized YAP/WWTR1 signal. * $P < 0.05$, ** $P < 0.01$. Scale bar = 24 μ m (**D**, main images and insets).

Inhibiting DNA Methylation Up-Regulated WWTR1 and YAP Expression and Nuclear Localization but Not TEAD Expression

Given the effect of DNA methylation inhibition on transcription of *CTGF* and *Bdnf* in rat obstruction *in vivo* and the role of the YAP/WWTR1 inhibition on their expression *in vitro*, it was speculated that DNA methylation would regulate *CTGF* and *Bdnf* indirectly through *YAP* and *WWTR1* expression during *in vitro* culture. *In vitro* treatment of BSMCs with DAC led to an increase in both YAP (2.2-fold, $P < 0.05$) (Figure 3I) and WWTR1 (1.67-fold, $P < 0.01$) (Figure 3J) expression. Finally, 6 hours of

mechanical stretch increased WWTR1 protein expression in human BSMCs by Western analysis as well (Figure 3K).

In vivo, PBO increased *Wwtr1* 2.3-fold above sham levels ($P < 0.05$) (Figure 4A), whereas PBO + DAC showed a trend toward increasing *Wwtr1* levels above PBO ($P < 0.10$). Nevertheless, PBO + DAC increased 4.9-fold compared with sham levels, with a *t*-test of $P < 0.05$ using ΔCt values. *Yap* expression was unchanged in mRNA expression (Figure 4B). TEADs are transcription factors that associate with YAP/WWTR1. *In vivo*, only *Tead-1* and *Tead-4* were detectable, but both remained unchanged during either obstruction or treatment (Supplemental Figure S10).

Figure 5 Brain-derived neurotrophic factor (BDNF) directly reduces smooth muscle cell (SMC) differentiation and growth. **A:** Treatment of human bladder SMCs with exogenous recombinant BDNF (10 ng/mL; Alamone Labs) leads to a decrease in smooth muscle myosin after 48 hours. **B:** After 24 hours, BDNF significantly increases the number of bladder SMCs by cell counting of more than three fields for four wells per treatment. **C:** After 48 hours, BDNF significantly increases proliferative marker PH3 in nuclei of bladder SMCs. BDNF directly affects SMC growth signaling. **D** and **E:** Exogenous BDNF increases extracellular signal-regulated kinase (ERK) and S6 kinase (S6K)1 signaling by Western blotting for phosphorylated ERK1/2 and S6K1, respectively. Total ERK1/2 and total S6K1, respectively, were used as reference proteins.¹⁵ **F** and **G:** BDNF and verteporfin interact to affect smooth muscle marker expression in human bladder smooth muscle after 24 hours. Human bladder SMCs plated on denatured collagen were treated for 24 hours with 10 ng/mL BDNF, verteporfin (VP; 0.1 $\mu\text{mol/L}$), or VP + BDNF. **F** and **G:** Cells were fixed in 4% paraformaldehyde and immunofluorescently stained for calponin (**F**) or smooth muscle myosin (**G**). Intensity of confocal images was analyzed on Velocity software. **G:** BDNF decreases calponin expression. In contrast, VP augments myosin expression in bladder SMCs, which is reduced by BDNF treatment. * $P < 0.05$. FCS, fetal calf serum.

Because protein degradation and nuclear localization play crucial roles in Yap and Wwtr1 function, Western blots and immunofluorescence were used to examine Yap and Wwtr1 quantity and localization in detrusor muscle (myosin positive) of sham or obstructed bladders with vehicle or decitabine (Figure 4, C and D). By Western blot, Yap and Wwtr1 expression could be differentiated *in vivo*, which demonstrated a moderate level of basal Yap and elevated Yap and Wwtr1 with obstruction with or without DAC (Figure 4C). Phosphorylated forms did not show high signal on the blots. Minimal immunofluorescence staining in sham or sham + DAC treated bladders was observed (Figure 4D). In PBO with vehicle, both cytoplasmic and nuclear expression was increased above sham levels. In contrast, nuclear localization was more prominent in DAC-treated PBO bladders, with higher-intensity signal in nuclei located in SMC bundles (Figure 4D). Minimal to no staining of cells between muscle bundles was noted in all groups. Conversely, the detection of the degraded form of YAP by immunofluorescence was minimal in obstruction groups with either vehicle or decitabine.

BDNF Protein Alters BSMC Growth, Growth Signaling Pathways, and SMC Marker Expression

Because Myh11 immunofluorescence was down-regulated, whereas BDNF increased during PBO and PBO plus DAC (Figure 1), it was determined if the SMC phenotype (SMC growth and SMC marker expression) was affected by treatment with exogenous recombinant BDNF (Figure 5). Human BSMCs were plated on tissue culture plastic (Figure 5, A–E) or denatured type I bovine collagen (Figure 5, F and G) at 4×10^4 cells/mL, left to attach overnight, and serum starved for 24 hours. By Western analysis, the highest tested dosage of BDNF (10 ng/mL) led to a reduction of smooth muscle myosin heavy chain (Figure 5A). In addition, BDNF increased growth of BSMCs, with cell counts 1.55-fold increased ($P < 0.05$)

(Figure 5B). Expression of the proliferative marker phospho-histone-3 also increased 13% in response to BDNF ($P < 0.05$) (Figure 5C). Moreover, S6 kinase [mammalian target of rapamycin (mTOR)] and extracellular signal-regulated kinase pathways, which have been previously implicated in mechanistic regulation of BSMC growth,^{11,12} were activated by high doses of BDNF (Figure 5, D and E).

Verteporfin-BDNF Antagonism Leads to Altered SMC Phenotype

Although VP treatment increased Myh11 expression (Figure 5G), VP also reduced BDNF expression (Figure 3E). To examine possible interactivity of BDNF with YAP/WWTR1 induction of SM markers, VP was added to exogenous BDNF-treated cells (Figure 5, F and G). Damaged matrix, which is induced during over-distension and stretch of bladder smooth muscle,^{11,12} is another surrogate of *in vivo* obstruction. BDNF treatment reduced protein expression of smooth muscle marker, calponin, on damaged matrix ($P < 0.05$) (Figure 5F). Also, Myh11 expression was affected in a different way: VP increased Myh11 expression in cells plated on damaged matrix ($P < 0.05$), but this increase was reduced by exogenous BDNF treatment ($P < 0.05$) (Figure 5G). This supports a role for BDNF both upstream of YAP/WWTR1 in calponin expression and downstream of YAP/WWTR1 in myosin expression.

BDNF Induces a Loss of Normal Contractile Force That Depends on BDNF Receptor Tyrosine Kinase Activity

Pretreatment of cultures of bladder smooth muscle cells with exogenous BDNF compared with vehicle was able to prevent normal levels of smooth muscle cell contraction in collagen gel contraction assays after 4 and 18 hours (by 14% and 23%, respectively). Interestingly, GNF 5837, an inhibitor of BDNF signaling through neurotrophic receptor

Figure 6 Smooth muscle contraction is inhibited by brain-derived neurotrophic factor (BDNF) treatment and reversed by neurotrophic receptor tyrosine kinase (NTRK)2 kinase inhibition. **A:** Human bladder smooth muscle cells (BSMCs) plated on collagen gels induce contraction, but it is inhibited by pretreatment of cells with 10 ng/mL BDNF. Addition of 10 μ mol/L GNF 5837, an inhibitor of NTRK2 kinase activity, reverses the relaxation response of BDNF and enhances the contractile response significantly. **B:** To examine *Ntrk2* expression levels during mechanical stretch, real-time quantitative PCR was performed on rat BSMCs that were mechanically stretched using a slowly ramping pattern to 5% elongation at 6 hours. Mechanical strain of rat bladder smooth muscle cells augments mRNA expression of *Ntrk2* full-length and truncated isoform. * $P < 0.05$, ** $P < 0.01$.

tyrosine kinase (NTRK)2, was able to reverse the lack of contraction, increasing the BDNF contractile response by 20.7%, 24.8%, and 31.8% at 2, 4, and 18 hours, respectively ($P < 0.005$) (Figure 6A). The GNF 5837 + BDNF–induced contraction was even more pronounced than vehicle, increasing 12.7% and 10.5% at 2 and 4 hours, respectively. Real-time quantitative PCR was performed to examine if *Ntrk2* was altered during obstruction *in vivo*. *Ntrk2* expression of neither the total nor the truncated (T1) isoform in bladder tissues was altered significantly during obstruction or DAC treatment (data not shown). Because the expression from whole bladder is a mix of cell lineages, *Ntrk2* isoforms were examined in rat bladder smooth muscle cells. Interestingly, total and T1 *Ntrk2* increased 1.8-fold ($P < 0.05$) and 20.2-fold ($P < 0.005$) during stretch versus no-stretch BSMCs (Figure 6B).

Discussion

In this study, pharmacologic inhibition (induced loss) of DNA methylation leads to frequent and inefficient voiding, as well as marked dilative and hypertrophic changes in the obstructed bladder. Our findings suggest that DNA methylation is crucial for regulation of bladder pathophysiology incited by PBO. Bladder myopathies characterized by obstruction and loss of controlled storage and emptying functions increase in aging populations who are prone to a global loss of DNA methylation.^{16,17} HT-qPCR of candidate genes revealed that *Bdnf*, *Cyr61*, and *Ctgf* comprised a small cluster of genes up-regulated by obstruction. Moreover, this obstruction-induced cluster was further amplified in the presence of DAC. *Bdnf* expression demonstrated a

quantitative link to several pathophysiologic changes relating to growth, SMC hypertrophy, and loss of voiding efficiency. *Bdnf* was initially selected as a relevant candidate gene in bladder pathophysiology because of its up-regulation during obstruction and implied association with the pathomechanics of obstruction. More specifically, it was found that *Bdnf* and *Ctgf* associate with physiological changes during obstruction and their expression is mechanically induced, which is dependent on YAP/WWTR1 signaling.

BDNF Is Regulated by DNA Methylation, WWTR1, and YAP

Regulation of BDNF expression relies heavily on CpG demethylation and methylation in concert with chromatin and transcription factor binding.^{10,15} However, *BDNF* promoter CpG DNA methylation in our samples did not reveal large changes in CpG methylation of the promoter region. The largely stable DNA methylation pattern in *BDNF* so far uncovered contrasts with the dynamically regulated *in vitro* BSMC DNA methylome. In a previous study, an induction of genome-wide methylation changes was observed in BSMCs after exposure to denatured extracellular matrix for only 2 days.³ Interestingly, there was a global hypomethylation of the BSMC DNA alongside localized hypermethylation.³ More important, this may relate to the known plasticity of SMCs. Mature normal SMCs demonstrate a high level of hydroxy-methyl-cytosine (HOMeCpG),^{18,19} similar to that of stem cells. SMCs have been suggested to use HO methylation as a mechanism to respond to highly changeable environments.^{18,19}

Figure 7 Schematic pathway describes the dependency of smooth muscle cell (SMC) dedifferentiation induced by obstructive bladder stimuli, such as strain on YAP/WWTR1 to produce brain-derived neurotrophic factor (BDNF), alongside CTGF and CYR61. Inhibition of DNA methylation leads to increased YAP and WWTR1, which leads to increased BDNF and CTGF. BDNF itself leads to increased SMC growth and loss of some myosin expression, in a time- and dose-dependent manner. Growth pathways, which have been linked to bladder SMC growth, are activated by BDNF. Inhibition of YAP/WWTR1 increases expression of SMC markers that are down-regulated by BDNF, suggesting some combined effects of YAP/WWTR1 and BDNF. Furthermore, blocking either BDNF receptors or YAP/WWTR1 leads to increase in SMC marker expression. ERK, extracellular signal-regulated kinase; mTOR, mammalian target of rapamycin.

Interestingly, decitabine can increase HO-mCpG levels in leukemic cells, Jurkat cells, and neural stem cells,^{20,21} but little is known about HO-mCpG during decitabine treatment of SMCs in obstruction.²² One focus of our future work centers on HO-mCpG and mCpG alterations of transcription factor binding in promoter regions of *BDNF*, *CYR61*, or *CTGF*, and the potential instability of HO methylation in PBO as a stepping stone to demethylation.

CpG methylation changes in transcriptional regulators may up-regulate expression of BDNF and *CYR61* + *CTGF*. To search for this mechanism of increased BDNF, *CYR61*, and *CTGF*, pathway analysis using TOPGO and OPOSSUM unveiled a common YAP/WWTR1 and TEAD binding element among the coregulated genes. DAC treatment greatly increases transcription of WWTR1 *in vitro* in conjunction with downstream targets, *CYR61* + *CTGF* and BDNF. Protein levels of BDNF by enzyme-linked immunosorbent assay also increased during obstruction plus treatment, but not as much as expected given the mRNA levels. This may be because of increased antisense RNA against *Bdnf* mRNA, which regulates transcript stability. Other transcriptional mechanisms altering *Bdnf* mRNA may include MECP2, which is inhibitory in general. MECP2's potential role in disinhibition of BDNF during obstruction with or without DAC is not consistent, because *Mecp2* mRNA levels increase during obstruction when *Bdnf* mRNA is increasing, yet decrease with DAC treatment during obstruction (Supplemental Figure S9). More important, WWTR1 mRNA levels themselves were altered by DAC treatment both *in vivo* and *in vitro* (Figures 3J and 4A), suggesting that the increase in the *Bdnf* and *Cyr61* + *Ctgf* may be related to the increased expression of their transcriptional cofactors. Interestingly, WWTR1 and YAP staining indicated that there is likely some regulation at the protein level, because a significant amount of protein was seen in the cytoplasm of the PBO + vehicle and sham + DAC groups. Because many transcription factors interact with WWTR1 and YAP to increase their effects downstream, TEAD expression, which is associated with YAP/WWTR1 activity, was examined. However, the TEADs detected are not significantly altered by obstruction or DAC. YAP and WWTR1 are also regulated post-translationally, which was addressed through an examination of YAP/WWTR1 expression and nuclear localization (Figures 3 and 4D). *In vivo*, Yap antibody staining showed that phosphorylated Yap, which is most often shunted for degradation, appeared to be reduced during obstruction. This was concurrent with increased nuclear localization in the obstructed groups, with the decitabine treatment increasing localization further. The implication is that DNA methylation normally restricts WWTR1 and YAP expression and localization patterns, but unrestricted YAP and WWTR1 expression leads to vastly up-regulated CTGF and BDNF, damaging the bladder. YAP and WWTR1 are coactivators, which associate with DNA only through the DNA binding

activity of other transcription factors, such as early growth response 1 (EGR1), T-box 5, β -catenin, and myocardin. Critically, YAP and WWTR1 are involved in the regulation of BDNF during mechanical stimulation through binding to its promoter. An EGR1 binding site in the *BDNF* promoter is one target for mechanosensitive binding of YAP/WWTR1 (Figure 3I). EGR1 is a transcription factor, which binds DNA and YAP during proliferation, mechanical stretch,²³ and inflammation.^{24,25} In summary, DNA methylation, in part, regulates BDNF through the regulation of the YAP and WWTR1 transcription and localization.

BDNF Regulates Smooth Muscle Differentiation

Although many studies have examined BDNF in terms of its effects on neuronal function in the bladder,^{4,5,26–28} few have examined its direct role in the BSMC phenotype. The myogenic hypothesis in obstructive and overactive bladder is supported by work examining direct effects of cytokines and other factors on the detrusor phenotype and function.^{29–31} However, BDNF alters survival, proliferation, synthetic activity, and contractile functions of many cell types from many organs. BDNF's role in lung and vascular SMC differentiation, contraction, and proliferation has been explored in a few limited studies. Together, this suggests that a more direct role for BDNF should be considered in the function of bladder smooth muscle than previously suggested,⁵ especially given the elevated level of BDNF in the detrusor. Interestingly, basal BDNF in the bladder exceeds basal expression of all other organs, even the brain.¹⁵ Furthermore, the PBO+DAC level of BDNF expression is 100-fold higher than in sham bladder. This high level of bladder BDNF could have widespread effects on neuronal and nonneuronal cell types. The high levels of BDNF immediately surrounding the smooth muscle bundles in the endomysial spaces in this study (Figure 7), during obstruction, suggest that BDNF may target muscle bundles and their surrounding cells. Further supporting a direct role of BDNF on SMC function, it was found that BDNF was able to alter markers of smooth muscle differentiation (eg, its smooth muscle myosin heavy chain and calponin expression), which was consistent with the loss of differentiation in the obstructed bladders.

BDNF Signals to Proproliferative Pathways in Bladder Smooth Muscle

BDNF can signal through the mTOR pathway, such as S6 kinase (S6K), elongation initiation factor 4E, mitogen-activated protein kinase-1, and mitogen-activated protein kinase-3,³² which are also activated in both the urothelium and mesenchyme during SMC strain and bladder obstruction.^{6,10,11} Previously, it was found that mTOR pathway is activated during PBO,⁶ and induces smooth muscle hypertrophy during PBO. SMC dedifferentiation is regulated by

S6K1 *in vitro*.^{11,33} Conversely vascular SMC differentiation is improved by inhibition of mTOR complex 1 through a combination of blocking S6K1, insulin receptor substrate-1/2, and GATA binding protein 6.^{11,33} However, inhibition of S6K as opposed to the entire mTOR pathway in mice during obstruction lead to worsening of bladder hypertrophy.⁷ Herein, it was observed that BDNF induces extracellular signal-regulated kinase and S6K1 and is associated with loss of differentiation in BSMCs, consistent with previous studies.¹⁰ In particular, high-dose sustained BDNF signaling may play a role in bladders by causing sustained extracellular signal-regulated kinase signaling, which is known to have differential effects on BSMC growth and neurons.¹⁰ Although the *in vivo* role of BDNF signaling in smooth muscle cells remains to be further elucidated, it is clear that DNA methylation is playing a role in BDNF regulation *in vivo* and *in vitro*.

Other pathways may also be regulating SMC marker genes and myocardin during decitabine treatment, including serum response factor.³⁴ In supplementary HT-qPCR data (Supplemental Figure S8), serum response factor was down-regulated by DAC treatment, showing the opposite pattern of expression to BDNF during obstruction and DAC treatment. Serum response factor increased nonsignificantly during obstruction, but decreased with DAC. MECP2 is also known to have a role in BDNF regulation,³⁵ but as mentioned above, MECP2 expression is not concordant with BDNF expression *in vivo* (Supplemental Figure S9).

YAP and WWTR1 Inhibition Improves Smooth Muscle Phenotype by Down-Regulating BDNF

For the first time, we observe that YAP/WWTR1 regulation of BDNF by mechanosensitive stimuli may be a crucial mechanism that leads to aberrant gene regulation of a subset of genes and discrete alterations in BSMC phenotype and function. As such, this is the first report to show that YAP and WWTR1 play a central role in the mechanoregulation of BDNF, CYR61, and CTGF in bladder smooth muscle. In addition, BDNF's effects were dose dependent, which may have an impact on the varying effects of BDNF *in vivo*. In some reports, BDNF has been suggested as a possible therapy,⁵ whereas in others, its association with hyperactivity is described.³⁶ The diverging results may relate to dosage of BDNF. Dose-dependent effects of BDNF have been noted in other cell types.³² In addition, locally expressed versus systemically introduced BDNF may have diverging effects on bladder pathophysiology. Systemic BDNF may be beneficial for neuronal control of the bladder, through improved neuronal sprouting, neuronal survival, cognition, recovery from neuronal trauma, and prevention of social stress, but these effects might not be beneficial in the local bladder setting. Local high levels of BDNF likely induce growth and loss of smooth muscle cell markers, as *in vitro* (Figure 5), which may affect the BDNF-correlated

pathophysiology, including micturition, residual volume, and hypertrophy.

BDNF Alters Contractile Function of Bladder Smooth Muscle through Nonkinase Activity of NTRK2

The loss of contractile function in response to BDNF was consistent with the effects of BDNF on differentiation of SMCs. It is known that BDNF can increase contractile frequency of bladder strips and the bladder *in vivo*, but the effect of BDNF on bladder smooth muscle phenotype shown herein provides new information that may support a role for loss of micturition efficiency (Figure 1). Interestingly, the nerve density (Supplemental Figure S11) coincided within the BDNF elevation during PBO + DAC versus PBO alone, although not during PBO versus sham. However, the role of the main BDNF receptor, NTRK2, has divergent effects on gel contraction (Figure 6A), as the isoforms of the receptor determine its signaling capacity (Figure 7). Inhibition of the tyrosine kinase of NTRK2 with GNF 5837 promotes gel contraction. This is consistent with the known activity of the partially truncated NTRK2 isoform T1, which lacks the kinase activity but retains Ca²⁺- and Rho kinase-mediated contractile signaling. Our results suggest that gel contraction is promoted by the nonkinase isoform of NTRK2 T1. However, other pathways (eg, muscarinic and adrenergic) participate in bladder smooth muscle contraction *in vivo*, although these ultimately feed into sarcoplasmic reticulum regulation of calcium. Furthermore, relaxation of BSMCs has been studied mainly as a consequence of inhibition of contraction, but in the heart, BDNF signaling through phospholipase C appears to have an important role in ensuring the relaxation phase.³⁷ Silencing the T1 isoform in the presence of BDNF overexpression is associated with reduced Rho kinase signaling and decreased activity, whereas BDNF overexpression can alter neuronal density and activity, leading to bladder hyperactivity. Although the mRNA of *Ntrk2* full-length and truncated isoforms was increased *in vitro* by stretch and NTRK was found to play a role in regulating relaxation and contraction *in vitro*, *Ntrk2* effects *in vivo* were not in the scope of this article, and form the focus of future studies. This study demonstrates the association of SMCs and gross bladder hypertrophy with BDNF as a downstream target of YAP and WWTR1.

Conclusions

Pathophysiologic parameters of obstruction are associated with expression of *Bdnf*, concordant with a direct role for BDNF in control of SMC phenotype. DNA methylation inhibition augments *Bdnf* expression in BSMCs *in vitro* and during partial obstruction of the bladder *in vivo*, suggesting *Bdnf* is normally repressed by DNA methylation.

Furthermore, Ctgf and Bdnf expression are modulated by WWTR1 and YAP, which in turn are epigenetically regulated by DNA methylation *in vitro* and *in vivo*.

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Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.06.024>.

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