

Original Article

The alteration of ER β 5 and collagen metabolism is relevant to the development of stress urinary incontinence

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Abstract: The primary purpose of this study was to identify the predominant isoform of ER β responsible for the development of stress urinary incontinence (SUI). In this study, anterior vaginal wall tissues were taken from five SUI patients and five women who served as a control. Fibroblast cells were isolated from the tissues and treated with the selective ER β antagonist (4-[2-phenyl-5,7-bis(trifluoromethyl) pyrazolo [1,5-a]pyrimidin-3-yl]phenol, PHTPP) or the agonist (diarylpropionitrile, DPN). The quantitative real-time PCR method was used to detect the mRNA expressions of ER β (ER β 1, 2, 3, and 5), MMP-1, TIMP-1, and collagens I and III. Immunohistochemistry staining was used to detect the protein expressions of ER β 5, MMP-1, TIMP-1, and collagens I and III. An enzyme-linked immunosorbent assay (ELISA) was used to detect the protein expressions of collagens I and III. The MTT method was used to determine the viability of fibroblast cells. Our results showed that it was the ER β 5 rather than the ER β 1, ER β 2, or ER β 3 that significantly decreased in the SUI patients. Alteration of collagen metabolism was noticed in the SUI patients characterized by a decrease of collagen I, collagen III, and TIMP-1 and an increase of MMP-1. An *in vitro* study showed that the mRNA and protein expressions of ER β 5, collagen I, collagen III, and TIMP-1 were decreased, and MMP-1 was increased after PHTPP treatment. The four measurements were changed in the opposite direction after DPN treatment. These results suggested that the alteration of ER β 5 and collagen metabolism may be relevant to the development of SUI, so ER β 5 is a potential target for SUI treatment.

Keywords: Stress urinary incontinence, estrogen receptor β , collagen metabolism

Introduction

Stress urinary incontinence (SUI) is a common urological disease defined as the involuntary leakage of urine under stress conditions such as coughing and sneezing [1, 2]. Activities such as birth trauma, menopause, and aging may result in the development of SUI [3, 4]. SUI is a bothersome condition and compromises the quality of life for affected women [5, 6]; although significant improvement has been made in its treatment, our comprehension of the underlying pathogenesis of this disease remains unclear.

Estrogen plays an important role in the female urogenital system. The biological effects of estrogen are manifested through its interaction

with the estrogen receptors (ERs). ER α and ER β , two predominant subtypes of ERs, have a high similarity in their DNA binding domains and significant differences in their ligand binding domains [7]. Evidence has suggested that ER α and ER β have different roles and expression modes in different organs, which ultimately result in distinct pathophysiological effects [8]. ER β exists as five distinct versions, namely ER β 1 to ER β 5 [9]; in contrast to ER α , the role of ER β in SUI is less clear.

Collagen forms the matrix of connective tissue which serves as a structural support, supporting the stability of the urinary tract. Collagen has a significant role in the maintenance of urinary continence; in a study performed by Han et al., the authors demonstrated that patients

Table 1. Primer sequences used for quantitative real-time PCR

TIMP-1	F	5'-TGTTGTTGCTGTGGCTGATAG-3'
	R	5'-GGTCTGGTTGACTTCTGGTGT-3'
MMP-1	F	5'-CTGTTCTGGGGTGTGGTGTGTC-3'
	R	5'-CTCCCATCATTCTTCAGTTGTAGT-3'
Collagen I	F	5'-CAAGGTGTTGTGCGATGACG-3'
	R	5'-TGGTCGGTGGGTGACTCTG-3'
Collagen III	F	5'-GCTCTGCTTCATCCCACTATTA-3'
	R	5'-GGCAAACCTGCACAACATTCTC-3'
ERβ-1	F	5'-ACCGATGCTTTGGTTTGG-3'
	R	5'-GGGAGCCCTCTTGGCTTTTA-3'
ERβ-2	F	5'-GACAGAGAAGTGCCGACGAG-3'
	R	5'-GGTCACTGCTCCATCGTTGC-3'
ERβ-3	F	5'-TCAACAGCCACGGGAATGAG-3'
	R	5'-CTTTCACCTGTCTTCAACCC-3'
ERβ-5	F	5'-GGCATCTCCTCCAGCAGCAAT-3'
	R	5'-TCCCATCCCAAGCCCAAGCAG-3'
β-actin	F	5'-GGCATGGGTGTCAGAAAGGAT-3'
	R	5'-ATGTCACGCACGATTCC-3'

with pelvic organ prolapse and SUI had lower expression levels of collagen I and collagen III than control and speculated that collagen changes may be associated with the development of SUI [10]. Therefore, exploring collagen metabolism, which is regulated by estrogen [11, 12], is of great importance for understanding the pathogenesis of SUI.

This study aimed to identify the predominant type of ERβ responsible for the development of SUI; moreover, we used an ERβ antagonist and an ERβ agonist to treat fibroblast cells isolated from SUI patients trying to determine the potential of ERβ as a target for SUI treatment.

Materials and methods

Patients

Women diagnosed with SUI according to the guidelines of the International Continence Society (ICS) who underwent tension-free vaginal tape surgery at the department of gynecology of our hospital between December 2015 and January 2017 were recruited for the study. Subjects without SUI or pelvic organ prolapse (POP) who underwent an intravaginal cystectomy for vaginal wall cysts were recruited as controls. The criteria for exclusion from the control group were: hormone replacement therapy wi-

thin the previous 3 months; signs of urinary infection; estrogen-related disease (endometriosis, myoma or functional ovarian tumor); and urge incontinence. Biopsy samples of the anterior vaginal wall were taken from the uterine cervix. A sample of tissue was fixed in neutral buffered formalin and embedded in paraffin for immunohistochemistry staining, and the remainder was frozen immediately at -80°C for quantitative real-time PCR and ELISA tests. The study was approved by the Institutional Review Committee of Zhejiang University School of Medicine. Written informed consent was provided by all participants prior to their biopsies.

Cell culture and treatment

Fibroblast cells isolated from the anterior vaginal wall tissues were cultured in plates in a complete medium at 37°C in a humidified 5% CO₂ atmosphere. Cells were seeded at a density of 2 × 10⁴/well in 96-well plates or 5 × 10⁵/well in 6-well plates. After 24 h culturing, selective ERβ antagonist PHTPP (1 μM; Tocris Biosciences, Minneapolis, MN, USA) or agonist DPN (1 μM; Sigma-Aldrich, St. Louis, MO, USA) were added. Cells were incubated for another 48 h. An MTT experiment was performed to detect the cellular activity. Quantitative real time-PCR was used to evaluate the mRNA expressions of ERβ (ERβ 1, 2, 3 and 5), matrix metalloproteinase-1 (MMP-1), tissue inhibitor of metalloproteinase-1 (TIMP-1), and collagens I and III. Immunohistochemistry staining was used to assess the protein expressions of ERβ5, MMP-1, TIMP-1, and collagens I and III. An ELISA assay was used to evaluate the concentrations of collagen I and collagen III.

Quantitative real-time PCR

Total RNA was extracted from tissues or fibroblast cells using the TaKaRa MiniBEST Universal RNA Extraction Kit (Takara, Dalian, China). cDNA was synthesized using the PrimeScript™ RT Reagent Kit (Takara; Dalian, China). Quantitative RT-PCR was performed on the ABI 7500 Fast Real-Time PCR System (Applied Biosystems; Carlsbad, New Mexico, USA). The relative expression of mRNA was quantified using the 2^{-ΔΔCT} method and normalized to β-actin [13]. The primer sequences used for quantitative RT-PCR are shown in **Table 1**. Each experiment was performed at least three times.

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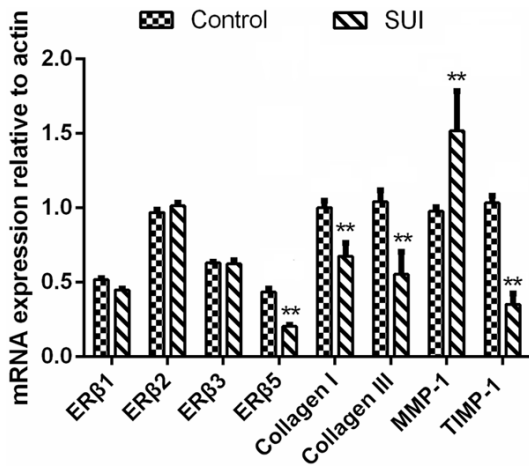


Figure 1. The mRNA expressions of ERβ1, ERβ2, ERβ3, ERβ5, collagen I, collagen III, MMP-1, and TIMP-1 in anterior vaginal wall tissues. SUI, stress urinary incontinence. vs. control, ** $P < 0.01$.

Immunohistochemistry staining

The anterior vaginal wall tissues or fibroblast cells were fixed in 4% paraformaldehyde, rinsed with water, dehydrated through a graded ethanol series, and embedded in paraffin. Sections, 4~7 μm thick, were deparaffinized. The sections were incubated respectively with the following primary antibodies: anti-ERβ5 (clone 5/25; Serotec; dilution 1:100), anti-MMP-1 (Proteintech Group, Inc; dilution 1:300), anti-TIMP-1 (Proteintech Group, Inc; dilution 1:200), anti-Collagen I (Boster Biological Technology, Inc; dilution 1:200) and anti-Collagen III (Boster Biological Technology, Inc; dilution 1:200), and then incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody (Dako A/S; Glostrup, Denmark). As for the negative control, immunostaining was performed by incubating the samples with PBS instead of with a primary antibody. After staining with DAB and counterstaining with hematoxylin, the sections were viewed and quantified under a DM-4000B microscope (Leica Microsystems; Wetzlar, Germany). Integrate optical density (IOD) per vision-field of immunohistochemistry photographs were taken with Image Pro plus software (Media Cybernetics Inc; Bethesda, Maryland, USA).

Enzyme-linked immunosorbent assay

The concentrations of collagen I and collagen III were measured using the Human Collagen Type

I ELISA KIT (MyBioSource; San Diego, California, USA) and the Human Collagen Type III ELISA KIT (MyBioSource; San Diego, California, USA), respectively. All procedures were performed according to the manufacturer's instructions.

Cell viability assay

After treatment with PHTPP and DPN, the cells were incubated for 4 h with 3-[4,5-dimethylthiazol-2-yl]-2,5-assay diphenyl-tetrazolium bromide (MTT) dye, which was converted by viable cells to blue formazan crystals. The crystals were solubilized by dimethyl sulfoxide (DMSO). The absorbance (OD value) was measured at 570 nm with a microplate reader (PerkinElmer, Inc.; Waltham, MA, USA). Each experiment was performed at least three times.

Statistical analysis

The statistical analyses were performed using SPSS version 20.0 software (IBM Corporation; Somers, NY, USA). Comparisons between groups were analyzed by independent *t*-tests or a one-way analysis of variance as appropriate. A *p* value < 0.05 was considered significant.

Results

The mRNA expressions of ERβ1, ERβ2, ERβ3, ERβ5, collagen I, collagen III, MMP-1, and TIMP-1 in the anterior vaginal wall tissues

From **Figure 1** we can see that, when compared with the control group, the mRNA expressions of ERβ1, ERβ2, and ERβ3 in the SUI group were almost unchanged ($P > 0.05$). The mRNA expressions of ERβ5, collagen I, collagen III, and TIMP-1 were significantly decreased in the SUI group compared to the control group ($P < 0.01$). Unlike ERβ5, collagen I, collagen III and TIMP-1, the mRNA expression of MMP-1 changed in the opposite direction, and it was considerably higher in the SUI group as compared with the control group ($P < 0.01$).

The protein expressions of ERβ5, collagen I, collagen III, MMP-1, and TIMP-1 in the anterior vaginal wall tissues

Considering that the mRNA expressions of ERβ1, ERβ2, and ERβ3 were almost unchanged in the anterior vaginal wall tissues, in this part, only one isoform of ERβ, i.e., ERβ5, was determined using immunohistochemistry staining.

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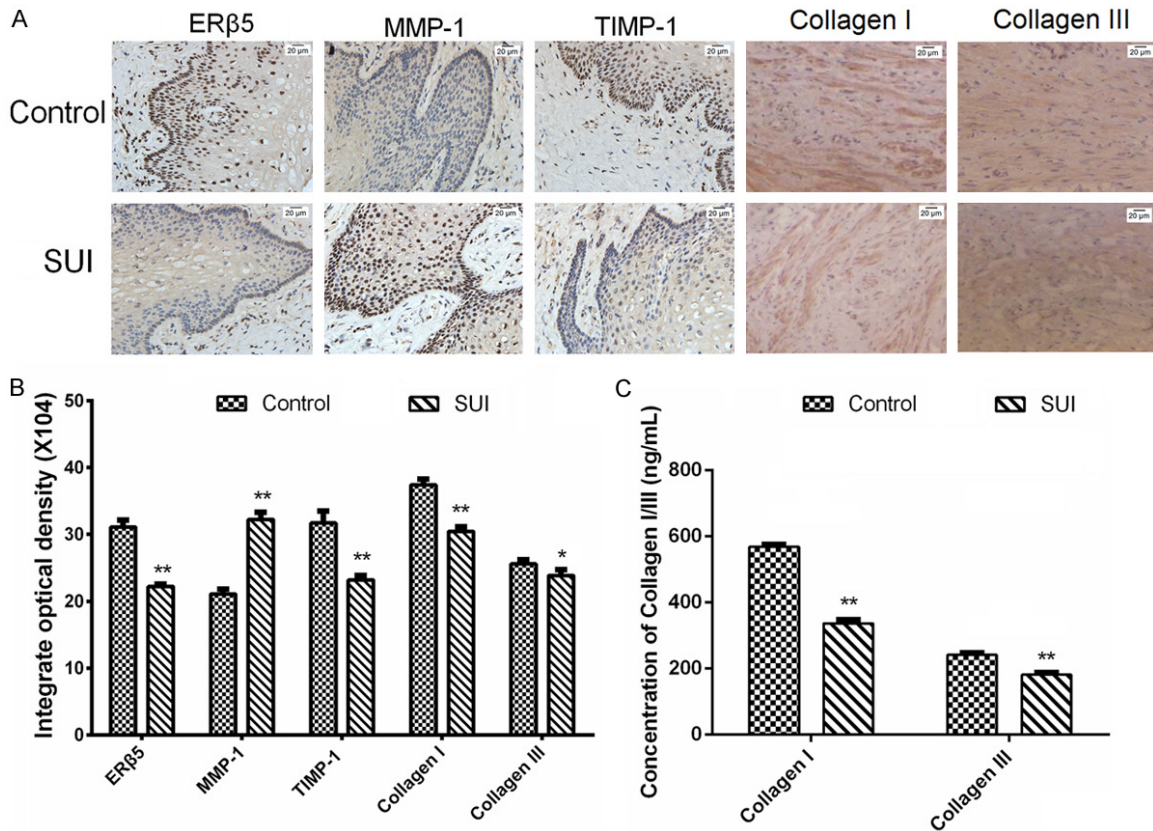


Figure 2. The protein expressions of ERβ5, MMP-1, TIMP-1, collagen I, and collagen III in the anterior vaginal wall tissues. A: Representative immunohistochemistry staining pictures; SUI, stress urinary incontinence; × 200, magnification bar 20 μm; B: Semi-quantitative of immunohistochemistry staining pictures by integrated optical density (IOD) value; C: Collagen I and collagen III concentrations determined by the ELISA method. vs. the control, *P < 0.05 and **P < 0.01.

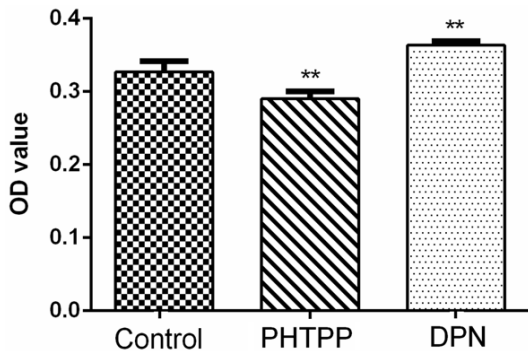


Figure 3. The Effects of PHTPP and DPN on the viability of fibroblast cells. PHTPP, 4-[2-phenyl-5,7-bis(trifluoromethyl) pyrazolo [1,5-a]pyrimidin-3-yl]phenol; diethylpropionitrile, DPN. vs. control, **P < 0.01.

As shown in **Figure 2A** and **2B**, the protein expressions of ERβ5 was significantly decreased in the SUI group as compared with the con-

trol group (P < 0.01). **Figure 2A** and **2B** showed that the protein expression of MMP-1 was increased, but TIMP-1, collagen I and collagen III were decreased in the SUI group as compared with the control group, and these differences were statistically significant (P < 0.01). The ELISA results showed that the concentrations of collagen I and collagen III were significantly decreased in the SUI group as compared with the control group (P < 0.01) (**Figure 2C**).

The effects of PHTPP and DPN on the viability of human fibroblast cells

As shown in **Figure 3**, when compared with the control group, the OD value of the PHTPP group was significantly decreased (P < 0.01); on the other hand, the OD value of the DPN group was significantly increased (P < 0.01). The MTT results suggest that ERβ affects the proliferation of human fibroblast cells.

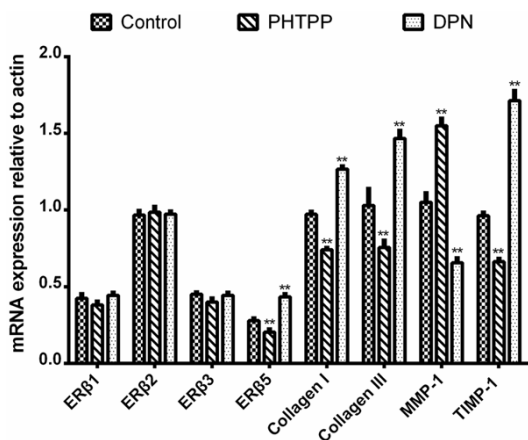


Figure 4. The mRNA expressions of ER β 1, ER β 2, ER β 3, ER β 5, collagen I, collagen III, MMP-1, and TIMP-1 in human fibroblast cells. The data shown as the means \pm SD. PHTPP, 4-[2-phenyl-5,7-bis(trifluoromethyl) pyrazolo [1,5-a]pyrimidin-3-yl]phenol; diarylpropionitrile, DPN. vs. control, ** $P < 0.01$.

The effects of PHTPP and DPN on the mRNA expressions of ER β 1, ER β 2, ER β 3, ER β 5, collagen I, collagen III, MMP-1, and TIMP-1 in human fibroblast cells

In **Figure 4** we can see that the mRNA expressions of ER β 1, ER β 2, and ER β 3 showed no significant change in the three groups ($P > 0.05$). The mRNA expressions of ER β 5, collagen I, collagen III, and TIMP-1 were decreased in the PHTPP group and increased in the DPN group as compared with the control group, and these differences were statistically significant ($P < 0.01$). The mRNA expression of MMP-1 was increased in the PHTPP group and decreased in the DPN group as compared with the control group ($P < 0.01$).

The effects of PHTPP and DPN on the protein expressions of ER β 5, collagen I, collagen III, MMP-1, and TIMP-1 in human fibroblast cells

As shown in **Figure 5**, the protein expressions of the ER β 5, MMP-1, TIMP-1, collagen I, and collagen III were similar with their mRNA expressions. For ER β 5, TIMP-1, collagen I and collagen III, a significant decrease in the PHTPP group and an increase in the DPN group were observed as compared with the control group ($P < 0.01$). The protein expression of MMP-1 was increased in the PHTPP group and decreased in the DPN group as compared with the control group ($P < 0.05$).

Discussion

The female genitourinary tract is supported by structures like the anterior vaginal wall, the pubovesical fascia, and the pubourethral ligaments. Defects in any of these structures may lead to a dysfunction of the genitourinary tracts. ERs have been found in these structures, highlighting the importance of ERs in maintaining the stability of the genitourinary tracts [8, 14]. A previous study demonstrated that ER β was decreased in the anterior vaginal wall of women with SUI and speculated that ER β may be involved in the occurrence of SUI [15]; however, ER β has five isoforms, and which isoform imposes a predominant influence on the development of SUI is still poorly understood. In the present study, we first demonstrated that it was the ER β 5, rather than ER β 1, ER β 2, or ER β 3 that was significantly decreased in the anterior vaginal walls of SUI patients.

Collagen plays an important role in the maintenance of urinary continence. The degradation of collagen I and III is closely related with the activity of matrix metalloproteinases (MMPs) such as MMP-1, MMP-2, and MMP-8 [16]. The activity of MMPs is down-regulated by the tissue inhibitors of the metalloproteinases (TIMPs) such as TIMP-1, TIMP-2, and TIMP-3 [17]. Collagens, MMPs, and TIMPs are usually used in the evaluation of the collagen metabolism [10, 18]. In a study performed by Chen et al., they found that MMP-1 mRNA expression was increased and TIMP-1 mRNA expression was decreased in the vaginal wall tissues of stress incontinent women [18]. Han et al. demonstrated that patients with pelvic organ prolapse (POP) and SUI had lower levels of type I and type III collagen than the controls [10]. Our results were similar to these two reports. In our study we found that, when compared with the control group, the mRNA and protein expressions of collagen I, collagen III, and TIMP-1 were significantly decreased, and the mRNA and protein expressions of MMP-1 were significantly increased in the anterior vaginal wall tissues of patients with SUI. These results indicated that alterations in collagen metabolism may relevant to the development of SUI.

Estrogen is the principal hormonal regulator of collagen metabolism and has a profound influence on pelvic collagen metabolism, both in synthesis and degradation [19, 20]. Estrogen

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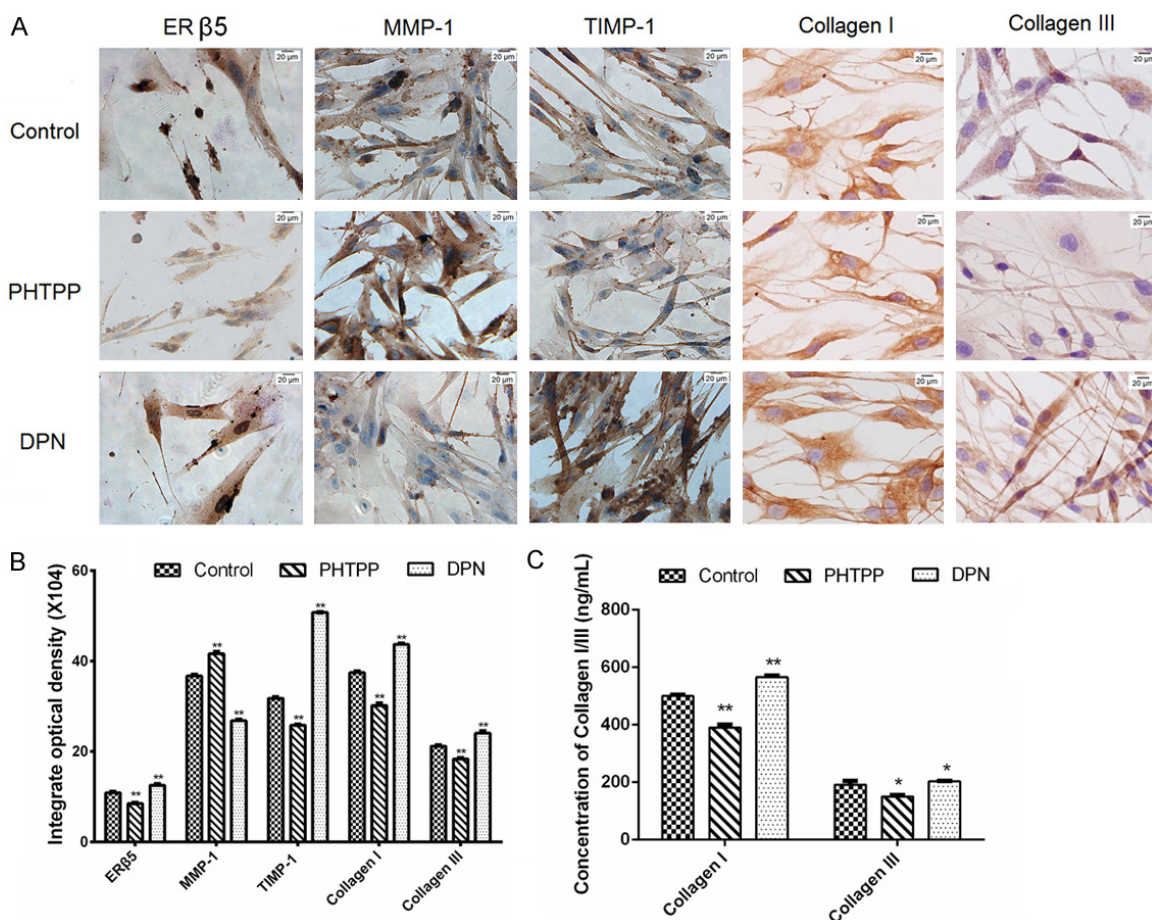


Figure 5. The protein expressions of ER β 5, MMP-1, TIMP-1, collagen I and collagen III in the human fibroblast cells. A: Representative immunohistochemistry staining pictures, $\times 200$, magnification bar 20 μm ; B: Semi-quantitative view of the immunohistochemistry staining pictures by integrated optical density (IOD) value; C: Collagen I and collagen III concentrations determined by the ELISA method. PHTPP, 4-[2-phenyl-5,7-bis(trifluoromethyl) pyrazolo [1,5-a] pyrimidin-3-yl]phenol; diarylpropionitrile, DPN. vs. control, * $P < 0.05$ and ** $P < 0.01$.

action is mediated by the ERs; therefore, intervention of the ERs with selective antagonists or agonists may influence collagen metabolism. To confirm this speculation, fibroblast cells, isolated from the anterior vaginal wall tissues of SUI patients, were treated with ER β antagonist (PHTPP) or agonist (DPN), and we found that the proliferation of fibroblast cells was inhibited by PHTPP and promoted by DPN; also, the mRNA and protein expression of ER β 5, collagen I, collagen III, and TIMP-1 were decreased and MMP-1 was increased after PHTPP treatment. In contrast to PHTPP, ER β 5, collagen I, collagen III, TIMP-1, and MMP-1 were changed in the opposite direction after DPN treatment. These results indicated that ER β 5 may be a potential target for SUI treatment.

In conclusion, our study demonstrated that it was ER β 5 rather than ER β 1, ER β 2, and ER β 3

that were significantly decreased in the anterior vaginal walls of SUI patients; the alteration of collagen metabolism may be relevant to the development of SUI; ER β 5 may be a potential target for SUI treatment. However, this study has some limitations. Firstly, only 10 tissues (5 vs. 5) were used in this study, so the number is quite limited. Second, the amplification of primers specific to ER β 4 failed, so we didn't determine the mRNA and protein expressions of ER β 4; Third, the protein expressions of ER β 5, MMP-1, and TIMP-1 were semi-quantitatively determined by immunohistochemistry, and quantitative methods such as Western blot and ELISA should be used. Fourth, in the cell experiment, we used selective ER β antagonist and agonist to confirm the potential value of ER β 5 in treating SUI. Ideally, methods specific to the ER β 5 gene such as RNA interference should be used. In the future, more comprehensive and

rigorous studies are needed to further confirm the roles of ER β in SUI.

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Disclosure of conflict of interest

None.

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