BASIC SCIENCE

miR-195-5p Regulates the Phenotype Switch of CCSM Cells by Targeting Smad7

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ABSTRACT

Introduction: Phenotype switch refers to the process in which smooth muscle cells change from contractile type to synthetic type and acquire the ability of proliferation. Phenotypic transformation involves many changes of cell function, such as collagen deposition and fibrosis, which affect the normal erectile function of penis.

Aim: To investigate the role of miR-195-5p in regulating the Phenotype switch of the corpus cavernosum smooth muscle (CCSM) cells.

Methods: A small mother against decapentaplegic 7(Smad7) virus vector and a miR-195-5p mimics or an si-Smad7 viral vector and a miR-195-5p inhibitor were transfected into CCSM cells. The cells were obtained by primary culture of rat corpus cavernosum smooth muscle tissue. Real-time polymerase chain reaction (PCR) experiments, Western blotting, hematoxylin-eosin (HE) staining, transwell experiments, MTT assays, and flow cytometry were used to detect miR-195-5p, Smad7, phenotype switch markers of CCSM cells and related protein expression, as well as changes in cell morphology, migration, proliferation and apoptosis.

Main Outcome Measure: To study the regulation of miR-195-5p in CCSM cells by overexpression and silencing strategies.

Results: Overexpressed miR-195-5p promoted the transformation of CCSM cells from a contractile type to a synthetic type. Meanwhile, the migration ability and proliferation ability of CCSM cells increased, and the apoptosis rate decreased. The expression-silencing of miR-195-5p gave rise to the opposite effect. The results of the rescue experiment demonstrated that overexpressed Smad7 rescued the inhibitory of the switch of the CCSM cell phenotype from the contractile type to the synthesis type caused by overexpression of miR-195-5p alone. Moreover, the enhancement effect of the migration ability and proliferation ability of CCSM cells was also eliminated, and the apoptosis rate was increased. Silencing miR-195-5p and Smad7 at the same time resulted in the opposite effect.

Conclusion: miR-195-5p may regulate the phenotype switch of CCSM cells by targeting Smad7. **Zhang J, Zhang X, Zhang J, et al. miR-195-5p Regulates the Phenotype Switch of CCSM Cells by Targeting Smad7. Sex Med 2021;9:100349.**

Key Words: Erectile Dysfunction (ED); miR-195-5p; Small Mother Against Decapentaplegic 7(Smad7); Corpus Cavernosum Smooth Muscle (CCSM); Phenotypic Transformation

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INTRODUCTION

Erectile dysfunction (ED) is defined inability to achieve and maintain a sufficient penile erection to successfully engage in sexual intercourse. ED is a common disease that is a serious problem for men. There are more than 100 million ED patients in the world. It is estimated that by 2025, there will be more than 300 million ED patients worldwide.[1] The data show that the total prevalence of ED in China is 26.1%, while that in men

over 40 years old is as high as 40.2%. ED seriously affects quality of life in men and is an important hidden danger to social stability. At present, the treatment of ED is insufficient, which is largely due to the complex pathogenesis of ED.

The pathogenesis of ED is complex and has not been fully elucidated. Research shows that the causes of ED mainly include vascular disease, hypogonadism, increased oxygen free radicals, and autonomic and peripheral nerve damage.[2-4] The main tissues exhibiting pathology are the penile vascular endothelium and cavernous smooth muscle. Pathology observed in patients with ED included a decrease in nitric oxide synthesis, impaired vascular endothelial function, a decrease in smooth muscle cells and an accumulation of collagen fibers.[3-5] There are many molecular mechanisms involved in ED, such as disruption of the endothelial nitric oxide synthase (eNOS)/cyclic guanosine monophosphate (cGMP)/cGMP-dependent protein kinase G (PKG) pathway, upregulation of the transforming growth factor-β1 (TGF-β1)/ Smad pathway, and upregulation of the RhoA pathway.[6] However, these findings have not completely solved clinical problems. It is still of great significance to further explore the pathogenesis of ED and find new therapeutic targets.

In the past, studies on the pathogenesis of ED have mainly focused on changes in the vascular endothelium and vasoactive factors. It is well known that a normal penile erection depends on a certain number of cavernous smooth muscle cells with diastolic function. It is worth noting that in most cases, ED eventually leads to changes in the structure of the cavernous body of the penis and to changes in specific functions, including different degrees of fibrosis, decreased cavernous smooth muscle cell number, and the loss of normal diastolic function in the cavernous body. In other words, collagen deposition and cavernous fibrosis are the common pathological outcomes of ED with different causes.

In recent years, the study of smooth muscle phenotype switching in cardiovascular disease has increased in depth. Vascular smooth muscle cells (VSMCs) have bidirectional differentiation functions. According to the differences in structure and function, VSMCs can be divided into contractile and synthetic phenotypes. The function of contractile cells is contraction, while the main functions of synthetic cells are proliferation, migration, and the regulation and secretion of extracellular matrix.[7,8] When blood vessels are damaged or VSMCs cultured in vitro are stimulated by certain factors, VSMCs can transform from the contractile phenotype to the synthetic phenotype and acquire the proliferative ability. This process of morphological, structural and functional changes is called phenotypic transformation. During the phenotypic switch, contractile phenotype markers such as α-smooth muscle actin (a-SMA), smooth muscle myosin heavy chain (SMMHC), smoothelin, smooth muscle 22α (SM22α) and calponin 1 are downregulated. Corpus cavernosum is considered a special vascular tissue. The smooth muscle cells in the trabeculae of the corpus cavernosum are similar to vascular smooth muscle cells (VSMCs) in function.[9] Cavernous smooth muscle (CCSM) is the structural basis of cavernous sinus space relaxation and penile erection, accounting for 40%-50% of the total composition of cavernous tissue. CCSM plays a key role in the hemodynamic changes of normal penile erections. Moreover, CCSM is the terminal tissue of various factors and plays an important role in the core position. When the number of CCSM cells is reduced or phenotypic transformation is induced by various causes, hemodynamic changes in the corpus cavernosum will be caused, and then the normal erectile function of the penis will be affected.[10-12] Previous studies have confirmed that in penile tissue, upregulation of the TGF-β1/Smad signaling pathway can promote the switching of the corpus cavernosum smooth muscle (CCSM) cell phenotype from a systolic type to a synthetic phenotype.[13] The increase in synthetic cells leads to an increase in collagen fibers, which leads to fibrosis of the penile cavernous body.[13] Smad7 belongs to the Smad family, which is an intermediary signaling molecule that helps TGF-β1 bind with its receptor and then transmit a signal from the cytoplasm to the nucleus. Smad7 is an inhibitory Smad protein and has been well established as a key negative regulator of TGF-β1/Smad signaling. Smad7 can competitively and tightly bind to the TGF-β1 receptor, thus blocking the TGF-β1 signal transduction pathway.[14,15]

MicroRNAs (miRNAs) are highly conserved in structure and have offered a new point for the study of disease mechanisms. According to previous studies, miRNAs are closely associated with the onset of diseaseand mainly regulate target genes at the post-transcriptional level.[16,17] Recently, functional research on miRNAs has been performed in many areas of life science, such as cardiovascular disease, nervous system diseases, and cancer.[18,19] miR-195-5p, a less well-studied miRNA, plays crucial roles in many diseases, including cancers[20-22], acute kidney injury[23] and gestational diabetes.[24] Only a few targets of miR-195-5p are known, such as *FKBP1A*[21], *MACC1*[25] and *VEGFA*[26]. It has been reported that miR-195-5p plays an important regulatory role in smooth muscle cells,[27-29] and smooth muscle is an important effector tissue of ED. However, no study has reported the role of miR-195-5p in ED. In this work, we carried out functional studies of miR-195-5p in the phenotypic switching of CCSM cells. We also identified Smad7 as a novel target of miR-195-5p in CCSM. Taken together, these data indicate that miR-195-5p may serve as a promising therapeutic target for the treatment of ED.

MATERIALS AND METHODS

Separating and Identifying Corpus Cavernosum Smooth Muscle (CCSM) Cells

Nanjing Medical University Animal Laboratory (Nanjing, China) provided us with male Sprague-Dawley rats. Then, following protocols from other authors, explant cell cultures were prepared.[30,31] The Ethics Committee of Nanjing Maternal and Child Health Hospital approved this study.

Experimental Grouping and Cell Transfection

The Smad7 overexpression vector, which was used to induce Smad7 overexpression (rescue experiment), and miR-195-5p

mimics were used to establish 5 groups: miR-195-5p mimics (mimics), Smad7 overexpression (op-Smad7), miR-195-5p mimics+Smad7 overexpression (MO), blank control (BC), and negative control (NC) groups. Then real-time PCR was used to determine whether mature miR-195-5p and Smad7 were overexpressed in the cells.

A Smad7 expression-silencing vector was constructed. Smad7 was silenced by miR-195-5p-mediated overexpression (rescue experiment), and 5 groups were established: the miR-195-5p (inhibitor), Smad7 expression-silencing (si-Smad7), miR-195-5p inhibitor +Smad7 expression-silencing (IS), blank control (BC), and negative control (NC) groups. Then, real-time PCR was applied to determine whether mature miR-195-5p and Smad7 expression in the cells was silenced.

Histopathological Evaluation

HE staining was performed on second-passage CCSM cells. Eighty percent of the cells were fused in the 6-well plate. The culture medium was removed. After washing them twice with PBS, 4% of the cells were fixed with POM. After 24 hours, hematoxylin and eosin staining was performed, and then the cells were observed under a microscope (Olympus; Tokyo, Japan).

MTT Assay of Cell Viability

IPEC-1 cells in the logarithmic growth phase were inoculated into 96-well plates at a density of 1.0×10^4 cells/well. 5% CO_2 was used to incubate the cells at 37°C for 24 hour and Se-AOS was exploited to treat them for 24 hours. Then, 0.5 mg/mL MTT solution replaced the medium. Incubation for 4 hour later, the 96-well plates were centrifuged at 4000 r/min for 10 minutes. Then we discarded the liquid in each well, added 150 μL of DMSO, and measured the OD values of each well at a wavelength of 570 nm.

Isolation of RNA and Quantitative Real-Time PCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to collect CCSM cells. According to the manufacturer's protocol, the total RNA was extracted and purified by RNeasy Mini Kit (Qiagen, Hilden, Germany). Then, an M-MLV Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA) was used to perform reverse transcription for miRNA analysis. SYBR Premix Ex Taq Reagent test kit (TaKaRa, Foster City, CA, USA) on a StepOne Real-Time PCR System (Life Technologies) were leveraged for performing qPCRs. Expression levels of miR-195-5p or mRNAs were standardized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or the levels of U6 expression. The results were analyzed using the ΔΔCT method. The primer sequences were as follows: mir-195-5p RT- CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCCAATAT; mir-195-5p F-CGCAGCACAGAAATATTGGC; mir-195-5p R- CTCAACTGGTGTCGTGGAGTC; Smad7 F- GGGCTTTCAGATTCCCAACTT; Smad7 R- GCCGATCTTGCTCCTCACTT; GAPDH F- CGCTAACATCAAATGGGGTG; GAPDH R- TTGCTGACAATCTTGAGGGA G; hU6 F- CCTGCTTCGGCAGCACAT; h-U6 R- AACGCTTCACGAATTTGCGT; R- OPN sense: AGCACACAAGCAGACGTTTTG; R- OPN antisense: GCAACTGGGATGACCTTGATAG; R- Calponin1 sense: AGCAGG AGCTGAGAGAGTGGAT; and R- Calponin1 antisense: TTCTC CAGCTGGTGCCAGTT.

Western Blotting

RIPA lysis buffer (ASPEN, NanTong, China) containing phenylmethylsulfonyl fluoride (Beyotime) and protease inhibitor cocktail (ASPEN, Pleasanton, CA, USA) were used to extract proteins. Then, the proteins were carried on a 10% SDS-PAGE gel via electrophoresis and transferred onto a polyvinylidene difluoride membrane. Five percent of skim milk was used to block the membrane for 2 hours and then together with an anti-Smad7 antibody were incubated (ab15116) for 24 hours and with secondary antibodies for 30 minutes. A developing agent was added to the membrane, and the results were recorded after X-ray exposure.

Apoptosis Induction and Cell Viability Assay

H_2O_2 was added to the culture medium at a final concentration of 200 μM to induce apoptosis in CCSMs. The cells were placed in a 6-well plate with 2×10^5 cells/cm^2 density, cultivated for 24 hours, and then the cell survival rate was determined. After rinsing the cells, they were cultivated with exosome-depleted media containing FBS with or without exosomes at 10 μg/mL or 20 μg/mL for 6 hours and then treated with H_2O_2 for 18 hours to induce apoptosis. Soon afterward, PBS was explored to collect and wash the cells twice, which was treated with Annexin-V-propidium iodide double staining. Cell viability was detected by a flow cytometer (BD FACS Canto, Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Statistical Analysis

The mean ± standard deviation (SD) was utilized to express the data. Comparison between 2 groups were analyzed with Student's 2-tailed t-tests, and comparisons among 3 or more groups were analyzed with 1-way ANOVA. $P < .05$ was considered statistically significant and is indicated in the figures.

RESULTS

Overexpression and Silencing of miR-195-5p Result in Phenotypic Switching of CCSM Cells

We examined whether miR-195-5p affected CCSM cells. The morphological structure of CCSM cells was observed through HE staining. As shown in Figure 1A, the overexpression and silencing of miR-195-5p had no significant effect on the cells. The miR-195-5p mimics group expressed a higher level of miR-195-5p ($P < .05$, Figure 1B) than the control group, and the miR-195-5p silencing group expressed a lower level of miR-195-

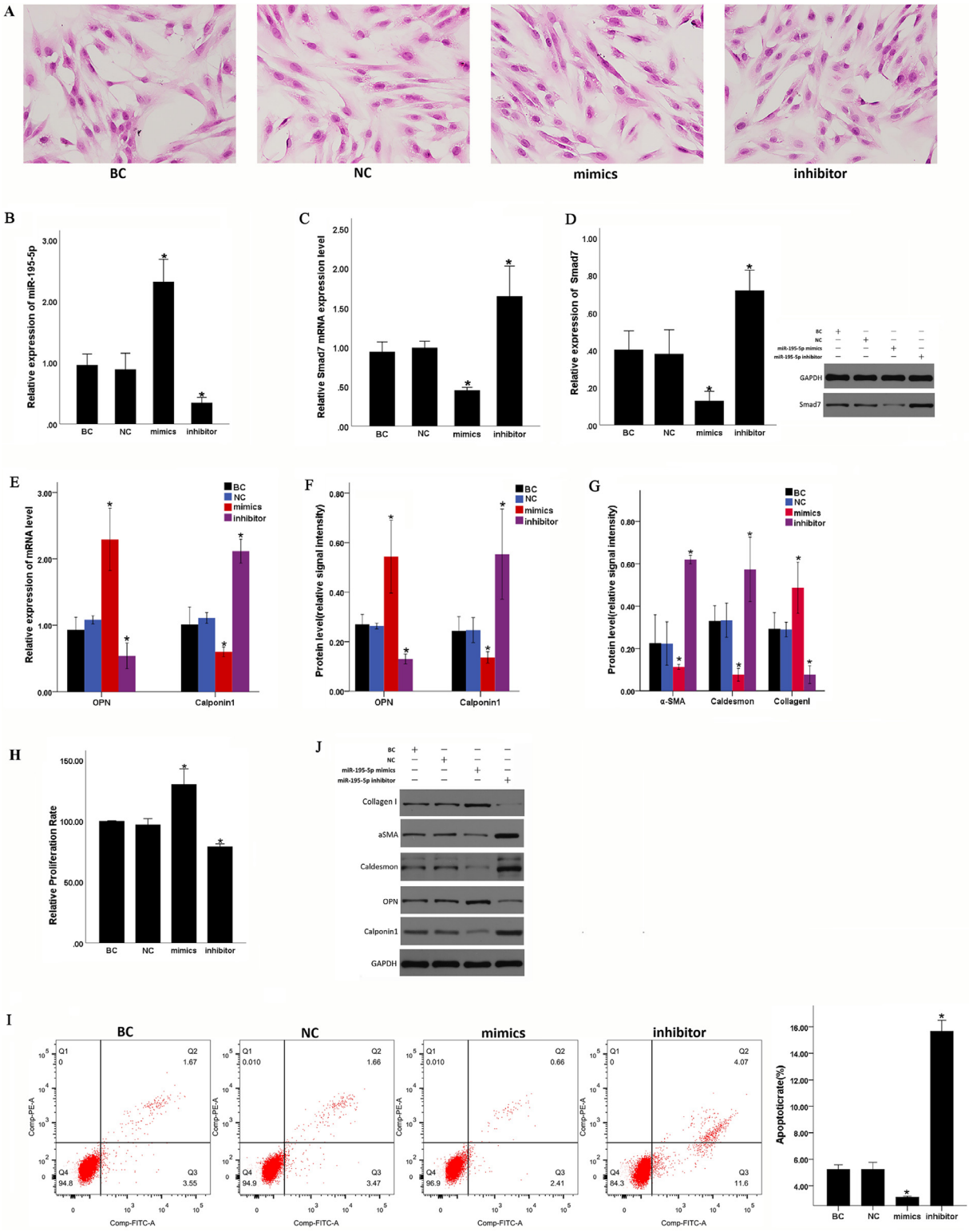

Figure 1. Overexpression and-silencing of miR-195-5p results in phenotypic switching of CCSM cells. (A) Histological changes in CCSM cells (400 × magnification) under hematoxylin and eosin (H&E) staining. (B) Efficiency of miR-195-5p overexpression or silencing. (C and D) Related protein and mRNA expression levels of Smad7. (E and F) Related protein and mRNA expression levels of Calponin1 and OPN (bar chart). (G) Related expression levels of proteins related to phenotypic transformation in CCSM cells (bar graph). (H) Cell proliferation measured by the MTT method, *$P < .05$. (I) CCSM cell apoptosis rate detected by flow cytometry, *$P < .05$. (J) Related expression levels of the proteins in CCSM cells (grayscale). NC = normal control; BC = blank control; mimics = miR-195-5p mimics; inhibitor = miR-195-5p inhibitor.

5p than the control group, as verified by qPCR. Then, PCR was applied to determine the expression level of Smad7 mRNA in CCSM cells after miR-195-5p overexpression and silencing. The miR-195-5p overexpression group expressed less Smad7 mRNA than the control group, while the miR-195-5p silencing group expressed more Smad7 mRNA than the control group (Figure 1C). Furthermore, the Smad7 protein level was detected by Western blotting. The results showed that the Smad7 protein level was lower in the miR-195-5p overexpression group, and higher in the miR-195-5p expression-silencing group (Figure 1D). The mRNA expression levels of Calponin1 and OPN in CCSM cells were detected by PCR after overexpression and silencing of miR-195-5p.

The mRNA and protein expression levels of Calponin1 and OPN in CCSM cells were detected separately by PCR and Western blotting. When miR-195-5p were overexpressed, the expression level of the CCSM contractile marker Calponin1 was less and the expression level of the CCSM synthetic marker OPN was higher in the miR-195-5p mimics group.When miR-195-5p was silenced, the expression level of the CCSM contractile marker Calponin1 was higher and the expression level of the CCSM synthetic marker OPN was lower than that in the control group (Figure 1E, F, and J). The relative expression of proteins related to CCSM phenotype transformation is shown in Figure 1G and Figure 1J. Compared with the negative and blank control groups, the miR-195-5p group showed reduced expression of α-SMA and caldesmon and increased collagen I expression. Compared with the negative and blank control groups, the miR-195-5p-silencing group showed significantly increased α-SMA and caldesmon expression and reduced collagen I expression.

The MTT assay results demonstrated that the miR-195-5p mimics group had a higher proliferation ability than the negative and blank control groups ($P < .05$), while the proliferation ability of the miR-195-5p silencing g group was lower than that of the negative and blank control groups. This result indicates that the proliferation of CCSM cells may be promoted by miR-195-5p (Figure 1H). Flow cytometry showed that the apoptosis rate was lower in the miR-195-5p mimics group than in the negative and blank control groups ($P < .05$), which suggested that overexpression of miR-195-5p inhibited apoptosis in CCSM cells (Figure 1I).

Overexpression of Smad7 Rescued the Inhibitory Effect of miR-195-5p Overexpression on the Phenotype in CCSM Cells

The morphological structure of CCSM cells was observed through HE staining. The influence of simultaneous overexpression of miR-195-5p and Smad7 was not statistically significant, as shown in Figure 2A.

To further investigate the possible mechanism of miR-195-5p in the phenotypic transformation of CCSM cells, we simultaneously overexpressed miR-195-5p and Smad7. Figure 2B and C present the qPCR verification of the overexpression of miR-195-5p and Smad7. restored Smad7 protein expression miR-195-5p significantly downregulated the Smad7 protein expression level, and Smad7 overexpression restored Smad7 protein expression (Figure 2D). In Figure 2E, F, and J, miR-195-5p overexpression led to a phenotypic switch in CCSM cells from the contractile phenotype to the synthetic phenotype. However, compared with the group in which of miR-195-5p alone was overexpressed, the group in which miR-195-5p and Smad7 were simultaneously overexpressed showed increased calponin 1 expression levels and reduced OPN expression levels, which inhibited the transformation of the CCSM cell phenotype from the contractile phenotype to the synthetic phenotype. The relative expression of proteins related to CCSM phenotype transformation is shown in Figure 2G and Figure 2J. Overexpression of both miR-195-5p and Smad7 rescued the decrease of α-SMA and caldesmon expression caused by the overexpression of miR-195-5p alone, and the expression levels of α-SMA and caldesmon were equal to those of the control group. Overexpression of both miR-195-5p and Smad7 also rescued the increase in collagen caused by the overexpression of miR-195-5p alone, but its expression level in the double overexpression group was still higher than that in the control group ($P < .05$).The MTT assay results showed that simultaneous overexpression of miR-195-5p and Smad7 reversed the increase in the apoptosis rate of CCSM cells that resulted from overexpression of miR-195-5p alone, but the apoptosis rate in the double overexpression group was still higherthan that inthe control group, although this difference was not statistically significant ($P < .05$) (Figure 2H). Flow cytometry showed that simultaneous overexpression of miR-195-5p and Smad7 reversed the decreased proliferation of CCSM cells caused by the overexpression of miR-195-5p alone(Figure 2I).

Simultaneous Silencing of miR-195-5p and Smad7 Rescued the Phenotypic Switch Effect Induced by miR-195-5p Silencing in CCSM Cells

Afterward, we simultaneously silenced miR-195-5p and Smad7. The silencing of miR-195-5p and Smad7 was verified by qPCR (Figure 3A). We discovered that silencing miR-195-5p alone significantly upregulated Smad7 protein expression, and additionally silencing Smad7 reversed this effect (Figure 3B).

Figure 3C, D, and H present the differential expression of Calponin1 and OPN in the group with simultaneous silencing of miR-195-5p and Smad7, as measured by quantitative polymerase chain reaction. When miR-195-5p and Smad7 were silenced at the same time, the Calponin1 expression level was significantly lower than that of the negative and blank control groups but more than that of the miR-195-5p-silenced group. The expression of OPN showed the opposite trend. The OPN expression level in the group with simultaneous silencing of miR-195-5p and Smad7 was higher than those in the normal

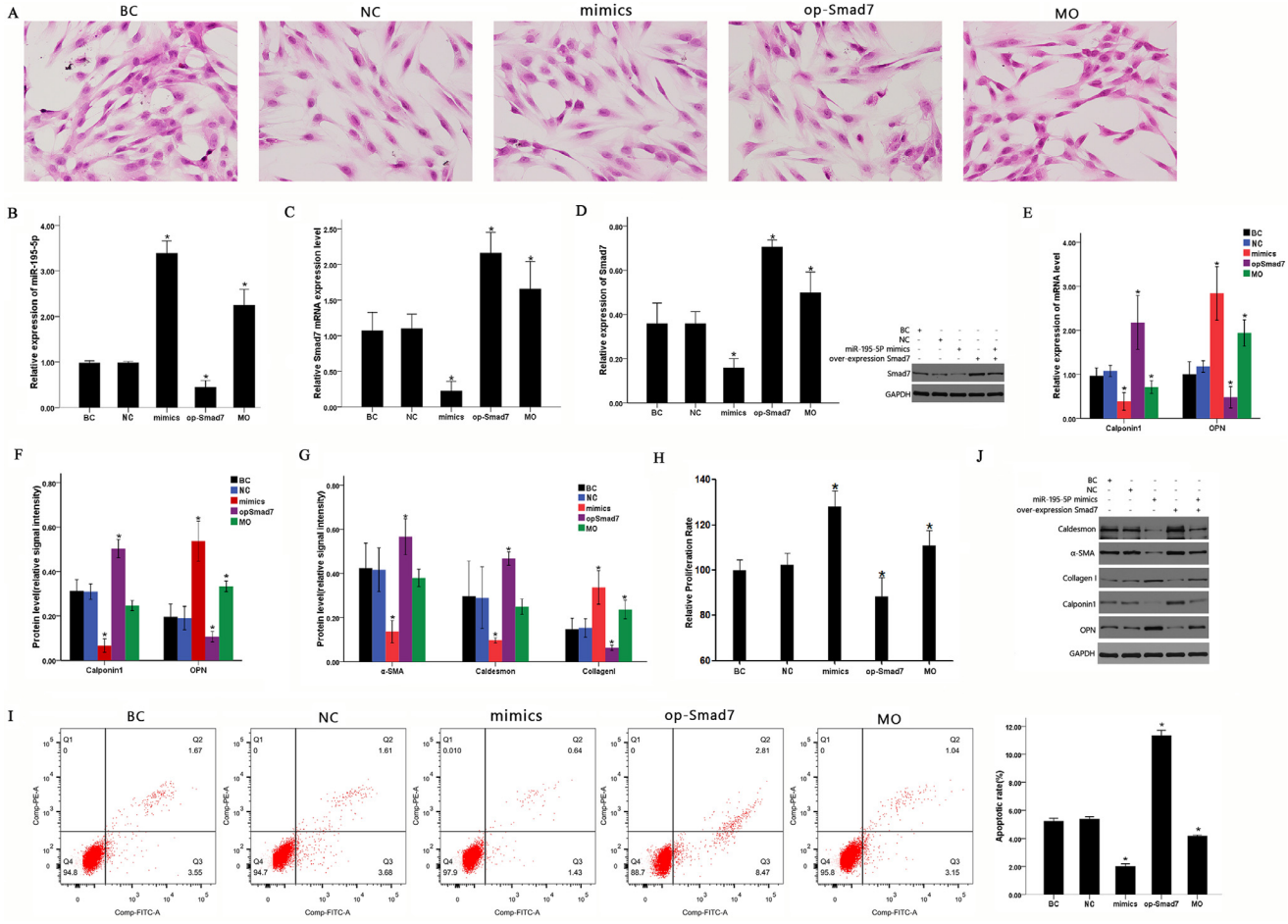

Figure 2. Overexpression of Smad7 and miR-195-5p rescued the inhibitory effect caused by overexpression of miR-195-5p alone on the CCSM cell phenotype, resulting in the transformation from the contractile phenotype to the synthetic phenotype. (A) Histological changes in CCSM cells (400 × magnification) under hematoxylin and eosin (H&E) staining. (B) Efficiency of miR-195-5p overexpression. (C and D) Related mRNA and protein expression levels of Smad7. (E and F) Related mRNA and protein expression levels of Calponin1 and OPN (bar chart). (G) Related expression levels of proteins related to phenotype switching in CCSM cells (bar graph). (H) Cell proliferation measured by the MTT method $P < .05$. (I) CCSM cell apoptosis rate detected by flow cytometry, * $P < .05$. (J) Related expression levels of the proteins in CCSM cells (grayscale).

and negative control groups but lower than that in the miR-195-5p- silenced group ($P < .05$).

The relative expression of proteins related to CCSM phenotype transformation is shown in Figure 3E and H. Simultaneous silencing of miR-195-5p and Smad7 reversed the increase in α-SMA and caldesmon expression caused by miR-195-5p silencing alone, and their expression was reduced to levels significantly lower than the control level. Simultaneous silencing of miR-195-5p and Smad7 reversed the decrease in collagen caused by miR-195-5p silencing alone, and its expression level was higher than the control level ($P < .05$).

The MTT assay results showed that simultaneous silencing of miR-195-5p and Smad7 reversed the increase in the apoptosis rate of CCSM cells caused by miR-195-5p silencing alone, but the apoptosis rate was still higher than that of the normal control cells ($P < .05$) (Figure 3G). Flow cytometry showed that simultaneous silencing of miR-195-5p and Smad7 reversed the decrease in the proliferative ability of CCSM cells caused by silencing of miR-195-5p alone, and the proliferation level was not significantly different from the control level (Figure 3F).

DISCUSSION

Erectile dysfunction is a serious problem for male patients. Because the pathogenesis of ED is very complex, there are many shortcomings in the current clinical treatment methods. For example, phosphodiesterase 5 (PDE5) inhibitors are commonly used in the clinical treatment of ED and ease the suffering of some patients.[32] However, for ED related to senility, diabetes, and nerve-damage, such treatments are not effective,[33,34] and the cost is high. PDE5-Is are mainly used to alleviate symptoms and cannot cure the condition, and their long-term safety is not fully

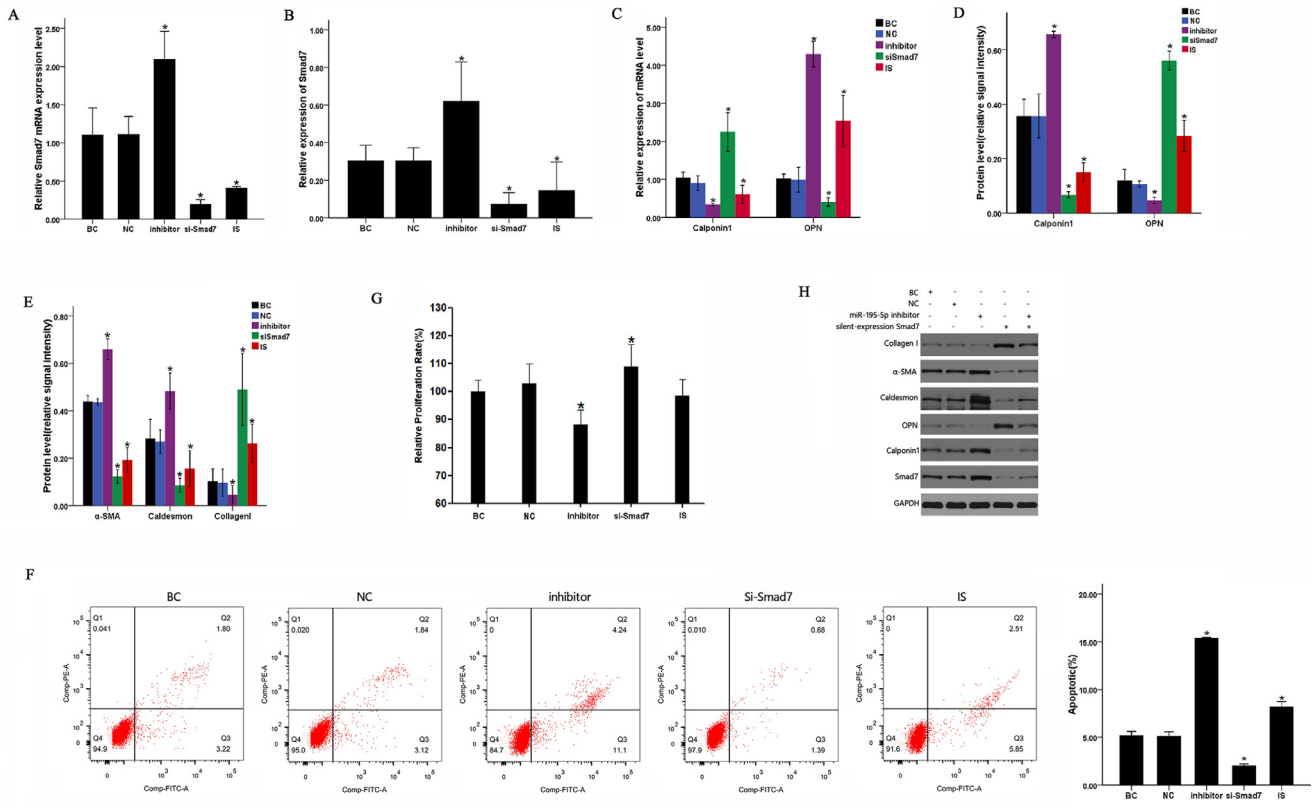

Figure 3. Simultaneous silencing of miR-195-5p and Smad7 reverses the phenotypic transformation of CCSM cells from synthetic to contractile induced by the silencing of miR-195-5p alone. (A and B) Related mRNA and protein expression levels of Smad7. (C and D) Related mRNA and protein expression levels of Calponin1 and OPN (bar chart). (E) Related expression levels of proteins related to phenotypic transformation in CCSM cells (bar graph). (F) CCSM cell apoptosis rate detected by flow cytometry, * $P < .05$. (G) Cell proliferation measured by the MTT method, * $P < .05$. (H) Related expression levels of the proteins in CCSM cells (grayscale).

understood. Studies have found that PDE5-Is may be related to hearing impairment.[35] Other methods currently used for the clinical treatment of ED, such as testosterone replacement therapy, sexual psychotherapy, and lifestyle improvement, also have limitations.[36,37] Therefore, exploring new mechanisms of ED pathogenesis and finding a more effective treatment and a cure for ED has been a popular area of research in the field of andrology.

Past research on the pathogenesis of ED has focused on changes in the vascular endothelium and vasoactive factors. However, normal penile erections require a specific number of CCSM cells with diastolic function. Changes in the structure of the cavernous body of the penis, which can occur via fibrosis and reduction of cavernous smooth muscle cells, will cause the cavernous body to lose its normal diastolic function, resulting in ED. ED is closely related to phenotypic transformation of the CCSM. CCSM accounts for 40%-50% of the entire penile corpus cavernosum tissue composition. The phenotypic transformation of CCSM cells refers to the change in CCSM cells from "contractile" to "synthetic" or "proliferative." Smooth muscle myosin heavy chain (SMMHC), osteopontin (OPN), calponin, α-smooth muscle actin (α-SMA), smoothelin, and desmin can be used as molecular markers of contractile CCSM cells; vimentin[38,39] and collagen I can be used as molecular markers of synthetic or proliferative CCSM cells. The 2 phenotypes of CCSM cells have an important effect on penile erectile function. The proper proportion of CCSM cells of different phenotypes must be maintained, or the conversion from the contractile phenotype to the synthetic phenotype will lead to ED.

Since the sex organs are situated on the superficial part of the body with relatively slow blood circulation, they may be a target for gene therapy.Recently, investigators found that miRNAs play a significant role during the onset of reproductive system diseases, cardiovascular diseases, cancer, and so on.[17,18] Therefore, determining the role of miRNAs in the diastolic function of CCSM cells is necessary.

In addition, miR-195-5p plays an essential role in the phenotypic transformation of CCSM cells, and Smad7 was confirmed to target miR-195-5p via prediction and detection. Therefore, this study explored the effect of miR-195-5p on Smad7 expression levels and the CCSM cell phenotype transformation via overexpression and inhibition experiments. Moreover, rescue experiments were performed to demonstrate the mechanism by

which miR-195-5p regulates CCSM phenotypic phenotype transformation through Smad7.

Our research found that after overexpressing miR-195-5p suggested that the CCSM contractile markers Calponin 1, α-SMA, caldesmon, and Smad7 protein expression levels were significantly lower than those of the control group, while the CCSM synthetic markers OPN and collagen1 protein expression levels have opposite effects. The results of miR-195-5p silencing were contrary to the above. The above findings indicated that overexpressed miR-195-5p can affect the phenotypic transformation of CCSM cells. Overexpressed miR-195-5p can lead to the transformation from contractile CCSM cells to syngeneic CCSM cells, increase the synthesis of the CCSM, and increase collagen, which weakens or eliminates the diastolic function of the cavernous body, thus regulating the process of ED by remodeling the cavernous tissue structure. The increase in the number of synthetic cells leads to proliferation, migration, secretion and degradation of extracellular proteins, which is consistent with our experimental results.

In the rescue experiment, we found that miR-195-5p overexpression significantly downregulated Smad7 expression at the protein level. When miR-195-5p and Smad7 were overexpressed at the same time, the expression level of Smad7 protein was increased in the double overexpression group compared to the control group but was still lower than that in the Smad7 overexpression group. When miR-195-5p and Smad7 were simultaneously silenced, the protein expression level of Smad7 in the double silencing group was significantly lower than that in the control group but was higher than that in the Smad7 silencing expression group. These results suggest that miR-195-5p downregulates Smad7 expression at the protein level. In the rescue experiment, detection of Calponin 1, OPN and proteins related to CCSM phenotype transformation also showed that simultaneous overexpression of miR-195-5p and Smad7 could rescue the increase in synthetic CCSM caused by miR-195-5p overexpression alone, and the number of contractile CCSM cells increased. Moreover, the proliferation capacity of CCSM cells was also reduced, and the rate of apoptosis increased. Under normal homeostatic conditions found in healthy vascular physiology, smooth muscle cells (SMCs) ordinarily have low baseline levels of proliferation[40-42]. SMCs with increased proliferation and decreased apoptosis may indicate a phenotypic switch from the contractile to the synthetic phenotype.In addition, the results of silencing miR-195-5p and Smad7 were the opposite. These results indicatethat miR-195-5p can regulate the phenotype switch of CCSM through Smad7.

Previous studies have confirmed that Smad7 belongs to the Smad family. At present, 8 kinds of Smad proteins have been found in mammals, and they can be divided into 3 categories: (i) receptor-regulated Smads (R-smads), including Smad l, Smad2, Smad3, Smad5 and Smad8; (ii) common Smads (co-Smads), including only Smad4 in mammals; and (iii) inhibitory Smads (I-Smads), including Smad6 and Smad7.[43,44] Smad proteins are signal-mediating molecules that can transmit signals from the cytoplasm to the nucleus after binding to TGF-β1 and its receptor. Smad7 is an inhibitory Smad protein and one of the main negative regulators of the TGF-β1/Smad signaling pathway. It can competitively and tightly bind to the TGF-β1 receptor, thus blocking the TGF-β1 signal transduction pathway.[14,15] TGF-β is a multifunctional mediator that regulates proliferation, differentiation, migration, adhesion and apoptosis in different cell types, such as macrophages, activated T and B cells, dendritic cells, and so on.[45,46] A great number of studies have indicated that disorder of the TGF-β1/Smad pathway can lead to tissue fibrosis, which is an essential pathogenic mechanism. As 2 major downstream regulators of TGF-β1 signaling, Smad2 and Smad3 promote TGF-β1-mediated tissue fibrosis, while Smad7 plays a protective role against TGF-β1-mediated fibrosis as a negative feedback regulator of the TGF-β1/Smad pathway.[47,48] In penile tissue, the upregulation of the TGF-β1/Smad signaling pathway can promote the transformation of the CCSM cell phenotype from the contractile phenotype to the synthetic phenotype. The function of contractile cells is contraction, while the main functions of synthetic cells are proliferation andmigration and the secretion and degradation of extracellular proteins. The increase in synthetic cells leads to an increase in collagen fibers, which leads to fibrosis of the corpus cavernosum.[13]

All the above consequences suggest that through Smad7, miR-195-5p may regulate the phenotypic switching switch of CCSM cells. Smad7 is one of the key proteins in the CCSM cell phenotype transformation process. The decreased expression of Smad7 in CCSM cells may give rise to a change in the CCSM cell phenotype from the contractile phenotype to the synthetic phenotype, which may lead to penile cavernous fibrosis, resulting in ED. However, it is worth noting that there are some limitations to this study. The CCSM cells employed in this study were cultured in vitro. Hence, the data are insufficient to illustrate the connection between real human penis CCSM cells and in vitro cells. Our study lacks in vivo experiments on the function of miR-195-5p and the relationship between miR-195-5p and Smad7. To verify the function of miR-195-5p, future studies need to be performed at the animal or human level.

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STATEMENT OF AUTHORSHIP

Jing Zhang: Conception and Design, Analysis and Interpretation of Data, Drafting the Article, Final Approval of the Completed Article; Jiehua Ma: Conception and Design, Analysis and Interpretation of Data, Revising It for Intellectual Content, Final Approval of the Completed Article; Lianjun Pan: Conception and Design, Analysis and Interpretation of Data, Revising It for Intellectual Content, Final Approval of the Completed Article; Aixia Zhang: Conception and Design, Revising It for Intellectual Content, Final Approval of the Completed Article; Jingjing Zhang: Acquisition of Data, Revising It for Intellectual Content; Final Approval of the Completed Article; Xingyuan Zhang: Acquisition of Data, Drafting the Article, Final Approval of the Completed Article; Shengnan Cong: Acquisition of Data, Analysis and Interpretation of Data, Revising It for Intellectual Content, Final Approval of the Completed Article. Lianjun Pan and Jiehua Ma conceived the idea, wrote the review, edited and approved the final version. Jing Zhang and Xingyuan Zhang wrote the manuscript.

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