Published in final edited form as:

J Urol. 2009 October ; 182(4 Suppl): 1775–1780. doi:10.1016/j.juro.2009.05.126.

Bladder Stromal Loss of Transforming Growth Factor Receptor II Decreases Fibrosis After Bladder Obstruction

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Abstract

Purpose—Transforming growth factor- β is a potent stimulator of extracellular matrix production. Several studies show that loss of transforming growth factor- β signaling decreases kidney, liver and lung fibrosis. However, the role of transforming growth factor- β signaling in bladder fibrosis is not entirely understood. We investigated the effect of stromal loss of such signaling in mice after partial bladder outlet obstruction.

Materials and Methods—We performed partial bladder outlet obstruction by urethral ligation in 5-week-old female *Tgfr2^{colTKO}* mice. These mice were compared to WT mice with partial bladder outlet obstruction and to WT nonobstructed controls. After 4 weeks and before sacrifice urodynamics were performed. Bladder tissue was harvested, and p-Smad2 and collagen (Masson's trichrome) staining were performed.

Results—Bladder compliance was increased in partially obstructed *Tgfr2^{colTKO}* mice and decreased in partially obstructed WT mice. The latter had increased smooth muscle hypertrophy and increased collagen deposition between smooth muscle bundles compared to those in *Tgfr2^{colTKO}* mice and nonobstructed controls. Transforming growth factor- β responsive collagen promoter activity was significantly decreased in *Tgfr2* knockout bladder stromal cells vs WT stromal cells.

Conclusions—Stromal loss of transforming growth factor- β signaling decreased collagen deposition after partial bladder outlet obstruction. In contrast to collagen production by recruited macrophages, stromal transforming growth factor- β signaling appears to be the primary source of fibrosis after partial bladder outlet obstruction. These findings further support the hypothesis that manipulating transforming growth factor- β signaling in bladder stromal cells would provide a future avenue for neuropathic bladder and bladder fibrosis treatment.

Keywords

urinary bladder; receptors; transforming growth factor beta; urinary bladder neck obstruction; collagen; fibrosis

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TGF β 1 is a 25 kDa homodimer and heterodimer present in a wide variety of tissues that interacts with specific cell membrane receptors on virtually all cell membranes. TGF- β has a pivotal role in diverse biological effects on cellular processes, including the regulation of cell growth and survival, cell and tissue differentiation, development, inflammation, immunity, and tissue remodeling and repair. TGF- β is an important mediator of multiple pathological states, including cancer and fibrotic disease of the skin, lung, liver and kidney.¹ TGF- β initiates signaling by binding to T β RII, which results in T β RI recruitment and phosphorylation. After activation T β RI propagates the signal to a family of intracellular signal mediators known as Smads. Smad proteins are classified according to their structure and function in signaling by TGF- β family members. Receptor regulated Smads (Smad2 and Smad3) are phosphorylated and activated by T β RI. They then form complexes with a central Smad (Smad4) and translocate to the nucleus to regulate target gene expression.² The multifunctional cytokine TGF- β has a key role in regulating connective tissue metabolism and exerts potent stimulatory effects on collagen synthesis. It also affects ECM accumulation by increasing the expression of ECM genes such as collagen and proteoglycan.³ TGF- β also interferes with ECM degradation by inhibiting the gene expression pathways involved in ECM degradation.⁴

An extensive number of studies indicate that TGF- β also has an important role in organ fibrosis. Fibrosis is a complex process comprising a sequence of events, including inflammatory cell infiltration and proliferation of matrix producing fibroblasts and myofibroblasts with increased ECM accumulation, which is partly due to uncontrolled synthesis and/or degradation.⁵ Bladder outlet obstruction induces smooth muscle remodeling in the bladder wall and leads to impaired ability of the bladder to store and empty urine. The mouse model of pBOO has been used to show that such obstruction induces ECM production and accumulation in the bladder.

Abnormal accumulation of ECM components is often observed during pathological processes. The pathogenesis of fibrotic disorders is mostly similar regardless of the tissue involved. We determined whether the loss of functional stromal TGF- β signaling in bladder stroma would decrease collagen deposition after pBOO. In contrast to collagen production by recruited macrophages, stromal TGF- β signaling appears to be the primary source of fibrosis after pBOO. These findings further support the hypothesis that manipulating stromal TGF- β signaling may provide a future therapeutic target for bladder fibrosis.

MATERIALS AND METHODS

All animal experiments and maintenance were performed in accordance with the Vanderbilt University institutional animal care and use committee. All surgery was done in 5-week-old female WT and Tgfr2^{colTKO} mice. Mice had free access to water and food (Harlan™ Teklad 2918).

Animal Care

Mice lacking stromal Tgfr2 were generated by homologous recombination between Tgfr2^{floxE2/floxE26} and Col 1 α 2-Cre-ER mice⁷ in the C57/Bl6 background and then

backcrossed into a pure C57BL/6 background to obtain $Tgfr2^{colTKO}$. Systemic administration of 4-OH-tamoxifen (Sigma®), the active metabolite of tamoxifen, enables the translocation of Cre-recombinase in a cell specific temporal manner. Administration of 4-OH-tamoxifen (10 mg/ml) activates Cre mediated recombination, yielding mice deficient in stromal $Tgfr2$ mediated cell signaling.

Animal Surgery

As previously described by Lemack et al,⁸ we performed pBOO surgery. Anesthesia was maintained using inhaled isoflurane delivered at 1.5% to 2% in pure oxygen flowing at 250 ml per minute. A 26 gauge angiocatheter was introduced into the bladder through the urethra under sterile conditions. A 1 cm lower midline incision was made. Blunt dissection was done to identify the urethra with the angiocatheter in place. After minimal dissection of surrounding tissues a curved clamp was passed posterior and a 4-zero silk tie was secured snugly but not tightly at the bladder neck. Sham operated mice underwent an identical procedure but the tie was not placed. Animals were observed for signs of discomfort during the study. Buprenorphine (0.1 mg/kg) was injected subcutaneously for postoperative pain control. Urodynamic measurements were made in all pBOO animals and controls 4 weeks after surgery and the mice were sacrificed.

Urodynamics

We used previously described urodynamic measurement methods⁹ with modifications. Mice were anesthetized using inhaled isoflurane at 1.5% to 2.0% in pure oxygen to maintain anesthesia for urodynamic measurements. The bladder, proximal rhabdosphincter and distal urethra were exposed. The bladder was accessed with a 26 gauge needle and emptied. Sterile saline was infused at 30 μ l per minute via a syringe pump and intravesical pressure was monitored. Transvesical cystometry was recorded using a Model 400 Digi-Med Low Pressure Analyzer (Micro-Med, Louisville, Kentucky). Continuous recordings were made using AcqKnowledgeTM 3.5.3 software. The flow rate was measured after the bladder was filled to capacity. The bladder was connected to a water column at a fixed height. Determining the time needed for a given volume of saline to flow through the bladder and exit the urethra allowed flow rate calculation. Maximal bladder pressure and flow rate were measured from the highest peak. The degree of urethral obstruction was determined by flow rate at fixed bladder pressure. Bladder compliance or elasticity was defined as the change in volume divided by the change in pressure during bladder filling.

Bladder Harvest and Processing

All experimental mice were sacrificed after 4 weeks by anesthetic overdose followed by cervical dislocation. Bladders were harvested and bivalved in a reproducible manner across the detrusor midsection. Specimens were fixed in 4% paraformaldehyde-phosphate buffered saline and embedded in paraffin wax with the detrusor midsection facing and lying parallel to the microtome blade. Sections (5 μ) were cut for staining and IHC.

Immunohistochemical Staining

For IHC all incubation was done at room temperature unless otherwise stated. Slides were deparaffinized and hydrated through xylene and graded alcohols. Antigen retrieval was performed on slides by the sodium citrate method by microwaving in 9 mM sodium citrate (pH 5.0) for 9 minutes and leaving them at room temperature for 1 hour. Endogenous peroxidase activity was blocked with peroxidase blocking reagent for 13 minutes and sections were washed in phosphate buffered saline for 4 minutes. IHC was performed using the Vectastain® Elite ABC peroxidase kit according to the manufacturer protocol. Gomori's trichrome staining was performed with a Masson's trichrome staining system (Dako, Glostrup, Denmark) and an automated stainer platform. Staining was performed at the mouse histology core facility at our institution.

Immunofluorescence Staining

Bladder stromal cells isolated from WT and T β R^{II} KO cells were grown in a Lab-Tek™ Chamber Slide™ system in RPMI 1640 medium supplemented with 10% FBS, penicillin (50 μ g ml⁻¹) and streptomycin (50 U ml⁻¹) at 37C in a humidified atmosphere (5% CO₂). Medium was replaced with 0.2% serum containing medium for 12 hours before treatment with or without TGF- β for 90 minutes. Preparations were fixed, permeabilized and then used for immunofluorescence staining with anti-p-Smad2 antibody. Stained cells were visualized by fluorescence microscope.

Transcriptional Response Assay

Bladder stromal cells from T β R^{II} WT or KO cells were isolated and maintained in RPMI 1640 medium supplemented with 10% FBS. Tgfr2 WT and KO stromal cells were transiently transfected with Renilla luciferase and Col1A2-luc reporter plasmids. Transfected cells were incubated in 5% CO₂ incubator at 37C for 48 hours. Cell lysates were used to measure luciferase and Renilla luciferase activity with a Stop & Glo® dual luciferase assay kit. Normalized luciferase activity was shown.

RESULTS

Urodynamic data were compared in WT vs Tgfr2 KO mice that underwent open pBOO surgery 4 weeks before harvest. Obstructed WT mice had significantly higher urethral resistance than unobstructed controls. Stromal Tgfr2 KO mice had significantly decreased leak point pressure than WT pBOO mice. Compliance, defined as capacity divided by leak point pressure, is another means of evaluating the relationship of pressure and volume in the bladder (see table). Compliance at 50% capacity was significantly decreased in WT pBOO mice vs controls without obstruction and stromal Tgfr2 KO mice showed significantly improved compliance vs WT pBOO mice (fig. 1).

To further investigate the role of stromal TGF- β signaling in the bladder fibrosis induced by pBOO bladders harvested 4 weeks after surgery were evaluated histologically. Hematoxylin and eosin staining of bladders from WT pBOO mice revealed smooth muscle hypertrophy and fibrotic lesions compared to control findings. Tgfr2 KO and WT pBOO mice had increased smooth muscle hypertrophy compared to WT control mice (fig. 2). To determine

the role of stromal TGF- β signaling in pBOO induced collagen synthesis Masson's trichrome staining was done. WT pBOO mice had significantly increased collagen deposition between smooth muscle bundles compared to that in controls (fig. 3). In contrast, Tgfr2 KO pBOO mice had decreased collagen staining and fibrosis compared to those in WT pBOO mice. Together urodynamic data and trichrome staining suggest that stromal specific TGF- β signaling has a major role in compliance by decreasing urethral resistance, significantly increasing the 50% compliance rate and attenuating fibrosis in the absence of TGF- β signaling.

To confirm Tgfr2 targeted deletion in bladder stromal cells in Tgfr2^{colTKO} mice in response to 4-OH-tamoxifen injection we performed p-Smad2 immunostaining. Positive identification of p-Smad2 staining in WT control and WT pBOO mice stroma and urothelium confirmed intact stromal TGF- β signaling. The exclusive loss of p-Smad2 staining in 4-OH-tamoxifen administered Tgfr2^{colTKO} mice stromal cells alone further confirmed the specific loss of TGF- β signaling in stromal cells not in the urothelium of Tgfr2^{colTKO} mice (fig. 4).

Stromal cell subcellular localization of p-Smad2 was done by immunofluorescence analysis, followed by in vitro stromal cell culture. p-Smad2 staining was predominantly located in the cytoplasm in WT and Tgfr2 KO stromal cells in the absence of TGF- β treatment. TGF- β treatment significantly increased nuclear translocation of p-Smad2 in WT stromal cells. However, p-Smad2 translocation was completely blocked by the loss of Tgfr2 signaling in Tgfr2 KO cells isolated from 4-OH-tamoxifen administered Tgfr2^{colTKO} mice under similar conditions (fig. 5, A). These results suggest that Tgfr2 loss inhibits TGF- β mediated nuclear translocation of p-Smad2 and supports the fact that 4-OH-tamoxifen administration inactivates Tgfr2 function in stromal cells in Tgfr2^{colTKO} mice. To further verify the role of stromal Tgfr2 signaling in collagen synthesis in response to TGF- β we performed transient transfection analysis using Col1A2-Luc reporter plasmids. TGF- β strongly induced reporter activity in WT stromal cells but TGF- β induced reporter activity was strongly suppressed in Tgfr2 KO stromal cells (fig. 5, B). These data suggest that stromal Tgfr2 signaling is needed for TGF- β mediated collagen regulation in response to pBOO in mice.

DISCUSSION

Genetically modified mice provide a unique opportunity to implicate a specific gene in the sequence of events by which pBOO results in specific categories of ECM modifications or detrusor responses. Several groups reported the development of mouse models of pBOO.^{10,11} Congenital or acquired bladder obstruction can result in a stiff-walled fibrotic organ characterized by trabeculation, infiltration of connective tissue in abnormal locations and low volume capacity at high pressure. Various neurological and obstructive conditions can cause the bladder wall to accumulate connective and fibrotic tissue.¹² Our data show that pBOO increased urethral resistance and decreased compliance (at 50% capacity). These effects could be partially reversed by stromal loss of Tgfr2 signaling in the bladder. Urodynamic studies using isoflurane anesthesia may not be optimal, especially in regard to the observation of detrusor contractions, but our initial objective was to collect data on bladder capacity and compliance only. Other studies may also have considered bladder

weight as a relevant factor in bladder function. However, increased bladder weight may not only result from obstruction. Other factors also significantly influence bladder weight, such as bladder volume, detrusor overactivity and methodological limitations, including variability in the number of different sites on the anterior bladder wall used to derive bladder weight.

Tissue fibrosis is the result of excessive ECM accumulation, which causes organ failure. Several studies show that TGF- β is essential for wound healing, serves as a mediator for scarring via the stimulation of matrix molecule deposition and angiogenesis, and is an essential mediator of pathological scarring in fibrotic disorders.^{13–16} In a newly developed 4-OH-tamoxifen inducible stromal specific Tgfr2^{colTKO} mouse model of pBOO we noted that the severity of obstruction induced fibrosis could be ameliorated by decreased TGF- β signaling in bladder stromal cells. The conditional activation of signaling using the Cre/loxP system with deletion of a transcription stop cassette allows high level activation that is transmissible to daughter cells upon mitosis and does not depend on the continued presence of the tamoxifen ligand. In parallel with our finding that stromal loss of Tgfr2 decreased collagen deposition after pBOO, TGF- β signaling was involved in increased collagen synthesis by bladder stromal fibroblasts in response to pBOO. TGF- β recruits fibroblasts from surrounding tissues, and stimulates their proliferation and differentiation, thus enhancing the synthesis of collagen, fibronectin and proteoglycan. These findings support the notion that TGF- β is an important mediator of bladder fibrosis that can be decreased by altering the TGF- β signaling pathway in stromal cells. Further refinement of these models is needed to develop molecular therapy to prevent or reverse fibrosis and the functional consequences of bladder outlet obstruction.

Our finding of increased collagen synthesis by bladder stromal fibroblasts in response to pBOO supports results in previous studies showing increased collagen synthesis and accumulation in cases of pulmonary fibrosis,¹⁷ liver fibrosis,¹⁸ myocardial fibrosis¹⁹ and radiation induced muscle fibrosis.²⁰ Furthermore, these studies show that fibrotic lesion formation in different organs is characterized by similar pathways of collagen synthesis and accumulation. The Col1A2 promoter contains Smad binding elements that are necessary and sufficient to mediate the transcriptional responses induced by TGF- β . The profibrotic effect of TGF- β in bladder fibroblasts induced by pBOO was efficiently antagonized by the stromal loss of TGF- β signaling induced by 4-OH-tamoxifen. Tgfr2 KO in stromal cells significantly suppressed TGF- β induced Col1A2 promoter activity, suggesting that in bladder stromal fibroblasts TGF- β signaling is required for the collagen regulation and fibrosis induced by pBOO. In our studies we have not seen any pathological changes on IHC in mice treated with 4-hydroxytamoxifen alone. Also, further studies of in vitro bladder contractile function may provide more valuable information on WT and Tgfr2 KO bladder function after obstruction.

CONCLUSIONS

Loss of stromal TGF- β signaling in mice decreased collagen deposition after pBOO. In contrast to collagen production by recruited macrophages, stromal TGF- β signaling appears to be the primary source of fibrosis after pBOO. These findings further support the

hypothesis that manipulating TGF- β signaling may provide a future avenue for treating neuropathic bladder and bladder fibrosis.

Acknowledgments

Study received Vanderbilt University animal care and use committee approval.

Supported by National Institutes of Health Grant 1RO1 DK068593-02 (JCP).

Dr. Poncelet, University of Washington School of Medicine, provided Col1A2-luc and Dr. Lynn L. Woo reviewed the manuscript. Cell culture medium was obtained from Gibco®.

Abbreviations and Acronyms

ECM	extracellular matrix
FBS	fetal bovine serum
IHC	immunohistochemical staining
KO	knockout
pBOO	partial bladder outlet obstruction
p-Smad2	phospho Smad 2
TβR	TGF receptor
TGF-β	transforming growth factor- β 1
Tgfr2	TGF- β 1 receptor type II
Tgfr2^{colTKO}	OH tamoxifen-inducible KO of stromal Tgfr2

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Figure 1.

Urodynamics in WT mice without obstruction, WT mice and stromal Tgfbr2 KO mice with open pBOO 4 weeks before harvest shows that stromal Tgfbr2 KO increases compliance in pBOO mice.

Figure 2. pBOO induced smooth muscle hypertrophy and fibrosis in mouse bladder. *A*, WT control. *B*, WT mouse with pBOO. *C*, Tgfr2 KO mouse without pBOO. *D*, Tgfr2 KO mouse with pBOO. H & E, scale bar indicates 50 μm.

Figure 3. Stromal loss of TGF- β signaling decreased collagen deposition. *A*, WT control. Scale bar indicates 50 μ m. *B*, WT mouse with pBOO. Arrows indicate collagen deposition. *C*, stromal Tgfb2 KO control. *D*, stromal Tgfb2 KO mouse with pBOO. Arrows indicate loss of collagen deposition. Trichrome stain.

Figure 4.

Confirmation of 4-OH-tamoxifen inducible stromal Tgfr2 KO by p-Smad2 staining.

Bladder tissue was harvested from Tgfr2^{colTKO} mice administered 4-OH-tamoxifen or oil.

A, control with p-Smad2 staining (arrows) in stroma (S) and urothelium. B, loss of p-Smad2 staining only in Tgfr2 KO bladder stromal cells. E, bladder epithelium. Scale bar indicates 25 μ m.

Figure 5.

A, p-Smad2 staining confirmed loss of Tgfb2 KO in bladder stromal cells in vitro. p-Smad2 localization was detected by IHC. Tgfb2 WT and KO mice bladder stromal cells were cultured in chamber slides, fixed and processed for immunofluorescence using anti-p-Smad2 antibodies. Fluorescence was visualized by antirabbit antibody conjugated to Cy3 (red areas) using Axioplan fluorescence microscope (Carl Zeiss, Jena, Germany). B, luciferase assay revealed that stromal loss of Tgfb2 did not respond to TGF-β induced collagen transcriptional response. WT and Tgfb2 KO bladder stromal cells were transiently cotransfected with Renilla and Col1A2-Luc plasmids. At 20 hours after transfection cells were treated with or without 5 ng/ml TGF-β for 22 hours in low serum (0.2% FBS) containing RPMI 1640 medium. Luciferase activity was normalized with Renilla luciferase activity. Relative luciferase activity is shown as mean ± SD of triplicate measurements. Asterisk indicates multiple comparison test with Bonferroni adjustment after data rank transformation $p < 0.005$ for all groups.

	Mean $\pm$ SD Capacity (μ l)	Mean $\pm$ SD Leak Point Pressure (cm H ₂ O)	Mean $\pm$ SD Compliance at 50% Capacity (μ l/cm H ₂ O)
Control	183 $\pm$ 15.0	49.2 $\pm$ 5.1	5.8 $\pm$ 2.1
WT pBOO	83 $\pm$ 20.1	79.5 $\pm$ 8.9	1.0 $\pm$ 1.7
Tgfr2 KO pBOO	163 $\pm$ 28.4	57.9 $\pm$ 6.4	4.6 $\pm$ 1.4