

miR-93-mediated collagen expression in stress urinary incontinence via calpain-2

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Abstract. The aim of the present study was to investigate the expression and mechanism of microRNA (miR)-93 in collagen expression in stress urinary incontinence (SUI). Vaginal tissue, primary fibroblasts and SUI primary fibroblasts were obtained to detect the expression of miR-93, interstitial collagenase (MMP1), collagen I and calpain-2. Reverse transcription-quantitative polymerase chain reaction analysis was performed to detect the levels of miR-93 and MMP1. Western blotting was used to evaluate the protein levels of calpain-2, MMP1 and collagen I. MMP1 and hydroxyproline levels in the supernatant were measured by ELISA. The association between miR-93 and calpain-2 was investigated by luciferase reporter assays. The expression of miR-93 and collagen I was significantly downregulated in the SUI group, while the expression of calpain-2 and MMP1 was significantly upregulated. ELISA analysis demonstrated that the MMP1 level increased and the hydroxyproline level decreased in the SUI group. Additionally, calpain-2 was identified to be a target of miR-93, and miR-93 was able to negatively regulate the expression of calpain-2. Restoration of calpain-2 in miR-93-overexpressing SUI primary fibroblasts reversed the alteration in hydroxyproline expression, indicating that calpain-2 was negatively associated with collagen expression. The results of the present study suggested that miR-93 regulated MMP1 and collagen I expression in fibroblasts via calpain-2. miR-93 mediated collagen expression in stress urinary incontinence via calpain-2.

Introduction

Stress urinary incontinence (SUI) is a functional disorder characterized by spontaneous flow of urine caused by a sudden increase in abdominal pressure with spontaneous contraction

of the bladder (1). SUI occurs when intraabdominal and intravesical pressure is increased beyond the capability of the closing mechanism without bladder contraction. When bladder contraction causes urinary incontinence, this is termed urgency urinary incontinence. SUI is a complex disease involving multiple factors and is frequently accompanied by female pelvic organ prolapse, and the severity increases with age (2,3). At present, the common theories for SUI pathogenesis are the pelvic floor theory and the hammock hypothesis, which consider that structural and functional defects of urethral support tissue, including the anterior vaginal wall, cause the occurrence of SUI (4). The primary components of the urethral support tissue consist of connective tissues containing a large amount of extracellular matrix (ECM) and a small number of cells (5). The primary component of the ECM is collagen, including collagen I and collagen III (6). In the urethral support tissue of patients with SUI, the collagen content has been demonstrated to be markedly reduced (7), which is of importance in examining the mechanism underlying SUI.

Calpain is a neutral protease that is common in human tissues, is associated with the degradation of various cytoskeletal proteins, and serves an important role in the degradation of myofibrils and other ECM components (8). The mRNA and protein expression levels of calpain in the urethral tissue of patients with SUI were demonstrated to be increased compared with a control group (9); therefore, calpain may serve a role in the pathogenesis of SUI.

MicroRNAs (miRNAs/miRs) are non-coding short-chain RNAs which are able to regulate cell proliferation and differentiation, protein synthesis, and the progression of diseases, including cancer (10). It has been previously demonstrated that the dysregulated expression of collagen may be mediated by abnormal miRNAs. miR-19b is frequently involved in cell proliferation, collagen synthesis and fibrogenesis (11,12). Beaumont *et al* (13) demonstrated that miR-19b expression was reduced in patients with aortic stenosis, which may be a potential biomarker of increased myocardial collagen. Xiao *et al* (15) observed that in hypertrophic scar fibroblasts, miR-185 regulated the concentration of transforming growth factor- β 1 and collagen I. Notably, Jing and Jiang (14) reported that miR-93 regulated collagen loss by targeting stromelysin-1 (MMP3) in human nucleus pulposus cells. Therefore, it was hypothesized that miR-93 may regulate collagen expression in SUI via calpain.

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The aim of the present study was to investigate the expression of miR-93, calpain-2, interstitial collagenase (MMP1) and collagen I in patients with SUI, and to detect how miR-93 regulated collagen expression. The expression of miR-93, calpain-2, MMP1 and collagen I was determined in SUI primary fibroblasts (an *in vitro* experiment was performed to examine the mechanism of miR-93 in collagen expression in SUI due to its good controllability) and vaginal wall tissue from patients with SUI, which may provide a theoretical basis for the development of novel treatments for SUI.

Materials and methods

Patients and samples. Patients with SUI were diagnosed using the criteria of the International Continence Society (Bristol, UK), and patients with moderate or severe pelvic organ prolapse were excluded. Full thickness of vaginal wall tissue specimens were collected from patients with SUI (SUI group; n=18), and from patients undergoing wound repair surgery for e.g., paraurethral cysts (control group; n=20) from July 2015 to August 2016. All specimens were kept in frozen (-80°C) until further use. All experiments were approved by the Ethics Committee of the Third Hospital of Hebei Medical University (Shijiazhuang, China). All patients gave informed written consent.

Cell culture. Primary fibroblasts and SUI primary fibroblasts were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in a 5% CO₂ incubator at 37°C, until cells became 80-90% confluent between passages 5 and 7.

Transfection of SUI primary fibroblasts. Cells were seeded at a density of 4x10⁴ cells/well. miR-93 mimics, inhibitors, negative controls, small interfering-calpain 2 (si-CAPN2) and pcDNA-CAPN2 were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). 100 nM of each miR or siRNA and cells were co-cultured with Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for cell transfections for 24 h, according to the manufacturer's protocol. Each cell transfection was performed in triplicate. The sequences of the miR-93 mimic, 5'-CAAAGUGCUGUUCGUGCAGGUAG-3'; miR-93 inhibitor, 5'-CUACCUGCAGAACAGCACUUUG-3' and si-CAPN2, 5'-CTGGAACATATAGACCCAGA-3'.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated from primary fibroblasts and vaginal wall tissue using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. miRNA reverse transcribed to cDNA was prepared using the TaqMan miRNA RT kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). The ABI Prism 5700 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for the qPCR. A total fluid volume of 25 µl and TaqMan qPCR Master Mix reagents (Applied Biosystems; Thermo Fisher Scientific, Inc.) were

used for amplification. RT-qPCR was conducted for 35 cycles in a LightCycler 480 machine (Roche Diagnostics, Basel, Switzerland). It consisted of a denaturing phase at 94°C for 1 min, 1.5 min at 60°C for primer annealing, and an extension temperature of 72°C for 2 min. All reactions were performed in triplicate. A 2-step cycle protocol was used to measure mRNA expression. Data were analyzed using ABI Prism 5700 SDS software (Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative expression of miR-93, CAPN2 and MMP1 genes was calculated via the comparative 2^{-ΔΔC_q} method (16). Primers of Calpain-2 forward, 5'-AAAAAAGCTTGGATGGCGGGC ATCGCGGC-3' and reverse, 5'-AAAAATCTAGAAAGTAC TGAGAAACAGAGCCAAGAGATAAGGTCG-3'; miR-93 forward, 5'-AGTCTCTGGGCTGACTACATCACAG-3' and reverse, 5'-CTACTCACAAAACAGGAGTGGAATC-3'; MMP1 forward, 5'-GAGCTCAACTTCCGGGTAGA-3' and reverse, 5'-CCCAAAGCGTGTGACAGTA-3'; and β-actin forward, 5'-CCACCATGTACCCAGGCATT-3' and reverse, 5'-AGGGTGTAAAACGCAGCTCA-3'.

Western blot analysis. The proteins from primary fibroblasts and vaginal wall tissue were extracted using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) and centrifuged at 12,000 x g for 15 min at 4°C. Protein concentrations were determined using a Bicinchoninic Acid Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Western blotting was performed according to standard procedures. Samples containing equal amounts of proteins (50 µg) were separated on a 10% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes (GE Healthcare, Chicago, IL, USA) which were blocked using 5% non-fat dried milk for 2 h at room temperature. Anti-calpain-2 (cat. no. PA5-17494; Invitrogen; Thermo Fisher Scientific, Inc.), anti-MMP1 (cat. no. MA1-771; Invitrogen; Thermo Fisher Scientific, Inc.), anti-β-actin (cat. no. PA1-46296; Invitrogen; Thermo Fisher Scientific, Inc.) and anti-collagen I (cat. no. PA1-26204; Invitrogen; Thermo Fisher Scientific, Inc.) were used as the primary antibodies at a 1:1,000 dilution at 4°C for 12 h. The corresponding horseradish peroxidase-conjugated secondary antibody (Pierce; Thermo Fisher Scientific, Inc., cat. no. 31491B) was incubated at room temperature for 1 h. Protein was detected by the enhanced chemiluminescence system (Roche Diagnostics, Basel, Switzerland), and analyzed by Image Lab 4.1 software. β-actin served as the internal control protein.

Luciferase reporter assays. 293T cells were used for transient transfections with Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. 293T cells were seeded in 24-well plates at a density of 5x10⁴ cells/well. The pMIR-REPORT-CAPN2-3'UTR plasmid was constructed by inserting the fragment of the 3'-untranslated region (UTR) of CAPN2 containing the miR-93 putative binding region into the pMIR-REPORT-CAPN2-3'UTR plasmid (Invitrogen; Thermo Fisher Scientific, Inc.). For the luciferase reporter assay, the reporter plasmid (pMIR-REPORT-CAPN2-3'UTR) and microRNAs (100 nM of miR-93 or negative control) were co-transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h. Cells were collected

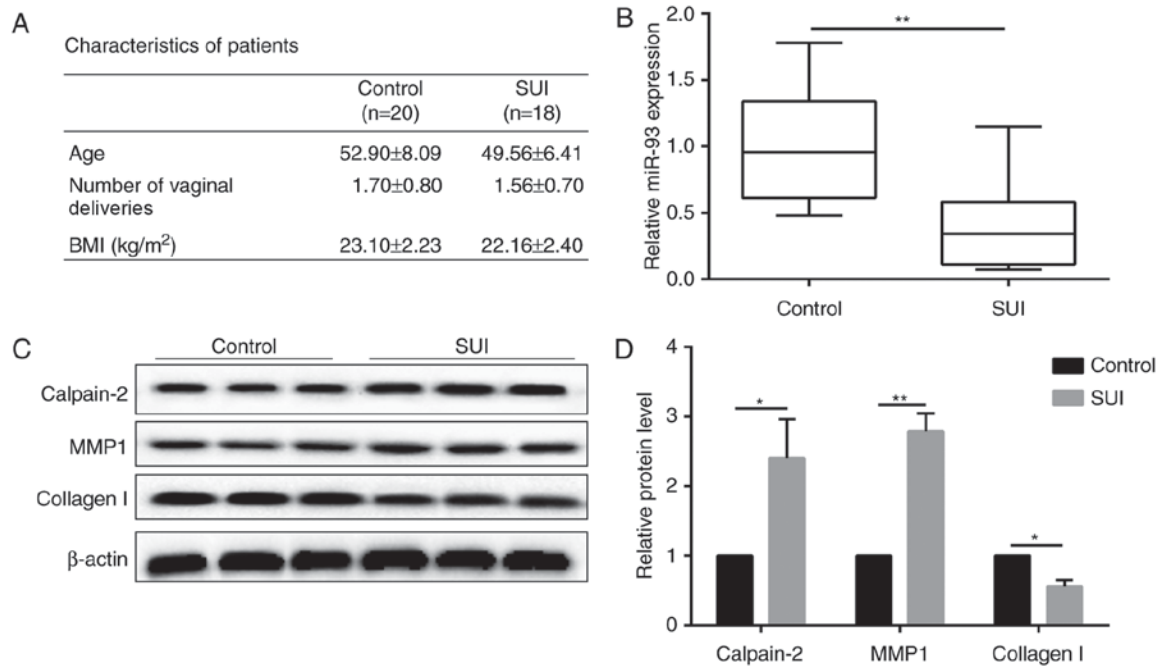


Figure 1. Expression of miR-93 and proteins of the vaginal wall tissue in patients with SUI. (A) Characteristics of patients in the control and SUI groups. (B) miR-93 expression in the control group was increased compared with the SUI group. The protein levels of calpain-2 and MMP1 were increased in the SUI group, while the collagen I level was decreased in the SUI group, as demonstrated by (C) western blotting and (D) densitometric analysis. * $P<0.05$; ** $P<0.01$. SUI, stress urinary incontinence; BMI, body mass index; miR, microRNA; MMP1, interstitial collagenase.

to measure luciferase activity using a dual luciferase detection system by comparison with *Renilla* luciferase activity (Promega Corporation, Madison, WI, USA).

ELISA analysis. The protein expression of MMP1 and hydroxyproline in the supernatant was quantified using a Human MMP1 ELISA kit (cat. no. ABIN625053; Abnova, Taipei, Taiwan) and a Hyp ELISA kit (D720160; Sangon Biotech Co., Ltd.), according to the manufacturer's protocols. The absorbance was measured at 450 nm using a microplate reader.

Statistical analysis. SPSS 18.0 (SPSS, Inc., Chicago, IL, USA) was used for the data analysis. Data were presented as the mean \pm standard deviation. The differences in mRNA expression and protein levels between the control and SUI group were analyzed by a paired t-test. The differences in relative luciferase activity, and relative mRNA and protein levels of calpain-2 among the different groups were analyzed using one-way analysis of variance. Student-Newman-Keuls test was used for post-hoc multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of miR-93 and proteins in vaginal wall tissue.

In order to investigate how miR-93 regulates collagen, the expression of miR-93, calpain-2, MMP1 and collagen I in vaginal wall tissue was detected in the SUI and control groups. Data about vaginal wall tissue specimens was collected from patients with SUI [SUI group; $n=18$; average age, 49.56 ± 6.41 ; number of vaginal deliveries, 1.56 ± 0.70 ; body mass index (BMI), 22.16 ± 2.40], and from patients with diseases, including

paraurethral cysts, requiring wound repair (control group; $n=20$; average age, 52.90 ± 8.09 ; number of vaginal deliveries, 1.70 ± 0.80 ; BMI, 23.10 ± 2.23) (Fig. 1A). miR-93 expression in the SUI group was decreased compared with the control group, as detected via RT-qPCR (Fig. 1B). The protein levels of calpain-2 and MMP1 were increased in the SUI group as detected by western blotting, while the collagen I level was decreased in SUI group (Fig. 1C and D). The results of the present study indicated that the decrease in miR-93 and increase in calpain-2 and MMP1 may contribute to collagen expression.

Expression of miR-93 and proteins in vitro. *In vitro* experiments were performed in the control (primary fibroblasts) and SUI (SUI primary fibroblasts) groups to verify the previously described expression patterns. It was observed that miR-93 expression in the SUI group was decreased compared with the control group (Fig. 2A), while MMP1 expression was increased in the SUI group (Fig. 2B). The protein expression of calpain-2 and MMP1 in the SUI group was upregulated, while collagen I expression was downregulated in the SUI group (Fig. 2C and D). ELISA analysis demonstrated that the MMP1 level in the supernatant in the SUI group was increased compared with the control group, while the hydroxyproline level in the supernatant in the SUI group was decreased (Fig. 2E and F).

Calpain-2 is a direct target of miR-93. The microRNA software (microRNA.org) was used in the present study and predicted that calpain may be a direct target of miR-93. The regulatory effect of miR-93 on calpain-2 was confirmed via a luciferase reporter assay in 293T cells. It was demonstrated that miR-93 was able to bind to the CAPN2 gene, and that overexpression of miR-93 decreased the calpain-2 expression level (Fig. 3A).

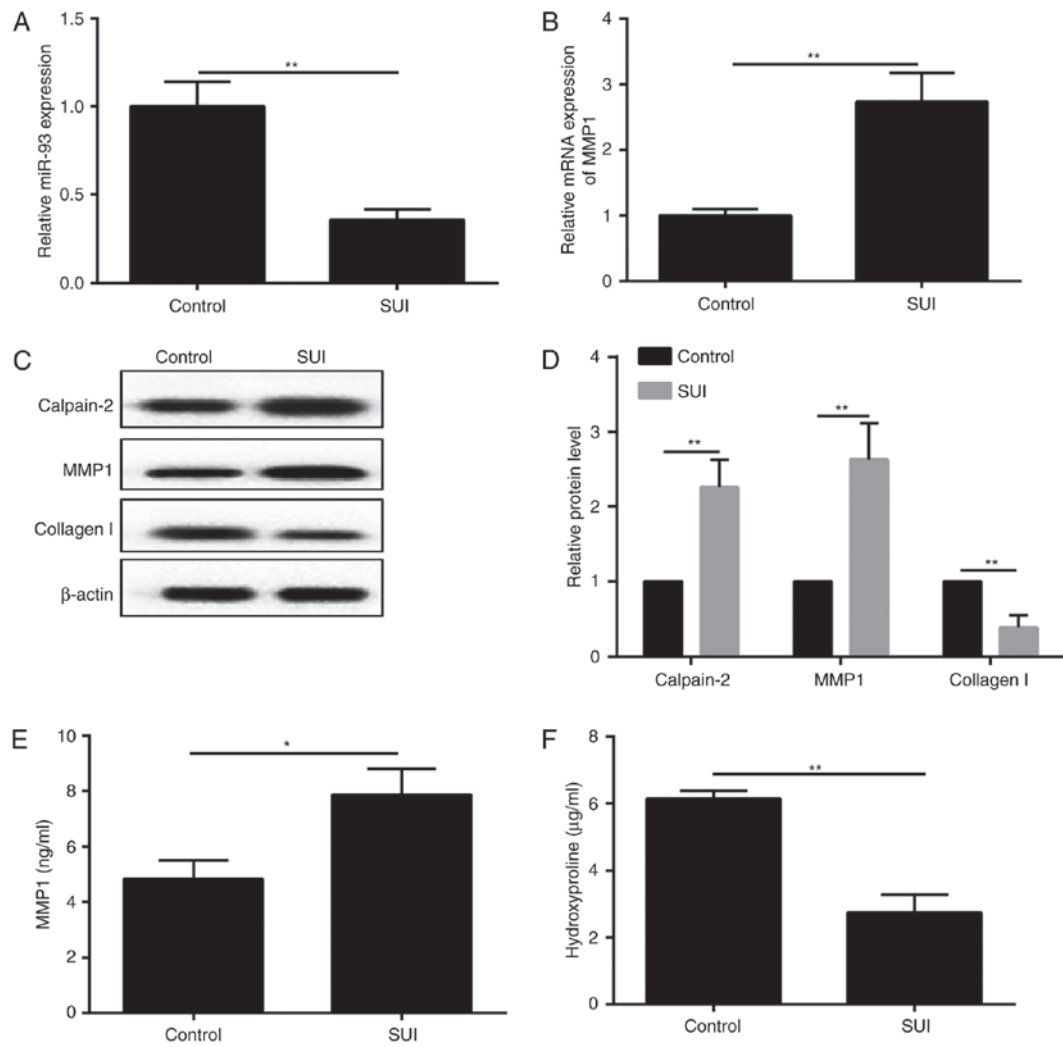


Figure 2. *In vitro* experiments in the control (primary fibroblasts) and SUI (SUI primary fibroblasts) groups. (A) Decreased expression of miR-93 in the SUI group. (B) MMP1 mRNA expression in the SUI group increased. The upregulation of calpain-2 and MMP1 protein expression in the SUI group, and the downregulation of collagen I in SUI group, was demonstrated via (C) western blotting and (D) densitometric analysis. (E) ELISA analysis demonstrated that the MMP1 level in the supernatant in the SUI group was increased compared with the control group. (F) Hydroxyproline level in the supernatant in SUI group was decreased compared with the control group. * $P < 0.05$; ** $P < 0.01$. SUI, stress urinary incontinence; miR, microRNA; MMP1, interstitial collagenase.

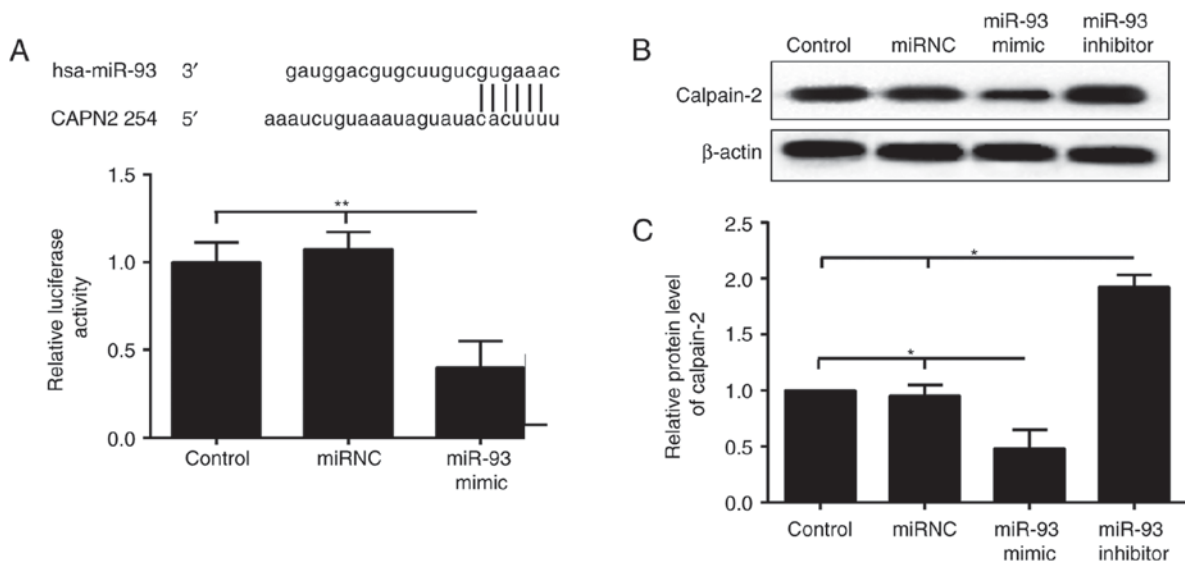


Figure 3. Calpain-2 is a direct target of miR-93. (A) Regulatory effect of miR-93 on calpain-2 was confirmed by luciferase reporter gene in 293T cells. The effects of miR-93 overexpression and miR-93 inhibition on the expression of calpain-2 were demonstrated via (B) western blotting and (C) densitometric analysis. * $P < 0.05$; ** $P < 0.01$. CAPN2, calpain-2 gene; miR, microRNA; NC, negative control.

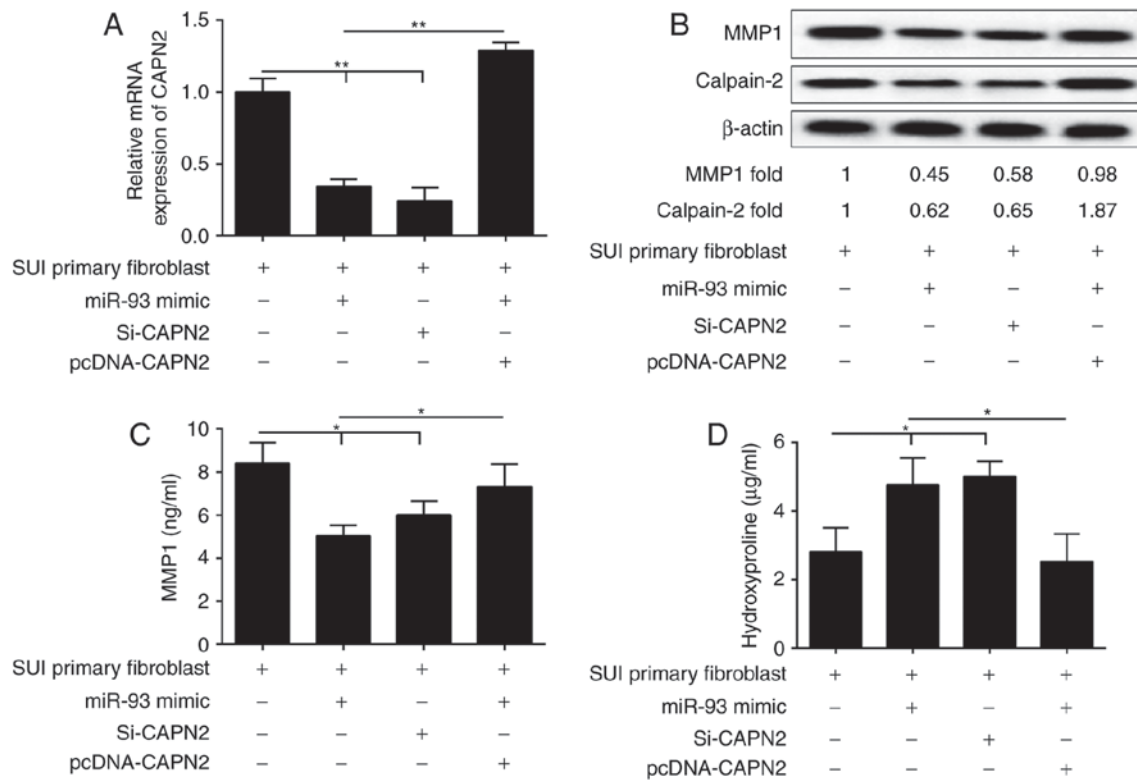


Figure 4. miR-93 regulates MMP1 and collagen I expression in fibroblasts via calpain-2. (A) The mRNA expression of CAPN2 decreased following miR-93 overexpression or CAPN2 silencing. (B) The protein levels of MMP1 and calpain-2 decreased following miR-93 overexpression or CAPN2 silencing. Calpain-2 overexpression reversed this effect. (C) The MMP1 level secreted by SUI primary fibroblasts decreased following miR-93 overexpression or CAPN2 silencing. Calpain-2 overexpression reversed this effect. (D) The hydroxyproline level in the supernatant increased following miR-93 overexpression or CAPN2 silencing. Calpain-2 overexpression reversed this effect. *P<0.05; **P<0.01. CAPN2, calpain-2 gene; SUI, stress urinary incontinence; miR, microRNA; si, small interfering; MMP1, interstitial collagenase.

In addition, the effects of miR-93 overexpression and miR-93 inhibition on the expression of calpain-2 were measured by western blotting, which further verified that overexpression of miR-93 decreased calpain-2 expression and that inhibition of miR-93 increased calpain-2 (Fig. 3B and C). The results of the present study demonstrated that calpain-2 was a direct target of miR-93.

miR-93 regulates MMP1 and collagen I expression via calpain-2. The mRNA expression of calpain-2 decreased following miR-93 overexpression or CAPN2 silencing. The protein levels of MMP1 and calpain-2 decreased following miR-93 overexpression or CAPN2 silencing, and CAPN2 overexpression reversed this effect (Fig. 4A and B). The MMP1 level secreted by SUI primary fibroblasts decreased following miR-93 overexpression or CAPN2 silencing, and calpain-2 overexpression reversed this effect (Fig. 4C). The hydroxyproline level in the supernatant increased following miR-93 overexpression or CAPN2 silencing, and calpain-2 overexpression reversed this effect (Fig. 4D).

Discussion

Previous studies have demonstrated that miRNAs exert important roles in regulating a variety of biological and pathological processes, including cell growth, differentiation and apoptosis (17,18). Numerous studies have demonstrated that miRNAs are involved in the progression of diseases that occur

in different tissues. For example, miR-139 was able to regulate pulmonary fibrosis via β -catenin (19). miR-122 has been demonstrated to be associated with iron overload-mediated hepatic inflammation (20). miRNA-155 may be considered to be a novel potential marker of subfertility in men with chronic kidney disease (21). It has additionally been reported that miRNAs serve roles in SUI. In 2014, Liu *et al* (22) reported that 12 miRNAs were abnormally expressed in SUI, including two groups: In the upregulated group were let-7a, miR-101, miR-125b-2, miR-190b and miR-892b; in the downregulated group were miR-124, miR-330-3p, miR-485-3p, miR-517b, miR-523, miR-589, and miR-93. In addition, miR-93 has been reported to be involved in a number of diseases and to be abnormally expressed in the progression and tumorigenesis of a number of types of cancer, including endometrial, lung, breast, hepatocellular, pancreatic, colorectal and ovarian cancer (23). However, the roles of miRNA in SUI remain unclear. The present study provided new evidence that miR-93 was able to regulate the expression of collagen in SUI via calpain-2, which enriches the literature and supports the hypothesis that miR-93 may serve a prominent role in SUI.

In the present study, it was observed that miR-93 expression was significantly downregulated in the vaginal wall tissues of patients with SUI and in SUI primary fibroblasts. In order to further investigate the function of miR-93 in the development of SUI, a luciferase reporter assay was performed to investigate its association with calpain-2. The results demonstrated that miR-93 was able to bind to CAPN2;

additionally, overexpression of miR-93 decreased the expression of calpain-2, which suggested that calpain-2 may be a direct target of miR-93. As the primary components of the urethral support tissues are connective tissues which contain a large number of ECM components, and ECM metabolism is regulated by degradative enzyme matrix metalloproteinases (MMPs) (23,24), degradative enzyme inhibitors and tissue inhibitors of metalloproteinases may be altered in patients with SUI. In the present study, it was observed that MMP1 was upregulated in the vaginal wall tissues of patients with SUI and in SUI primary fibroblasts, suggesting that MMP1 may be negatively regulated by miR-93, which was consistent with a previous report (14).

A previous study demonstrated that collagen (collagen I and III) degradation may be associated with the pathogenesis of SUI, and have indicated that dysregulation of collagen metabolism may be involved in the development of SUI (24). Hydroxyproline is a primary component of collagen and has been used as a biochemical marker for collagen content (25). The results of the present study revealed that the expression of hydroxyproline was downregulated in SUI primary fibroblasts, which indicated that collagen expression was downregulated in the SUI group. The hydroxyproline level was additionally detected when miR-93 was overexpressed or CAPN2 was silenced, and it was demonstrated that the hydroxyproline level was increased, while CAPN2 overexpression reversed this effect, indicating that calpain-2 was negatively associated with collagen expression. The present findings suggested that the decrease in collagen expression mediated by the downregulation of miR-93 may be involved in the development of SUI. In addition, miR-93 may bind to CAPN2, and miR-93 exhibited a marked negative association with calpain-2, indicating that miR-93 may mediate collagen expression in SUI via calpain-2.

In conclusion, the results of the present study indicated that miR-93 was downregulated in the vaginal wall tissues of patients with SUI and in SUI primary fibroblasts, and its level was associated with collagen expression. Additionally, miR-93 overexpression increased the expression of collagen by targeting calpain-2. The present findings further clarified the role of miR-93 and provided a theoretical basis for the development of novel treatments for SUI.

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