Effect of integrin β1 in the treatment of stress urinary incontinence by electrical stimulation

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Abstract. The aim of the present study was to investigate the protective effect of integrin β1 in the treatment of stress urinary incontinence (SUI) by electrical stimulation, and the underlying mechanisms by which electrical stimulation regulates the collagen metabolism of female vaginal wall fibroblasts (FVWFs). FVWFs obtained from the vaginal wall tissue of patients with (Ingelman-Sundberg scale; grade II, n=8; grade III, n=10) or without (n=8) SUI during gynecological operations were isolated by enzymatic digestion and subsequently identified by immunocytochemistry. Following this, cultured FVWFs were treated with an inhibitor of integrin β1, recombinant human integrin β1 and electrical stimulation (100 mv/mm, 2 h, 20 Hz), followed by total mRNA and protein extraction. mRNA and protein expression levels of integrin β1, transforming growth factor (TGF)-β1 and collagen (COL) I and III in FVWFs were quantified by reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis respectively. Integrin β1, TGF-β1 and COL I and III expression levels were decreased in patients with SUI compared with healthy controls, and the grade III group had lower levels than the grade II group. Following electrical stimulation treatment, the expression levels of TGF-β1, COL I and III were enhanced in the grade II group, but not in the grade III group. Nevertheless, the inhibitor of integrin β1 reduced the protective effect of electrical stimulation in the grade II group. In addition, electrical stimulation combined with recombinant human integrin β1 could also protect cells from SUI in the grade III group. The present study provides evidence for the increased degradation of the extracellular matrix and integrin β1 in the vaginal wall tissues of patients with SUI, and the protective effect of electrical stimulation against SUI via integrin β1. These results provide a novel mechanism for the treatment of SUI using electrical stimulation.

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Introduction

Stress urinary incontinence (SUI) is defined as the involuntary leakage of urine during laughing, coughing, sneezing or physical exercise (1), and is the most common type of urinary incontinence affecting up to 35% of women (2). The predisposing risk factors include pregnancy, vaginal delivery, obesity, long-term high abdominal pressure and age (3). It is known to have adverse effects on quality of life in ~54.3% of all pregnant women in four aspects: Physical activity, travel, social relationships and emotional health (4). These aspects lead women to isolate themselves and to stop participating in the routine activities of daily living. In 1995 it was estimated that more than $16 billion was spent on SUI (5); this figure may take into account the costs of nursing home admissions, incontinence pads, medical and surgical treatment and time lost from work. With an increase in the incidence of SUI among middle-aged and elderly women, the social and economic burden of SUI may be more serious in a context of increased global life expectancy (6,7).

Currently, surgical and non-surgical strategies are used to treat SUI. The use of a midurethral sling (MUS) is the preferred surgical treatment for women with SUI (8). MUS is a one-time, effective and long-term treatment of SUI, but it carries a greater risk of complications (9). MUS is not suitable for patients with mild SUI. Treatment of SUI must balance the efficacy, adverse events and costs. Pelvic electrical stimulation (PES) is a conservative treatment strategy that can inhibit the parasympathetic motor nerve to relax the bladder and promote repeated pelvic floor muscle contraction to enhance muscle contraction while strengthening a full bladder (10,11). Numerous reports have confirmed that PES can treat SUI and relieve symptoms (12-15). A large clinical, randomized controlled study published by the UK National Health Service in 2016 showed that the cure rate for PES treatment combined with pelvic floor muscle exercises was 40% higher than that of simple pelvic floor exercises (14). However, the specific mechanism of action of PES in treating SUI remains unknown.

Previous studies have confirmed that extracellular matrix (ECM) remodeling is an important step in the pathogenesis of pelvic floor dysfunction (15,16). A large number of studies

suggest that integrin β1 is a key component of the cytoskeleton and is involved in the regulation of cell adhesion, migration, proliferation and apoptosis; however, to the best of our knowledge there is no research about the role of integrin β1 in the treatment of SUI by electrical stimulation. In this study, the mechanism of electrical stimulation in the treatment of SUI through integrin β1/transforming growth factor (TGF)-β/ECM was investigated, providing a theoretical basis for the prevention and treatment of SUI.

Materials and methods

Patient selection and tissue collection. All protocols were approved by the Ethics Committee of Renmin Hospital of Wuhan University. All samples were obtained from patients undergoing routine hysterectomy after obtaining signed informed consent forms. A total of 8 patients (age between 55 to 65) who underwent hysterectomy surgery (for reasons excluding the presence of malignant tumors and SUI) served as controls, and 18 patients (age between 55 to 65) who underwent gynecological surgery only for SUI (grade II and III) in the Obstetric and Gynecological Department of Renmin Hospital of Wuhan University between January 2017 and January 2018, were enrolled in the present study. SUI severity was classified using the Ingelman-Sundberg scale: Grade I urinary incontinence was characterized as being induced by coughing or sneezing, grade II urinary incontinence was induced by running or picking up an object from the floor and grade III urinary incontinence was induced by walking or climbing stairs (17). If a patient had overlapping symptoms, the higher grade was chosen as the severity of urinary incontinence. None of the women recruited had any connective tissue diseases, pathologically confirmed endometriosis, estrogen-associated ovarian tumors, pelvic inflammatory conditions, or advanced pelvic organ prolapse [greater than stage II by the Pelvic Organ Prolapse Quantification scale (18)]. Furthermore, these patients were free from any complications that may lead to diseases associated with collagen depletion, including diabetes and hyperthyroidism. Patients who had received any pelvic surgery or had a history of estrogen application within the past three months were excluded from the present study. All participants were postmenopausal. After informed consent was obtained, approximately 1 cm^2 of a full-thickness excision was made of the periurethral vaginal wall 1 cm lateral to the urethrovesical junction (identified by a Foley balloon) from patients undergoing surgery for SUI. Smaller 0.5-cm^2 biopsy samples of the vaginal wall from a similar area were excised in postmenopausal continent control subjects who underwent benign gynecologic surgeries for fibroids, dysfunctional bleeding and ovarian cysts. The epithelial layer was removed with a razor blade when the tissues were collected, after which primary cell culture was performed immediately. The remainder of the tissue was frozen immediately in liquid nitrogen and then stored at -80˚C for further processing.

Primary cell culture. A modified enzyme digestion method was used in the present study for the establishment of the primary cell culture, which was similar to previous research methods (19). The tissues were washed 3-5 times with PBS (HyClone; GE Healthcare Life Sciences) containing 1% double-antibiotic solution (100K U/ml penicillin G and 100 mg/ml streptomycin; Hangzhou Ginom Biomedical Technology Co., Ltd.) in order to clear the underlying blood, axungia and necrotic tissue. Tissues were then sectioned into 1 mm^3 fragments. Sections were digested with 1% collagenase I (Invitrogen; Thermo Fisher Scientific, Inc.) for 3 h at 37˚C in 5% CO_2, followed by further digestion with 0.25% trypsin (Sigma-Aldrich; Merck KGaA) for 5 min. Subsequently, 2 ml of FBS (Gibco; Thermo Fisher Scientific, Inc.) was added to stop digestion. DMEM (Hangzhou Ginom Biomedical Technology Co., Ltd.) supplemented with 15% FBS was slowly added to the culture flask. The culture medium was replaced every two days and FVWFs were cultured to 70% confluence for passage. The stable primary cells were obtained after ~15 days. The FVWFs were used at passages 4-8 for subsequent experiments.

Immunofluorescence staining. Vimentin is commonly used as a fibroblast marker to confirm the purity of cultured fibroblasts, as described in a previous study (19). After cells had migrated out of the vaginal wall tissue and become stable, the cells were applied to a glass slide. The cells were then fixed with 4% paraformaldehyde (Sinopharm Chemical Reagents Co., Ltd.) for 15 min at 4˚C and 0.5% Triton X-100 (Beyotime Institute of Biotechnology) for 20 min at 4˚C. After washing with PBS and blocking with 5% goat serum for 30 min at room temperature (cat. no. AR1009; Wuhan Boster Biological Technology, Ltd.), the cells were incubated with rabbit anti-vimentin (1:50; cat. no. SAB1305433; Sigma-Aldrich; Merck KGaA) and mouse anti-cytokeratin-19 (1:50; cat. no. ab7755; Abcam) overnight at 4˚C, followed by fluorescence-marked goat anti-rabbit IgG (FITC, 1:200; cat. no. BA1032; Wuhan Boster Biological Technology, Ltd.) and goat anti-mouse (Cy3, 1:200; cat. no. BA1101; Wuhan Boster Biological Technology, Ltd.) at room temperature for 1 h. DAPI (1 μg/ml, Beyotime Institute of Biotechnology) staining conducted for 5 min at room temperature was used to observe the nuclei. Finally, images (magnification, x100) were captured using a fluorescence microscope (Olympus BX53; Olympus Corporation).

Electrical stimulation and drug incubation. The model of cellular electrical stimulation was modified from Song *et al* (20). The cover glass (24x50 mm; thickness, 0.13-0.17 mm) was cut into two equal parts as a sealing strip. Dow Corning 4 (Dow Silicones Corp.) electrical insulating compound was applied under the sealing strip to form a rectangular area between the sealing strips that was used to inoculate cells. Then, DC4 was used to connect the sealing strip and the inner edge of the culture plate to block the free flow of the culture medium and to prepare the electric chamber (Fig. 1B). Agar salt bridges (2%) of 15 cm in length were used to connect Ag/AgCl electrodes in beakers of saturated KCl solution to the chamber of culture medium. Direct current was provided by a direct current power supply (Ever Prosperous Instruments, Inc.), which was connected with the Ag/AgCl electrodes in the beakers (Fig. 1A). The FVWFs were exposed to the electrical stimulation at 100 mV/mm and incubated in a 5% CO_2 incubator at 37˚C for 2 h. Cells without exposure to electrical stimulation served as controls. In order to inhibit integrin β1, anti-integrin β1 antibody (10 μg/ml; cat. no. ab24693; Abcam) was added to the FVWF culture medium both 2 h prior to

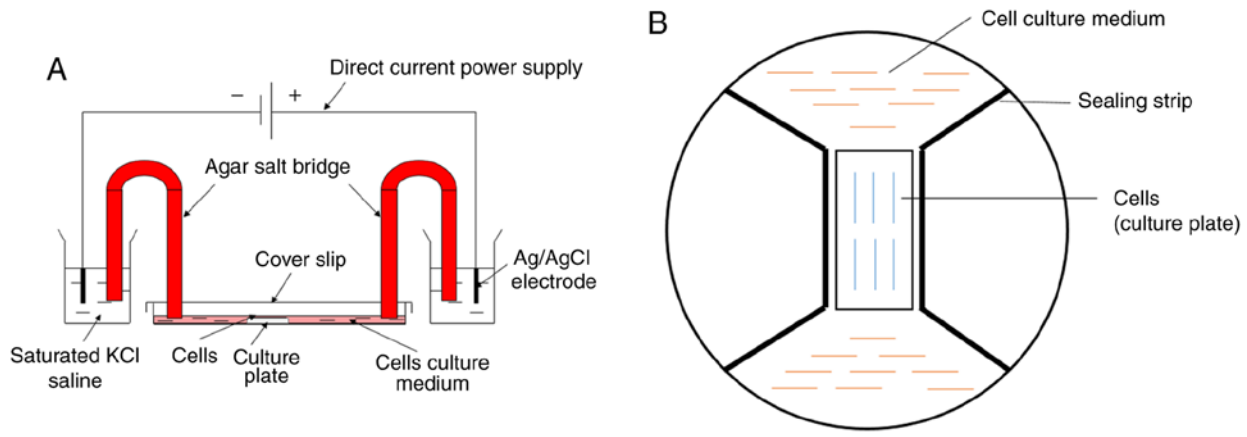

Figure 1. Electrical stimulation diagram. (A) Cell electrical stimulation apparatus. (B) Electric chamber.

and during the treatment with electrical stimulation in the electrostatic chamber. For the non-specific antibody control, Purified Mouse Monoclonal IgG 1 (10 μg/ml; cat. no. ab91353; Abcam) was used. In addition, recombinant human integrin β1 (0.1 μg/ml; cat. no. NBP2-59570; Novus Biologicals, LLC) was also used for incubation with FVWFs for 24 h in a 5% CO_2 cell incubator at 37˚C.

Western blot analysis. After exposure to electrical stimulation and/or the inhibitor, FVWFs were harvested using RIPA lysis buffer containing a proteinase inhibitor (cat. no. AR0102 and AR1178; Wuhan Boster Biological Technology, Ltd.) for 30 min on ice. Following this, total protein was extracted by centrifugation for 10 min at 4˚C and 12,000 x g, followed by protein concentration quantification with a bicinchoninic acid assay kit (cat. no. P0010; Beyotime Institute of Biotechnology). A total of 20 μg of total cellular protein was mixed with gel loading buffer, separated by 10% SDS-PAGE and transferred onto PVDF membrane (Merck KGaA). Membranes were blocked in 5% non-fat milk in TBS with 0.1% Tween 20 (TBS-T) for 1 h at room temperature and washed with TBS-T twice. Membranes were incubated with the following rabbit primary antibodies: Anti-integrin β1 (1:1,000; cat. no. 34971; Cell Signaling Technology, Inc.), anti-TGF-β1 (1:1,000; cat. no. ab92486; Abcam), anti- collagen (COL) I (1:1,000; cat. no. ab34710; Abcam) and anti-COL III (1:1,000; cat. no. ab7778; Abcam), overnight at 4˚C, followed by incubation with goat anti-rabbit fluorescence-labeled secondary antibodies (1:10,000; IRDye700 and IRDye800; cat. no. 926-32211; LI-COR Biosciences) for 1 h at 37˚C after washing. A rabbit anti-GAPDH primary antibody (1:2,000; cat. no. ab9485; Abcam) served as an internal reference control. The reactive bands were detected with an Odyssey® infrared imaging system (LI-COR Biosciences). For each sample, the band densities were quantified using LI-COR Odyssey v.3.0 software (ODY-0102; LI-COR Biosciences) were normalized against that of GAPDH and the data was obtained from three experiments.

Reverse transcription-quantitative (RT-q) PCR. The mRNA expression levels of integrin β1, TGF-β1, and COL I and III in cells were evaluated by RT-qPCR after electrical stimulation or/and drug incubation. The primers used for amplification were purchased from Beijing SBS Genetech Co., Ltd. Total RNA from FVWFs was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), following the manufacturer's protocol. RNA (2 μg) was reverse transcribed to cDNA (n=3; temperature protocol: 25˚C for 5 min, 42˚C for 30 min and 85˚C for 5 min) using a Revert Aid First Strand cDNA Synthesis kit (cat. no. k1622; Thermo Fisher Scientific, Inc.) and reaction mixture aliquots (1 μl) were used as templates for PCR. Primer sequences for TIMP-1, MMP-2, MMP-9, COL I and III, and GAPDH were purchased from SBS Genetech Co., Ltd. (Table I). qPCR was performed using SYBR® Premix Ex Taq™ reagent (cat. no. DRR041; Takara Bio, Inc.) and an Applied Biosystems 7500 Real-Time system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were: 40 cycles of initial denaturation, 95˚C for 5 min; denaturation, 95˚C for 10 sec; anneal, 55˚C for 20 sec; and extension, 72˚C for 20 sec. Normalized quantification cycle (Cq) values were used for comparison (21). Each sample was analyzed in triplicate.

Statistical analysis. Data are presented as the mean ± SD for each group and were analyzed by one-way ANOVA using GraphPad Prism 5.0 (GraphPad Software, Inc.). Multiple means were compared by Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of FVWFs. A previous study by the authors accurately identified stable fibroblasts by positive staining of vimentin and negative staining of cytokeratin (22) (Fig. 2A-D). As observed by light microscopy, the cells had long spindles contacting each other to form a network structure with a similar behavior to fibroblasts (Fig. 2E).

Response of FVWFs to electrical stimulation. To verify the protective effect of electrical stimulation on FVWFs, protein and mRNA expression of TGF-β1, COL I and III were analyzed before and after electrical stimulation. When compared to the control group, the protein and mRNA expression of TGF-β1, COL I and III in the grade II and grade III groups decreased

Table I. Primer sequences for reverse transcription-quantitative PCR.

Gene	Species	Forward primer; 5'-3'	Reverse primer; 5'-3'
GAPDH	Human	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
Integrin β1	Human	GCCTGTTTACAAGGAGCTGAA	CTGACAATTTGCCGTTTTCC
TGF-β1	Human	ACCTGAACCCGTGTTGCTCT	CTAAGGCGAAA GCCCTCAAT
COL I	Human	CAAGACGAAGACATCCCACCAATC	ACAGATCACGTCATCGCACAACA
COL III	Human	TCGCTCTGCTTCATCCCACTAT	CTTCCAGACATCTCTATCCGCAT

TGF-β1, transforming growth factor-β1; COL, collagen.

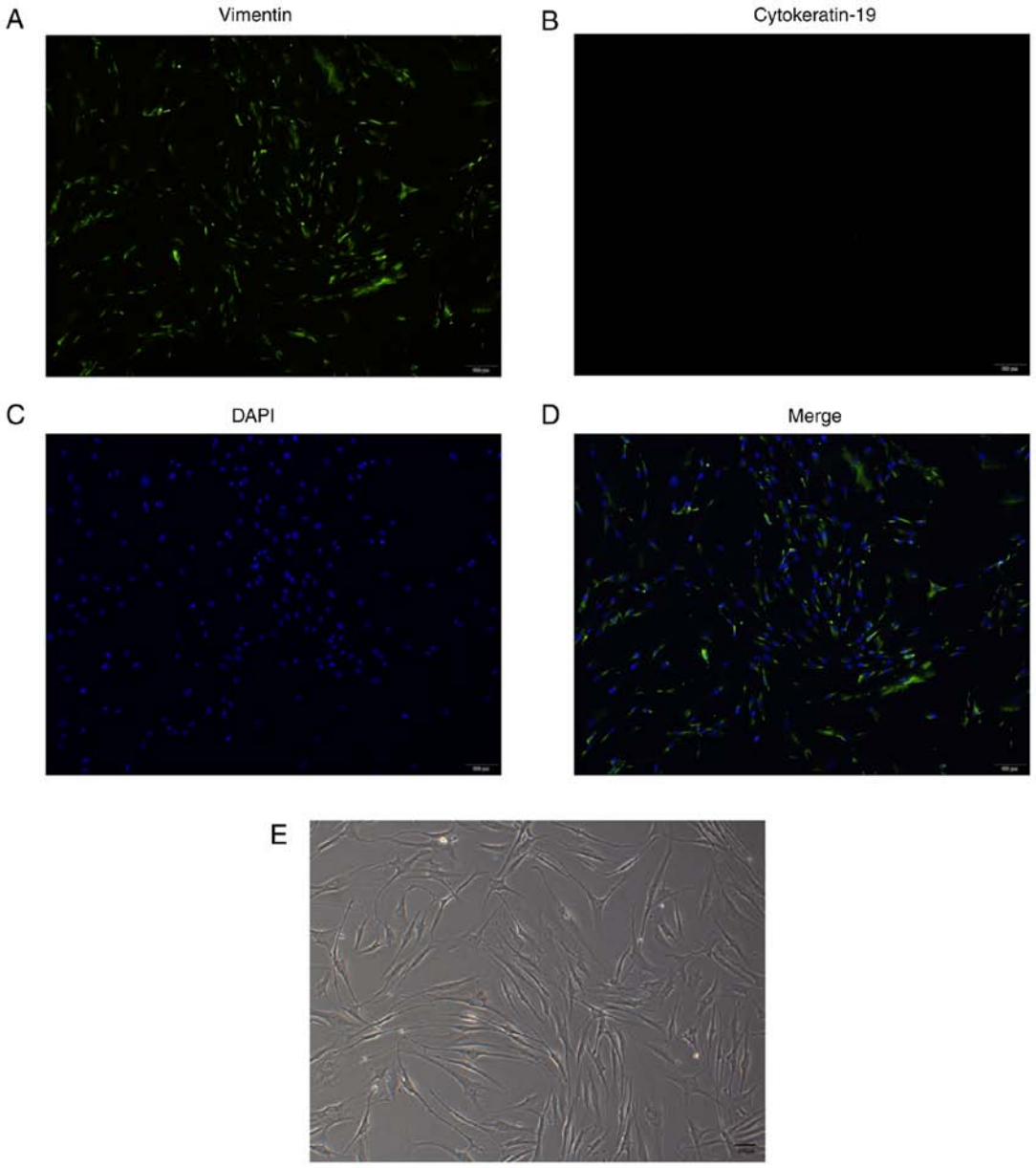

Figure 2. Identification of FVWFs. Immunohistochemical staining for (A) vimentin, (B) cytokeratin 19, (C) DAPI and (D) merged in cultured FVWFs (magnification, x100). (E) Primary FWVF visualized by light microscopy. FVWF, female vaginal wall fibroblasts.

significantly, with the reduction being more severe in the grade III group (Fig. 3). The results showed that the protein expression levels of TGF-β1, COL I and III were increased in the grade II group after electrical stimulation. Therefore, electrical stimulation could reverse the reduction of TGF-β1, COL I and III at the mRNA and protein level in the grade II

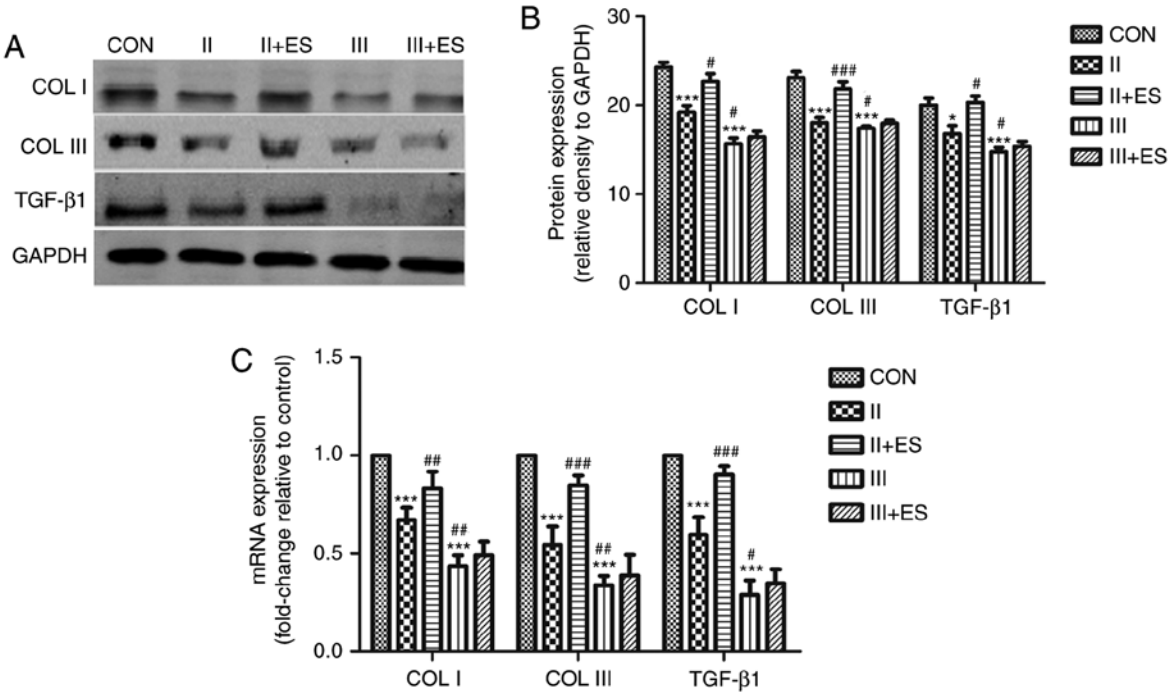

Figure 3. Protein and mRNA expression of TGF-β1, COL I and III before and after electrical stimulation. (A and B) Western blotting and (C) reverse transcription quantitative PCR analysis was used to measure the different expression levels of TGF-β1, COL I and III in five groups. Data are presented as the mean ± SD (n=5). *P<0.05 and ***P<0.001 vs. respective CON; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. respective group II. TGF-β1, transforming growth factor-β1; COL, collagen; CON, control group without SUI; II, grade II group; III, grade III group; ES, electrical stimulation.

group. However, the protein and mRNA expression levels of TGF-β1, COL I and III were not changed by electrical stimulation in the grade III group. This is consistent with the fact that electrical stimulation is an effective treatment strategy for mild and moderate SUI, but not for severe SUI.

Integrin β1 plays an important role in the treatment of SUI by electrical stimulation. Integrins are cell surface receptors with diverse functions that allow cells to bind to and respond to each other and to the ECM (23). Integrins also contribute to the control of cell proliferation, shape and motility (24). Integrin β1 plays an important role in the activation of downstream signals that regulate several cellular mechanisms. Previously, studies found that integrin β1 and TGF-β1 are involved in a variety of diseases, including fibrosis. TGF-β1 can induce the expression of integrin β1 and the activation of integrin β1 can enhance the effect of TGF-β1-mediated collagen synthesis (25,26). In a study investigating the central nervous system, oligodendrocyte progenitor cells were exposed to physiological levels of electrical stimulation and were found to display a marked electrotactic response that was dependent on integrin β1 (27). In the present study, it was found that the levels of integrin β1 were lower in the grade II and grade III groups compared to the control group, while the grade III group displayed a more marked reduction than that in the grade II group (Fig. 4A). Therefore, a link between integrin β1 and the electrical stimulation-guided synthesis of the ECM was examined. It was found that inhibiting integrin β1 led to a reduction in the protein levels of TGF-β1, COL I and III, with a reduction at the mRNA level also observed in the grade II group (Fig. 4B). The protective effect of electrical stimulation was suppressed by the inhibitor of integrin β1. These results show that integrin β1 is important for the electrical stimulation-guided synthesis of the ECM. To further investigate the role of integrin β1, recombinant human integrin β1 was incubated with FVWFs from the grade III group. A protective effect of electrical stimulation in the grade III group was observed. After incubation with recombinant human integrin β1 followed by electrical stimulation, the FVWFs of the grade III group showed a significant increase in the expression of TGF-β1, COL I and III (Fig. 4C).

Discussion

Electrical stimulation is a versatile treatment with a poorly understood therapeutic mechanism. Pelvic floor electrical stimulation is one of the first-line conservative treatments for female urinary incontinence. The effects of electrical stimulation on collagen metabolism have been extensively studied in many fields, including wound healing (28,29), rehabilitation of osteoarthritis and fractures (30), and periodontal tissue remodeling (31). Furthermore, studies reported that electrical stimulation could upregulate the expression of collagen (15,32). In the present study, the importance of integrin β1 in the treatment of SUI via TGF-β1/collagen in electrical stimulation was demonstrated.

The pathophysiology of SUI involves defects in the supporting tissues, including the suburethral vaginal wall, pelvic floor muscles, fascia and pubo-urethral ligaments (33). The connective tissue linking these structures is also an important factor (34). The pelvic supporting tissues are composed primarily of connective tissue in which collagen and elastic fibers are the predominant ECM components. Altered collagen and elastin metabolism has been documented in tissues from women with SUI (35). Collagen provides strength and stability

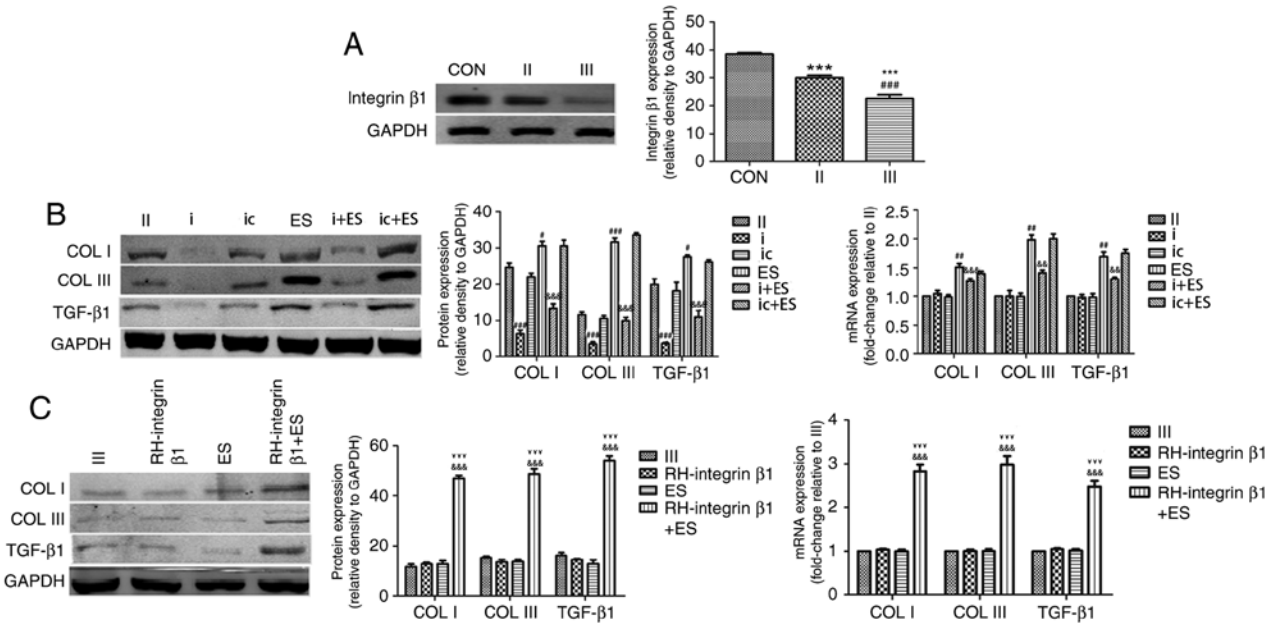

Figure 4. Effect of integrin β1 in the treatment of SUI by electrical stimulation. (A) Western blot analysis was used to detect the different expression levels of integrin β1 in three groups. (B) The proteins and mRNA levels of TGF-β1, COL I and III were measured after incubation with the inhibitor of integrin β1 and/or electrical stimulation in the grade II group. (C) After incubation with recombinant human integrin β1 and/or electrical stimulation in the grade III group, the expression levels of TGF-β1, COL I and III were analyzed. ***P<0.001 vs. CON, $^{\#}$P<0.05 $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. group II., $^{\&\&}$P<0.01 and $^{\&\&\&}$P<0.001 vs. ES. $^{\forall\forall\forall}$P<0.001 vs. group III. TGF-β1, transforming growth factor-β1; COL I and III, collagen I and III; CON, control group without SUI; SUI, stress urinary incontinence; II, grade II group; III, grade III group; i, inhibitor (anti-integrin β1 antibody); ic, inhibitor control (non-specific antibody control); ES, electrical stimulation; RH-integrin β1, recombinant human integrin β1.

to the supporting tissue. Therefore, the collagens in pelvic tissue, COL I and III, provide powerful tension and strength for the pelvic tissue. In addition, fibroblasts serve a pivotal role, particularly in pelvic connective tissue, ECM remodeling and mechanical force resistance, by regulating the balance between collagen synthesis and degradation following tissue injury (36,37). Therefore, the present study selected FVWFs to examine the phenotype of COL I and III. In this study, ECM metabolism and associated molecules including TGF-β1, COL I and III were altered in the FVWFs of SUI patients compared to patients without SUI. Loss of the ECM leads to the dysfunction of attachments and support of pelvic connective tissue (38,39). In addition, a previous study demonstrated that the expression of COL I and III are decreased in SUI in the periurethral vaginal wall tissue (16).

TGF-β1 is a key factor in ECM metabolism and is involved in the pathomechanism of SUI (40,41). Low expression of TGF-β1 is closely related to the occurrence of retrograde venereal degeneration of pelvic support structures such as in SUI (42). A recent study reported that the TGF-β1 pathway is activated and the expression of COL I and III are significantly decreased in rats with SUI (40). Integrins were originally named to denote their role as integral membrane complexes linking the ECM to the actin cytoskeleton. However, it is now clear that integrins alone or in combination with other cell surface receptors mediate many key intracellular signals and are important for survival. Integrins exist in several activation states on the cell surface, where activation induces integrin clustering that leads to the recruitment of multiple signaling molecules and the regulation of different signaling pathways (43). The regulatory function of integrin can be achieved in several ways, including ligand engagement and binding of intracellular proteins. Evidence indicates that there is extensive crosstalk between integrins and TGF-β. Wang *et al* (40) demonstrated that a subset of integrins is responsible for a large proportion of TGF-β activation in the epithelial-mesenchymal trophic unit. In addition, it has been reported that blocking integrin β1 function inhibits TGF-β-mediated mitogen activated protein kinase 1/p38 activation and epithelial to mesenchymal trans-differentiation progression. Therefore, integrin β1 can affect the activation and expression of TGF-β1. In a study by Li *et al* (41), inhibition of TGF-β1 signaling by SB-431542 blocked electrical stimulation-driven condensation and significantly suppressed electrical stimulation-driven increases in COL II A1, aggrecan, and SOX9 expression in mesenchymal stem cells (40-44). The present study showed that electrical stimulation with decreased integrin β1 could not upregulate the expression of TGF-β1 in the grade III group. However, the expression levels of TGF-β1, COL I and III were enhanced by electrical stimulation after adding recombinant human integrin β1. Furthermore, electrical stimulation had no effect on the ECM when integrin β1 was inhibited by an antibody in the grade II group. These data suggest that the upregulation of TGF-β1 and the ECM by electrical stimulation may be dependent on integrin β1.

Electrical stimulation not only induces a directed accumulation of the integrin β1 subunit in human retinal pigment epithelial cells, but also upregulates its mRNA and protein levels (45). Furthermore, integrin-mediated TGF-β activity could promote enhanced collagen deposition in epithelial cells (46). In the present study, electrical stimulation reversed the reduction of the ECM in the grade II group. However, this was only effective when electrical stimulation was combined with recombinant

human integrin β1 in the grade III group. Endogenous integrin β1 alone is not sufficient to improve the expression of the ECM under conditions of electrical stimulation. These data suggest that the lack of integrin β1 may be a reason why electrical stimulation is ineffective at treating severe SUI.

In conclusion, it has been shown that electrical stimulation can improve collagen expression when integrin β1 levels are sufficient via the integrin β1/TGF-β1 pathway, which may play an important role in the treatment of SUI by electrical stimulation. This work offers a mechanistic insight into SUI that may facilitate therapeutic intervention and contribute to the efficient prevention and treatment of SUI.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

YL was involved in project development, data collection, analysis and manuscript writing. BSL, CL, SSH and JM were involved with study development, supervision, manuscript editing. MH, JMT and STL were involved with manuscript editing and data analysis. TTW and HXZ were involved with project development, data collection and analysis. LH was involved with project development, manuscript editing and supervision.

Ethics approval and consent to participate

The study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Renmin Hospital of Wuhan University. The participants provided written informed consent to participate in this study.

Patient consent for publication

Consent for publication was obtained from all participants.

Competing interests

The authors declare that they have no competing interests.

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