

Proteomic analysis of urethral protein expression in an estrogen receptor α -deficient murine model of stress urinary incontinence

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Abstract

Purpose The roles of estrogen receptor α (ER α) in stress urinary incontinence (SUI) remain elusive. This study was conducted to understand the molecular mechanism of ER α against SUI.

Methods Wild-type (ER $\alpha^{+/+}$) and ACTB-cre ER α knockout (ER $\alpha^{-/-}$) female mice were generated. Urethral function and protein expression were measured. Leak point pressures (LPP) and maximum urethral closure pressure (MUCP) were assessed in mice under urethane anesthesia. After the measurements, the urethras were removed for proteomic analysis using the two-dimensional differential gel electrophoresis and liquid chromatography–mass spectrometry technology. Interaction between these ER α pathway proteins was further analyzed by using MetaCore. Lastly, Western blot and immunohistochemistry (IHC) were

used to confirm the candidate protein expression levels and locations, respectively.

Results Compared with the ER $\alpha^{+/+}$ group, the LPP and MUCP values of the ER $\alpha^{-/-}$ group were significantly decreased. Additionally, we identified 11 differentially expressed proteins in the urethra of ER $\alpha^{-/-}$ female mice; five proteins were down-regulated and six were up-regulated. The majority of the ER α knockout-modified proteins were involved in muscle development, contraction, and regulation, as well as immune response (amphotericin signaling and phagocytosis), proteolysis, and cell adhesion (platelet aggregation and integrin-mediated cell–matrix adhesion). IHC and Western blot confirmed the down-regulation of tropomyosin and up-regulation of myosin in urethra.

Conclusions This is the first study to estimate protein expression changes in urethras from ER $\alpha^{-/-}$ female mice. These changes could be related to the molecular mechanism of ER α in SUI.

Y.-H. Chen and C.-J. Chen contributed equally to this study.

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Keywords Estrogen receptor α · Leak point pressures · Maximum urethral closure pressure · Proteomics · Stress urinary incontinence

Introduction

Stress urinary incontinence (SUI) is defined as the involuntary leakage of urine under stress conditions such as coughing and sneezing [1, 2]. The effects of birth trauma, menopause, and aging may contribute to the development of SUI [3]. Estrogen exerts a variety of regulatory functions in the female urogenital system. Estrogen actions are mediated by estrogen receptors (ERs), encoded by two distinct genes, ER α and ER β [4]. Estrogen has many roles in the reproduction and urinary systems, and the generation of the ER α and ER β knockout mice has further illustrated its roles. It has been shown that ER α is more critical than ER β in mediation of the estrogen actions necessary for maintenance of healthy somatic cell function [5]. In the female rodent, the infertility of the ER α knockout mouse is due in part to the insensitivity of the uterus to the mitogenic and different actions of estrogen [6]. In urogynecology, the efficacy of estrogen for SUI in postmenopausal women is still controversial [7]. Therefore, the specific roles of estrogen and ER α in SUI remained elusive.

Because of the limited availability of human tissue for study, animal models are an important adjunct in improving our understanding of SUI [8]. Vaginal distension and pudendal nerve transaction have been used for creation of SUI in rats, as evidenced by lowered leak point pressures (LPP) on urodynamic testing. The use of mice in various lines of translational research has made available transgenic and knockout technologies for conducting mechanistic studies of various target diseases [9]. The C57BL/6 mouse, for example, has been widely used for genetic manipulation in previous studies concerning urinary and pelvic disorders [10]. Interestingly, the decrease of ER in the pelvic floor tissues in pelvic organ prolapse (POP) patients may be closely related to the occurrence of SUI [3].

Proteomics approaches to identify and quantify the entire protein content of a tissue at a given time may provide insights into the mechanisms of diseases [11]. Our aim was to understand the molecular mechanism of ER α in SUI, and in this study using two-dimensional differential gel electrophoresis (2D DIGE) and liquid chromatography–mass spectrometry (LC–MS/MS) technology, we identified ER α pathway candidate proteins in urethra by comparing urethra tissues collected from ER α deficient and age-matched wild-type female mice.

Materials and methods

ER α knockout mice and genotyping

The ACTB-Cre/ER α knockout (ER $\alpha^{-/-}$) mice were generated via the Cre-loxP system by mating the floxed ER α homozygous female mice with ACTB-Cre transgenic male mice [12, 13]. After two generations of mating, wild-type (ER $\alpha^{+/+}$) heterozygote (ER $\alpha^{+/-}$) and knockout (ER $\alpha^{-/-}$) mice were developed. Genomic DNA was isolated from tail biopsies and used as a template for PCR genotyping. The primers used for ACTB-ER α mutant mice were as follows: P1, 5'-AGG CTT TGT CTC GCT TTC C-3', P2, 5'-GAT CAT TCA GAG AGA CAA GAG GAA CC-3'; and Cre, sense, 5'-AGG TGT AGA GAA GGC ACT TAG C-3'; antisense, 5'-CTA ATC GCC ATC TTC CAG G-3'. The genotyping of ACTB-ER α mutant mice was determined by PCR using the P1, P2, and Cre primer mixture. The size of P1-P2 fragments for wild-type and knockout allele was 741 and 223 bp, respectively. The size of Cre fragments was 411 bp (Fig. 1) [12, 13].

The mice underwent suprapubic bladder tubing (SPT) placement [12, 13], and LPP and maximum urethral closure

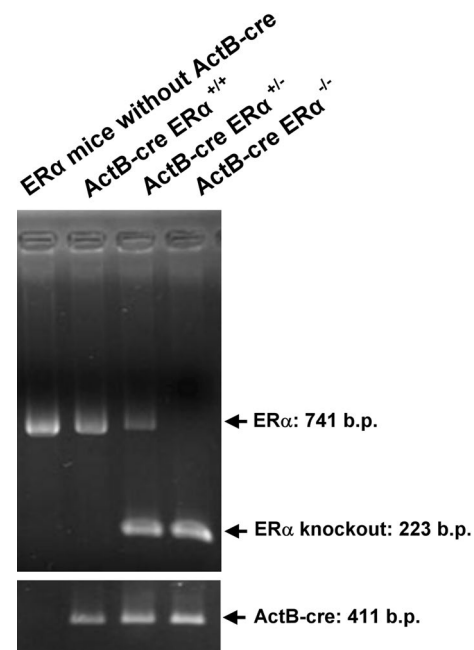


Fig. 1 Genotyping of different ER α mutant mice. Lane 1 ER α mice without ACTB-cre, Lane 2 ActB-cre transgenic mice (ActB-cre ER $\alpha^{+/+}$), the size of cre fragment was 411 bp, Lane 3 heterozygote ER α knockout mice (ActB-cre ER $\alpha^{+/-}$), in which the size of floxed ER α allele was reduced to 223 bp after the deletion of intervening DNA via ActB-cre recombinase, Lane 4 homozygote ER α knockout mice (ActB-cre ER $\alpha^{-/-}$), in which the size of both floxed ER α alleles was reduced to 223 bp by ActB-cre recombinase and ER α band was absent

pressure (MUCP) were assessed in these mice under urethane (1 g/kg, i.p.) anesthesia ($n = 6$ for each group). After measurements, the animals were killed, and the urethras were removed for further analyses. Since the external urethral sphincter is mostly responsible to maintain the continence mechanism lies in the mid-urethra, the middle-one-third of urethras harvested from six rats for each group were used for proteomic analysis, immunohistochemistry (IHC), and Western blot. The tissue samples were divided into two parts, one part ($n = 3$) was frozen and homogenized for proteomic and Western blot analyses, and the other part ($n = 3$) was fixed with 4 % paraformaldehyde for further IHC analyses. All experimental protocols were approved by the Institutional Animal Care and Use Committee of China Medical University (Reference number: 101-201-N).

Suprapubic tube implantation

The surgical procedure was carried out under 1.5 % isoflurane anesthesia [12, 13]. An SPT (PE-10 tubing, Clay Adams, Parsippany, NJ, USA) was implanted in the bladder.

LPP measurement

Two days after implanting the bladder catheter, the LPP was assessed. The bladder catheter was connected to both a syringe pump and a pressure transducer. Pressure and force transducer signals were amplified and digitized for computer data collection at 10 samples per second (PowerLabs, AD Instruments, Bella Vista, Australia). The average bladder capacity of each mouse was determined after 3–5 voiding cycles. Subsequently, the LPP was measured [12, 13].

Urethral pressure profile (UPP)

UPP was assessed in these mice under urethane anesthesia. The bladder catheter was connected to a syringe pump with room temperature saline at 1 ml/h. The urethral catheter was connected to a pressure transducer. A withdrawal speed of 10 μm per minute was used. Pressure and force transducer signals were amplified and digitized for computer data collection at 10 samples per second. Three successive profiles were obtained in the supine position. The urethral closure pressure (P_{close}) is the difference between the urethral pressure (P_{ure}) and the bladder pressure (P_{ves}): $P_{\text{close}} = P_{\text{ure}} - P_{\text{ves}}$. Maximum urethral pressure and MUCP were determined from the UPP measurements taken [12, 13]. LPP and MUCP were performed by two independent examiners to avoid the possible operator-dependent bias.

Two-dimensional differential gel electrophoresis

Frozen pieces of urethra were homogenized. Protein was extracted, and the concentrations were determined [14].

Fifty micrograms of protein samples from vaginal tissue of ER $\alpha^{-/-}$ mice was pooled and labeled with CyDye 3 (Cy 3) (400 pmol/ μl) (GE Healthcare, Waukesha, WI, USA), those from wild-type mice were pooled and labeled with Cy 5, and internal standards were labeled by mixing of Cy 2 with 50 μg of protein samples (25 μg from treated mice plus 25 μg from control mice). The three labeled protein samples were mixed and denatured in 450 μl rehydration buffer, and the mixed sample was applied to a 3–10 24 cm IPG strip (GE Healthcare), followed with IEF as rehydration for 12 h, 500 V for 0.5 h, 1,000 V for 1 h, 8,000 V for 3 h, and 8,000 V for 24,000 Vhr. The two-dimensional electrophoresis was performed at 17 W until the bromophenol blue dye reached the bottom of the gel [14].

Protein spot analysis

The labeled gels were scanned using a Typhoon 9410 imager (GE Healthcare) with specific excitation and emission wavelengths. Difference in-gel analysis (DeCyder, v6.5, GE Healthcare) was used for intra-gel analysis. Biological variance analysis was used for inter-gel matching and statistical analysis [14].

In-gel digestion

The labeled gels were stained using silver staining for gel spots excision. The gel pieces were dehydrated with 100 % acetonitrile and rehydrated with 25 mM ammonium bicarbonate (pH 8.2) solution. After drying in a centrifugal concentrator, the gel pieces were rehydrated in 25 mM ammonium bicarbonate containing 1 ng/ μl sequencing grade modified trypsin (Promega) for 1 h at 4 °C and then incubated at 37 °C overnight. After digestion, the tryptic peptides were extracted from the gel using 1 % FA in 50 % ACN [14].

Protein identification by LC–MS/MS

LC–MS/MS was performed using an ion trap mass spectrometer (HCTultra PTM Discovery, Bruker, Germany) coupled online with an Ultimate 3000 nanoLC system (Dionex). Peptide fragment mass spectra were acquired in data-dependent Auto-MS/MS mode with a scan range of 100–2,500 m/z , two averages, and five precursor ions selected from the MS scan range of 400–1,600 m/z . Peptide peaks were detected and deconvoluted automatically using Data Analysis 4.0 software (Bruker). Mass lists in the form of MASCOT Generic Files were created automatically and used as the input for MASCOT MS/MS ions searches of the NCBI database (release 20101220) using an in-house MASCOT 2.2.04 server. Search parameters were selected as taxonomy–mouse; enzyme–trypsin; fixed modifications–carbamidomethyl (C); variable modifications–oxidation

(M); precursor peptide tolerance ± 0.5 Da; MS/MS tolerance ± 0.5 Da; significant threshold $P < 0.05$ [14].

Networks analysis using MetaCore

MetaCore (GeneGo, St. Joseph, MI, USA) was used to map the differentially expressed proteins into biological networks. Differentially expressed proteins were converted into gene symbols and uploaded into MetaCore for analysis. The biological process enrichment was analyzed based on Gene Ontology processes [14].

Immunohistochemistry (IHC) staining

The urethras were histologically examined with IHC staining for indicating the protein expression regions of the urethra. The sections were then incubated with the primary antibody derived from rabbit TPM3 and anti-myosin heavy chain antibodies (clone A4.1025, myosin, heavy polypeptide 1), 1:200 dilution, Millipore, MA, USA], followed by incubation with avidin–biotin–horseradish peroxidase complex, with 0.5 mg/ml of 3,3'-diaminobenzidine as chromogen (Vector Lab, USA).

Western blot analysis

Detection of proteins was performed with the use of the antibody derived from rabbit (anti-TPM3 and anti-myosin antibodies, Millipore, MA, USA). The intensity of each band was quantified using a densitometer (Molecular Dynamics, Sunnyvale, CA, USA) [15, 16].

Statistical analysis

Data are expressed as mean \pm standard deviation (SD) of six individual mice. Student's *t* test was used to compare two different groups of sample, and one-way analysis of variance (ANOVA) was used to compare data from more than two groups. *P* value < 0.05 was considered statistically significant. All calculations were performed using the Statistical Package for Social Sciences (SPSS for Windows, SPSS Inc, Chicago, IL, USA).

Results

Decreased LPP and MUCP in $ER\alpha^{-/-}$ mice

The ACTB-Cre/ $ER\alpha$ knockout ($ER\alpha^{-/-}$) mice were generated via the Cre-loxP system by mating the floxed $ER\alpha$ homozygous female mice with ACTB-Cre transgenic male mice [12]. When female mice reach 8 weeks old, leak point pressures (LPP) and maximum urethral closure pressure

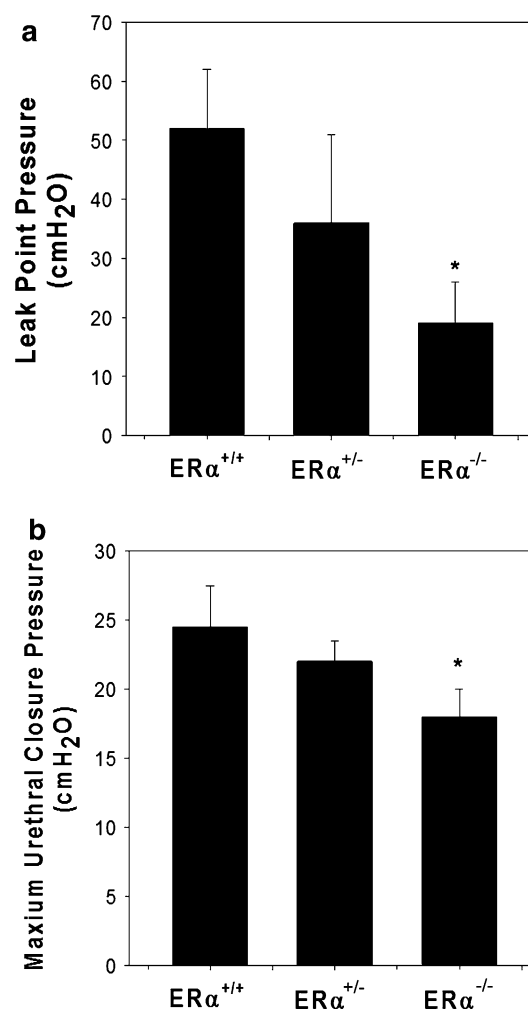


Fig. 2 LPP (a) and MUCP (b) value in the different groups. After SPT placement, the LPP and MUCP were assessed in mice under urethane anesthesia. LPP and MUCP were performed by two independent examiners to avoid the possible operator-dependent bias. Each bar represents the mean \pm standard deviation of six individual mice. * $P < 0.05$ different from the value in the $ER\alpha^{+/+}$ group with one-way ANOVA

(MUCP) were assessed in wild-type ($ER\alpha^{+/+}$), heterozygote ($ER\alpha^{+/-}$) and knockout ($ER\alpha^{-/-}$) female mice under urethane anesthesia.

Compared to $ER\alpha^{+/+}$ group, our data showed that LPP and MUCP values were slightly decreased in the $ER\alpha^{+/-}$ heterozygote female group without statistical significance. By contrast, LPP and MUCP values were significantly decreased in the $ER\alpha^{-/-}$ group compared with the $ER\alpha^{+/+}$ group (Fig. 2a, b).

Protein expression profile from proteomic analysis

After the LPP and MUCP measurements, the urethras were removed for proteomic analysis using the 2D DIGE

Table 1 Differentially expressed proteins in the urethra of ER $\alpha^{-/-}$ mice

Entry name	Protein name	Ratio	P value
<i>Down-regulation</i>			
TPM3_MOUSE	Tropomyosin α -3 chain	-1.44	0.022
DDAH2_MOUSE	N(G),N(G)-dimethylarginine dimethylaminohydrolase 2	-1.58	0.027
DESM_MOUSE	Desmin	-1.76	0.0048
TCTP_MOUSE	Translationally controlled tumor protein	-2.59	0.018
CAPR2_MOUSE	Caprin-2	-5.62	0.03
<i>Up-regulation</i>			
MYL1_MOUSE	Myosin light chain 1/3	13.94	0.043
MLRS_MOUSE	Myosin regulatory light chain 2	8.82	0.0055
MYL3_MOUSE	Myosin light chain 3	7.19	0.013
NDUS8_MOUSE	NADH dehydrogenase iron-sulfur protein 8	6.09	0.0054
MYL1_MOUSE	Myosin light chain 1/3	4.55	0.016
UCHL1_MOUSE	Ubiquitin carboxyl-terminal hydrolase isozyme L1	2.25	0.031

and LC–MS/MS technology. As there was no significant difference of LPP and MUCP measurements between ER $\alpha^{+/+}$ and ER $\alpha^{-/-}$ group, we only compared the protein expression profile between ER $\alpha^{-/-}$ and ER $\alpha^{+/+}$ group. We identified 11 urethra proteins differentially expressed with statistical significance in ER $\alpha^{+/+}$ and ER $\alpha^{-/-}$ female mice. Additionally, five proteins were down-regulated and six were up-regulated (Table 1; Fig. 3). The majority of the ER α knockout-modified proteins were involved in muscle development, contraction, and regulation, as well as immune response (amphoterin signaling and phagocytosis), proteolysis, and cell adhesion (platelet aggregation and integrin-mediated cell–matrix adhesion) (Fig. 4a, b).

Tropomyosin and myosin and expressions in the urethra of ER $\alpha^{-/-}$ mice

We further focused on urethral contraction-related proteins including tropomyosin and myosin [17]. Since there are many different types (including various heavy and light chains) of tropomyosin and myosin, it is difficult to analyze all the protein subtypes, therefore, the most common commercially available antibodies, including anti-tropomyosin α -3 chain and anti-myosin heavy chain were chosen for the subsequent analyses. The urethras were histologically examined with IHC staining for indicating the protein expression regions of the urethra (Figs. 6a, 7a). Among these differentially expressed proteins of urethra, tropomyosin and myosin were chosen for further confirmation. From 2D DIGE (Fig. 5), and Western blot analyses, tropomyosin (Fig. 6b) in the urethra was significantly decreased in the ER $\alpha^{-/-}$ group as compared with that in the ER $\alpha^{+/+}$ group. By contrast, myosin (Fig. 7b) expression in the urethra was significantly increased in the ER $\alpha^{-/-}$ group.

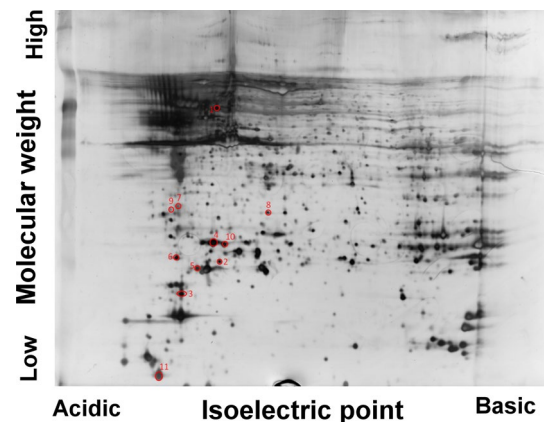


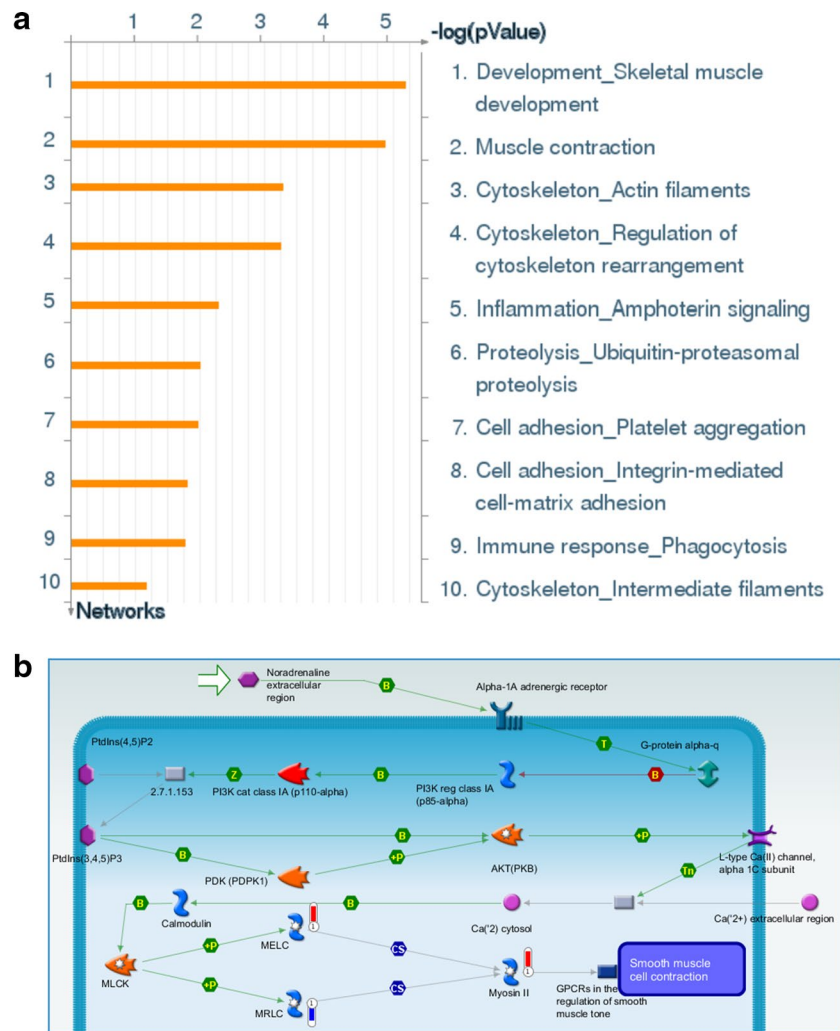
Fig. 3 Two-dimensional electrophoresis images of urethra from ER $\alpha^{-/-}$ female mice. The urethras were removed for proteomic analysis using the 2D DIGE and LC–MS/MS technology. We identified 11 urethra proteins (in red) differentially expressed with statistical significance in ER $\alpha^{+/+}$ and ER $\alpha^{-/-}$ female mice

Discussion

Estrogen actions mediated by ERs are known to modulate lower urinary tract (LUT) trophicity [18]. In the present study, no obvious structural differences were found between the wild-type and ER $\alpha^{-/-}$ mice, but LPP and MUCP values were significantly decreased in the ER $\alpha^{-/-}$ group compared with the ER $\alpha^{+/+}$ group, indicating an important role of ER α in SUI. Further explicit assessments of degree of inflammation and fibrosis may differ between these two groups.

This is the first study that estimates the changes in protein expression related to ER α in SUI. We found 11 differentially expressed proteins between ER $\alpha^{+/+}$ and ER $\alpha^{-/-}$ female mice. The majority of the identified ER α

Fig. 4 Biological network analysis (a) for differentially expressed proteins of urethra from $ER\alpha^{-/-}$ female mice using MetaCore mapping tool (b). The network was generated using shortest path algorithm to map interaction between the proteins. The majority of the $ER\alpha$ knockout-modified proteins were involved in muscle development, contraction, and regulation, immune response (amphoterin signaling and phagocytosis), proteolysis, and cell adhesion (platelet aggregation and integrin-mediated cell–matrix adhesion)



knockout-modified proteins were involved in muscle development, contraction, and regulation, as well as immune response (amphoterin signaling and phagocytosis), proteolysis, and cell adhesion (platelet aggregation and integrin-mediated cell–matrix adhesion). We further focused on tropomyosin and myosin and explored their expressions by IHC and Western blot analyses.

Tropomyosin α -3 chain (TPM3), which was underexpressed in $ER\alpha^{-/-}$ female mice by 1.44-fold, is a member of the tropomyosin family of actin-binding proteins involved in the contraction of striated and smooth muscles by controlling the binding of myosin heads to the actin filament [19]. Since tropomyosin is responsible for muscle contraction, this down-regulation of tropomyosin may be a mechanism of the loss of normal urethral function (lower LPP and MUCP values) in SUI.

Among 11 differentially expressed proteins between $ER\alpha^{+/+}$ and $ER\alpha^{-/-}$ female mice, three of the proteins that exhibited overexpression in $ER\alpha^{-/-}$ female mice were related to myosin, including myosin regulatory light chain

2, myosin light chain 3 and myosin light chain 1/3. Myosin proteins are composed of both heavy and light chains and are essential components of muscles. Myosin heavy chains help determine the speed of muscle contraction; in contrast, the role of myosin light chains (whose expression was 4.55-fold higher in $ER\alpha^{-/-}$ female mice) is unclear. Since myosin is responsible for muscle contraction, this overexpression of myosin light chains may be a mechanism to counteract the loss of normal urethral function in SUI. This finding is consistent with previous work showing 4.4-fold overexpression of the myosin light chains in the pubococcygeus muscle of women with POP [20].

The expression of NADH dehydrogenase iron-sulfur protein 8 was significantly 6.09-fold higher in $ER\alpha^{-/-}$ female mice than in $ER\alpha^{+/+}$ female mice. NADH dehydrogenase iron-sulfur protein 8 is a subunit of mitochondrial enzyme involved in the electron transfer process [21]. The expression of ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1) was significantly 2.25-fold higher in $ER\alpha^{-/-}$ female mice than in wild-type

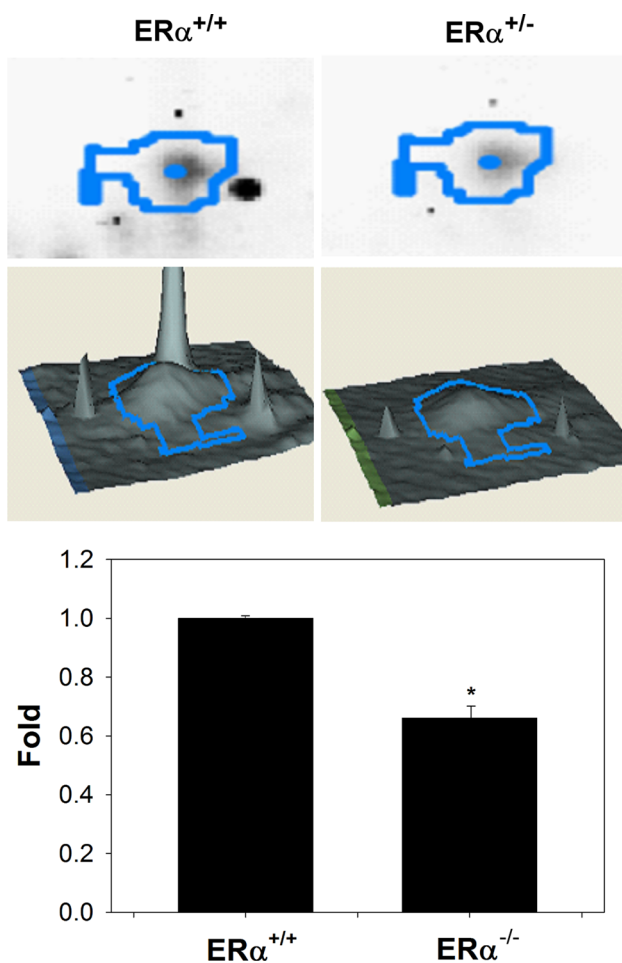


Fig. 5 Three-dimensional simulation figures of protein spots acquired gel images by 2D-DGE DeCyder 6.5 software. The labeled gels were scanned using a Typhoon 9410 imager with specific excitation and emission wavelengths. Difference in-gel analysis was used for intra-gel analysis. Biological variance analysis was used for inter-gel matching and statistical analysis. The data show spot identified as tropomyosin down-regulated in urethra of ERα^{-/-} female mice

female mice. UCH-L1 is a deubiquitinating enzyme that is responsible for making ubiquitin, which is required to target proteins for degradation by the ubiquitin–proteasome pathway [22].

Desmin, which was underexpressed in ERα^{-/-} female mice by 1.76-fold, is a type III intermediate filament found near the Z line in sarcomeres [23]. Desmin down-regulation may induce muscle damage during contraction in the urethra [24], leading to lower LPP and MUCP values in ERα^{-/-} female mice. Translationally controlled tumor protein (TCTP), which was underexpressed in ERα^{-/-} female mice by 2.59-fold, is involved in a variety of cellular activities, including microtubule stabilization, calcium-binding activities, and apoptosis [25]. TCTP down-regulation may induce apoptosis. *N(G),N(G)*-dimethylarginine dimethylamino-hydrolase 2 (DDAH II), which was underexpressed

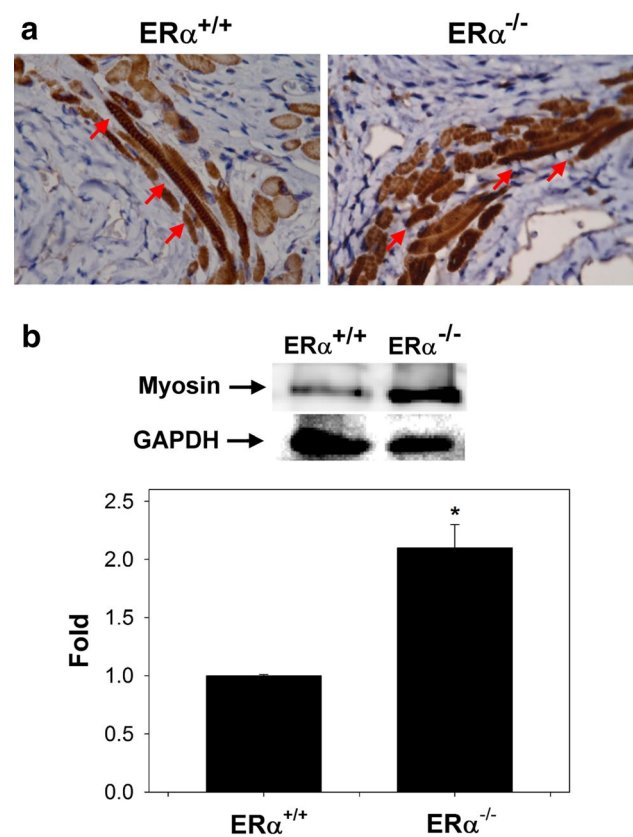


Fig. 6 Alterations of tropomyosin expression in urethra as indicated by IHC staining (a) and Western blot (b) analyses. The urethra was histologically examined with IHC staining for indicating the protein expression regions of the urethra. The sections were then incubated with the primary antibody derived from rabbit [anti-tropomyosin α-3 chain (TPM3)]. Western blot analysis was performed with the use of the antibody. The intensity of each band was quantified using a densitometer. The values are calculated by fold and expressed as mean ± SD of at least three individual samples. *Significantly different from the value in the control group ($P < 0.05$) with Student's *t* test

in ERα^{-/-} female mice by 1.58-fold, is important in removing methylarginines, generated by protein degradation, from accumulating and inhibiting the generation of nitric oxide [26]. DDAH II down-regulation may induce the generation of nitric oxide in the urethra, leading to lower LPP and MUCP values in ERα^{-/-} female mice. Caprin-2, which was underexpressed in ERα^{-/-} female mice by 5.62-fold, may regulate the transport and translation of mRNAs, modulating for instance the expression of proteins involved in synaptic plasticity in neurons [27].

Our study has certain limitations. First, the pelvic floor structure of the mouse, which is a quadruped and has a lax abdominal wall, is different from that of a human female; therefore, the results of this study need to be carefully applied to human subjects. Second, urodynamic studies were conducted under anesthesia; fortunately, none of our subjects manifested any evidence of

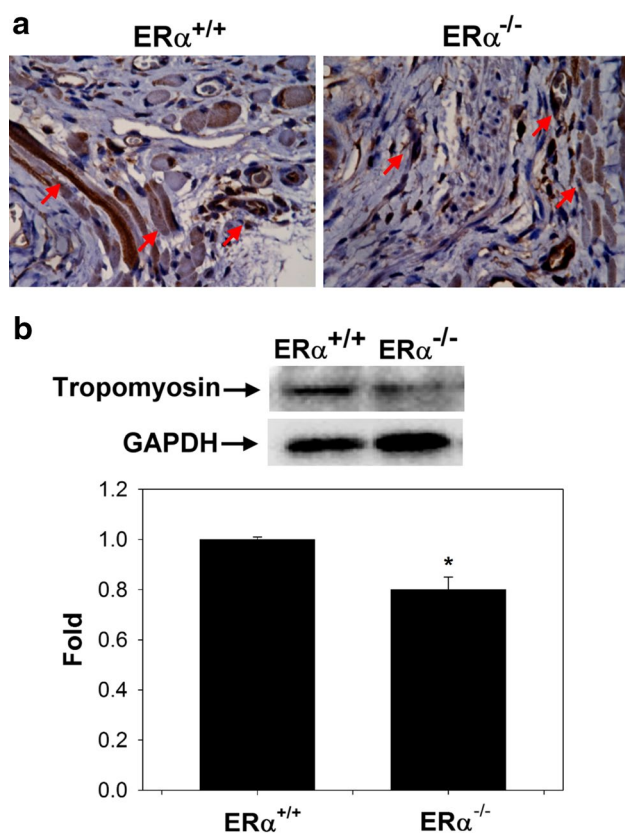


Fig. 7 Alterations of myosin expression in urethra as indicated by IHC staining (a) and Western blot (b) analyses. The urethra was histologically examined with IHC staining for indicating the protein expression regions of the urethra. The sections were then incubated with the primary antibody derived from rabbit [anti-myosin heavy chain antibodies (clone A4.1025, myosin, heavy polypeptide 1)]. Western blot analysis was performed with the use of the antibody. The intensity of each band was quantified using a densitometer. The values are calculated by fold and expressed as mean \pm SD of at least three individual samples. *Significantly different from the value in the control group ($P < 0.05$) with Student's *t* test

bladder instability, implying that detrusor overactivity was not present, and giving credence to our interpretation of fluid expulsion in the absence of increased bladder pressure as evidence of SUI. Third, since too large profiles were found from proteomic study, we could not use Western blot to confirm all the expression levels of potential target proteins in urethras from mice. Fourth, the experimental conditions and the sample preparation techniques could have affected the detection of some proteins. For example, urethras harvested after LPP and MUCP measurements might cause the urethra fatigue and then alter the urethral function, thus possibly leading to biased results. By contrast, advanced analytic methods such as electromyography can be used to replace the LPP method [28].

Conclusions

The results suggest a role for $ER\alpha$ in SUI. This study is the first one to estimate protein expression changes in urethras from $ER\alpha^{+/+}$ and $ER\alpha^{-/-}$ female mice. These changes could be related to the molecular mechanism of $ER\alpha$ functions in SUI.

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Conflict of interest The authors have no conflicts of interest to disclose.

Ethical standard The procedures of the animal study received approval from the ethical committee of the Institutional Animal Care and Use Committee of China Medical University (Reference number: 101-201-N, Date: Feb. 3, 2012).

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