Application of estrogen for the treatment of stress urinary incontinence in mice

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Abstract
Background Stress urinary incontinence (SUI) is a pervasive health tissue among women, which seriously affects the quality of life. The etiology of SUI is complex and diverse in women, with past studies having demonstrated that estrogen deficiency plays an important role in pelvic floor muscle atrophy and urethral degeneration. We comprehensively investigated the effects of estrogen in the treatment of SUI in female mice at cellular and animal levels.

Methods L929 fibroblasts mechanical injury model was established by four-point bending device, and SUI mouse model was established by vaginal dilation method commonly used to simulate labor injury. After estrogen treatment, the expressions of Collagen I, Collagen III, Elastin, TIMP-1, TIMP-2, MMP-2, and MMP-9 were detected, the leak point pressure (LPP) and abdominal leak point pressure (ALPP) of mice in each group were detected, and both the effect of estrogen on extracellular matrix remodeling of mouse urethra and anterior vaginal wall was observed from the histological level.

Results The results revealed that an appropriate amount of estrogen can promote the expression of Collagen I, Collagen III, Elastin, TIMP-1, and TIMP-2, decrease the expression of MMP-2 and MMP-9, and maintain the dynamic balance of MMPs/TIMPs at both cellular and animal levels. Meanwhile, we determined that estrogen can increase the LPP and ALPP values of SUI mice. The collagen fibers’ content in the mice treated with estrogen was significantly greater than in the control group mice.

Conclusions The estrogen may alleviate the symptoms of SUI by reconstituting ECM, thus laying a solid foundation for further exploration of estrogen therapy.

Keywords Estrogen · Extracellular matrix · Stress urinary incontinence

Introduction
Stress urinary incontinence (SUI) is a usual problem affecting women [1], which refers to a type of pelvic floor dysfunction disease wherein urine leaks involuntarily from the external urethra at enhanced abdominal pressure due to activities such as coughing, exercising, laughing, or sneezing [2, 3]. SUI has a prevalence ranging from 5 to 69% in women depending on the population studied, with most studies in the range of 25–45% [4]. SUI may lead to adverse physical, psychic, and social effects, seriously affecting the quality of life [5], especially sexual dysfunction [6]. Since women with SUI tend to avoid sexual intercourse on account of leakage during intercourse, wetness at night, embarrassment, and depression [7]. Moreover, disorders of desire, arousal and lubrication, anorgasmia, and dyspareunia were also reported in women suffered from SUI, which may contribute to sexual dysfunction [8]. The etiology of SUI is complex and diverse for women, including obesity, vaginal delivery, aging, decreased ovarian function, and estrogen deficiency [9–11]. Previous studies have indicated that estrogen deficiency is an important cause of pelvic floor muscle atrophy and urethral degeneration [12]. The Phase III clinical trial of the estrogen receptor blocker levormeloxifene revealed that its main side effect was to induce SUI [13]. In addition, the incidence rate of postmenopausal SUI was significantly higher than others [14].
In recent years, with the development of the social economy, the diagnosis and treatment of SUI have attracted more and more attention, a few newer diagnoses and treatment methods have emerged. Presently, conservative therapies, including biofeedback therapy [15], pelvic floor muscle training [16], and drug therapy such as topical estrogen therapy [17], were usually used in the treatment of mild-to-moderate cases. Severe cases of SUI usually require surgical intervention, including mesh vaginal surgery [18], placement of synthetic slings [19], such as single-incision mini-sling (SIMS), tension-free vaginal tape (TVT), and transobturator tape (TOT) [20], local injection of bulking agents, and implantation of artificial urinary sphincters [21, 22]. However, some patients submitted to surgical correction of SUI using suburethral slings suffered from sexual function, including reduction of libido, dyspareunia, or sexual inactivity [23]. For patients with mild-to-moderate SUI, topical estrogen therapy is usually preferred owing to its convenience, fewer side effects, and low price. However, for a long time, the basic research of estrogen in the treatment of SUI is still relatively lacking.

Extracellular matrix (ECM) is an essential component of the connective tissues of the pelvic floor. It is a complex long-chain protein mixture, composed of collagen, elastin, and proteoglycan [24]. Previous studies have confirmed that ECM remodeling is a significant association in the pathogenesis of SUI [25]. It has been found that estrogen affects the transcription and expression of collagen, which is the main component of ECM [26]. Besides, some symptoms and signs related to menopause are caused by insufficient estrogen secretion [27]. Estrogen replacement therapy (ERT) has been reported to reshape collagen in the urogenital tissues of postmenopausal women by changing its quantity and quality [28]. Pentenyl flavonoids and phytoestrogens play a potent role as estrogen receptor-α selective agonists in postmenopausal sexually active women affected by postmenopausal urethral and vulvovaginal atrophy, possibly counteracting the effects of postmenopausal estrogen loss [29]. Animal experiments have proven that estrogen can increase the composition of loose connective tissues in the urethra, which is rich in blood vessels [30], and at the same time, increase the distribution density of sympathetic nerves in the pelvic floor tissues and regulate neurotrophy [31]. Therefore, the supplementation of exogenous estrogen in postmenopausal SUI patients can effectively alleviate the symptoms of SUI [32].

Although the application of estrogen is clinically effective in the treatment of SUI, the basic research of estrogen in the treatment of SUI is relatively lacking. Meanwhile, reports on the remodeling of ECM caused by estrogen are not quite systematic. Therefore, in this article, according to our previous experience [25], we constructed the animal model of SUI by vaginal dilation, and correspondingly, we constructed the mechanical injury model of L929 fibroblasts [33]. We have described the effects of topical estrogen to treat SUI from the animal and cellular level to investigate the reasons for the good efficacy of topical estrogen in the treatment of SUI, the mechanism by which estrogen affects the expression of ECM, to provide further evidence for the clinical efficacy of estrogen on women with SUI.

Materials and methods

Cell experimental design and cell culture

Mouse L929 fibroblasts were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China). Fibroblasts were fostered in Dulbecco’s modified Eagle’s medium (DMEM) (Procell Life Science & Technology Co., Ltd.) (supplemented with 4.5 g/L d-glucose, without Phenol red indicator) with 10% fetal bovine serum (FBS; Gemini Bio-Products, California, USA) and 1% antibiotics (100 KU/mL penicillin G, 100 mg/mL streptomycin; Genom Biotech Ltd.) in an incubator at 37 °C under 5% CO2 atmosphere.

To explore the expression of ECM without or with estrogen therapy before and after cell stress loading, we categorized the cells with the same growth status into 4 groups: normal cells without estrogen therapy (Group C), normal cells with estrogen therapy (Group E2), cells that had undergone cyclic mechanical strain with estrogen therapy (Group S + E2), and cells that had undergone cyclic mechanical strain without estrogen therapy (Group S). For cells treated with estrogen, the concentration of estrogen was 10⁻⁵ nmol/L, which was based on cell proliferation and apoptosis experiments. These four groups of cells were cultured in an incubator for 4 h after their respective treatments, followed by collection in 6-well plates for subsequent experiments.

Cyclic mechanical strain

The L929 fibroblasts were collected on four-point bending cell strain gauges (Miracle Technology Co. Ltd., Chengdu, China) at a density of 1 × 10⁵ cells/mL, and 2-mL cell suspension was uniformly spread on each of the strain gauges. Next, 4 pieces of stress gauges were laid each time, and only 2 of them were subjected to mechanical stress loading. The cells were continuously cultured in an incubator with 5% CO2 at the temperature of 37 °C for 24 h to prepare the cell slide. While the cells adhered firmly and grew up to a sub-fusion state, the slide was placed in the strain state containing 65 mL of complete culture medium and then stretched for mechanical strain loading, as shown in Fig. 1b. The parameters of the four-point bending facility parameters (Miracle Technology Co. Ltd., Chengdu, China) were adjusted to a frequency of 1 Hz for 4 h and the cells were...
subjected to 5333 µ as described in our previous research, using 0 µ strain as the control strain [36]. At the end of the cyclic mechanical strain, the cells on the slide were cultured for another 4 h, as shown in Fig. 1c. Then, all the cells were collected to detect the corresponding indicators.

**Cell proliferation**

β-Estradiol (E8875; Sigma) was dissolved in anhydrous ethanol to a concentration of 1 M, which was further diluted in DMEM (4.5 g/L d-glucose, without Phenol red indicator) to make the final concentration of β-estradiol to 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, and 10⁻⁸ nmol/L, respectively. The L929 fibroblasts were uniformly cultured in 96-well plates at the density of 5000 cells per well and fostered in a medium containing different concentrations of estradiol (100 µL medium per well). The proliferation of L929 fibroblasts was investigated using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Shanghai, China) for 24 h. The medium of each well was discarded, and replaced with a mixture containing 90 µL serum-free medium and 10 µL of CCK-8 reagent to each well. After 2 h, the absorbance was measured by spectrophotometer at 450 nm (5 holes in each group).

**Western blotting**

After applying the design-based treatment, the proteins were successfully obtained from L929 cells, the urethra, and the anterior vaginal wall of mice with RIPA lysate supplemented with phenylmethanesulfonyl fluoride (PMSF, 1 mM; Servicebio, Wuhan, China). Western blotting was performed as described previously [34]. The antibodies used are listed below: anti-Collagen type I antibody (1:1000, Abcam, ab88147), anti-Collagen type III antibody (1:500, Abcam, ab7778), anti-Elastin antibody (1:1000, Abcam, ab2173560), anti-MMP-2 antibody (1:1000, Abcam, ab86607), anti-MMP-9 antibody (1:1000, Abcam, ab228402), anti-TIMP-1 antibody (1:500, Santa, sc-21734), anti-TIMP-2 antibody (1:500, Santa, sc-21735), and anti-GAPDH antibody (1:1000, Abcam, ab8245).

**Quantitative real-time polymerase chain reaction (Q-PCR)**

Q-PCR was performed to investigate the expression of related genes in our experimental design. The reaction was performed as described elsewhere [34]. All the primers used in the study were obtained from Sangon Biotech (Shanghai, China; listed in Table 1).

**Animal experiment design and treatment**

All of our animal experimental protocols and implementations were conducted based on the institutional guidelines of the Institutional Review Committee and authorized by the Ethics Committee of the Institutional Animal Care and Use Committee of Renmin Hospital of Wuhan University (20210306).

We purchased wild-type C57BL/6 female mice (8–9-week-old) from the Center for Animal Experiment of Wuhan University. A total of 60 wild-type C57BL/6 female mice were stochastically separated into 4 groups of 15 mice prior to treatment begin, briefly as follows: control group (Group C), estrogen-alone group (Group E2), vaginal dilatation group (Group VD), and vaginal dilatation with estrogen group (Group VD + E2). The groups VD and VD + E2 were subjected to vaginal dilatation on the same day. From the second day of vaginal dilatation, conjugated estrogen cream (CEEs; Xinjiang Xinziyuan Bio-Pharmaceutical Co. Ltd, Yili, China) was applied to the vagina of the mice in groups E2 and VD + E2 with a 1-mL syringe. The calculation of vaginal CEE dose was based on the comparison of body weight and human dose, as mentioned in a previous study [35]. The usual vaginal
maintenance dose (1 g cream) for a 60-kg woman could produce 625 μg of CEEs. By comparison with the weight, the CEE dose for a 20-g C57BL/6 female mouse was calculated to be 0.21 μg.

After 7 days of continuous exposition of CEE to the mice in the two groups, the suprapubic canal was implanted in all mice in 4 groups, and the leakage point pressure (LPP) and abdominal pressure LPP (ALPP) were measured on the same day. Then, the anterior vaginal wall and the urethra of innamate mice were obtained for Western blotting, Q-PCR and section staining.

Masson trichrome staining

A portion of the animal samples was intercalated into paraffin, sliced into transverse sections of thickness 4 μm, and stained with the Masson Kit HT15 (Sigma, USA) following the manufacturer’s protocol. The mean optical density (MOD) value gathered by the ImageJ software was considered as the quantitative index to assess the content of collagen fibers.

Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analysis was conducted with the GraphPad Prism software version 8 (San Diego, CA, USA). One-way analysis of variance (ANOVA) was employed for several comparisons in three or more groups. P < 0.05 was regarded as statistically significant.

Results

Estrogen promotes the proliferation of L929 fibroblasts

The proliferation effect of β-estradiol at different concentrations on L929 fibroblasts was detected by the CCK8 assay. As shown in Fig. 1a, with the increase of concentration in a certain range, the proliferation effect of β-estradiol on L929 fibroblast became more and more significant. Specifically, at the concentration of 10⁻⁵ nmol/L, the L929 fibroblast showed the maximum proliferation effect. Therefore, we selected 10⁻⁵ nmol/L as the optimal concentration of β-estradiol to treat L929 fibroblast for the subsequent experiments.

The promoting effect of estrogen on the expression of ECM for L929 fibroblasts

ECM is a complex long-chain protein mixture composed of collagen, elastin, and proteoglycan. Among these constituents, Collagen I and Collagen III are the main collagen types in the pelvic floor. They are of vital importance for maintaining the elasticity and toughness of the pelvic floor support system. To reveal the effect of estrogen on ECM, we treated L929 fibroblasts according to the cell experiment design, after which the ECM production of L929 fibroblast was investigated by Western blotting and

| Table 1 A list of primers used for the quantitative real-time polymerase chain reaction in this study |
|-----------------------------------------------|----------------|----------------|---------------|
| Gene name | Gene ID  | Primer sequence (5´–3´) | Ampli-con size (bp) |
| M-GAPDH  | NM_008084.3 | F: TGAAGGGTTGGACCAAAAAG | 227 |
| M-COL1A1 | NM_007742.4  | F: CGCTGATAGGGAGGGAG | 116 |
| M-Col3a1 | NM_009930  | F: GGTATGTAATTTTCTGAGG | 298 |
| M-MMP2   | NM_008610.2 | F: GAAATGGATATCGAATTGTGA | 127 |
| M-MMP9   | NM_013599.3 | F: CGTCGACGCTCCTGAGG | 149 |
| M-TIMP-1 | NM_053819.1 | F: CCAGAGATATCGAGACCA | 201 |
| M-TIMP-2 | NM_011594.3 | F: GCAGTCTAGATCACC | 140 |
| M-Elastin | NM_007925.4 | F: ACCACACAGCAAGGAAATCA | 177 |
Q-PCR. As can be seen from Figs. 2a–c and 3a–c, both the protein and mRNA expressions of COL1A1, COL3A1, and Elastin in L929 fibroblasts, which are the main components of ECM. d, e Estrogen inhibited the expression of MMP-2 and MMP-9 in L929 fibroblasts. f–g Estrogen promoted the expression of TIMP-1 and TIMP-2 in L929 fibroblasts. h Protein electrophoresis bands. (C: normal cells without estrogen therapy; E2: normal cells with estrogen therapy; S: cells undergone cyclic mechanical strain without estrogen therapy; S + E2: cells undergone cyclic mechanical strain with estrogen therapy. *compared with C, p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; #compared with E2, p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001; $compared with S, p < 0.05, $$$p < 0.01, $$$$p < 0.001, $$$$$p < 0.0001. Every experiment was repeated thrice.)

**Estrogen can maintain a balance of TIMP/MMP to stabilize the ECM homeostasis**

Under normal physiological conditions, MMPs and TIMPs maintain a state of dynamic balance, which is essential for maintaining ECM homeostasis. MMPs mainly hydrolyze collagen and the basement membrane, while TIMPs inhibit the breakdown of ECM and the basement membrane [36]. We conducted western blotting and Q-PCR to investigate the expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 in the L929 fibroblasts among the 4 groups. As shown in Figs. 2d–g and 3d–g, both the protein and mRNA expressions of MMP-2 and MMP-9 were significantly reduced in the Group E2 cells when compared with that in the Group C cells. Both the protein and mRNA expressions of TIMP-1 and TIMP-2 showed dramatic improvement in the Group S + E2 cells relative to that in the Group S cells. These data together suggest that moderate β-estradiol can increase the expression of MMPs while decreasing the expression of TIMPs, thereby maintaining a balance of TIMP/MMP to stabilize the ECM homeostasis.

**Estrogen improved the urodynamic parameters of LPP and ALPP in female SUI mice**

To further corroborate the results of the in vitro studies mentioned above, we employed the vaginal dilation method for establishing a mouse model of SUI disease. The computer, physiological recorder, pressure sensor, micro-injection pump, and other devices were used to ascertain that the urodynamic parameters were connected to obtain a urodynamic curve. For instance, the LPPS and ALPPs of mice in Group C and E2 are shown in Table 2. The LPPS and ALPPs of mice in Group VD and Group VD + E2 are shown in Table 3.
The quantitative analyses of the parameters are indicated in Tables 2 and 3. As can be seen from these tables, the levels of LPPS and ALPPs were almost identical between Group C and Group E2. However, the LPPs and ALPPs in the Group VD + E2 were significantly higher than those in the Group VD. These data suggest that, for female mice with SUI, vaginal topical application of estrogen can improve the symptoms of SUI. However, for normal mice, the vaginal topical application of estrogen showed no significant effect on the assessed urodynamic parameters.

The effect of estrogen on the remodeling of ECM in the anterior vaginal wall and the urethra of female SUI mice

To reveal the therapeutic effect of estrogen on female SUI mice, we collected samples from the urethra and anterior vaginal wall of each group of mice to investigate the expression of ECM-related genes by Western blotting and Q-PCR. As shown in Figs. 4a–c and 5a–c, both the protein
and mRNA expressions of COL1A1, COL3A1, and Elastin were dramatically enhanced in the Group E2 mice when compared with those in the Group C mice. Analogously, both the protein and mRNA expressions of COL1A1, COL3A1, and Elastin were significantly enhanced in the Group VD + E2 mice relative to those in the Group VD mice. As shown in Figs. 4d–g and 5d–g, the protein and mRNA expressions of MMP-2 and MMP-9 in Group E2 mice were greatly reduced when compared to those in the Group C mice. Analogously, the protein and mRNA expressions of MMP-2 and MMP-9 in the Group VD + E2 mice were significantly lower when compared to the Group VD mice. As for TIMP-1 and TIMP-2, their protein and mRNA expressions were remarkably higher in Group E2 mice than in the Group C mice. Finally, both the protein and mRNA expressions of TIMP-1 and TIMP-2 were significantly higher in Group VD + E2 mice than in Group VD mice. These data together suggest that the effect of estrogen on the remodeling of ECM in the urethra and the anterior vaginal wall of female SUI mice is similar to the effect of estrogen on the remodeling of ECM secreted by L929 fibroblasts.

Histological observation on the effect of estrogen on ECM remodeling of the urethra and anterior vaginal wall in SUI mice

To further reveal the effect of estrogen on the remodeling of ECM in the pelvic floor of mice with SUI, samples from the anterior vaginal wall and the urethra of inanimate mice were subjected to Masson staining and then photography (images shown in Fig. 6a–d). In these sections, the collagen fibers were blue and the muscle fibers were red. The mean optical density (MOD) value obtained by Image J was regarded as the quantitative index to assess the collagen fibers’ content, as shown in Fig. 6e. The collagen fibers’ content in the Group E2 mice was more abundant than in the Group C mice. Similarly, the collagen fibers’ content in the Group VD + E2 mice was also significantly greater than those in the Group VD mice. These data together indicated that estrogen could effectively upregulate the expression of collagen fibers, which may explain why estrogen could alleviate the symptoms of mice with SUI.
Fig. 5 Estrogen promoted the mRNA expression levels of Collagen I, Collagen III, and Elastin related to the ECM in the anterior vaginal wall and the urethra of SUI mice. a-e Estrogen promoted the mRNA expression levels of Collagen I, Collagen III, and Elastin in the anterior vaginal wall and the urethra of SUI mice. d, e Estrogen inhibited the mRNA expression levels of MMP-2 and MMP-9 in the anterior vaginal wall and the urethra of SUI mice. f, g Estrogen promoted the mRNA expression levels of TIMP-1 and TIMP-2 in the anterior vaginal wall and the urethra of SUI mice. (C: control group; E2: estrogen-alone group; VD: vaginal dilation group; VD+E2: vaginal dilation with the estrogen group. *compared with C, \( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \), ****\( p < 0.0001 \); #compared with E2, \( p < 0.05 \), ##\( p < 0.01 \), ###\( p < 0.001 \), ####\( p < 0.0001 \); $compared with VD, \( p < 0.05 \), $$\( p < 0.01 \), $$$\( p < 0.001 \), $$$$_{\text{S}}$$\( p < 0.0001 \). Every experiment was repeated thrice.)

Fig. 6 Histological observation of the effect of estrogen on ECM remodeling of the urethra and the anterior vaginal wall in SUI mice. a The collagen fibers’ content in Group C mice. b The collagen fibers’ content in Group E2 mice. c The collagen fibers’ content in Group VD mice. d The collagen fibers’ content in Group VD+E2 mice. e The MODs of collagen fibers after Masson staining. (C: control group; E2: estrogen-alone group; VD: vaginal dilatation group; VD+E2: vaginal dilatation with the estrogen group. *compared with C, \( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \), ****\( p < 0.0001 \); #compared with E2, \( p < 0.05 \), ##\( p < 0.01 \), ###\( p < 0.001 \), ####\( p < 0.0001 \); $compared with VD, \( p < 0.05 \), $$\( p < 0.01 \), $$$\( p < 0.001 \), $$$$_{\text{S}}$$\( p < 0.0001 \).)
Discussion

SUI is a common disease affecting women. SUI is reported to be due to an imbalance of ECM remodeling in the urethra and anterior vaginal [37]. ECM reconstruction is mainly regulated by fibroblasts present in the pelvic floor [38]. Based on the present and previous study results, we selected L929 fibroblasts as our experimental cells [33]. Collagen I and Collagen III are the main types of pelvic floor collagen, while Elastin is the key component of connective tissues that provide elasticity and support to the pelvic floor tissues [39]. In this study, we found that the expressions of Collagen I, Collagen III, and Elastin were lower in the urethra and anterior vaginal wall of mice with SUI when compared with those in the control group mice. Based on the above results, we obviously speculated that the restoration of ECM may facilitate SUI treatment.

McKenzie et al. reported that estrogen affects the gene transcription and the expression of collagen, which is the main component of ECM [26]. ERT has been proven to improve certain aspects of the skin, such as by enhancing the morphology and elasticity of elastic fibers, Collagen III, and hyaluronic acid to reduce wrinkles [40]. Past studies have demonstrated that conjugated equine estrogen can improve bone quality as it increases the amount of Collagen I [41]. Other past studies have shown that estrogen plays a positive role in protecting glaucoma. Specifically, estrogen activates the synthesis of collagen and enhances the flexibility of the eyes, thereby reducing the intraocular pressure [42]. Past studies reported that local estrogen treatment increased the content of total collagen and cross-linked collagen in the menopausal rat model, which significantly stimulated the expression of collagen mRNA in a dose-dependent manner [35]. Based on the above findings, we attempted to evaluate the efficacy of estrogen for the treatment of SUI mice. The results revealed that both the LPP and ALPP of SUI mice treated with estrogen were higher than those of the control mice. Moreover, the urethra and anterior vaginal wall of SUI mice expressed higher levels of Collagen I, Collagen III, Elastin, TIMP-1, and TIMP-2 and lower levels of MMP-2 and MMP-9 relative to those of the control group mice. Our in vitro experiments revealed the similar outcomes, both the L929 fibroblasts after cyclic loading and the L929 fibroblasts without cyclic loading expressed higher levels of Collagen I, Collagen III, Elastin, TIMP-1, and TIMP-2 and lower levels of MMP-2 and MMP-9 in the presence of estrogen when compared with fibroblasts without estrogen treatment. These results together suggest that estrogen may alleviate the symptoms of SUI by reconstituting ECM.

The guidelines toward the diagnosis and treatment of female SUI (2017) in China indicated that vaginal local estrogen therapy can relieve some postmenopausal SUI symptoms and lower urinary tract symptoms. However, the reason why estrogen can be used to treat female SUI continues to remain unclear, and past studies have not been systematic in this regard. Although this study confirmed the effect of estrogen on ECM remodeling for the treatment of female SUI, the specific mechanism remains to be explored. The classic pathway of estrogen action is the estrogen receptor (ER) pathway. Previous studies have demonstrated that 17β-estradiol treatment in cells can activate ER [43]. The ER subtypes mainly include ERα and Erβ, with past studies which indicated that, in cardiac fibroblasts, 17β-estradiol has a sex-specific activation effect on ERα and Erβ [44]. Moreover, previous research demonstrated that 17-β estradiol can significantly reduce the stimulation of TGF-β on collagen synthesis as well as reduce the activation of the classic TGF-β/Smad2/3 signaling pathway in the skin fibroblasts [45]. Therefore, we speculated that estrogen may have other pathways to mediate ECM remodeling, planning to focus on the same in our future work.

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Author contributions LL: study design, data curation, formal analysis, and manuscript writing; SS: protocol development and data analysis; YL: cell experiment, data collection, and analysis; XL: mouse modeling, project administration, data collection, and analysis; JF: tissue sampling of mice and data collection; LY: molecular experiment, data collection, and analysis; LH: conceptualization, funding acquisition, and final approval.

Data availability statement The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors have declared that they have no conflict of interest.

Ethics approval All of our animal experimental protocols and implementations were conducted based on the institutional guidelines of the Institutional Review Committee and authorized by the Ethics Committee of the Institutional Animal Care and Use Committee of Renmin Hospital of Wuhan University (20210306).

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