Published in final edited form as:

Neurol Urodyn. 2017 February ; 36(2): 286–292. doi:10.1002/nau.22941.

A novel role for follistatin in hypersensitivity following cystitis

Amber D. Shaffer, PhD^{*}, Bin Feng, PhD^{+,#}, Jun-Ho La, PhD⁺, Sonali C. Joyce, BS, and G. F. Gebhart, PhD

Center for Pain Research, Department of Anesthesiology, University of Pittsburgh, Pittsburgh, PA, USA

Abstract

AIMS—Previous studies have shown that the activin-binding protein follistatin reduces inflammation in several mouse models of colitis. To determine whether follistatin also has a beneficial effect following bladder inflammation, we induced cystitis in mice using cyclophosphamide (CYP) and examined the relationship between bladder hypersensitivity and bladder follistatin expression.

METHODS—Adult female C57BL/6 mice were treated with CYP (100 mg/kg) or vehicle (saline) 3 times over 5 days. Bladder hypersensitivity was assessed by recording the visceromotor response (VMR) to urinary bladder distension and *in vitro* single-fiber bladder afferent recording. Follistatin gene expression was measured using qRT-PCR. Immunohistochemistry was employed for further characterization.

RESULTS—Bladder hypersensitivity was established by day 6 and persisted to day 14 in CYP-treated mice. On day 14, hypersensitivity was accompanied by increases in follistatin gene expression in the bladder. Follistatin-like immunoreactivity colocalized with laminin, and the percentage of structures in the lamina propria that were follistatin-positive was increased in CYP-treated mice. Exogenous follistatin increased VMR and afferent responses to bladder distension in CYP- but not vehicle-treated mice.

CONCLUSIONS—Chronic bladder pain following CYP treatment is associated with increased follistatin expression in the bladder. These results suggest a novel, pro-nociceptive role for follistatin in cystitis, in contrast with its proposed therapeutic role in colitis. This protein has exciting potential as a biomarker and therapeutic target for bladder hypersensitivity.

Keywords

Cystitis; Interstitial; Cyclophosphamide; Activins; Transforming Growth Factor beta; Visceral Pain; Single-Fiber Recording

^{*}Correspondence should be sent to: Amber D. Shaffer, One Children's Hospital Drive, 4401 Penn Avenue, Faculty Pavilion, 7th Floor, Pittsburgh, PA 15224, Phone: 412-692-6874, Fax: 412-692-6074, shafferad@upmc.edu.

⁺Authors contributed equally to this work.

[#]Current address: Department of Biomedical Engineering, University of Connecticut, Storrs, CT, USA

INTRODUCTION

The cytokine activin A is a member of the transforming growth factor β (TGF β) superfamily involved in the growth and differentiation of epithelial cells. It plays an important role in a variety of biologic processes including embryonic development, wound healing, hormone secretion, and nociception (1,2), and is upregulated early in the inflammatory cascade following systemic inflammation (3,4). The endogenous antagonist for activin A, follistatin, is an autocrine glycoprotein that binds to and neutralizes activin A with high affinity (5). There is increasing evidence that follistatin may play an anti-inflammatory role in colitis. For example, there is a remarkable increase in expression of the gene encoding activin A, *Inh β A*, in the gut of patients with inflammatory bowel disease (6,7). Activins are associated with colitis in several mouse models, and follistatin can reverse signs of this inflammation (8,9). In the bladder, activin A and activin receptor Type II have been found in transitional urothelium, smooth muscle, and endothelial cells (10). However, no studies to date have examined follistatin in the bladder.

The antineoplastic drug cyclophosphamide (CYP) induces hemorrhagic cystitis in humans and thus is widely used to model cystitis and associated pain in rodents. To determine if the protective effects of follistatin observed in colitis also occur during cystitis, saline (vehicle) or CYP (100 mg/kg) was intraperitoneally (i.p.) administered on days 1, 3, and 5 (11). The visceromotor response (VMR) to urinary bladder distension (UBD) was recorded on days 6 (d6) and 14 (d14). Subsequently, the bladders of these mice were collected and the gene expression of follistatin was measured using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Location and abundance of follistatin protein were examined through immunohistochemistry (IHC), and function was examined through VMR and single-fiber recordings after exogenous administration of follistatin. Portions of these data were previously presented in abstract form (12).

MATERIALS AND METHODS

Animals

Adult female C57BL/6 mice (18–21 g; 5–10 weeks; Jackson Laboratories, Bar Harbor, ME) were used. All protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Treatment

On days 1, 3, and 5, animals were anesthetized with 4% isoflurane, and vehicle (saline) or CYP (100 mg/kg) was administered i.p. Testing and tissue collection occurred on d6 and d14 (Figure 1A).

VMR Testing

Ten vehicle-treated mice and 12 CYP-treated mice underwent VMR testing followed by collection of bladders on d6. A separate set of 6 vehicle-treated mice and 7 CYP-treated mice underwent VMR testing followed by collection of bladders on d14. Mice were anesthetized with isoflurane (4% induction, 2% maintenance, 1% testing), and the urinary

bladder was catheterized with a 24-gauge angiocatheter *via* the urethra. Body temperature was maintained at approximately 37°C. Platinum wire electrodes were inserted into the left external oblique muscle. Electromyographic (EMG) activity was differentially amplified, rectified, and saved using Spike 2 software (Cambridge Electronic Design [CED], Cambridge, UK). Phasic UBD was delivered by pressurized air *via* the angiocatheter. EMG activity was recorded for 10 s before (baseline) and during 10 s of UBD. The VMR was quantified as: rectified EMG activity during UBD minus rectified baseline EMG. To pre-condition the bladder and optimize recording settings, one distension at 60 mmHg was performed. Next, responses to graded UBD at 15, 30, 45, or 60 mmHg were recorded with 2 repetitions at each pressure (4 min intervals).

qRT-PCR

Following VMR testing, bladders were removed. Tissue was homogenized in TRIzol® (Invitrogen, Carlsbad, CA, USA). RNA was precipitated with isopropanol, washed with 70% ethanol, and resuspended in RNase-free water. Genomic DNA was removed from 5 µg RNA through DNase (Invitrogen). A 2 µg RNA template underwent random-hexamer/oligo-d(T)-primed reverse transcription using Superscript II (Invitrogen). Gene expression of follistatin and GAPDH was measured by qRT-PCR on a CFX Connect Real Time Cycler (Bio-Rad, Hercules, CA, USA) using SYBR Green Master Mix (Bio-Rad) (primer sequences in Table I). Transcripts were quantified by linear regression of individual amplification curves using LinRegPCR (13) and normalized to GAPDH. Follistatin gene expression was reported as a percentage of the average follistatin expression in vehicle controls.

Histology

One mouse each from vehicle, d6 CYP, and d14 CYP treatments were deeply anesthetized with isoflurane and perfused with Lana's fixative. Bladders were removed, post-fixed in Lana's fixative, cryoprotected in 10 and 20% sucrose, and embedded in Tissue-Tek O.C.T. compound (Sakura, Torrance, CA). Longitudinal sections (20 µm) were stained with hematoxylin & eosin (H&E). Slides were cover-slipped with DPX mounting media (Sigma-Aldrich, St. Louis, MO) and examined using a Leica DM4000 B microscope and DFC300 FX camera (Leica Microsystems, Buffalo Grove, IL).

Immunostaining

Bladders from 3 vehicle-treated and 3 CYP-treated mice were collected as described above. Nonadjacent sections (20 µm, 60 µm apart) were incubated with polyclonal goat anti-follistatin antibody (1:20, R&D systems, Minneapolis, MN) and polyclonal rabbit anti-laminin antibody (1:4000, Abcam, Cambridge, MA) followed by donkey antisera against goat IgG (1:200, Cy3-conjugated, Jackson ImmunoResearch, West Grove, PA) and rabbit IgG (1:200, AlexaFluor®488-conjugated, Invitrogen). Slides were cover-slipped with Fluoromount (Sigma-Aldrich). Staining was visualized using a Leica DMI6000 B microscope. Images of the region with the highest density of follistatin-positive structures were captured with a DFC340 FX camera (Leica Microsystems) and viewed with ImageJ (National Institutes of Health, Bethesda, MD). The number of follistatin and laminin-immunoreactive (IR) structures in the lamina propria were counted in 17 sections from vehicle-treated mice and 26 sections from CYP-treated mice by a reviewer blinded to

treatment. Structures were only counted if they were larger than 3 μm in diameter and if the intensity of staining intensity was more than 2x background. The number of follistatin-IR structures was expressed as the percentage of total laminin-IR structures. Follistatin staining was verified through primary antibody omission and preadsorption (Supplemental Figure 1).

***In Vivo* Follistatin Treatment**

Recombinant mouse follistatin (5 μg in 100 μl , R&D systems) was administered i.p. 30 min prior to VMR testing in 8 vehicle-treated mice and 6 CYP-treated mice. This dose has been demonstrated to be sufficient for reducing colitis in several mouse models (8). Phosphate-buffered saline (PBS) vehicle was administered i.p. 30 min prior to VMR testing in 7 vehicle-treated mice and 6 CYP-treated mice.

***In Vitro* Single-Fiber Recordings from Bladder Afferents**

Afferent responses to UBD from 6 vehicle-treated and 6 CYP-treated mice were analyzed. Mice were euthanized *via* isoflurane inhalation followed by exsanguination. The bladder was removed with major pelvic ganglion and pelvic nerve (PN) attached and transferred to ice-cold modified Krebs's solution (in mM: 117.9 NaCl, 4.7 KCl, 25 NaHCO₃, 1.3 NaH₂PO₄, 1.2 MgSO₄·7H₂O, 2.5 CaCl₂, 11.1 D-Glucose) bubbled with 95% O₂ and 5% CO₂. After carefully removing attached tissue from the neurovascular bundle, the bladder and PN were transferred to an acrylic organ bath consisting of two adjacent chambers. The PN was laid onto a mirror in the recording chamber filled with paraffin oil (Thermo Fisher Scientific, Waltham, MA). The tissue chamber with the bladder was superfused with modified Krebs's solution (~32°C). Bundles of nerve fibers ~10 μm thick were individually placed onto a platinum-iridium recording electrode. Due to difficulty in eliminating air leakage in this *ex-vivo* preparation when distension was delivered *via* a transurethral catheter, air pressure was instead delivered *via* a 27 gauge syringe needle inserted into the dome of the bladder. The insertion point was sealed with cyanoacrylate, and the urethral outlet was ligated with silk suture (6-0, Ethicon US, LLC). Action potentials (APs) were recorded extracellularly using a low-noise AC differential amplifier (DAM80, WPI, FL). Activity was monitored on-line, filtered (0.3 to 10 kHz), amplified ($\times 10,000$), digitized at 20 kHz using a 1401 interface (CED), and stored on a PC. APs were discriminated off-line using Spike 2 software (CED). To avoid erroneous discrimination, no more than two clearly discriminable units in any record were studied.

The volume of the tissue chamber was restricted to contain ~5 ml of Krebs solution. Phasic UBD was delivered by pressurized air *via* the needle in the dome of the bladder. Baseline responses of a single afferent fiber to UBD at 10, 20, 40, 60, and 80 mmHg were recorded (10 sec duration, 100 sec intervals). Next, the effect of exposure to Krebs vehicle was assessed by stopping the flow of Krebs solution for 10 min. Finally, the Krebs solution in the tissue chamber was replaced with 5 ml Krebs containing 5 μg follistatin for 10 min. UBD was repeated immediately and after 20 min of washout. For each group, mean spike numbers were normalized to baseline responses at 80 mmHg distension.

Statistics

GraphPad Prism (GraphPad Software, La Jolla, CA) was used for all statistical analyses ($\alpha=0.05$). VMR and single-fiber responses were assessed using two-way ANOVAs. Area under the curve (AUC) of baseline afferent activity in vehicle- and CYP-treated mice was compared using a t-test, and post-hoc tests with the Bonferroni correction were used to compare afferent responses before and after exposure to follistatin. Differences in follistatin gene expression between vehicle- and CYP-treated mice were assessed using t-tests. Pearson correlation coefficients were used to calculate and determine the significance of correlations. A t-test was used to compare the number of follistatin and laminin-IR structures in bladder sections from vehicle- and CYP-treated mice.

RESULTS

Visceromotor Responses to Urinary Bladder Distension

VMR testing revealed bladder hypersensitivity in CYP-treated mice that was established by d6 and persisted to d14. On d6 (Figure 1B; $F(1,80)=8.775$, $p=0.004$) and d14 (Figure 1C; $F(1,44)=9.329$, $p=0.003$), CYP significantly increased VMR magnitude compared with vehicle.

Follistatin Gene Expression in the Bladder

Consistent with previous reports (11,14), H&E staining revealed edema and ulceration of the lamina propria and longitudinal muscle on d6 in a CYP-treated mouse (Figure 2A). By d14, edema had resolved and ulceration was greatly reduced. On d6 (Figure 2B,C), follistatin expression was similar in CYP- and vehicle-treated mice, and expression was not correlated with VMR magnitude. On d14 (Figure 2D), CYP increased follistatin compared with vehicle ($219\pm30\%$ vs. $100\pm9\%$, $p=0.004$). This increase was positively correlated with VMR magnitude (Figure 2E; $r^2=0.472$, $p=0.010$).

Immunolocalization of Follistatin in the Bladder

To determine whether upregulation of follistatin in the bladder is associated with changes in follistatin protein, we examined longitudinal bladder sections from CYP-treated and vehicle-treated mice. Follistatin immunoreactivity (IR) was colocalized with the basal lamina marker laminin adjacent to urothelium and endothelium (Figure 3A). CYP increased the number of follistatin-IR structures (arrowheads). In vehicle-treated mice, $34\pm4\%$ of structures were follistatin-IR, while in CYP-treated mice, $50\pm3\%$ of structures were follistatin-IR (Figure 3B; $p=0.005$). In other words, CYP treatment increased the percentage of follistatin-IR structures in CYP-treated mice to 147% of vehicle-treated mice.

Effect of Exogenous Follistatin on VMR to UBD

To assess the function of follistatin, recombinant mouse follistatin or vehicle (PBS) was administered i.p. 30 minutes prior to VMR testing. On d14, exogenous follistatin significantly increased bladder hypersensitivity in mice with CYP-induced cystitis (Figure 4B; $F(1,40)=19.49$, $p<0.0001$) but not in saline-treated controls (Figure 4A).

Effect of Exogenous Follistatin on Afferent Responses to UBD

To evaluate the effect of follistatin on bladder afferents, *in vitro* single-fiber recordings were undertaken on d14. Figure 5A shows representative recordings from a CYP-treated mouse of responses of UBD-sensitive pelvic nerve afferents to UBD, while mean normalized spike numbers for vehicle- and CYP-treated mice are shown in 5B and 5C, respectively. Some afferents displayed spontaneously activity, but afferents reliably responding to bladder distension were generally quiescent in the absence of distension. Therefore, we did not subtract spontaneous firing from total spike numbers during distension. Baseline (ctrl) responses to distension, before normalization, of vehicle and CYP-treated animals were not significantly different. The effect of follistatin treatment was significant for CYP- [F(3,90)=4.880, p=0.003] but not vehicle-treated mice. In CYP-treated mice, responses after 10 min exposure to follistatin (FST 10min, p=0.014) and 20 min washout (FST 30min, p=0.039) were significantly greater than baseline (ctrl) responses. Therefore, in agreement with its effect on UBD-induced VMR, follistatin is capable of sensitizing bladder afferents in CYP- but not vehicle-treated mice.

DISCUSSION

The present study tested the hypothesis that follistatin reduces hypersensitivity following chronic CYP treatment. We confirmed previous reports that CYP-induced cystitis leads to persistent bladder hypersensitivity. Hypersensitivity was accompanied by a significant increase in bladder follistatin gene expression which correlated with VMR magnitude. In the bladder, follistatin protein was localized to the basal lamina adjacent to urothelium and endothelium. Surprisingly, exogenous follistatin further sensitized nociceptive responses and bladder afferent firing to bladder distension in CYP-treated mice, contradicting our hypothesis. These experiments demonstrated for the first time that follistatin may contribute to bladder hypersensitivity after the resolution of bladder inflammation.

Using the same chronic treatment regimen employed in the present study, Brumovsky et al. (11) demonstrated increased chemosensitivity of stretch-sensitive bladder afferents on d6 of CYP treatment, accompanied by modest edema and infiltration of inflammatory cells to the bladder. More recently, DeBerry et al. (14) reported increased VMRs to UBD on d6 that persisted to d13. Bladder hypersensitivity was accompanied by modest edema and urothelial ulceration on d6 in the absence of plasma extravasation and neutrophil infiltration; histological changes resolved by d13. These findings were replicated in the present study, in which edema and ulceration in the bladder wall were largely resolved by d14, but bladder hypersensitivity persisted. DeBerry et al. found long-lasting changes in bladder afferent expression of functional transient receptor potential and ankyrin-1 (TRPA1), which could contribute to prolonged bladder hypersensitivity (14). Future studies are needed to define the role of central sensitization and descending modulation in this model.

Follistatin expression was significantly increased in CYP-treated mice on d14 but not d6. This timing indicates that follistatin may also play a role in the maintenance of bladder hypersensitivity following chronic CYP-induced cystitis. Previous studies demonstrated that acute or chronic CYP treatment can increase gene and protein expression of various other cytokines within 48 hours (15–22). These cytokines could contribute to inflammation

resulting in the histological damage found on d6. However, preliminary screening of several prototypical pro- and anti-inflammatory cytokines, including TGF β family members TGF β and Inh β A, did not reveal any significant changes in gene expression on d6 (data not shown).

The present study also revealed the presence of follistatin-like IR in the bladder. Follistatin-like IR was localized to connective tissue in the basal lamina adjacent to the urothelium and presumptive blood vessels. This is in agreement with previous studies demonstrating lipopolysaccharide-induced release of follistatin from endothelial cells (23,24). The percentage of structures in the lamina propria with follistatin-like IR was increased in CYP-treated mice, suggesting that the increase in gene expression revealed by qRT-PCR translated to an increase in protein. However, the increase in protein was modest (147% of vehicle) compared with the increase in gene expression (219% of vehicle). This may have been due to instability of follistatin mRNA leading to only a portion being translated. In addition, protein was quantified by counting follistatin IR-structures, which may have underestimated follistatin protein content because the amount of protein in each of these structures, as well as the amount of protein in the urothelium, may have increased.

Anti-inflammatory effects in colitis (8,9) led us to expect that follistatin may also have an anti-inflammatory and anti-nociceptive effect in the bladder through the neutralization of activin A. In order to determine how follistatin affects bladder sensitivity, we administered recombinant follistatin prior to VMR testing. Surprisingly, follistatin increased bladder hypersensitivity in CYP-treated mice, although it did not alter VMRs in vehicle-treated controls. These data suggest that follistatin may act in a pro-nociceptive manner in the urinary bladder following chronic CYP treatment. Future cystometric studies should examine whether follistatin has a similar effect on bladder function.

Exogenous administration of follistatin could increase bladder hypersensitivity in CYP-treated mice either by activating or sensitizing bladder afferents or by increasing peritoneal inflammation. This was examined by recording single-fiber activity of bladder afferents after bathing the serosal surface of the bladder in follistatin. In agreement with previous findings (11), baseline responses of afferents to bladder distension, prior to exposure to follistatin, were not different between vehicle- and CYP-treated mice. This is in contrast to the hypersensitivity observed during VMR testing. Treatment with CYP may evoke bladder hypersensitivity by increasing the number of afferents responding to bladder distention rather than the magnitude of their responses. Alternatively, similar baseline responses in vehicle- and CYP-treated mice may be due to the absence of sensitizing molecules or central processes in the *in vitro* preparation that normally contribute to hypersensitivity in CYP-treated mice. It should also be noted that air was delivered through a transurethral catheter during VMR testing and through a needle inserted into the dome of the bladder during single-fiber recording. However, the impact of this difference in methodology should be minimal because both methods produced reliable distension of the bladder. Most importantly, exposure to follistatin significantly increased the responses of bladder afferents to graded distension of the bladder in CYP-treated mice. This suggests that follistatin can sensitize peripheral terminals of bladder afferents in behaviorally hypersensitive mice.

In summary, CYP treatment is required to expose the pro-nociceptive effect of follistatin observed during VMR and *in vitro* single-fiber recordings. Given that the primary function of follistatin is sequestration of molecules belonging to the TGF β superfamily (activins and BMPs), our findings suggest that after CYP-induced cystitis, those TGF β superfamily molecules may locally activate an anti-nociceptive system, e.g. increasing opioid peptides as reported in the spinal cord (25). However, the rapid effect of follistatin and sensitization of bladder afferents *in vitro* also suggests that follistatin may sensitize bladder afferents by a novel mechanism unrelated to neutralization of TGF β superfamily molecules, possibly through an unidentified receptor. Future studies examining the expression of activin A and its impact on bladder sensitivity will help determine whether bladder hypersensitivity following follistatin is activin-dependent.

CONCLUSIONS

The principal finding of the present study was increased follistatin in the bladder in CYP-treated mice, which was associated with bladder hypersensitivity. Administration of exogenous follistatin sensitized bladder afferents to stretch and further increased VMR to UBD in CYP-treated mice, suggesting a novel, pro-nociceptive role for follistatin. This protein has exciting potential as a therapeutic target for the relief of chronic bladder hypersensitivity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by an IASP John J. Bonica Trainee Fellowship grant (ADS) and NIH award NS035790 (GFG). We thank Michael Burcham for assistance in preparation of the figures.

References

1. Munz B, Hübner G, Tretter Y, Alzheimer C, Werner S. A novel role of activin in inflammation and repair. *J Endocrinol*. 1999 May; 161(2):187–93. [PubMed: 10320815]
2. Xu P, Van Slambrouck C, Berti-Mattera L, Hall AK. Activin induces tactile allodynia and increases calcitonin gene-related peptide after peripheral inflammation. *J Neurosci*. 2005 Oct; 25(40):9227–35. [PubMed: 16207882]
3. Jones KL, Brauman JN, Groome NP, de Kretser DM, Phillips DJ. Activin A release into the circulation is an early event in systemic inflammation and precedes the release of follistatin. *Endocrinology*. 2000 May; 141:1905–8. [PubMed: 10803603]
4. Jones KL, de Kretser DM, Patella S, Phillips DJ. Activin A and follistatin in systemic inflammation. *Mol Cell Endocrinol*. 2004 Oct; 225(1–2):119–25. [PubMed: 15451576]
5. Nakamura T, Takio K, Eto Y, Shibai H, Titani K, Sugino H. Activin-binding protein from rat ovary is follistatin. *Science*. 1990 Feb; 247(4944):836–8. [PubMed: 2106159]
6. Hübner G, Brauchle M, Gregor M, Werner S. Activin A: a novel player and inflammatory marker in inflammatory bowel disease? *Lab Invest*. 1997 Oct; 77(4):311–8. [PubMed: 9354766]
7. Dignass AU, Jung S, Harder-d'Heureuse J, Wiedenmann B. Functional relevance of activin A in the intestinal epithelium. *Scan J Gastroenterol*. 2002 Aug; 37(8):936–43.

8. Dohi T, Ejima C, Kato R, Kawamura YI, Kawashima R, Mizutani N, Tabuchi Y, Kojima I. Therapeutic potential of follistatin for colonic inflammation in mice. *Gastroenterology*. 2005 Feb; 128(2):411–23. [PubMed: 15685552]
9. Zhang YQ, Resta S, Jung B, Barrett KE, Sarvetnick N. Upregulation of activin signaling in experimental colitis. *Am J Physiol Gastrointest Liver Physiol*. 2009 Oct; 297(4):G768–80. [PubMed: 19643954]
10. Ying SY, Zhang Z. Activin and activin receptors in the normal urinary bladder: immunohistochemistry, in situ hybridization, and RT-PCR. *Life Sci*. 1995 Sep; 57(17):1599–603. [PubMed: 7564907]
11. Brumovsky PR, Feng B, Xu L, McCarthy CJ, Gebhart GF. Cystitis increases colorectal afferent sensitivity in the mouse. *Am J Physiol Gastrointest Liver Physiol*. 2009 Dec; 297(6):G1250–8. [PubMed: 19779012]
12. Shaffer, AD., La, JH., Gebhart, GF. Altered cytokine expression contributes to bladder hypersensitivity following cystitis. Washington, DC: Society for Neuroscience; 2014 Nov. Program No 628.21Online
13. Ramakers C, Ruijter JM, Deprez RH, Moorman AF. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett*. 2003 Mar; 339(1):62–6. [PubMed: 12618301]
14. DeBerry JJ, Schwartz ES, Davis BM. TRPA1 mediates bladder hyperalgesia in a mouse model of cystitis. *Pain*. 2014 Jul; 155(7):1280–7. [PubMed: 24704367]
15. Arms L, Girard BM, Malley SE, Vizzard MA. Expression and function of CCL2/CCR2 in rat micturition reflexes and somatic sensitivity with urinary bladder inflammation. *Am J Physiol Renal Physiol*. 2013 Jul; 305(1):F111–22. [PubMed: 23594826]
16. Arms L, Girard BM, Vizzard MA. Expression and function of CXCL12/CXCR4 in rat urinary bladder with cyclophosphamide-induced cystitis. *Am J Physiol Renal Physiol*. 2010 Mar; 298(3):F589–600. [PubMed: 20032115]
17. Girard BM, Cheppurdira BP, Malley SE, Schutz KC, May V, Vizzard MA. Increased expression of interleukin-6 family members and receptors in urinary bladder with cyclophosphamide-induced bladder inflammation in female rats. *Front Neurosci*. 2011 Feb; 5:20. [PubMed: 21373362]
18. Gonzalez EJ, Girard BM, Vizzard MA. Expression and function of transforming growth factor- β isoforms and cognate receptors in the rat urinary bladder following cyclophosphamide-induced cystitis. *Am J Physiol Renal Physiol*. 2013 Nov; 305(9):F1265–76. [PubMed: 23926183]
19. Golubeva AV, Zhdanov AV, Mallel G, Dinan TG, Cryan JF. The mouse cyclophosphamide model of bladder pain syndrome: tissue characterization, immune profiling, and relationship to metabotropic glutamate receptors. *Physiol Rep*. 2014 Mar; 2(3):e00260. [PubMed: 24760514]
20. Malley SE, Vizzard MA. Changes in urinary bladder cytokine mRNA and protein after cyclophosphamide-induced cystitis. *Physiol Genomics*. 2002 Jan; 9(1):5–13. [PubMed: 11948286]
21. Smaldone MC, Vodovotz Y, Tyagi V, Barclay D, Phillips BJ, Yoshimura N, Chancellor MB, Tyagi P. Multiplex analysis of urinary cytokine levels in rat model of cyclophosphamide-induced cystitis. *Urology*. 2009 Feb; 73(2):421–6. [PubMed: 18848347]
22. Vera PL, Wang X, Meyer-Siegler KL. Upregulation of macrophage migration inhibitory factor (MIF) and CD74, receptor for MIF, in rat bladder during persistent cyclophosphamide-induced inflammation. *Exp Biol Med (Maywood)*. 2008 May; 233(5):620–6. [PubMed: 18375833]
23. Michel U, Schneider O, Kirchhof C, Meisel S, Smirnov A, Wiltfang J, Rieckmann P. Production of follistatin in porcine endothelial cells: differential regulation by bacterial compounds and the synthetic glucocorticoid RU 28362. *Endocrinology*. 1996 Nov; 137(11):4925–34. [PubMed: 8895365]
24. Hübner G, Hu Q, Smola H, Werner S. Strong induction of activin expression after injury suggests an important role of activin in wound repair. *Dev Biol*. 1996 Feb; 173(2):490–8. [PubMed: 8606007]
25. Tramullas M, Lantero A, Díaz A, Morchón N, Merino D, Villar A, Buscher D, Merino R, Hurlé JM, Izpisua-Belmonte JC, Hurlé MA. BAMBI (bone morphogenic protein and activin membrane-bound inhibitor) reveals the involvement of the transforming growth factor-beta family in pain modulation. *J Neurosci*. 2010 Jan; 30(4):1502–11. [PubMed: 20107078]

Figure 1. Experimental design and visceromotor responses (VMRs) to graded urinary bladder distension (UBD)

(A) Vehicle (saline) or CYP (100 mg/kg) was administered i.p. on days 1, 3, and 5. On d6 and d14 in different groups of mice, VMR testing was conducted followed by collection of bladders for measurement of follistatin gene expression. In addition, in separate groups of mice, bladders were collected on d6 and d14 for histological assessment. Follistatin IHC and VMR and single-fiber recordings following exogenous administration of follistatin were conducted only on d14. (B) CYP significantly increased VMRs to UBD on d6 compared with vehicle (veh) controls. (C) CYP significantly increased VMRs to UBD on d14 compared with vehicle. * $p < 0.01$, ANOVA

Figure 2. Follistatin gene expression in the bladder

(A) Representative H&E staining of bladder sections from vehicle- and CYP-treated mice on d6 and d14. Edema and ulceration of the lamina propria and longitudinal muscle were apparent on d6. By d14, edema had resolved, and ulceration was greatly reduced. Scale bar=100 μ m. (B) On d6, follistatin expression in CYP-treated mice (n=12) was not significantly different from that in vehicle-treated mice (n=10). (C) Also on d6, follistatin expression in all mice was not significantly correlated with VMR magnitude at 60 mmHg. (D) On d14, CYP (n=7) significantly increased follistatin (FST) expression in the bladder compared with vehicle (n=6). (E) Also on d14, follistatin expression in the bladder was positively correlated with VMR magnitude at 60 mmHg UBD (p = 0.010, Pearson correlation coefficient). *p=0.004, t-test

Figure 3. Immunostaining for follistatin and colocalization with laminin on d14

(A) Representative bladder tissue sections demonstrating an increase in the number of lamina propria structures immunoreactive to both follistatin and laminin in CYP-treated mice relative to vehicle (arrowheads). Scale bar=100 μ m. (B) This increase was quantified by counting the number of follistatin- and laminin-IR structures. The percentage of follistatin-IR structures that were also positive for laminin was significantly increased in CYP-treated mice. n represents number of sections counted. *p=0.005, t-test

Figure 4. Effect of follistatin on visceromotor responses (VMRs) to graded urinary bladder distension (UBD)

Follistatin (FST) or phosphate buffered saline (PBS) was injected i.p. at least 30 min prior to testing on d14 after CYP treatment. Follistatin significantly increased VMRs to UBD in CYP- but not vehicle-treated mice. * $p < 0.0001$, ANOVA

Figure 5. Effect of follistatin on responses of single-fiber pelvic nerve bladder afferents to stretch Bladders were removed with the pelvic nerve attached. Single-fiber responses to UBD were recorded at baseline (control, ctrl), after 10 min exposure to Krebs (veh), after 10 min exposure to follistatin (FST 10min), and after 20 min washout (FST 30min). Follistatin sensitized bladder afferents in CYP- but not in vehicle-treated mice. Responses at 10 min and 30 min were significantly greater than baseline responses. * $p < 0.05$ vs. ctrl, Bonferroni post-hoc test

Table 1

Primer sequences (5' → 3')

	Forward	Reverse	Accession #	Size (bp)
GAPDH	GTTTGTGATGGGTGTGAACCAC	TGGATGCAGGGATGATGTTCTG	NM_008084	263
FST	TGGATCTTGCAACTCCATCTCGGA	TGCCCAAAGGCTATGTCAAACTG	NM_008046.2	137

Abbreviations: GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; FST, Follistatin