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Electrical stimulation activates calpain 2 and subsequently upregulates collagens via the integrin β1/TGF-β1 signaling pathway

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ABSTRACT

Stress urinary incontinence (SUI) is a public health issue attributed to weakened pelvic supporting tissues. Electrical stimulation (ES) is one of the first-line conservative treatments for SUI. However, the underlying mechanism of ES in the treatment of SUI is not clear. Here, we show that ES suppresses cell apoptosis and upregulates collagen expression by functioning as a cell growth inducer to activate the calpain 2/talin 1/integrin β1/transforming growth factor (TGF)-β1 axis. Specifically, ES promoted Ca^{2+} to flow into the cytoplasm through the calcium channel, Cav 3.2, thereby activating calpain 2. Then, the activated calpain 2 cleaved talin 1, which induced the activation of integrin β1 and upregulated the TGF-β1-mediated transcription of collagen I and III. Notably, blocking Cav 3.2 suppressed calcium influx and inhibited the activation of downstream proteins. Furthermore, the knockdown of calpain 2 resulted in the reduction of cleaved talin 1, and the shRNA-integrin β1 treatment downregulated the level of activated integrin β1 and the expression of TGF-β1-induced collagen I and III. An association of the ES-modulated collagen I and III upregulation with the therapeutic effect of the ES-Ca^{2+}/calpain 2/talin 1/integrin β1/TGF-β1 axis was demonstrated in mouse fibroblast and mouse SUI models established through vaginal distension (VD). This outcome provides insight into clinical diagnosis and treatment.

1. Introduction

Stress urinary incontinence (SUI) is involuntary urine leakage that occurs when abdominal pressure is raised, such as during a sneeze, cough, laugh or exercise, and affects up to 35% of women [1]. Urodynamic examination showed that when the pressure during bladder filling was measured, involuntary urine leakage occurred when abdominal pressure increased without detrusor contraction. SUI is the most common type of urinary incontinence (UI). Vaginal delivery, age and obesity are the predisposing risk factors of SUI [2]. SUI has many negative effects on four aspects of the quality of life of approximately 54.3% of pregnant women, including physical activity, travel, social relationships and emotional health [3], making it difficult for some of these pregnant women to socialize and increasing the likelihood that they will become withdrawn. SUI has become a common problem that significantly impacts the quality of life (QOL), and it is a public health issue due to its high prevalence.

There arevarious treatments for SUI, including surgical treatment and conservative treatments. Surgery has a long-term and positive effect on most patients with SUI. However, patient trauma and the risks of postoperative dysuria, increased urination urgency and organ injury make surgery unsuitable for some mild and moderate SUI patients. Therefore, conservative treatments play an important role in some mild to moderate SUI patients. Pelvic electrical stimulation (PES), one of the first-line conservative treatments for female urinary incontinence, could inhibit the parasympathetic motor nerve to relax the bladder and promote repeated pelvic floor muscle contraction to enhance muscle contraction ability and to strengthen the bladder[4,5]. Numerous studies have suggested that PES can cure SUI and alleviate symptoms [6–8]. In a clinical project involving 207 patients with SUI, intravaginal electrical stimulation showed a cure rate of 65.7%, and the QOL of patients improved notably [9]. Furthermore, in a single-blind randomized control trial, electrical stimulation was an effective treatment for patients with urodynamic SUI, with greatly improved outcomes compared with no treatment [10].

Although numerous studies have strongly suggested that ES is effective in treating SUI, the underlying mechanism of ES in the treatment of SUI is not clear. Here, our current study aimed to confirm that PES

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could enhance the synthesis of collagens through the Ca^{2+}/calpain 2/talin 1/integrin β1/TGF-β1 axis to cure SUI.

2. Materials and methods

2.1. Animals and management

Our study was approved by the Institutional Animal Care and Ethics Committee of the Renmin Hospital of Wuhan University (20140305) and followed the institutional guidelines of the Institutional Review Board. Eight- to 10-week-old virgin female C57BL/6 mice were selected for this study and were provided by the Experimental Animal Center of the Renmin Hospital of Wuhan University. The Cav $3.2^{-/-}$ mice were obtained from Jackson Laboratory (stock number 013707). There were a total of 6 groups, the noninstrumented control (NC) groups (WT-NC and KO-NC), the vaginal distension (VD) groups (WT-VD and KO-VD) and the VD + PES groups (WT-VD + PES and KO-VD + PES), with 15 mice per group. As described in our previous study[11,12], the SUI mice were established using the vaginal distension method that imitates human vaginal birth. Seven days after vaginal distention, suprapubic tube implantations were performed, and then leak point pressure (LPP) and maximum cystometric capacity (MCC) were measured to identify the success of the SUI model. Finally, the anterior vaginal tissues were harvested after sacrificing the mice for the following experiments.

2.2. Pelvic electrical stimulation (PES)

In the PES groups, one day after VD, PES was administered to the mouse vagina for 15 min per day for 7 days at a frequency of 50 Hz. The electrodes were connected to Power lab 35 (AD Instruments – Australia) that delivered 2 mA isolation pulses with individual pulse duration of 0.2-millisecond (ms) (Fig. 1c, d), as described in our previous study [12].

2.3. Cells electrical stimulation (ES)

The model of cellular electrical stimulation used a modified method based on the research of Song [13]. The cover glass (24 mm × 50 mm, thickness 0.13–0.17 mm) was cut into two equal parts as a sealing strip. Dow Corning 4 (DC4, Dow Corning, Midland, Mich., USA) electrical insulating compound was daubed under the sealing strip to form a rectangular area between the sealing strips, which 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%) 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 Instrument, New Taipei City, Taiwan), which was connected to the Ag/AgCl electrodes in the beakers (Fig. 1A). L929 cells, mouse fibroblasts, were exposed to the direct current at 100 mV/mm and incubated in a 5% CO_2 incubator at 37 °C for 2 h. The control cells were not exposed to direct current. To inhibit the calcium channel, cells were incubated with mibefradil (T-type calcium channel inhibitor, 2.5 μM) for the 2 h prior to and during the ES treatment.

2.4. Cell culture and transduction

L929, a kind of mouse fibroblast cell, was purchased from China Center for Type Culture Collection (Wuhan) and cultured in RPMI 1640 (Genom Biotech Ltd., Hangzhou, China) supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products, California, USA) and 1% antibiotics (100 KU/ml penicillin G and 100 mg/ml streptomycin; Genom Biotech Ltd.) at 37 °C with 5% CO_2. The culture medium was replaced with fresh complete medium every two days, and the cells were cultured to 70% confluence before each passage.

Cells with 70% confluence were suspended at a density of 3–5 * 10^4 cells/ml, and 2 ml of the suspension was inoculated into 6-well plates. After the cells adhered to the wall, HitransG (40 μl), a kind of infection reagent, and lentivirus were added into FBS-free culture medium for

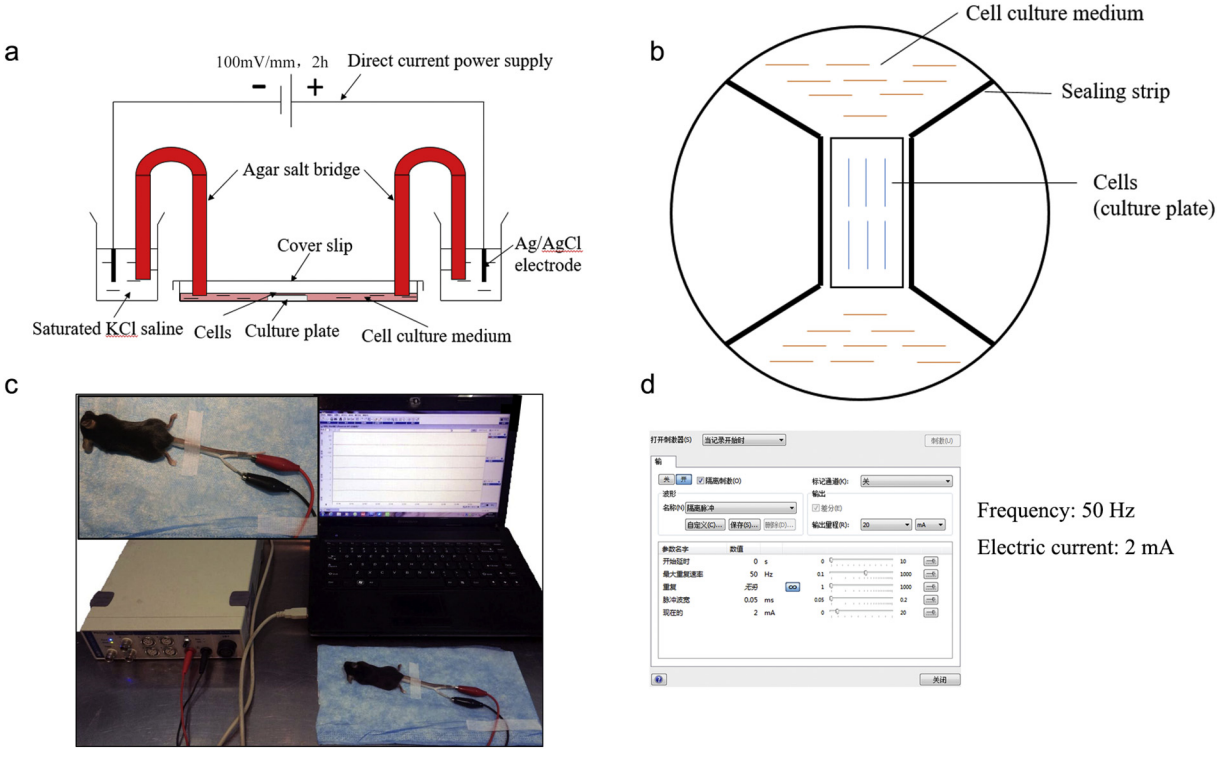

Fig. 1. Electrical stimulation diagram. a, Cell electrical stimulation apparatus. b, Electric chamber. c, Mouse pelvic electrical stimulation. d, The parameters of PES.

12 h and then replaced by complete medium. At 72 h post-transduction, the efficiency of infection was detected, and the cells were used for subsequent experiments. shRNAs specific to the mouse integrin β1 and calpain 2 gene (gene silencing) were constructed and purchased from Shanghai GeneChem Co., Ltd. (Shanghai, China). Equal amounts of lenti-integrin β1 (shRNA sequence: gcACGATGTGATGATTTAGAA) and calpain 2-shRNA (shRNA sequence: gcGGTCAGATACCTTCATTAA) or lenti-control-shRNA (as a negative control) at a multiplicity of infection (MOI) of 50 were transduced into L929 cells once they reached 20–30% confluence. In addition, a lentiviral vector (LV-Itgb1; Genechem, Shanghai, China) was used to establish a stably transfected L929 cell line expressing integrin β1 (integrin β1 overexpressing) at MOI 40.

2.5. Cell proliferation assay

The proliferation of L929 following PES was detected using Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Inc., Shanghai, China). Cells were digested from culture plate and seeded into 96-well plates after PES and cultured for 8 h. Next, the culture medium was removed and replaced with 110 μl mixture contained 100 μl of fresh medium and 10 μl of CCK-8 reagent. After 1 h, the absorbance was measured at wavelength of 450 nm using a spectrophotometric microplate reader. Each group was repeated in 5 wells.

2.6. Intracellular calcium detection

Cells were seeded into 6-well cell culture plates with a cover glass in each well. When the cell density reached 70%, cells were incubated with mibefradil or Ca^{2+}-free culture medium (HBSS) before and during ES, then removed and washed with Hank's Balanced Salt Solution (HBSS, without phenolsulfonphthalein, Procell Life Science & Technology Co., Ltd. Wuhan, China) 3 times, followed by incubation with Fluo 3-AM (2.5 mM, Sigma-Aldrich, Merck KGaA.) for 45 min at 37 °C with 5% CO_2. Then, the cells were incubated with HBSS for 20 min after washing. The stained cells were observed with fluorescence microscopy at an excitation of 488 nm and an emission of 525 nm.

2.7. Western blotting

Total protein was isolated using RIPA Lysis Buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, Servicebio, Wuhan, China) with phenylmethanesulfonyl fluoride (PMSF, 1 mM, Servicebio, Wuhan, China), and the protein concentration was detected using a BCA protein assay kit (Beyotime Institute of Biotechnology, Haimen, China). Protein samples (20 μg) were mixed with loading buffer separated by SDS-PAGE (10%) and transferred to polyvinylidene fluoride membranes (Merck KGaA). Then, the protein membranes were blocked with skim milk for 1 h at room temperature and incubated with rabbit primary antibodies overnight at 4 °C. Anti-calpain 2 (1:1000, catalog no. NBP2–15675) was purchased from Novus Biologicals (USA). Anti-talin 1 (1:1000, catalog no. 4021 s) and anti-integrin β1 (1:1000, catalog no. 34971 s) were purchased from Cell Signaling Technology (USA). Collagen I (1:2000, catalog no. ab34710) and collagen III (1:2000, catalog no. ab7778) were purchased from Abcam (USA). The primary antibody incubation was followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:3000, Servicebio, Wuhan, China) for 1 h at room temperature after washing. A rabbit anti-GAPDH primary antibody (1:2500, catalog no. ab9485, Abcam) served as an internal reference control. Then, the immunoreactive bands were treated with an enhanced chemiluminescent substrate (Pierce™ Fast Western Blot Kit, catalog no. 35055, Thermo Scientific, Waltham, MA, USA) and analyzed using the ChemiDoc™ Imaging System (Bio-Rad Laboratories, Inc., California, USA).

2.8. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and then reverse-transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (catalog no. k1622; Thermo Fisher Scientific, Inc.). The primers for calpain 2, talin 1, integrin β1, TGF-β1, GAPDH, collagen I and collagen III were obtained from SBS Genetech Co., Ltd. Primer sequences are listed in Table S2. The SYBR® Premix Ex Taq™ reagent was used for qRT-PCR purchased from Takara Bio, Inc., and an Applied Biosystems 7500 Real-Time system (Applied Biosystems, Thermo Fisher Scientific, Inc.) was used for analysis. The relative mRNA levels were calculated by the $2^{-\triangle\triangle Ct}$ method, and GAPDH was used as the internal control. The results are shown as the fold change.

2.9. Immunofluorescence

Cells were seeded into 6-well cell culture plates with a cover glass in each well. When the cell density reached 70%, cells were removed and treated with PES or left untreated. After washing 3 times with PBS, cells were fixed with 4% formaldehyde for 15 min, permeabilized with 0.5% Triton X-100 for 5 min, and then blocked with 5% goat serum for 30 min. Then, the cells were incubated with primary antibodies overnight at 4 °C, washed, and further incubated with FITC-conjugated goat anti-rabbit IgG (1:200, Servicebio, Wuhan, China). The primary antibodies were rabbit anti-calpain 2 (1:100), anti-talin 1 (1:50), anti-integrin β1 (1:100), collagen I (1:200) and collagen III (1:100). The cell nuclei were stained using 4′,6′-diamidino-2-phenylindole (DAPI, ready-to-use, Servicebio, Wuhan, China). Images were collected by fluorescence microscopy and analyzed by ImageJ 1.48r software (NIH, Bethesda, MD, USA).

2.10. Immunohistochemistry

The paraffin sections of the mouse anterior vaginal wall-urethra tissue samples were baked in an oven at 60 °C for 1 h and the wax was removed with water, xylene and different concentrations of ethanol (100%/90%/80%70%). Then, antigen retrieval was performed with microwave or citrate antigen retrieval solution. After blocking with 1% bull serum albumin (BSA), the tissue sections were incubated with anti-calpain 2 (1:100), anti-talin 1 (1:50), anti-integrin β1 (1:100), collagen I (1:100) and collagen III (1 μg/ml) overnight at 4 °C. Then, the tissue was incubated with the secondary antibody (Maxim Biotechnologies, Fuzhou, China) labeled with biotin for 1 h at room temperature after washing 3 times with PBS and stained by a DAB kit (Maxim Biotechnologies, Fuzhou, China). The nuclei were stained with hematoxylin for 5 min at room temperature. After dehydration to transparency, the tissue sections were sealed with neutral gum. Images were captured with light microscopy.

2.11. Masson's trichrome staining

A subset of the specimens were embedded in paraffin, cut into 4 μm transverse sections and stained with Masson's trichrome staining (Masson Kit HT15, Sigma, USA) according to the standard protocol. The MOD (mean optical density) value collected by ImageJ was selected as the quantitative index to evaluate the content of collagen fibers.

2.12. Statistical analysis

All data are expressed as the mean ± SD. Statistical analysis was performed using SPSS version 16.0 software (SPSS Inc., Chicago, IL, USA). Normality of distribution was assessed using the Kolmogorov–Smirnov test. Unpaired Student's *t*-test or Mann–Whitney *U* test was used to compare two groups, while one-way analysis of variance followed by Bonferroni post hoc test was used to compare

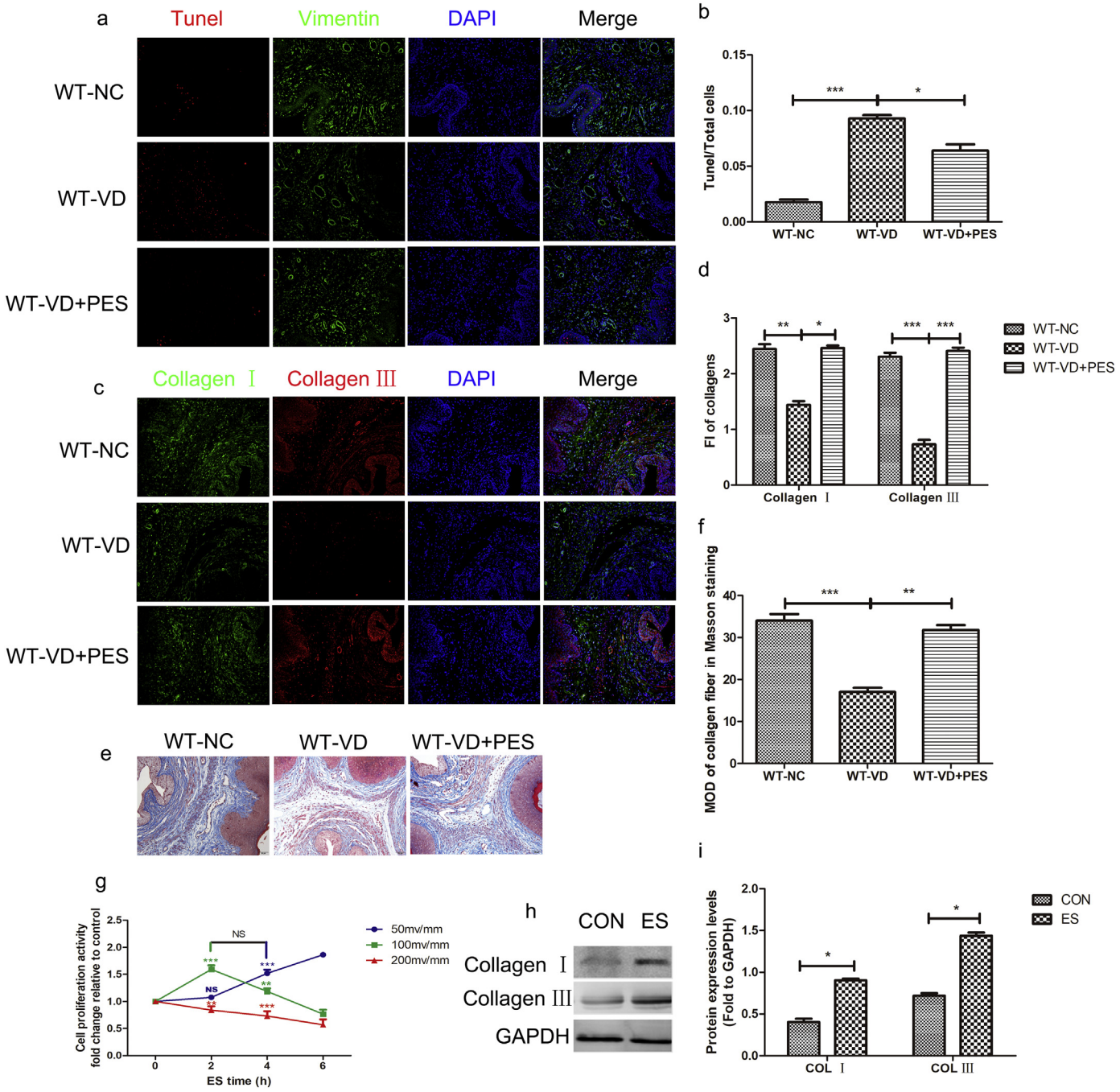

Fig. 2. Electrical stimulation upregulates collagen expression and exhibits a cell proliferation-promoting effect. a, Tunel staining of anterior vaginal tissues, magnification: ×200; b, The ratio of Tunel-positive cells to the total number of cells; c, Collagen I and III immunofluorescence staining of anterior vaginal tissues, magnification: ×200; d, Fluorescence intensity of collagen I and III; e, Masson's trichrome staining of anterior vaginal tissues, magnification: ×200; f, The MODs of collagen fibers after Masson's trichrome staining; g, Cell proliferation activity after electrical stimulation under different conditions; h and i, Collagen I and III protein expression in L929 cells after electrical stimulation at 100 mV/mm for 2 h. * represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$; NS represents no significance, every experiment was repeated for 3 times. (WT-NC: wild type normal control mice; WT-VD: wild type mice treated with vaginal distension; WT-VD + PES: wild type mice treated with vaginal distension combined with pelvic electrical stimulation; CON: normal L929 cells; ES: normal L929 cells treated with electrical stimulation).

groups. *P*-values of < 0.05 were considered statistically significant.

3. Results

3.1. Electrical stimulation potently induces expression of collagens and suppresses cell apoptosis

We observed that the anterior vaginal tissues of WT-VD group mice presented an increased rate of cell apoptosis and decreased expression of collagen I and III compared to the tissues of normal mice. However, PES suppressed cell apoptosis and upregulated the levels of collagen I and III (Fig. 2a–d). Notably, Masson's trichrome staining showed that the collagen fibers were long, well organized, and abundant in the normal mice, but they were fragmented, sparse, and disordered in the VD group. Importantly, PES potently reversed the reduction in collagen fibers and improved the structural damage of collagen fibers (Fig. 2e, f).

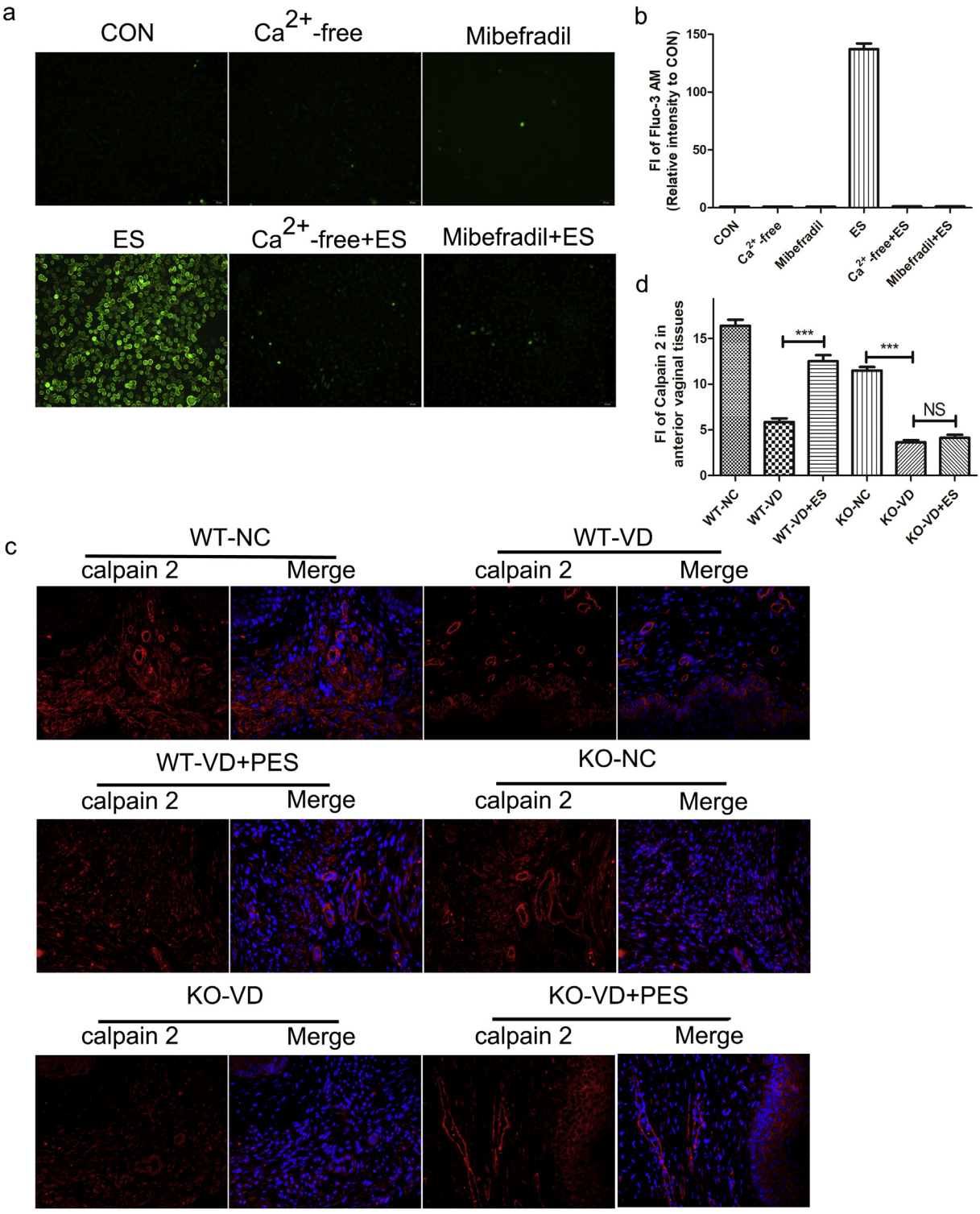

Fig. 3. Electrical stimulation activates calpain 2 via increasing intracellular Ca^{2+}. a, Fluorescence staining of Ca^{2+}, magnification: ×200; b, The green fluorescence intensity of fluo-3; c, Calpain 2 immunofluorescence staining of anterior vaginal tissues, magnification: ×200; d, Fluorescence intensity of calpain 2. *** represents $p < 0.001$, NS represents no significance, every experiment was repeated for 3 times. (WT-NC: wild type normal control mice; WT-VD: wild type mice treated with vaginal distension; WT-VD + PES: wild type mice treated with vaginal distension combined with pelvic electrical stimulation; KO-NC: noninstrumented control group of Cav 3.2 knockout mice; KO-VD: Cav 3.2 knockout mice treated with vaginal distension; KO-VD + PES: Cav 3.2 knockout mice treated with vaginal distension combined with pelvic electrical stimulation; CON: normal L929 cells; ES: normal L929 cells treated with electrical stimulation; Ca^{2+}-free: normal L929 cells treated with Ca^{2+}-free medium; Mibefradil: normal L929 cells treated with mibefradil; Ca^{2+}-free + ES: L929 cells treated with Ca^{2+}-free medium combined with ES; Mibefradil + ES: L929 cells treated with mibefradil combined with ES). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Furthermore, in cell experiments, the appropriate intensity of ES could improve the proliferation activity of fibroblasts, and we chose 100 mv/mm for 2 h as the ES condition for the subsequent experiment (Fig. 2g). In addition, collagen I and III showed a dramatic structural improvement after ES in fibroblasts (Fig. 2h, i). These data suggest that electrical stimulation potently upregulates the expression of collagen I and III and improves cell proliferation activity.

3.2. Electrical stimulation activates calpain 2 by increasing intracellular calcium concentration

We then sought to identify the signaling molecule(s) involved in the ES-induced upregulation of collagens. Following ES treatment, the intracellular Ca^{2+} concentration of L929 was increased significantly. However, ES could not increase the intracellular calcium levels when the cells were exposed to Ca^{2+}-free medium or mibefradil (Fig. 3a, b). We further found that the anterior vaginal tissues of SUI mice, which were composed mainly of fibroblasts, had lower calpain 2 activity than the tissues of wild type normal mice and that PES enhanced calpain 2 activity in SUI mice. Interestingly, in Cav 3.2 knock-out mice, although we administered the same PES to the SUI mice, PES did not improve calpain 2 activity of fibroblasts in anterior vaginal walls (consisting mainly of fibroblasts) (Fig. 3c, d). Furthermore, the MCC and LPP of SUI mice were lower than those of wild type normal mice, and PES clearly alleviated the damage. In addition, the potently protective effect was inhibited when the Cav 3.2 gene was knocked out (Table S1). The apoptosis rate couldn't be decreased by PES and the contents of collagen fibers were similar between the KO-VD group and the KO-VD + PES group in mouse anterior vaginal walls (Fig. S2). It is suggested that electrical stimulation has a significant effect on promoting the flow of calcium ions into cells. Similarly, calpain 2 activity was notably improved by ES in L929 cells, but mibefradil greatly inhibited the effect of ES (Fig. 4a, c). A similar result appeared in the Western blotting analyses (Fig. 4b, d). Collectively, these data strongly suggest that calpain 2 activity is a Ca^{2+}-dependent protein and that ES activates calpain 2 via increasing intracellular Ca^{2+}.

3.3. Calpain 2 is essential for the potent ES-induced upregulation of integrin β1

Next, we sought to validate the potent protein cleaving effect of calpain 2 induced by ES, which resulted in the upregulation of cleaved talin 1 expression and subsequently increased the activity of integrin β1. Calpain 2 is a calcium-modulated protease that respond to Ca^{2+} signals by removing specific portions of protein substrates, thereby irreversibly modifying their functions. Silencing calpain 2 strongly inhibited the activity of integrin β1, and weakened the ability of ES to improve integrin β1 function (Fig. 5a–d). Moreover, talin 1 (47 kDa) was decreased in the silenced sh-capn2 group and the sh-capn2 + ES group (Fig. 5a–d). The full-length talin 1 protein contains 2541 residues, and the molecular weight of the monomer is approximately 270 kDa. Talin 1 is connected by the N-terminal head domain (1–400 a.a., with a molecular weight of approximately 47 kDa) through a flexible region and the C-terminal ROD tail domain (482–2541a.a, with a molecular weight of approximately 220 kDa). The head domain of talin 1 contains a F0 structural domain and a FERM (Four-point-one-protein/ezrin/radixin/moesin) structural domain (consisting of the linear array F1, F2 and F3) [14], in which the PTB-like (phosphotyrosine-binding domain-like) structural domain of F3 can combine with the intracellular tail of beta integrin, thus inducing integrin allosterism, and activating integrin. The presence of F0 and F1 is also conducive to the realization of the maximum activation of integin [15]. Continuous alkaline residues distributed on the side of the FERM domain can bind to acidic membrane phospholipids, which is an interaction that also indispensable for the activation of integrins [16]. Talin 1, as the molecule directly upstream of integrin, plays an important role in the activation of integrin β1. Furthermore, recent studies have suggested that calpain 2 cleaves talin 1 at both the N- terminal and the C-terminal [17], and the talin 1 head liberated by calpain 2 cleavage has a function independent of full-length talin 1 [18]. These results suggest that the ES-induced activation of calpain 2 cleaves the N-terminal head domain of talin 1, and thus activates integrin β1.

3.4. Integrin β1 upregulates collagen I and III through TGF-β1 via electrical stimulation

In support of the effect of increasing collagen levels, the overexpression and silencing of integrin β1 were established in L929 cells. As shown, the mRNA and protein expression levels of collagen I, collagen III and TGF-β1 were enhanced by ES (Fig. 6a-e). However, the upregulation induced by ES was inhibited by integrin β1 silencing (Fig. 6a-e). Furthermore, integrin β1 overexpression mediated increase in collagen I, collagen III and TGF-β1, and this effect was further increased when overexpression was combined with ES (Fig. 7a-e). It is known that the principal ligands of integrins are the latency-associated peptides (LAPs) of TGF-β1, and these integrins play significant roles in the activation of the latent forms of this growth factor that are stored in the ECM in most healthy adult tissues[19,20]. At the same time, the inhibition of both integrin $\alpha_v\beta_6$ and $\alpha_v\beta_8$ recapitulates all of the developmental phenotypes of TGF-β1 and TGF-β3 [21]. Together, the above data reveal that integrin β1 downregulation significantly contributes to the decrease of TGF-β1 activity induced by ES and the subsequent inhibition of collagen I and III, and integrin β1 overexpression has the opposite effect. Thus, integrin β1 is a key factor in the collagen upregulation induced by electrical stimulation.

4. Discussion

ECM remodeling is an important pathomechanism of mechanical damage-induced SUI and has been confirmed by numerous studies [11]. The pelvic supporting tissues are composed mainly 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 [22]. Furthermore, in this study, the levels of collagen I and III were lower in the anterior vaginal wall of SUI mice than in that of control mouse. Thus, it is possible to treat SUI by restoring collagen expression.

Studies have shown that electrical stimulation can change intracellular Ca^{2+} concentrations. Ca^{2+}, as a second messenger widely distributed in cells, can regulate cell proliferation, differentiation, survival, death and other biological activities [23]. There are several ways to increase intracellular Ca^{2+} concentrations, such as calcium release from the endoplasmic reticulum and calcium influx through calcium ion channels. Voltage-gated calcium channels (VGCC) can be divided into L, N, P/Q, R, T, etc. subtypes and are mainly controlled by changes in cell membrane voltage [24]. In fibroblasts, the potential fluctuation of membrane is smaller than that of excitatory cells, which suggests that T-type calcium channels are more likely to be activated and play a role in electrophysiological activities than L-type calcium channels [25]. T-type calcium channels include the Cav 3.1, Cav 3.2 and Cav 3.3 subtypes, with Cav 3.2 widely distributed in tissues and involved in muscle excitation-contraction coupling and cell growth regulation [26]. Our previous study revealed that Cav3.2 showed the highest expression in the anterior vaginal wall, followed by Cav3.1; in contrast, the expression of Cav3.3 was extremely low [12]. Furthermore, in this study, ES greatly increased the intracellular Ca^{2+} concentration, which indicates that Cav3.2 is involved in this process.

Calpain 2, a Ca^{2+} concentration-dependent cysteine heterodimer protease, regulates the biological functions of other proteins by limiting the enzymatic hydrolysis of various enzymes and cytoskeleton protein systems in cells. Calpain 2 also plays an important role in cell stimulation response, proliferation, differentiation, death and other processes

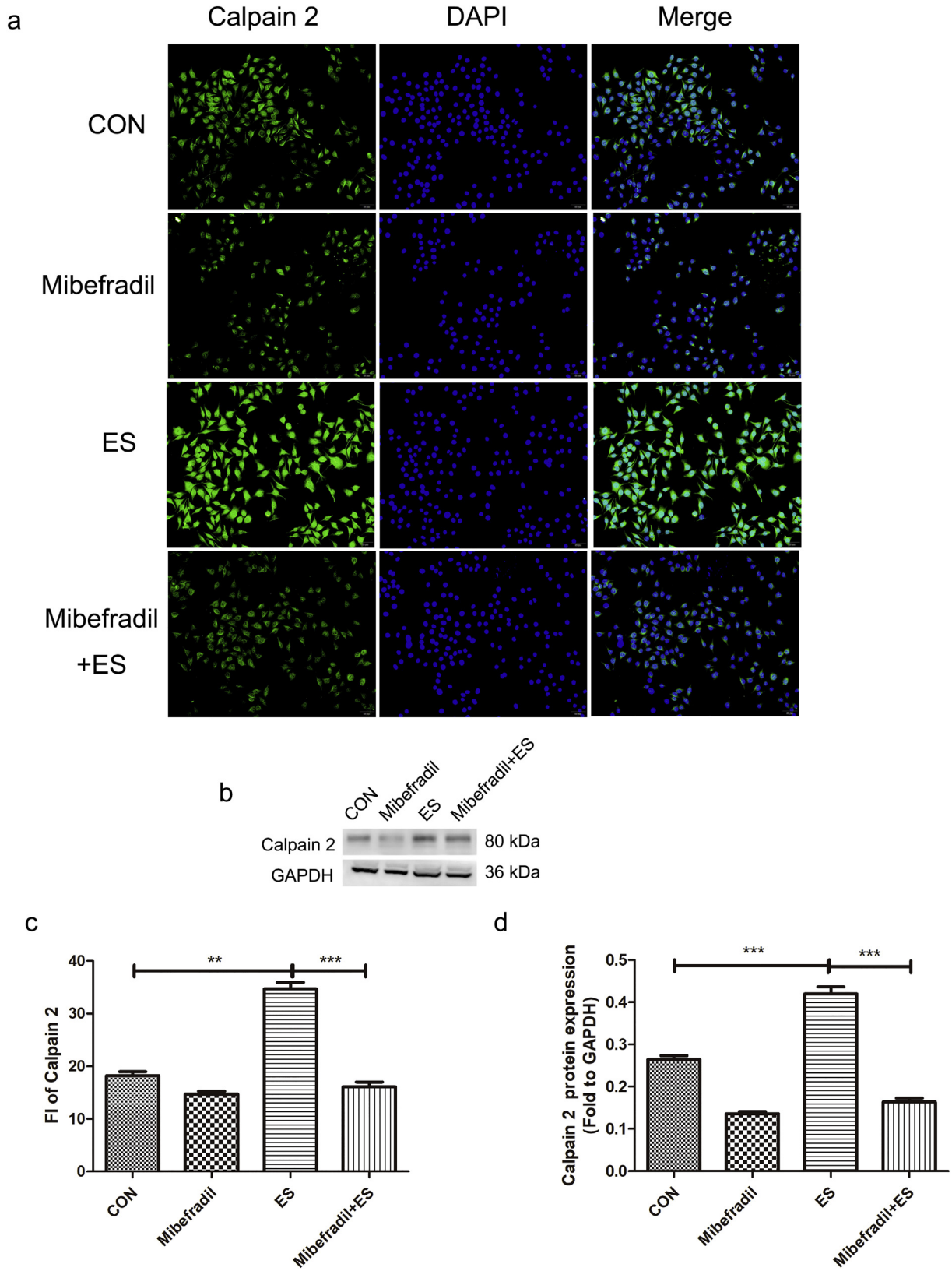

Fig. 4. Blocking calcium channels inhibits the activation of calpain 2 by electrical stimulation. a, Calpain 2 immunofluorescence staining of cells after different treatments, magnification: ×200; b and d, Calpain 2 protein expression of cells after different treatments; c, Fluorescence intensity of calpain 2. ** represents $p < 0.01$; *** represents $p < 0.001$, every experiment was repeated for 3 times. (CON: normal L929 cells; ES: normal L929 cells treated with electrical stimulation; Mibefradil: normal L929 cells treated with mibefradil; Mibefradil + ES: L929 cells treated with mibefradil combined with ES).

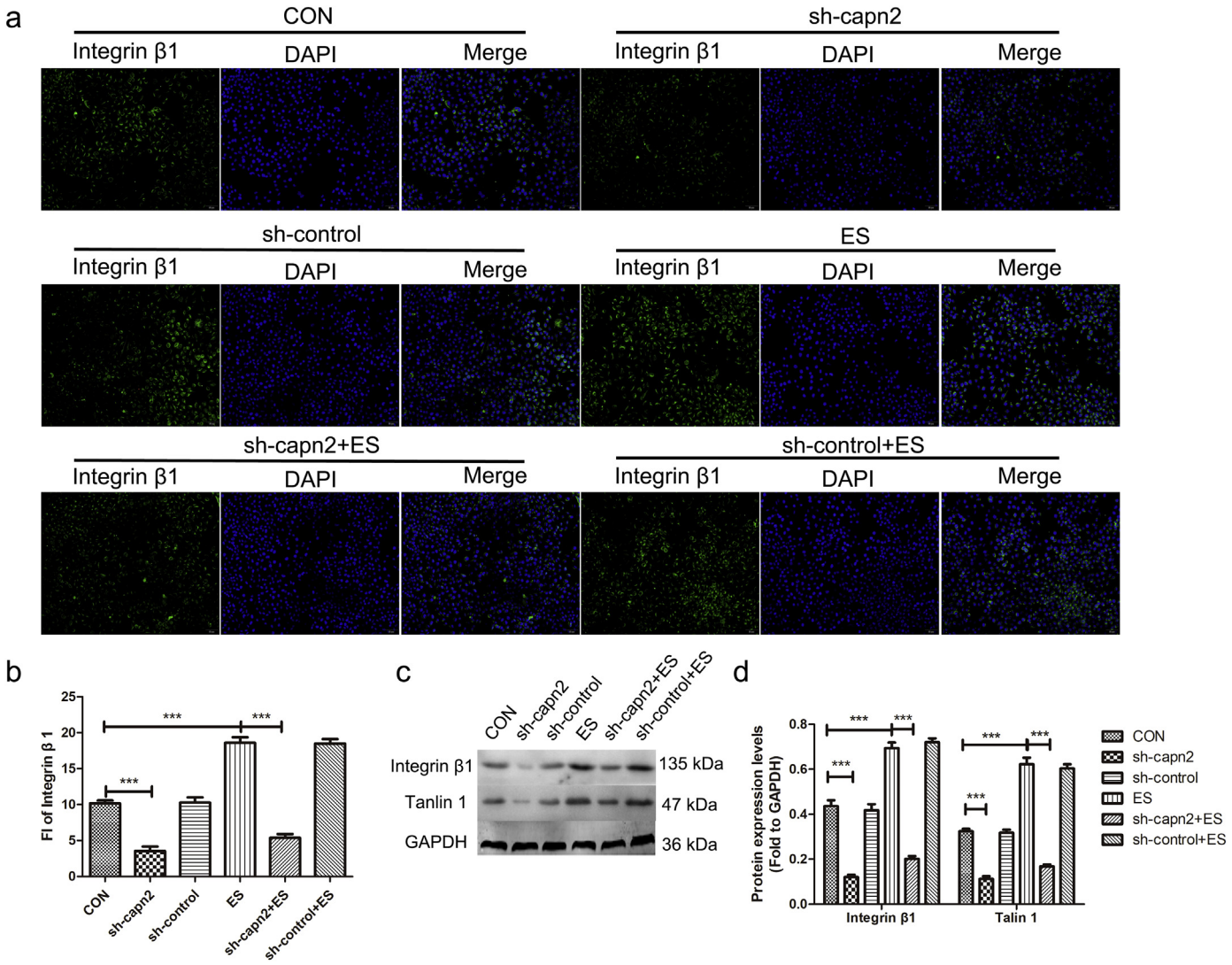

Fig. 5. ES induces the upregulation of integrin β1 via calpain 2 and cleaved talin 1. a, Integrin β1 immunofluorescence staining of cells after different treatments, magnification: ×200; b , Fluorescence intensity of integrin β1; c and d, Integrin β1 protein expression of cells after different treatments. *** represents $p < 0.001$, every experiment was repeated for 3 times. (CON: normal L929 cells; ES: normal L929 cells treated with electrical stimulation; sh-capn2: Lv-shcapn2 transfection established calpain 2 silencing in L929 cells; sh-control: negative control shRNA transfected into L929 cells; sh-capn2 + ES: sh-capn2 cells treated with ES; sh-control +ES: sh-control cells treated with ES).

[27]. In the present study, it was shown that the intracellular Ca^{2+} concentration was enhanced markedly by ES leading to the activation of calpain 2. Furthermore, talin 1 was cleaved by activated calpain 2. Additionally, the talin gene has two subtypes, talin 1 and talin 2, in which talin 1 is the main component, and the proteins encoded by them are 74% similar [28]. Cleaved talin 1 directly destroys the salt bond between Arg995 and Asp723 after binding to the intracellular integrin subunit, resulting in rapid helical dissociation of the alpha and beta subunits that are oriented across the membrane domain, which subsequently rotate 90 degrees in relation to the membrane. Thus, integrin exposes the binding sites between the extracellular domain and ligand, which leads to a change in the extracellular domain structure and the activation of integrin. Ultimately, the affinity between the extracellular portion of integrin and its ligand is increased [15].

A large amount of evidence indicates that there is extensive cross-talk between integrins and TGF-β. A subset of integrins are responsible for almost all TGF-β activation in the epithelial-mesenchymal trophic unit [29]. Integrin $\alpha_v\beta_1$ has high expression in activated fibroblasts, and it binds to the LAP of TGF-β1 and mediates TGF-β1 activation [30]. In addition, it has been reported that blocking integrin β1 function inhibits TGF-β-mediated p38/MAPK activation and epithelial to mesenchymal transdifferentiation progression [31]. These previous data suggest that integrin indeed exerts distinct biological effects on TGF-β. In the present study, integrin β1 overexpression and silencing positively regulated and negatively regulated TGF-β1, respectively. It is therefore of great significance to reveal that ES could regulate TGF-β1 through integrin β1. In a previous study, we have shown that mechanical stress and H_2O_2 overexposure inhibit cell proliferation and remodel the ECM network via the regulation of the TGF-β1/Smad 3 signaling pathway [32,33]. Here, we also demonstrate that collagens and TGF showed the same pattern of change under electrical stimulation. Considering the important role of integrin β1 in collagen metabolism, our observation that calpain 2 directly cleaved talin 1 to activate integrin β1 provides new insights into the clinical application of electrical stimulation therapy.

In this study, we first established 3 models to investigate the mechanism of electrical stimulation in treatment of SUI: SUI murine model, ES cell model and PES mouse model. In addition, our findings revealed a novel signaling pathway in which electrical stimulation enhanced collagen expression, which may help us improve the

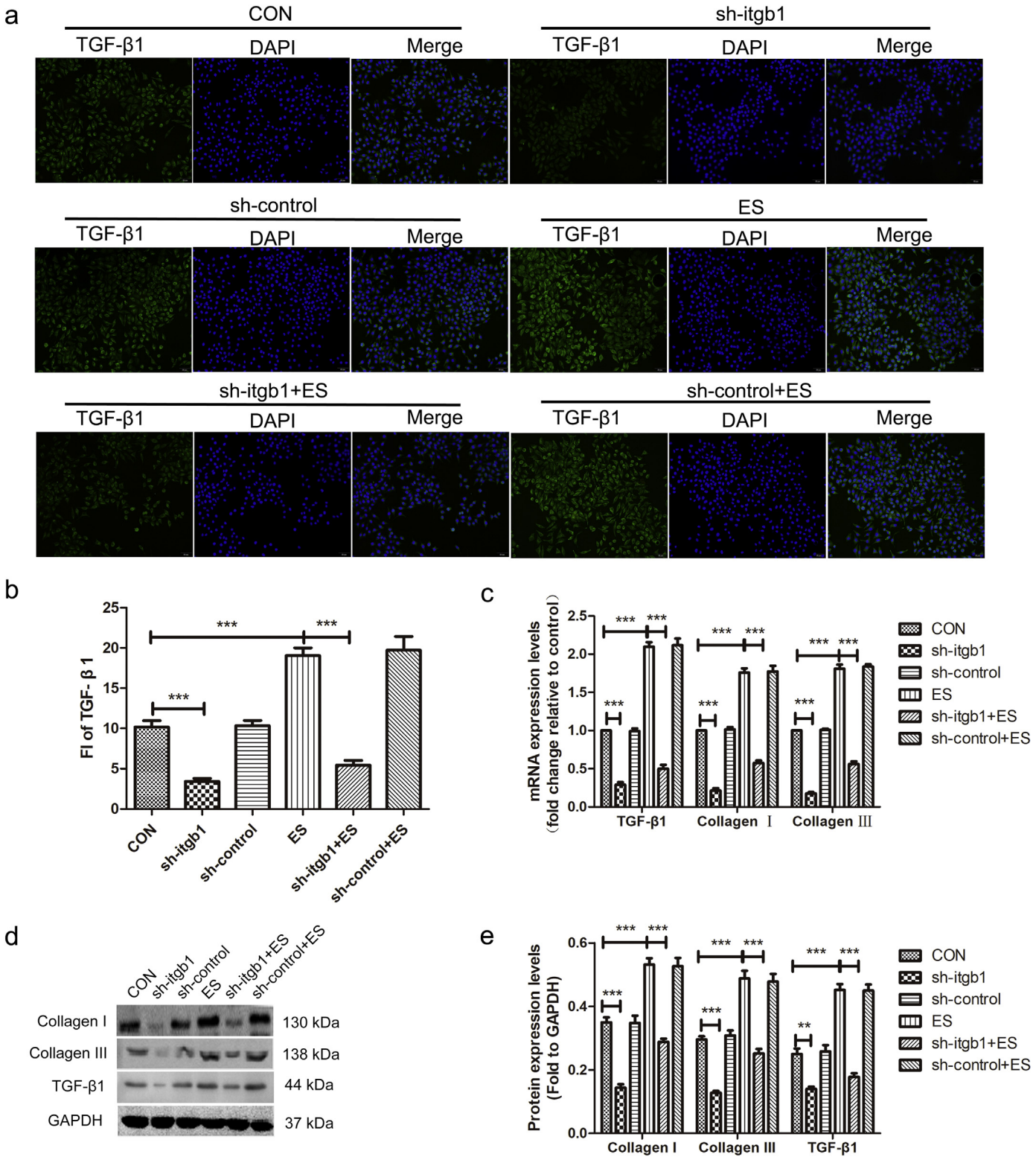

Fig. 6. The upregulation of collagens is restrained by integrin β1 silencing. a, TGF-β1 immunofluorescence staining of cells after different treatments, magnification: ×200; b , Fluorescence intensity of TGF-β1; c, mRNA expression of TGF-β1, collagen I and collagen III; d and e, TGF-β1, collagen I and collagen III protein expression in cells after different treatments. ** represents $p < 0.01$; *** represents $p < 0.001$, every experiment was repeated for 3 times. (CON: normal L929 cells; ES: normal L929 cells treated with electrical stimulation; sh-itgb1: Lv-shitgb1 transfection established integrin β1 silencing in L929 cells; sh-control: negative control shRNA transfected into L929 cells; sh-itgb1 + ES: sh-itgb1 cells treated with ES; sh-control + ES: sh-control cells treated with ES).

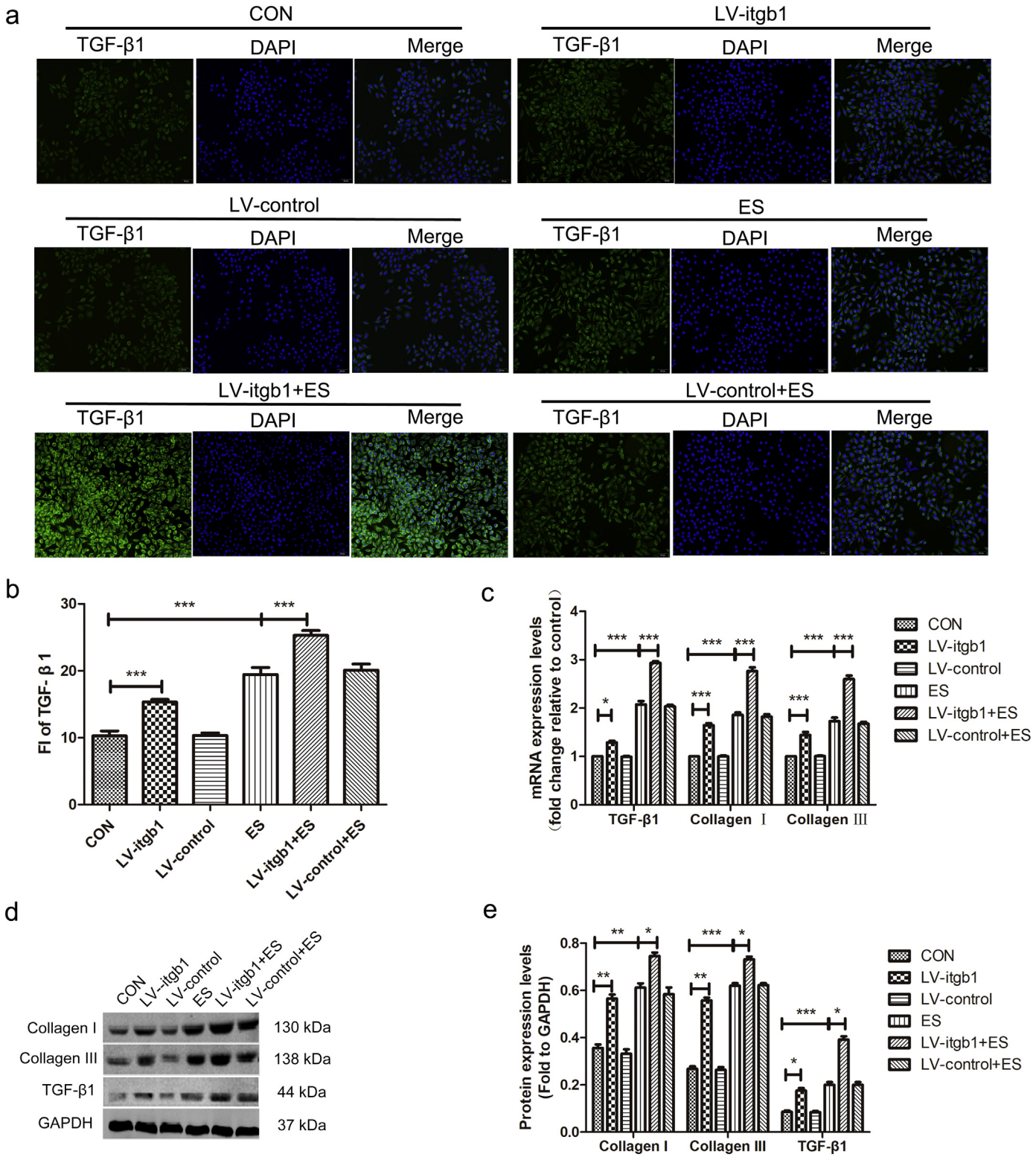

Fig. 7. The expression of collagens is enhanced by integrin β1 overexpression. a, TGF-β1 immunofluorescence staining of cells after different treatments, magnification: ×200; b , Fluorescence intensity of TGF-β1; c, mRNA expression of TGF-β1, collagen I and collagen III; d and e, TGF-β1, collagen I and collagen III protein expression of cells after different treatments. * represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$, every experiment was repeated for 3 times (CON: normal L929 cells; ES: normal L929 cells treated with electrical stimulation; LV-itgb1: Lv-itgb1 transfection established integrin β1 overexpressing L929 cells; LV-control: empty vector transfected into L929 cells; LV-itgb1 + ES: LV-itgb1 cells treated with ES; LV-control + ES: LV-control cells treated with ES).

therapeutic effect of electrical stimulation in the treatment of SUI.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.03.023.

References

[1] L. Wilson, J.S. Brown, G.P. Shin, K.O. Luc, L.L. Subak, Annual direct cost of urinary incontinence, Obstet. Gynecol. 98 (2001) 398–406.
[2] K.D. Sievert, B. Amend, P.A. Toomey, D. Robinson, I. Milsom, H. Koelbl, P. Abrams, L. Cardozo, A. Wein, A.L. Smith, D.K. Newman, Can we prevent incontinence? ICI-RS 2011, Neurourol. Urodyn. 31 (2012) 390–399.
[3] L.M. Dolan, D. Walsh, S. Hamilton, K. Marshall, K. Thompson, R.G. Ashe, A study of quality of life in primigravidae with urinary incontinence, Int. Urogynecol. J. Pelvic Floor Dysfunct. 15 (2004) 160–164.
[4] A.K. Monga, M.R. Tracey, J. Subbaroyan, A systematic review of clinical studies of electrical stimulation for treatment of lower urinary tract dysfunction, Int. Urogynecol. J. 23 (2012) 993–1005.
[5] A.P. Schmidt, P.R. Sanches, D.J. Silva, J.G. Ramos, P. Nohama, A new pelvic muscle trainer for the treatment of urinary incontinence, Int. J. Gynaecol. Obstet. 105 (2009) 218–222.
[6] R. Terlikowski, B. Dobrzycka, M. Kinalski, A. Kuryliszyn-Moskal, S.J. Terlikowski, Transvaginal electrical stimulation with surface-EMG biofeedback in managing stress urinary incontinence in women of premenopausal age: a double-blind, placebo-controlled, randomized clinical trial, Int. Urogynecol. J. 24 (2013) 1631–1638.
[7] T. Shamliyan, J. Wyman, R.L. Kane, Nonsurgical Treatments for Urinary Incontinence in Adult Women: Diagnosis and Comparative Effectiveness, Agency for Healthcare Research and Quality (US), Rockville (MD), 2012.
[8] C.F. Richmond, D.K. Martin, S.O. Yip, M.A. Dick, E.A. Erekson, Effect of supervised pelvic floor biofeedback and electrical stimulation in women with mixed and stress urinary incontinence, Female Pelvic Med. Reconstr. Surg. 22 (2016) 324–327.
[9] G. Chene, A. Mansoor, B. Jacquetin, G. Mellier, S. Douvier, F. Sergent, Y. Aubard, P. Seffert, Female urinary incontinence and intravaginal electrical stimulation: an observational prospective study, Eur. J. Obstet. Gynecol. Reprod. Biol. 170 (2013) 275–280.
[10] R.A. Castro, R.M. Arruda, M.R. Zanetti, P.D. Santos, M.G. Sartori, M.J. Girao, Single-blind, randomized, controlled trial of pelvic floor muscle training, electrical stimulation, vaginal cones, and no active treatment in the management of stress urinary incontinence, Clinics (Sao Paulo) 63 (2008) 465–472.
[11] J. Tang, B. Li, C. Liu, Y. Li, Q. Li, L. Wang, J. Min, M. Hu, S. Hong, L. Hong, Mechanism of mechanical trauma-induced extracellular matrix remodeling of fibroblasts in association with Nrf2/ARE signaling suppression mediating TGF-beta1/Smad3 signaling inhibition, Oxidative Med. Cell. Longev. 2017 (2017) 8524353.
[12] J. Min, B. Li, C. Liu, S. Hong, J. Tang, M. Hu, Y. Liu, S. Li, L. Hong, Therapeutic effect and mechanism of electrical stimulation in female stress urinary incontinence, Urology 104 (2017) 45–51.
[13] B. Song, Y. Gu, J. Pu, B. Reid, Z. Zhao, M. Zhao, Application of direct current electric fields to cells and tissues in vitro and modulation of wound electric field in vivo, Nat. Protoc. 2 (2007) 1479–1489.
[14] B.T. Goult, M. Bouaouina, P.R. Elliott, N. Bate, B. Patel, A.R. Gingras, J.G. Grossmann, G.C. Roberts, D.A. Calderwood, D.R. Critchley, I.L. Barsukov, Structure of a double ubiquitin-like domain in the Talin head: a role in integrin activation, EMBO J. 29 (2010) 1069–1080.
[15] K.L. Wegener, A.W. Partridge, J. Han, A.R. Pickford, R.C. Liddington, M.H. Ginsberg, I.D. Campbell, Structural basis of integrin activation by Talin, Cell 128 (2007) 171–182.
[16] M. Bouaouina, Y. Lad, D.A. Calderwood, The N-terminal domains of Talin cooperate with the phosphotyrosine binding-like domain to activate beta1 and beta3 integrins, J. Biol. Chem. 283 (2008) 6118–6125.
[17] N. Bate, A.R. Gingras, A. Bachir, R. Horwitz, F. Ye, B. Patel, B.T. Goult, D.R. Critchley, Talin contains a C-terminal calpain2 cleavage site important in focal adhesion dynamics, PLoS One 7 (2012) e34461.
[18] C. Huang, Z. Rajfur, N. Yousefi, Z. Chen, K. Jacobson, M.H. Ginsberg, Talin phosphorylation by Cdk5 regulates Smurf1-mediated Talin head ubiquitylation and cell migration, Nat. Cell Biol. 11 (2009) 624–630.
[19] J.S. Munger, X. Huang, H. Kawakatsu, M.J. Griffiths, S.L. Dalton, J. Wu, J.F. Pittet, N. Kaminski, C. Garat, M.A. Matthay, D.B. Rifkin, D. Sheppard, The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis, Cell 96 (1999) 319–328.
[20] D. Mu, S. Cambier, L. Fjellbirkeland, J.L. Baron, J.S. Munger, H. Kawakatsu, D. Sheppard, V.C. Broaddus, S.L. Nishimura, The integrin alpha(v)beta8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-beta1, J. Cell Biol. 157 (2002) 493–507.
[21] P. Aluwihare, Z. Mu, Z. Zhao, D. Yu, P.H. Weinreb, G.S. Horan, S.M. Violette, J.S. Munger, Mice that lack activity of alphavbeta6- and alphavbeta8-integrins reproduce the abnormalities of Tgfb1- and Tgfb3-null mice, J. Cell Sci. 122 (2009) 227–232.
[22] B. Chen, Y. Wen, X. Yu, M.L. Polan, Elastin metabolism in pelvic tissues: is it modulated by reproductive hormones? Am. J. Obstet. Gynecol. 192 (2005) 1605–1613.
[23] D.E. Clapham, Calcium signaling, Cell 131 (2007) 1047–1058.
[24] P. FATT, B. KATZ, The electrical properties of crustacean muscle fibres, J. Physiol. 120 (1953) 171–204.
[25] M.W. Strobeck, M. Okuda, H. Yamaguchi, A. Schwartz, K. Fukasawa, Morphological transformation induced by activation of the mitogen-activated protein kinase pathway requires suppression of the T-type Ca2+ channel, J. Biol. Chem. 274 (1999) 15694–15700.
[26] A.C. Dolphin, Voltage-gated calcium channels and their auxiliary subunits: physiology and pathophysiology and pharmacology, J. Physiol. 594 (2016) 5369–5390.
[27] G.P. Pal, J.S. Elce, Z. Jia, Dissociation and aggregation of calpain in the presence of calcium, J. Biol. Chem. 276 (2001) 47233–47238.
[28] D.R. Critchley, A.R. Gingras, Talin at a glance, J. Cell Sci. 121 (2008) 1345–1347.
[29] J. Araya, S. Cambier, A. Morris, W. Finkbeiner, S.L. Nishimura, Integrin-mediated transforming growth factor-beta activation regulates homeostasis of the pulmonary epithelial-mesenchymal trophic unit, Am. J. Pathol. 169 (2006) 405–415.
[30] N.I. Reed, H. Jo, C. Chen, K. Tsujino, T.D. Arnold, W.F. DeGrado, D. Sheppard, The alphavbeta1 integrin plays a critical in vivo role in tissue fibrosis, Sci. Transl. Med. 7 (2015) 288ra79.
[31] N.A. Bhowmick, R. Zent, M. Ghiassi, M. McDonnell, H.L. Moses, Integrin beta 1 signaling is necessary for transforming growth factor-beta activation of p38MAPK and epithelial plasticity, J. Biol. Chem. 276 (2001) 46707–46713.
[32] Q. Zhang, C. Liu, S. Hong, J. Min, Q. Yang, M. Hu, Y. Zhao, L. Hong, Excess mechanical stress and hydrogen peroxide remodel extracellular matrix of cultured human uterosacral ligament fibroblasts by disturbing the balance of MMPs/TIMPs via the regulation of TGFbeta1 signaling pathway, Mol. Med. Rep. 15 (2017) 423–430.
[33] C. Liu, Y. Wang, B.S. Li, Q. Yang, J.M. Tang, J. Min, S.S. Hong, W.J. Guo, L. Hong, Role of transforming growth factor beta1 in the pathogenesis of pelvic organ prolapse: a potential therapeutic target, Int. J. Mol. Med. 40 (2017) 347–356.