Metabolic, Endocrine, and Genitourinary Pathobiology

Molecular Mechanisms of Bladder Outlet Obstruction in Transgenic Male Mice Overexpressing Aromatase (*Cyp19a1*)

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We investigated the etiology and molecular mechanisms of bladder outlet obstruction (BOO). Transgenic (Tg) male mice overexpressing aromatase (*Cyp19a1*) under the ubiquitin C promoter in the estrogen-susceptible C57Bl/6J genetic background (AROM+/6J) developed inguinal hernia by 2 months and severe BOO by 9 to 10 months, with 100% penetrance. These mice gradually developed uremia, renal failure, renal retention, and finally died. The BOO bladders were threefold larger than in age-matched wild-type (WT) males and were filled with urine on necropsy. Hypotrophic smooth muscle cells formed the thin detrusor urinae muscle, and collagen III accumulation contributed to the reduced compliance of the bladder. p-AKT and ER α expression were up-regulated and Pten expression was down-regulated in the BOO bladder urothelium. Expression of only ER α in the intradetrusor fibroblasts suggests a specific role of this estrogen receptor form in urothelial proliferation. Inactivation of Pten, which in turn activated the p-AKT pathway, was strictly related to the activation of the ER α pathway in the BOO bladders. Human relevance for these

findings was provided by increased expression of p-AKT, PCNA, and ER α and decreased expression of PTEN in severe human BOO samples, compared with subnormal to mild samples. These findings clarify the involvement of estrogen excess and/or imbalance of the androgen/estrogen ratio in the molecular pathogenetic mechanisms of BOO and provide a novel lead into potential treatment strategies for BOO. (Am J Pathol 2011; 178:1233–1244; DOI: 10.1016/j.ajpath.2010.11.056)

A close interrelationship between lower urinary tract (bladder and urethra) symptoms, bladder outlet obstruction (BOO), and benign prostatic hyperplasia has been shown in aging men.^{1–4} These symptoms, which include increased voiding frequency and urgency, nocturia, incomplete bladder emptying, hesitancy, weak stream, and straining, occur in mild to severe form in 50% to 85% of men over 50 years of age.^{5–8} BOO, which reduces or prevents the flow of urine into the urethra, and urinary tract infection, bladder cancer, and incontinence comprise the major causes of lower urinary tract symptoms.^{9,10} Congenital or acquired BOO can result in a stiff-walled, fibrotic bladder with low capacity, high pressure, and noncompliance, which may ultimately damage the kidneys.¹⁰ The incidence of lower urinary tract symp-

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toms was shown to be higher among men with prostatic enlargement, which can narrow the urethral lumen.^{9,11} Despite the higher prevalence of benign prostatic hyperplasia, the detailed pathogenetic role of benign prostatic hyperplasia in inducing BOO remains unknown. A number of studies have demonstrated that androgens and estrogens play an important role in the development of both benign prostatic hyperplasia and BOO.^{12–16} Epidemiologically, BOO mainly occurs in men.¹⁷

The balance between the systemic levels of androgens and estrogens is altered in aging men.^{18–21} With increasing age, there is a gradual decline of androgen levels, especially the free and bioavailable testosterone.^{18,19} Simultaneously, estradiol (E_2) concentrations increase, especially in obese men.^{20,21} Increased prevalence of BOO with aging suggests that estrogen (or, more precisely, the altered androgen/estrogen balance) may be involved in its pathogenesis.²² Neonatally estrogenized male mice have increased voiding frequency and a decreased ratio of urinary flow rate to bladder pressure.²³ We have shown previously that male mice expressing the human *Cyp19a1* gene under the human ubiquitin C promoter in the FVB/N genetic background (AROM+/N) present with severe subfertility and cryptorchidism,²⁴ as well as with higher mean maximal bladder pressure and decreased mean maximal flow rate, consistent with the presence of mild infravesical obstruction, but never clear signs of BOO.²⁵

The underlying etiology of the higher prevalence of BOO with age in human remains poorly understood.²² We hypothesized that estrogen excess and/or imbalance of the androgen/estrogen ratio might have an effect on the molecular mechanisms of BOO. The only animal models reported to date are the surgically induced partial BOO animal model²⁶ and the Noble rat model, in which combined testosterone and E_2 treatment induced prostatic inflammation and obstructive voiding.²⁷ Differential effects of estrogens on the male urogenital tract and spermatogenesis have been demonstrated in mice with different genetic backgrounds.²⁸ Because the C57Bl/6J mouse strain is reported to be more estrogen-sensitive than other strains,²⁸ we hypothesized that Tg overexpression of *Cyp19a1* in this background (AROM+/6J) could intensify the previously documented mild bladder intravesical obstruction observed in the FVB/N background (AROM+/N), and would lead to full-blown BOO, allowing us to investigate the etiology and molecular mechanisms of this condition. To demonstrate and test the relevance of the AROM+/6J murine model to human BOO disease, we also studied severe human BOO samples for the molecular changes observed in the AROM+/6J bladders.

Materials and Methods

Transgenic Mice Expressing Human Cytochrome P450arom (CYP19A1)

Transgenic (Tg) mice expressing human cytochrome P450arom cDNA under the control of the ubiquitin C

promoter in the C57Bl/6J genetic background (AROM+/6J) were generated as described previously.²⁹ Generation of the new Tg line is described in the *Results* section. AROM+/6J female mice were phenotypically normal and were used as breeders. Wild-type (WT) littermate mice (C57Bl/6J) were used as controls in each age group ($n = 5$). We characterized the Tg mice specifically for the BOO phenotype at three time points: 2 months ($n = 6$), 4 months ($n = 6$), and 10 months ($n = 10$). For routine genotyping, PCR analysis was performed as described previously,²⁹ using DNA extracts from ear biopsies. After weaning at the age of 21 days, the mice were housed two to four per cage, females and males separately, in a room with controlled light (12 hours of light and 12 hours of darkness) and temperature ($21 \pm 1^\circ\text{C}$). They were fed with soy-free mouse chow SDS RM-3 (Whitham, Essex, United Kingdom) and tap water *ad libitum*. The Ethics Committee for animal experimentation of the China Agriculture University approved all of the animal experiments.

Morphological and Histological Analyses

Blood from WT and AROM+/6J mice was collected by cardiac puncture, and tissues were dissected for macroscopic analyses. For histological evaluation, the bladders were fixed in 4% paraformaldehyde. In addition to bladder, we took the testes, prostate, kidneys, adrenals, pituitary, spleen, and lungs. The tissues were first fixed, then dehydrated, embedded in paraffin, and sectioned. The sections (5 μm thick) were deparaffinized in xylene and stained with H&E. For detecting collagen fibers, the sections were stained with Van Gieson's solution.

Semiquantitative RT-PCR

Total RNA was isolated from bladder, testis, and fibroblast using the acid phenol method, and RT-PCR was performed. One μg of total RNA was incubated with 10 IU avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) at 37°C for 1 hour. The cDNAs were then denatured at 95°C for 5 minutes and amplified through 25 cycles of PCR, using the following conditions: 94°C for 30 seconds, 56°C to 61°C for 45 seconds, and 72°C for 45 seconds. An aliquot of the RT-PCR product was subjected to agarose gel electrophoresis and was visualized by ethidium bromide staining. Three individual experiments were conducted. As an internal control for the total amount of RNA used, a 406-bp fragment of the ribosomal protein L19 gene was amplified. The primers used for the various RT-PCR analyses are given in Table 1. The density of the gel bands was quantified by using ImageJ software version 1.34 (Image processing and analysis in Java, NIH, Bethesda, MD; <http://rsb.info.nih.gov/ij/download.html>).

Whole Bladder Tissue Culture

Whole bladders of 10-month-old WT and AROM+/6J male mice ($n = 5$ per group) were dissected out and were quickly washed in PBS. The bladders were sheared to ~ 1 cm each and then were cultured in Dulbecco's

Table 1. Primers Used for RT-PCR Analyses

Primer name	Primer sequence	Annealing temperature (°C)	Plateau phase	Product size (bp)
L19	F: 5'-GAAATCGCCAATGCCAACT-3' R: 5'-TGAGACTCGCAGGTCTAAGA-3'	50	18	406
Aromatase	F: 5'-ACTTCCCTAAGCCCAATG-3' R: 5'-CGCCACCTACTCGATAAA-3'	56	30	441
ER α	F: 5'-GGTCCAATTCTGACAATCGAC-3' R: 5'-TTTCGTATCCCGCCTTTCATC-3'	53	36	308
ER β	F: 5'-CTTGGTCACGTACCCCTTAC-3' R: 5'-GTATCGCGTCACCTTTCCTTT-3'	55	32	226
Col3a1	F: 5'-CTGAAGATGTCGTTGATGTGCAGC-3' R: 5'-GATCAGGACCCCAATGTGCATAGG-3'	57	22	343
PTEN	F: 5'-TGGATTGCACTTAGACTTGACCT-3' R: 5'-GCGGTGTCATAATGTCTCTCAG-3'	50	36	180
PCNA	F: 5'-CAACTTGAATCCCAGAAC-3' R: 5'-AGACAGTGGAGTGGCTTTT-3'	56	28	294
GPR30	F: 5'-GTCTTTCCGTCACGCCTAC-3' R: 5'-GCTCGTCTTCTGCTCCACA-3'	55	38	145

F, forward; R, reverse.

modified Eagle's medium (without fetal bovine serum). After 48 hours, the tissues were adherent to the wall. Then 17 β E₂ at 10 nmol/L concentration was added to the media. The cultured bladder RNA was extracted 24 hours later, and semiquantitative RT-PCR was done as described previously.²⁹⁻³¹

Embryonic Fibroblast Cell Culture

Embryonic fibroblast cells were isolated from fetuses of C57Bl mice and were maintained as described previously.³² E₂ and ICI 182,780 (ICI), PI3K inhibitor (PI3KI) were purchased from Sigma-Aldrich (St. Louis, MO). 4,4',4''-[4-Propyl-(1*H*)-pyrazole-1,3,5-triyl]trisphenol (PPT; an ER α -selective agonist) and 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN; an ER β -selective agonist) were purchased from Tocris Biosciences (Tocris Biosciences, Ellisville, MO). Before ligand treatment, the medium was replaced with phenol red-free Dulbecco's modified Eagle's medium supplemented with 5% dextran charcoal-stripped fetal bovine serum for 48 hours. Fibroblast cells were treated with either 0.01% ethanol (vehicle control), 10 nmol/L E₂, 10 nmol/L PPT, 10 nmol/L DPN, 100 nmol/L ICI, or 10 nmol/L of P13KI alone or in combination as E₂ + ICI, E₂ + P13KI, or E₂ + P13KI + ICI for 48 hours. For the indicated experiments, cells were pretreated with ICI for 8 hours before other treatments.

Immunohistochemistry

Sections (5 μ m thick) were cut from paraffin-embedded tissues and mounted on slides. After deparaffinization and rehydration in xylene and ethanol, sections were placed in 10 mmol/L citrate buffer (pH 6.0), followed by heating in a microwave oven for antigen retrieval. For this step, three periods of 5 minutes each were used, after which the sections were treated with 3% H₂O₂ in PBS (pH 7.6) for 10 minutes and blocked with normal goat serum (1:200 dilution) for 10 minutes. The sections were then incubated at 37°C for 1 to 2 hours with one of the follow-

ing antibodies: p-AKT (rabbit polyclonal IgG, 1:200 dilution; sc-81433; Santa Cruz Biotechnology, Santa Cruz, CA), AKT (rabbit polyclonal IgG, 1:300 dilution; sc-5298; Santa Cruz Biotechnology), or PCNA (goat polyclonal IgG, 1:200 dilution; sc-25280; Santa Cruz Biotechnology), PTEN (rabbit polyclonal IgG, 1:200 dilution; 21057-2/Ab-370; Signalway Antibody, Pearland, TX), ER α (rabbit polyclonal IgG, 1:500 dilution; Santa Cruz Biotechnology), and FSP1 (goat polyclonal IgG, 1:400 dilution; Santa Cruz Biotechnology). The primary antibody bound was detected by using biotinylated goat anti-rabbit IgG (1:200 dilution), followed by incubation with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). Specific binding was visualized by using 3',3'-diaminobenzidine tetrahydrochloride. Sections were slightly counterstained with Mayer's hematoxylin.

Western Blot

Total protein was extracted in extraction buffer [20% glycerol, 50 mmol/L Tris-HCl (pH 6.8) and 0.5% (v/v) Tween 20] and protease inhibitor cocktail (Sigma-Aldrich) was added, followed by centrifugation at 12,000 rpm (6439 g) for 10 minutes to remove nondissolved material. Protein concentration in the supernatant was measured using a Tiangen protein assay kit (Tiangen Biotech, Beijing, China). Aliquots of protein extract containing 30 μ g of total protein were electrophoresed in a 10% SDS-polyacrylamide gel electrophoresis gel and transferred to P-membrane for 3 hours with transfer buffer [25 mmol/L Tris, 192 mmol/L glycine, and 20% (v/v) methanol]. The membrane was blocked with 2% dried nonfat milk, and incubated with antibody against β -actin, ER α (as above), ER β (rabbit polyclonal IgG; ab3577; Abcam, Cambridge, MA), p-AKT (as above), AKT (as above), PCNA (as above), FSP1 (as above), PTEN (see above), or Col3a1 (mouse monoclonal IgG; sc-80564, Santa Cruz Biotechnology) in concentrations specified by the manufacturer's protocol. After treatment with primary antibody, the membrane was washed in Tris-buffered saline-Tween buffer (20 mmol/L Tris, 500 mmol/L NaCl, 0.05%

Tween 20) and incubated with secondary antibody (goat anti-rabbit IgG at 1:3000). Final exposure was obtained using enzymatic chemiluminescence (Amersham Pharmacia, GE Healthcare, Chalfont St Giles, UK). Finally, the films were scanned and quantified using ImageJ software, version 1.34 (NIH).

Human Bladder Specimens

Twenty-six human bladder paraffin-block samples from bladder cancer patients (age, 50 to 70 years) were selected from the Pathology Department of the First Hospital of Peking University. These samples consisted of 6 cases of subnormal/mild BOO (Urodynamic system grading 0–2)³³ and 20 severe BOO (Urodynamic system grading 6)³³ from male bladder cancer patients. Because of the difficulties in obtaining normal human bladder samples, we used the subnormal/mild BOO specimens as controls. Slides were analyzed by two expert pathologists on the basis of criteria adopted at the Department of Urology, Peking University First Hospital, Beijing, China, on the basis of an earlier report (J.L., D.Z.).³³ Sections (5 μ m thick) were cut from paraffin-embedded tissues and mounted on slides for further analyses.

Measurement of Serum Hormones

Serum testosterone was measured by radioimmunoassay after diethyl ether extraction, as described previously.³⁴ Concentrations of E₂ were measured using a commercial radioimmunoassay kit (Immunotech; Beckman Coulter, Marseille, France).

Statistical Analysis

One-way analysis of variance and Tukey's and Dunnett's post hoc tests were used for statistical analyses (SPSS 12.0.1 statistical package software; SPSS, Chicago, IL). *P* values of <0.05 were regarded as statistically significant. All values are presented as means $\pm$ SEM.

Results

Cyp19a1-Overexpressing Mice in the C57Bl/6J Genetic Background (AROM+/6J)

We generated Tg mice expressing the human *CYP19A1* cDNA under control of the human ubiquitin C promoter in the C57Bl/6J background. The same gene construct has been used previously to generate Tg mice in the FVB/N genetic background.¹⁵ Standard pronuclear injection techniques were used for production of the transgenic mice, as described previously.²⁹ Two AROM+/6J founders were produced, of which one was a severely subfertile male (small number of pups, and the positive ones were always cannibalized; the male died of bladder outlet obstruction at 10 months of age) and one fertile female. All AROM+/6J males of the F₁ generation and thereafter were severely subfertile and in general failed to produce offspring. The Tg females were fertile and were used for establishing a Tg

Figure 1. Phenotype of transgenic (Tg) mice expressing human *CYP19A1* under the ubiquitin C promoter in the C57Bl/6J genetic background (AROM+/6J). **A:** Inguinal herniae were observed at 2 months of age in AROM+/6J males with 100% penetrance (*n* = 16). **Insets:** Representative mouse of each group during the longitudinal laparotomy at necropsy. **B** and **C:** Complete bladder obstruction (BOO) was observed in 10-month-old male AROM+/6J mice with 100% penetrance (*n* = 10). Note dilated bladder filled with urine, renal hydronephrosis, and significantly smaller dark colored testes (**C**), compared with wild type (**B**). 1, adrenals; 2, kidneys; 3, seminiferous tubules; 4, bladder; 5, testes. Organs in panel **B** are from a WT mouse (*n* = 5) during necropsy.

line. The subfertile AROM+/6J males showed no signs of abnormalities until puberty; severe inguinal herniae were evident at 2 months of age (Figure 1A). Apart from the bladder phenotype, no phenotypic differences could be found between the 2-month-old, 4-month-old, and 10-month-old AROM+/6J males. Of the ten 10-month-old AROM+/6J males, five died between 9 and 10 months; the remaining five mice were sacrificed at 10 months as planned.

At necropsy, the Tg mice presented with dilated bladder filled with urine, resembling hydronephrosis, which is the equivalent extreme clinical condition of BOO in humans (see Supplementary Figure S1 at <http://ajp.amjpathol.org>). In addition, the Tg mice had significantly smaller and dark-colored testes, compared with WT controls [75 ± 21 (Tg) vs 181 ± 30 mg (WT) mg, *P* < 0.05] (Figure 1, B and C). There were no statistically significant differences in testis weight at 2 and 4 months of age. Serum E₂ levels were significantly higher in the AROM+/6J at 2 months [95 ± 35 (Tg) vs 10 ± 1.6 (WT) pg/ml, *P* < 0.05], 4 months [115 ± 38.2 (Tg) vs 12 ± 2.1 (WT) pg/ml, *P* < 0.05], and 10 months [131 ± 35 (Tg) vs 9.7 ± 1.8 (WT) pg/ml, *P* < 0.05] (*n* = 5 to 6 for WT and *n* = 6 to 10 for Tg). Serum testosterone levels inversely correlated with those of E₂ and were significantly decreased in the Tg males [0.28 ± 0.33 (Tg) vs 2.1 ± 1.41 (WT) ng/ml, *P* < 0.05], 4 months [0.43 ± 0.38 (Tg) vs 6.6 ± 4.82 (Tg) ng/ml, *P* < 0.05], and 10 months [0.38 ± 0.48 (Tg) vs 7.97 ± 5.03 (WT) ng/ml, *P* < 0.05] (*n* = 5 to 6 for WT and *n* = 6 to 10 for Tg). Serum testosterone and E₂ values of AROM+/6J males showed hormonal levels similar to those previously reported for AROM+/N mice.^{29,31,35}

The small testes of the 10-month-old AROM+/6J males had a phenotype identical to that previously described in AROM+/N mice, including macrophage activation, Leydig cell depletion, dysmorphic seminiferous tubules, and disrupted spermatogenesis.^{29,35} In contrast to the males, no major phenotypic alterations were found in the AROM+/6J females, and they presented with normal fertility with normal pregnancies, pup delivery, litter size, nursing, and offspring development. Macroscopically, the prostates of the 10-month-old AROM+/6J males were significantly smaller, compared with WT males.

Histopathological and Immunohistochemical Characterization of BOO in Lower Urinary Tract in AROM+/6J Male Mice

Because urinary bladder hypertrophy is regarded as a general consequence of BOO, we measured the bladder weights at different ages (2, 4, and 10 months) in the AROM+/6J and WT males. There was no significant difference at 2 months [15.8 ± 1.0 (Tg) vs 16.1 ± 1.0 (WT) mg, $n = 5$ to 6], but we observed a progressive increase in bladder weight from 4 months (27.4 ± 5.1 vs 16.6 ± 1.8 mg, AROM+/6J vs WT, $P < 0.05$, $n = 5$ to 6) to 10 months (77.6 ± 15.2 vs 19.4 ± 2.8 g, AROM+/6J vs WT, $P < 0.05$, $n = 6$ for WT and $n = 10$ for Tg) in AROM+/6J male mice.

The increased bladder weight could be due to hyperproliferation of the urothelium of bladder and detrusor urinae muscle. On histopathological analysis, the bladder showed three- to fourfold thicker lamina propria of the detrusor in Tg males at 10 months, compared with age-matched WT controls, but the smooth muscle layers of the detrusor of bladder were 70% thinner (Figure 2, A–D) (see also Supplementary Figure S1 at <http://ajp.amjpathol.org>). The detrusor in AROM+/6J males also lost its normal smooth muscle layers (internal longitudinal, outer circular, and outermost longitudinal) (Figure 2, E and F). Moreover, proliferative spindle-shaped fibroblast cells were observed to penetrate into the smooth muscle layers of the detrusor in Tg males (Figure 2F) of 10-month-old AROM+/6J males, a feature absent in age-matched WT controls (Figure 2, A, C, and E). Collagen staining was abundant in the lamina propria of 10-month-old AROM+/6J males (Figure 2D), but was rather scant in WT males (Figure 2C). Histopathological analysis further demonstrated that the thickness of the striated muscle layer of the proximal rhabdosphincter in Tg mice was significantly (70%) decreased in thickness, compared with age-matched controls (Figure 2, C and D).

Next, we checked proliferating cell nuclear antigen (Pcna) protein expression immunohistochemically as a marker for cell proliferation, to test whether the estrogen excess resulted in increased cell proliferation in the AROM+/6J bladders. Abundant Pcna expression was found in the layers of bladder urothelial cells and in the spindle-like cells within the AROM+/6J detrusor (Figure 2, G–J). Pcna expression also confirmed that the proliferative spindle-shaped fibroblast cells penetrated to the smooth muscle layers of the bladder detrusor in 10-

Figure 2. Histopathological and immunohistochemical (Pcna) analysis of bladder and fibrotic detrusor urinae muscle in 10-month-old WT (left panels) and AROM+/6J males with BOO (right panels). **A** and **B**: H&E staining reveals smooth muscle of the internal longitudinal, outer circular, and outermost longitudinal layers of the detrusor (line indicated by arrow), which are 70% thinner in Tg mice (**B**) than in age-matched WT controls (**A**). **C** and **D**: The laminae propriae (short lines indicated by short arrows) are four times thicker in AROM+/6J mice (**D**) than in WT mice (**C**), but fibrosis, characterized by pink-red collagen by Van Gieson staining, was observed in the AROM+/6J mice (**D**) both in the lamina propria (short line) and throughout the detrusor (long line indicated by long arrow). **E** and **F**: H&E staining reveals abundant fibrosis in disorganized, extensively shortened, and hard-to-distinguish layers of lamina propria of smooth muscle layers of detrusor in 10-month-old AROM+/6J mice (**F**), compared with WT (**E**). Dashed line indicates the muscle thickness and layer arrangement (**E**). **G–J**: Immunohistochemistry for Pcna in urothelium and intradetrusor fibroblasts of bladder in 10-month-old AROM+/6J Tg and WT mice. Abundant nuclear staining of Pcna protein expression (arrows indicate a representative selection of the abundant positive staining) was found in multilayers of bladder urothelial cells and in Tg mice proliferative spindle-shaped fibroblast cells (**H**) penetrating the smooth muscle layers of the bladder detrusor in Tg males (**J**). Scant Pcna stained cells could be found in WT detrusor (**G**), but not in fibroblasts (**I**). Scale bars: 50 μ m (**A–F**); 20 μ m (**G–J**).

month-old AROM+/6J males (Figure 2, H and J). Scant Pcna-stained cells could be found only in WT detrusor, not in fibroblasts (Figure 2, G and I). Morphometric analysis with ImageJ software revealed that ~20% of cells

Figure 3. Expression of selected estrogen-related genes in the bladder of 10-month-old wild-type (WT) and transgenic AROM+/6J males at mRNA (A–D, F–H) and protein (A–G) levels. The **upper blots** show RT-PCR mRNA data for aromatase (*Cyp19a1*) (A), *Esr1* (alias *ERα*) (B), *Pten* (C), *Pcna* (D), *Fsp1* (F), *Col3a1* (G), *Esr2* (alias *Erβ*), *Serpinb1* (alias *Hsp47*), and *Gpr30* (H), with *L19* as internal control. Data shown are for three WT and transgenic mice, using 1 μg of RNA extract per lane, and are representative data from one of three similar experiments. The **center graphs** (A–G) show densitometric quantification of the specific mRNA amplicons corrected for intensity of the *L19* amplicon. Each bar represents the mean ± SEM of three independent experiments in triplicates. A.D.U., arbitrary densitometric units. **P* < 0.05 (10-month-old AROM+/6J vs age-matched WT male bladder). The **lower blots** (A–D, F, and G) show protein bands by Western blot analysis, with molecular weight indicated (kDa). In E, the **upper blots** display bands for phosphorylated AKT (p-AKT). Protein extracts (30 μg per lane) from WT and AROM+/6J male mouse bladders (*n* = 3 for each) were analyzed and each experiment was repeated three times. Representative data from one of three similar experiments are shown. β-actin was applied as internal control, and expression levels were normalized to β-actin protein. **P* < 0.05 (WT vs AROM+/6J).

stained positive for Pcn α /total cells in AROM+6J male bladders, compared with ~2% staining positive for Pcn α cells in the WT control ($n = 6$ /group). The proportion of Pcn α -positive cells out of total cells in detrusor was 0.268 ± 0.009 in AROM+6J, compared with 0.019 ± 0.003 in WT ($P < 0.05$).

Differential Gene Expression in AROM+6J Male Bladder and Molecular Mechanisms of BOO

We first confirmed *Cyp19a1* expression in the urogenital tract, in total RNA isolated from testis, bladder, and urethra. As in a previous study,²⁹ Tg *Cyp19a1* mRNA was detected in the bladder (Figure 3A), testis, and prostate of AROM+6J males. To determine the local hormonal factors thought to be involved in the bladder phenotype of the Tg males, we checked the expression of nuclear estrogen receptors α and β in the bladder. At both the mRNA and the protein level, ER α was markedly up-regulated in the Tg male bladder at 4 and 10 months (Figure 3B). No significant differences in ER β levels in the bladders were found between the AROM+6J and WT controls, or between 4-month-old and 10-month-old AROM +/- 6J male bladders (Figure 3H) (see also Supplementary Figure S2 at <http://ajp.amjpathol.org>). Pcn α mRNA and protein were significantly up-regulated in both 4-month-old and 10-month-old AROM+6J male bladders (Figure 3C).

We also measured expression in the bladder of the G protein-coupled receptor 30 gene (*Gpr30*), which has been shown to be a relevant candidate to mediate nongenomic estrogen actions.³⁶ No significant differences in *Gpr30* mRNA expression levels were found between the AROM+6J and WT controls (see Supplementary Figure S2 at <http://ajp.amjpathol.org>). Next, we checked whether *Pten* expression was altered, because this gene is important in estrogen-dependent cell survival and apoptosis^{16,17} and is frequently mutated in estrogen-dependent diseases.^{16,18} A significant down-regulation or total loss of *Pten* expression was found in both the 4-month-old and the 10-month-old BOO AROM+6J male bladders (Figure 3D). Because PI3K/AKT is the direct downstream target for PTEN in cytoplasm, the next step was to investigate whether the PI3K/AKT signaling pathway (which is suppressed by PTEN through estrogen-induced action) was altered. As expected, Western blotting showed that p-AKT protein expression was significantly up-regulated in both 4-month-old and 10-month-old Tg male bladders (Figure 3E), but no significant changes were observed in total Akt between the groups (Figure 3E).

Because activated fibroblasts are the key mediators of fibrosis, we investigated the induction of fibroblasts during the development of fibrosis in the detrusor, by analyzing fibroblast-specific markers HSP47 (encoded by the *Serpinh1* gene) and FSP1 (encoded by the *S100a4* gene) in the bladder. FSP1 was significantly up-regulated in the detrusor (Figure 3F), but no differ-

Figure 4. Immunohistochemical analyses for p-AKT (A and B) and FSP1 (C and D) in 10-month-old AROM+/6J (B and D) and age-matched WT (A and C) male bladder. **A:** Only a few single weak p-AKT-positive stained cells were detected in WT age-matched control littermate bladder urothelia. **B:** Abundant p-AKT protein (arrows) nuclear staining expression in the hyperproliferated urothelial cells of 10-month-old AROM+/6J male bladder could be found, but not in spindle-shaped fibroblast cells. **C:** No positive stained cells could be detected in WT age-matched control littermates. **D:** FSP1 protein (arrows) nuclear staining was expressed only in the proliferative spindle-shaped fibroblast cells which penetrated to the smooth muscle layers of the bladder in 10-month-old AROM+/6J males. Scale bars = 20 μ m.

ences were found in HSP47 between Tg and WT tissues (Figure 3H).

Type I and III collagens are most abundant in the bladder wall. Because these proteins are the major tension-bearing structural elements in the urinary bladder, a proper type I/type III ratio is critical for normal function of the bladder. Type III collagen was significantly up-regulated in the Tg bladder samples (Figure 3G).

Immunohistochemical staining revealed abundant p-AKT protein in the hyperproliferated urothelial cells but not in the spindle-shaped fibroblast cells of 10-month-old Tg male bladder (Figure 4B), which supports the findings on p-AKT by Western blotting (Figure 3G). Only scattered p-AKT-positive cells were detected in age-matched WT samples (Figure 4A). Immunohistochemistry for FSP1 was consistent with the Western blotting analysis (Figure 3E), further confirming the location of spindle-shaped FSP1-positive cells only in the Tg detrusor (Figure 4D).

Mechanisms of Estrogen Action in Vitro in the Bladder

We evaluated in primary culture the expression of a subset of genes with possible relevance to the transcriptional events in response to local estrogen action in the bladder. The experiments were performed 2 days after plating of the 10-month-old AROM+/6J bladder cells. We have previously tested different doses of E₂ (1 nmol/L, 10 nmol/L, 100 nmol/L), and the 10 nmol/L dose was chosen for further experiments, based on earlier findings²⁶ and also because it was

Figure 5. Expression of estrogen-related genes at mRNA (A–D) and protein (A–F) levels in cultured 10-month-old AROM+/6J male bladder with different treatments: C, vehicle; ICI, estrogen receptor antagonist; PPT, ERα agonist; DPN, ERβ agonist. **A–D:** The upper blots show representative RT-PCR mRNA data for *Esr1* (alias ERα) (A), *Esr2* (alias ERβ) (B), *Pcnα* (C), and *Pten* (D), with L19 as internal control. The remaining blots (lower for A–D; upper for E and F) show protein bands by Western blot analysis, with molecular weight indicated (kDa). The graphs show the densitometric quantification. A.D.U., arbitrary densitometric units. Other details are as for Figure 3. **P* < 0.05, for PPT vs C, E₂ vs C, ICI vs C (ERα and Pcnα in A and C); for DPN vs C (ERβ in B); for PPT vs C, E₂ vs C (Pten and Col3a1 in D and F); and for E₂ vs C (p-AKT in E).

most effective in up-regulating Cyr61 expression (see Supplementary Figure S3 at <http://ajp.amjpathol.org>). Treatment of the cultures with E₂ and the ERα agonist PPT (10 nmol/L) for 24 hours resulted in up-regulation of ERα, whereas the ERβ agonist DPN (10 nmol/L) had no effect (Figure 5A). ERβ was up-regulated only by the latter compound, and not by any other treatments (Figure 5B). ERα and ERβ expressions were significantly down-regulated by the combined ERα and ERβ inhibitor ICI 182,780 (100 nmol/L) (Figure 5, A and B). The addition of ICI blocked the up-regulation of ERα expression induced by E₂ and PPT (*P* < 0.05, E₂ + ICI vs E₂ or PPT) (Figure 5A), but had no effect on ERβ expression (Figure 5B). In response to the stimulations, the *Pcnα* gene showed identical expression pattern with ERα (Figure 5C), which may implicate *Pcnα* as the direct gene target for estrogen receptors (more precisely, ERα). Moreover, *Pten* expression was significantly down-regulated with E₂ and/or the ERα agonist PPT (Figure 5D). When stimulated for a short period (30 minutes), E₂ had no effect, suggesting that the rapid nongenomic regulatory pathway for AKT phosphorylation was not activated. p-AKT protein expression was up-regulated significantly in the bladder cells after 24 hours of culture in the presence of E₂ and/or the ERα agonist PPT (Figure 5E), but no significant differences

were observed in total AKT between the groups (Figure 5E). Moreover, E₂ significantly up-regulated *Col3a1* expression in the cultures, and this effect was suppressed by cotreatment with ICI (Figure 5F).

Estrogen Induces Fibroblast Activation in Vitro

We isolated embryonic fibroblasts from WT C57Bl/6J fetuses as described previously³² and cultured them *in vitro*. p-AKT protein expression was not found in either AROM+/6J or WT C57Bl/6J embryonic fibroblast. Both E₂ and the ERα agonist PPT significantly up-regulated ERα expression, but this effect was lost in the presence of ICI or ICI + PI3KI (Ly294002 or PI3KI; PI3K inhibitor) (Figure 6A). PPT also significantly up-regulated ERα mRNA expression, and addition of ICI + PI3KI to E₂ blocked this effect (Figure 6A). Consistent with the data on primary bladder cultures, E₂ and PPT, but not DPN, significantly induced cell proliferation (up-regulation of Pcnα) (Figure 6B). This E₂ effect was profoundly suppressed by ICI or ICI + PI3KI (Figure 6B).

To verify whether the fibroblast proliferation correlated with E₂ stimulated accumulation of collagen, we examined *Col1* and *Col3a1* expression. E₂ (as well as PPT, but not DPN) significantly up-regulated *Col3a1* mRNA and protein expression, and this E₂ effect was significantly

Figure 6. Expression of estrogen-related genes at mRNA (A–C) and protein (A–D) levels in cultured embryonic fibroblasts from C57Bl mouse fetuses with different treatments. **A–C:** The upper blots show representative RT-PCR mRNA data for ERα (A), PcnA (B), and Col3a1 (C), with L19 as internal control, from three individual fetal fibroblast cultures treated with different compounds: C, vehicle; ICI, estrogen receptor antagonist; PPT, ERα agonist; DPN, ERβ agonist; P13KI (Ly294002), PI3K antagonist. The remaining blots (lower for A–C; upper for D) show protein bands by Western blot analysis, with molecular weight indicated (kDa). The graphs show the densitometric quantification. A.D.U., arbitrary densitometric units. **P* < 0.05, for PPT vs C (ERα and PcnA in A and B); for PPT vs C, E₂ vs C and E₂ + P13KI vs C (Col3a1 in C); and for AROM+/6J bladder vs C (p-AKT in D).

suppressed by ICI. E₂-mediated and/or PPT-mediated fibroblast proliferation and fibrogenesis did not have any effect on the p-AKT and/or total AKT protein levels in the embryonic fibroblast cultures (Figure 6D). Consistent with the lack of p-AKT protein expression in the spindle-shaped fibroblast cells of 10-month-old Tg male bladder (Figure 6A), the gene expression changes measured in primary fibroblast cell culture exposed to E₂ stimulation *in vitro* were mediated mainly by estrogen receptors, and most predominantly via ERα, whereas no further activation of p-AKT took place.

Similarities in Gene Expression Changes between Murine AROM+/6J and Human Male BOO Bladders

To demonstrate the relevance of the AROM+/6J murine model for human BOO disease, we analyzed the same molecular changes observed in the AROM+/6J in human BOO samples. We analyzed the protein expression of ERα, PTEN, PCNA, and p-AKT immunohistochemically in severe (*n* = 20) and subnormal/mild (*n* = 6) human BOO bladder paraffin-block samples. The ERα-positive urothelial cells were markedly increased in severe BOO male bladders (Figure 7B), compared with subnormal/mild bladder samples (Figure 7A). PCNA-positive cells were markedly increased in the severe BOO male bladder samples (Figure 7D), compared with the subnormal ones

(Figure 7C). We were not able to detect any staining for PTEN-positive cells in either subnormal/mild or severe BOO male bladders (data not shown). Because PI3K/AKT is the direct downstream target for PTEN in cytoplasm, the next step was to investigate whether the PI3K/AKT signaling pathway was altered in human BOO patients. As expected, immunohistochemical staining showed abundant p-AKT expression in the hyperproliferated urothelial cells, but not in the spindle-shaped fibroblast cells in severe human male BOO (Figure 7F), and only a few single cells staining positive were found in the subnormal/mild group (Figure 7E).

Discussion

The present study showed that AROM+/6J males presented with severe inguinal herniae at 2 months of age, with urine-filled dilated bladder and hydronephrosis, reduced testis size, and finally with complete BOO at 10 months of age. The prostates of these mice were significantly smaller, so BOO could not be due to any mechanical obstruction of urethra due to benign prostatic hyperplasia and consequent dilatation of bladder. In 10-month-old AROM+/6J males, histopathological analyses revealed hypertrophic bladder with thicker lamina propria and detrusor atrophy with disorganized muscle layers. Moreover, the fibrosis penetrated throughout detrusor of the bladder. These results are in

Figure 7. Immunohistochemical analyses for ER α , PCNA, and p-AKT in severe and subnormal/mild human male BOO bladder paraffin-block samples. Representative data from a total of 6 subnormal/mild and 20 severe BOO samples are presented. The ER α -positive urothelial cells were markedly increased in number in all severe BOO male bladders (B), compared with subnormal/mild bladder samples (A). PCNA-positive nuclear stained cells were markedly increased in the severe BOO male bladder (D), compared with subnormal ones (C). Abundant p-AKT expression in the hyperproliferated urothelial cells, but not in the spindle-shaped fibroblast cells in severe human male BOO (F), whereas only a few single cells stained positive in the subnormal/mild group (E). Arrows indicate positively stained cells (in severe BOO samples, only a representative selection of the abundant positively stained cells). Scale bar = 20 μ m.

agreement with prior studies and indicate disorganized detrusor structures in BOO.^{36,37} Abundant expression of the cell proliferation marker PcnA supported the histopathological signs of hyperplasia of the bladder urothelium and fibroblasts, responsible for the increased bladder weight. This confirmed findings from earlier studies, in which hyperplasia of the epithelium and fibroblasts were observed in animals with BOO.^{14,37}

Under normal conditions, urothelium belongs to the slowest growing epithelial cell types in the renal pelvis, urethra, and bladder (despite reports of estrogen-induced proliferation^{38,39}). Our data showed estrogen-induced urothelial cell proliferation both *in vivo* and *in vitro*. In contrast to the muscle cell hyperplasia frequently observed in BOO,^{40,41} the detrusor in AROM+/6J mice undergoes atrophy at 10 months of age. Similarly to AROM+/N males,^{29,35} the AROM+/6J males showed high serum E₂ and low testosterone levels, decreased androgen/estrogen ratio, and disrupted reproductive development and spermatogenesis, putatively due to estrogen susceptibility of the C57Bl/6J genetic background.²⁸ Androgen receptor has been detected in bladder detru-

sor,⁴² and muscle cells showed androgen response. Thus, the detrusor muscular atrophy in the AROM+/6J mice could have also resulted from androgen deficiency and decreased androgen/estrogen ratio.

Expression of ER β is predominantly distributed in the male urinary tract, such as the bladder, urethra, and prostate, whereas ER α expression is scarcely detectable in the rodent male bladder.^{23,42,43} No abnormalities in voiding function could be found in ER $\alpha^{-/-}$, ER $\beta^{-/-}$, or in estrogen receptor double-knockout (ie, both ER α and ER β) male mice.⁴⁴ In the present study, we found that ER α was up-regulated in 10-month-old AROM+/6J male obstructed bladder, suggesting a possible local estrogen action resulting in bladder malfunction via ER α counteracting androgen effects in the bladder locally. In turn, this possibly caused chronic irritation and consequent development of infravesical obstruction in the lower urinary tract of the AROM+/6J males at 10 months of age.

In addition to the well-characterized classical nuclear receptors, a growing body of evidence suggests that rapid estrogen actions are mediated by estrogen-binding proteins on the plasma membrane.³⁶ An orphan G protein-coupled receptor, GPR30, has been identified as a relevant candidate for the estrogen membrane receptor.³⁶ Estrogens could activate the cytosolic PI3K/AKT signaling pathway, leading to increased level of p-AKT. We were unable to detect differential expression of Gpr30 in AROM+/6J bladder, both *in vivo* and *in vitro*. Moreover, in both WT and AROM+/6J bladder the known Gpr30 agonist ICI was also unable to induce Gpr30 expression *in vitro*.

Loss of PTEN expression or PTEN mutations have been shown in estrogen related cancers.^{45–47} The prevalent paradigm is that AKT phosphorylation is negatively regulated by PTEN. E₂-induced reduction of PTEN expression has been reported in hepatocytes by Marino et al.⁴⁸ Their studies showed that ER-dependent AKT phosphorylation is paralleled by a decrease of PTEN level. In the present study, we showed clear down-regulation of Pten expression both in AROM+/6J bladder tissue and in estrogen-treated bladder cultures *in vitro*, confirming the role of PTEN in mediating estrogen action. Blockage of the ER α pathway inactivated the PTEN/p-AKT pathway in the BOO bladders. In bladder culture, we demonstrated that E₂ action in the intradetrusor fibroblast proliferation was mediated through ER α only. Our findings are in agreement with a previous study in ER $\alpha^{-/-}$ mice, in which estrogen/ER α did activate the phosphorylation of AKT and lack of ER α did not produce phosphorylation of AKT in endometrial cells.⁴⁹

We also demonstrated that estrogen remodels the bladder detrusor through fibroblast activation, as characterized by increased fibroblast cell proliferation and altered collagen III synthesis. E₂ could modulate fibroblast activation via estrogen receptor, which was supported by the result that ICI could prevent the E₂-induced activation in fibroblast culture. However, the mechanism how E₂ could activate fibroblasts remains unknown and calls for studies with other coregulators involved in E₂ and estrogen receptor activity. The imbalance of collagen III/I synthesis may play a major

role in bladder smooth muscle remodeling. Decreases in type III/type I ratio are reported to alter the fiber size in detrusor, resulting in a more compliant bladder in collagen-deficient mice.⁵⁰ Some clinical studies have reported type III collagen up-regulation and an increase in type III/I ratio in noncompliant fibrotic human bladders.^{51,52} Nonetheless, how type III collagen up-regulation could lead to bladder dysfunction remains controversial. In the present study, we demonstrated that estrogen activated intradetrusor fibroblasts, which in turn, increased the proliferation of fibroblasts and up-regulated collagen III expression and may have caused bladder deformation and dysfunction in BOO of the AROM+/6J males.

In accord with a recent study on sex steroid receptors in male human bladder by Chavalmane et al,⁵³ we also detected ER α expression in human bladder. The number of ER α -positive urothelial cells was markedly increased in severe BOO human male bladders, compared with subnormal/mild human BOO bladders. Also, the number of PCNA-positive cells was markedly increased in severe BOO male bladders. We could not, however, detect any PTEN-positive cells in either subnormal/mild or severe human BOO male bladders. A plausible explanation for the lack of PTEN expression could be that all patients whose samples were studied had bladder cancer. The molecular pathogenesis of the hyperproliferation of urothelial cells in bladder cancer, although showing signs of increased estrogen action, may not be identical with that observed in our mouse model. As expected, abundant p-AKT-positive cells were found in the hyperproliferated urothelium in severe human BOO, but not among the spindle-shaped fibroblast cells. These findings are in agreement with observations in our AROM+/6J murine model, supporting the human relevance of the model. Although it might be too early to draw definitive conclusions about the etiological role of estrogens in human BOO, larger-scale clinical investigations on the relationship would be appropriate, on the basis of the present findings.

Fibroblast proliferation was specifically triggered by E₂ causing accumulation of collagen in primary fibroblast culture. E₂ (through ER α) significantly up-regulated *Col3a1*, and this E₂-mediated effect was suppressed by ICI in the embryonic fibroblasts *in vitro*. E₂ and/or ER α agonist PPT did not have any effect on the p-AKT protein levels in the embryonic fibroblast cultures, whereas E₂ and PPT significantly up-regulated the phosphorylation of AKT in whole bladder culture. These data suggest that the gene expression changes observed in primary fibroblast culture exposed to E₂ stimulation *in vitro* are mediated mainly by estrogen receptors, and most predominantly via ER α , without the activation of p-AKT. This was further confirmed by immunohistological finding of p-AKT-positive cells detected only in the urothelia of AROM+/6J, but not in the spindle-like fibroblasts.

Taken together, our data showed that increased local estrogen action through *Cyp19a1* overexpression, promoted murine urothelial cell proliferation *in vivo* and *in vitro* (see Supplementary Figure S4 at <http://ajp.amjpathol.org>). This proliferative effect was elicited via ER α -mediated sig-

naling. The activated estrogen/ER α pathway inhibited *PTEN* expression, which in turn activated the PI3K/AKT pathway, finally resulting in urothelial cell proliferation in BOO. Local E₂/ER α promoted intradetrusor fibroblast activation without the activation of p-AKT and further enhanced fibroblast hyperproliferation and impaired the accumulation of type III collagen within the detrusor. These findings suggest that excess estrogen action on the bladder may function as the etiological signal leading to BOO through modification of the smooth muscle layer and urothelium. AROM+/6J mice may serve as an ideal experimental model to study the molecular mechanisms involved in the development of non-neurogenic BOO, and drugs such as CYP19A1 inhibitors, selective estrogen and androgen receptor modulators, or PI3K inhibitors for the treatment of BOO.

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