BASIC SCIENCE

Overexpressing miR-122-5p Inhibits the Relaxation of Vaginal Smooth Muscle in Female Sexual Arousal Disorder by Targeting Vasoactive Intestinal Peptide Receptor 1

Shengnan Cong, MS,[1,#] Tao Gui PhD,[2,#] Qinchuan Shi MSc,[3] Jingjing Zhang MSc,[3] Jingyi Feng, MS,[4] Lianjun Pan, MD,[3] Jiehua Ma, PhD,[3] and Aixia Zhang, MD[1,3]

ABSTRACT

Introduction: Female sexual arousal disorder (FSAD) is a common issue causing physical and psychological pain, but it has no standard diagnostic criteria or treatment. So its pathogenesis desiderates to be explored.

Aim: To investigate the specific function of miR-122-5p in FSAD.

Methods: 18 subjects were grouped into FSAD and normal control groups according to the Chinese version of the Female Sexual Function Index, and the expression levels of miR-122-5p and vasoactive intestinal peptide receptor 1 (VIPR1) protein in their tissue were verified through real-time quantitative polymerase chain reaction (qRT-PCR) and western blot (WB) analysis. Then in *vitro* experiment, miR-122-5p was overexpressed or inhibited in rat vaginal smooth muscle cells (SMCs). The relaxation of rat vaginal SMCs was reflected by the cell morphology, intracellular free cytosolic calcium ion (Ca^{2+}) levels, cell proliferation and apoptosis, together with the cyclic adenosine monophosphate (cAMP) concentration and protein kinase A (PKA) activities. Additionally, the expression levels of relaxation-related proteins, including VIPR1, stimulatory G protein (Gs), adenylate cyclase (AC), and PKA, were detected based on WB analysis. Furthermore, a rescue experiment that simultaneously overexpressed or silenced miR-122-5p and VIPR1 was conducted, and all the indicators were evaluated.

Main Outcomes Measure: The expression level of VIPR1 and downstream proteins, cell morphology, cell proliferation and apoptosis, and intracellular free Ca^{2+} levels were examined.

Results: We verified that women with FSAD had higher miR-122-5p and lower VIPR1 protein. Then overexpressing miR-122-5p decreased relaxation of rat vaginal SMCs, which was manifested as a contractile morphology of cells, an increased intracellular free Ca^{2+} concentration, and lower cAMP concentration and PKA activity. Moreover, by rescue experiments, we inferred that VIPR1 was the target of miR-122-5p and affected the relaxation function of vaginal SMCs.

Conclusion: miR-122-5p regulates the relaxation of vaginal SMCs in FSAD by targeting VIPR1, ulteriorly providing an underlying diagnostic and therapeutic target for FSAD. **Cong S, Gui T, Shi Q, et al. Overexpressing miR-122-5p Inhibits the Relaxation of Vaginal Smooth Muscle in Female Sexual Arousal Disorder by Targeting Vasoactive Intestinal Peptide Receptor 1. Sex Med 2021;9:100390.**

Key Words: Female sexual arousal disorder; Vaginal smooth muscle cell; miR-122-5p; Vasoactive intestinal peptide receptor 1

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[1]School of Nursing, Nanjing Medical University, Jiangsu, China;

[2]Jiangsu Province Academy of Traditional Chinese Medicine, Nanjing, China;

[3]Women's Hospital of Nanjing Medical University (Nanjing Maternity and Child Health Care Hospital), Nanjing, China;

[4]High School Affiliated to Nanjing Normal University International Department, Nanjing, China

[#]These authors contributed equally to this work.

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INTRODUCTION

Female sexual dysfunction (FSD) is a common public problem that harms women's health and affects family and social stability.[1] FSD can be subdivided into four domains: hypoactive sexual desire, female sexual arousal disorder (FSAD), sexual pain and insufficient orgasm.[2-4] The prevalence of FSAD is the highest among these 4 types.[5] FSAD is defined as an inability to obtain or maintain sufficient sexual excitement to an extent that it causes personal distress.[6,7] As previous studies reported, the prevalence of FSAD was 28.3% in China.[2] However, considering differences in cultures, diagnostic criteria and data collection methods, the reported prevalence of FSAD differs considerably, and many cases may go unnoticed.[7,8] Furthermore, its clinical treatment currently depends on cognitive behavior therapy and other non-drug therapies. These existing treatments not only require many medical resources but also have poor efficacy, which increases the social and economic burden.[7,9] Therefore, it is necessary to conduct an in-depth study on FSAD to explore its potential mechanism and seek effective diagnosis and treatment.

Female sexual arousal is a neurovascular-mediated process characterized by an increase in vaginal diameter and vaginal wall thickness, genital tissue swelling, clitoral congestion and vaginal lubricating mucus secretion.[8,10] The possible mechanism of these main physiological changes is neurotransmitter-mediated smooth muscle (SM) relaxation together with increased blood flow to the clitoris, vagina and external genitalia.[10,11] At the same time, the vasodilatory function of smooth muscle will affect changes in blood flow.[12,13] Therefore, smooth muscle relaxation plays a vital role in FSAD and provides an entry point for the clinical treatment of FSAD, such as pelvic floor rehabilitation therapy.[4] However, the therapeutic effect of pelvic floor muscle training is still uncertain due to the absence of sufficient evidence.[14,15] Thus, there are still many gaps of knowledge hindering the clinical treatment of FSAD, and the regulatory mechanism of smooth muscle relaxation in the vagina needs to be further explored.

In the past few years, micro(mi)RNA has been a focus of study, and its functional exploration provides a novel perspective for the study of diseases. Mature miRNAs are a group of small non-coding RNAs that can bind to the 3′ untranslated region of mRNAs and then regulate gene expression by degrading mRNAs or inhibiting the progression of protein translation.[16-19] Recently, the potential therapeutic effect of miRNA has been examined in tumors, cardiovascular diseases, and other diseases,[20-22] involving its role in smooth muscle.[23-25] For instance, as a specific marker for the early prognosis of acute myocardial infarction,[26] miR-122-5p can participate in the proliferation and apoptosis of vascular smooth muscle cells (SMCs).[27] Moreover, Lazzarini R et al.[28] found that miR-122-5p was upregulated in leiomyoma progenitor cells relative to myometrial progenitor cells. These results indicate that miR-122-5p plays an essential role in regulating smooth muscle proliferation and apoptosis in many diseases. Nevertheless, the specific role and possible targets of miR-122-5p in smooth muscle relaxation in the vagina and FSAD remain to be explored.

In this work, by searching miRBase, we found that vasoactive intestinal peptide receptor 1 (VIPR1) may be the target of miR-122-5p. VIPR1 is one of the receptors of the vasoactive intestinal polypeptide (VIP), and it has a high affinity for VIP.[29] VIP was previously proven to have the main function of relaxing the blood vessels and non-vascular smooth muscles of the vaginal wall, causing local vasodilation and vaginal lubrication.[30,31] These findings indicate that by being targeted by VIP, VIPR1 is involved in the regulation of smooth muscle relaxation, and defects in VIPR1 may be the basis of the pathophysiology of FSAD.[31]

Therefore, in this study, we aimed to verify the expression levels of miR-122-5p and VIPR1 protein in vaginal tissue from FSAD through comparison with normal controls. Subsequently, by overexpressing and silencing miR-122-5p in rat SMCs, we explored its function in FSAD and then further investigated whether its function is mediated by targeting VIPR1.

MATERIALS AND METHODS

Collection of Clinical Specimens

Patients undergoing vaginal hysterectomy or with severe vaginal wall prolapse who required surgical treatment in the Women's Hospital of Nanjing Medical University were selected as the research subjects. The patients meeting the following criteria were enrolled: (i) adult Han women with medium or above educational levels; (ii) providing informed consent. Simultaneously, patients were excluded if they (i) had a serious organic disease, such as cardiovascular disease; (ii) had an asexual history; or (iii) their partners had sexual dysfunction. Before the operation, 18 subjects were evaluated according to the Chinese version of the Female Sexual Function Index (CVFSFI). In accordance with the cut-off value reported by Ma J et al,[2] 9 women were split into the FSAD group (with CVFSFI total score<23.45, arousal disorder score≤3.15 and lubrication disorder score≤4.05) and 9 women were split into the normal control (NC) group (with CVFSFI total score>23.45). After the operation, their vaginal tissues were collected and saved, which were exploited to preliminarily validate the differences in miR-122-5p and VIPR1 protein expression levels between FSAD and normal controls. The Institutional Review Board at Women's Hospital of Nanjing Medical University (Nanjing Maternity and Child Health Care Hospital) approved and supervised this study.

Isolation and Culture of Primary Rat Vaginal SMCs

Anterior vaginal wall tissues were separated from the uterus of 8-week-old normal control female Sprague Dawley (SD) rats. At the anterior midline portion under sterile conditions, full-thickness tissues of the anterior vaginal wall (0.4 × 0.4 cm) were obtained from the vaginal cuff and the

primary culture of rat smooth muscle cells was performed as previously described.[32] Tissues were cultivated in DMEM/F-12 (Invitrogen, Carlsbad, CA, USA) medium supplemented with 10% fetal bovine serum (FBS) (Gibico, Carlsbad, CA, USA), 1 mM glutamine (Invitrogen, Carlsbad, CA), 0.075% Na2HCO3, 100 U/mL penicillin and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA). The tissue was minced together with mechanically dissociated and PBS was simultaneously exploited to wash fragments of the tissue 3 times. Then they were plated onto fibronectin-coated dishes (Corning, NY, USA). After the initial outgrowth, clones with a morphology resembling the smooth muscle phenotype were patch cloned and propagated in a complete medium. The cultured rat vaginal SMCs were identified by immunocytochemical staining for alpha-smooth muscle actin (SMA) as described previously.[33]

miR-122-5p Overexpression and Inhibition

MiR-Up agomir (UGGAGUGUGACAAUGGUGUUUG) and MiR-Down antagomir (CAAACACCAUUGUCACACUCCA) products were purchased from GenePharma, Co., Ltd. (Shanghai, China) and used for miR-122-5p overexpression and inhibition, respectively. Primary rat SMCs were transfected with agomir or antagomir using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.[34] TRIzol reagent (Invitrogen, Carlsbade, CA) was leveraged to isolate total RNA from rat SMCs for the miRNA assay following the manufacturer's instructions. The level of intracellular miR-122-5p expression was examined with a U6 snRNA Normalization and Hairpin-it microRNA RT-PCR Quantitation Kit (GenePharma, Co., Ltd.) in line with the instructions. The miR-122-5p level was detected by stem loop real-time RT-PCR. Shortly, miR-122-5p was first reverse transcribed by specific stem-loop RT primers (GTCGTATCCAGTGCAGGGTCCGAGGTATT CGCACTGGATACGACCAAACA), and then quantitated by qPCR primers (forward: CGCGTGGAGTGTGACAATGG; reverse: AGTGCAGGGTCCGAGGTATT).

VIPR1 Knockdown and Overexpression

In rat vaginal SMCs, a lentiviral short hairpin RNA (shRNA) delivery system was leveraged to knock down VIPR1 expression. Shortly, 5×10^6 HEK293T cells were plated into 10 cm cell culture dishes and incubated overnight for 16 hours at 37°C and 5% CO_2. Then co-transfection of HEK293T cells at 80% confluence with the lentiviral expression constructs pLKO.1-shRNA, viral envelope plasmid (pMD2.G), and viral packaging plasmid (psPAX2) using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) were conducted based on the manufacturer's protocols. Lentiviral expression constructs with scrambled shRNA were used as a control (specified as Scr). The sequence of the Scr shRNA was: CCTAAGGTTAAGTCGCCCTCG. Meanwhile, lentiviral expression constructs with VIPR1 shRNA were used for VIPR1 knockdown, and VIPR1 shRNA sequence was: GCAGTTGATTGAGATACAGCG. Viral supernatants were collected at 72 hours post-transfection, filtered through 0.22 μm filters, and stored at -80°C until use. 2×10^6 Primary SMCs cells were seeded and incubated overnight prior to infection. The medium was replaced with a 1:3 dilution of viral supernatant supplemented with 10 μg/mL polybrene, and incubated for 24 hours, followed by replacement with normal growth medium. Stable VIPR1 knockdown cell lines were selected using 1 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO). VIPR1 overexpression in rat SMCs by a constructed eukaryotic expression vector of pcDNA3.1-VIPR1, and the empty pcDNA3.1 vector were used as vector controls.

Table 1. Sequences of the primers used for qRT-PCR analysis

Gene symbol	Direction	Sequence (5′-3′)
VIPR1	Forward	AAGCTGCACTGTACCCGAAA
	Reverse	CGCTGTTGAAGAGGGCCATA
Gs	Forward	TCAACGACTGCCGTGACATC
	Reverse	TGGGGAGAAAAGGGAAAGGT
AC	Forward	ACGGAGTCAACCGTTTCGGGAG
	Reverse	GGTCGGGTGAGAGTGGCAGG
PKA	Forward	TGCAATGTTTCCAGTCTCCTTT
	Reverse	GGTCAATGCCCCACAGTTTC
GAPDH	Forward	GCGAGATCCCGCTAACATC
	Reverse	CTCGTGGTTCACACCCATCA

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from human vaginal tissue and primary cell lines for qRT-PCR by using TRIzol reagent following the manufacturer's instructions. A HiScriptIII Reverse Transcriptase Kit (Vazyme, Nanjing, China) was utilized to synthesize complementary DNA (cDNA) for qRT-PCR. The thermocycling conditions of the reverse transcription were as follows: genomic DNA was removed with gDNA wiper at 42°C for 2 minutes; then, first strand cDNA was synthesized at 25°C for 5 min, 37°C for 45seconds and 85°C for 5seconds. According to the manufacturer's instructions, qPCR was subsequently conducted using AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China). Experiments were performed in an ABI7500 system (Applied Biosystems). The relative mRNA expression levels were normalized to the reference gene GAPDH and calculated using the $2^{-\Delta\Delta CT}$ method. The qRT-PCR reactions were carried out in triplicate in 96-well optical reaction plates. Table 1 displays the sequences of the primers for qRT-PCR.

Western Blot Analysis

With cell lysis buffer for Western (Beyotime, P0013), total protein was isolated from the cells and tissues. All proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and followed by semi-dry blotted onto PVDF membranes. Primary antibodies

against VIPR1 (Abcam, ab123517, dilution, 1:1000), stimulatory G protein (Gs) (Abcam, ab101736, dilution, 1:1000), adenylate cyclase (AC) (Abcam, ab14788, 1:1000), protein kinase A (PKA) (CST, #5842T, dilution, 1:1000) and GAPDH (MBL, M171-3MS, dilution, 1:3000) were used. HRP-conjugated rabbit anti-goat (ZSGB-Bio Technology Co., Ltd, 1:5,000; ZB-2306) and goat anti-rabbit IgG (Abconal, AS014, dilution, 1:10000) as the secondary antibody were used. The blots were developed with Western ECL Blotting Substrate (Tanon) and visualized by exposure to X-ray film, and the films were scanned with an Epson Perfection V700 Photo Scanner.

Relaxation Functional Analysis of Rat Vaginal SMCs

After overexpressing or silencing, 100 nM VIP (Sigma, St. Louis, MO, USA) was added to both the experimental group and the control group for eliciting the relaxation response. 60 seconds later, the reaction was interrupted by adding 80 μl of 2.5% glutaraldehyde. The morphology of rat vaginal SMCs was identified by inverted phase-contrast microscopy (Olympus IX70, JAPAN) and the individual cell length was measured with a graticule fitted on the inverted microscope. The average length of rat SMCs in the control state or experiment group was obtained from 10 cells from 1×10^4 rat vaginal SMCs randomly. The relaxation response was reflected by the increase in the average length of the 10 SMCs after treatment compared with the NC group.[35,36]

Cell Counting Kit-8 (CCK8) Assay

Cells were seeded in 96-well plates at a concentration of 2000 cells per well, CCK8 assay (Vazyme, Nanjing, China) was carried out after incubation for 4 days. At an absorbance of 450 nm, a Tecan Infinite M200 plate reader (Crailsheim, Germany) was leveraged to read the optical density (OD). All of the above assays were performed independently at least three times.

Cell Apoptosis Analysis

Using Annexin V-FITC/PI Apoptosis Detection Kit (Keygen, Nanjing, China), apoptosis was determined. Shortly, 1×10^6 cells were collected and washed twice in PBS, then 500 μl of binding buffer containing 5 μl Annexin V-FITC antibody and 5 μl Propidium Iodide (PI) was added to resuspend the cells for 10 minutes at room temperature while protected from light. The samples were subsequently analyzed by BD FACScalibur Flow cytometer. Also, Terminal deoxynucleotidyl transferase-mediated biotinylated UTP-nick end labeling (TUNEL) assay was performed for experimental verification. The cells were fixed, permeabilized, and labeled by following the protocol using In Situ Cell Death Detection Kit (Roche, Mannheim, Germany). Then, the slides were examined under a fluorescence microscope (magnified 200 ×).

Cyclic Adenosine Monophosphate (cAMP) Levels Assay

The levels of cAMP were detected in the cells by using a cAMP Assay Kit (ab234585, Abcam, Cambridge, UK) in accordance with the manufacturer's protocol. Briefly, we first plated the cells overnight in growth medium at 5000 cells/well in a 96-well plate, aspirated off the cell growth medium when cultures reached 90% confluence, added 100 μl/well of cell lysis buffer, and incubated them at room temperature for 10 minutes. Then, the lysates were centrifuged for 10 minutes at 13,000 rpm and the clear supernatant extract was analyzed for cAMP levels following the manufacturer's protocol. Finally, the intracellular levels of cAMP were determined by measuring the absorbance at wave length of 405 nm using a Tecan Infinite M200 plate reader (Crailsheim, Germany).

PKA Kinase Activity Assay

The activities of PKA were examined with a PKA enzyme activity kit (ab139435, Abcam, Cambridge, UK). Briefly, 1×10^6 cells were collected and washed twice in ice-cold phosphate-buffered saline (PBS), and then the cells were lysed on ice for 15 minutes. The lysates were centrifuged for 20 minutes at 13,000 rpm, and the clear supernatant extract was analyzed for kinase activity in line with the manufacturer's protocol. Finally, measure absorbance increase on a microplate reader at a wave length of 450 nm using a Tecan Infinite M200 plate reader (Crailsheim, Germany).

Intracellular Free Calcium Ion (Ca^{2+}) Content Assay

The intracellular free cytosolic calcium ion (Ca^{2+}) levels were tested with the cell-permeable calcium-sensitive fluorescent indicator Fluo-3 AM (S016, Beyotime Institute of Biotechnology, Haimen, China). A total of 1×10^6 cells were collected and washed twice in PBS, and then they were incubated with 5 μM Fluo-3 AM at 37°C for 30 minutes in the dark. Subsequently, the cells were washed with PBS three times, and the fluorescence intensity of Fluo-3 AM combined with cytosolic calcium was analyzed by flow cytometry (FASCalibur, California, USA). The mean fluorescence intensity of 10000 cells was recorded for Ca^{2+} content analysis.

Statistical Analysis

The results were presented as mean ± standard error of the mean (SEM) from three duplicates. All statistical analyses were performed with SPSS 19.0 statistical software, and P values < 0.05 were considered statistically significant. Comparisons between two groups use paired or unpaired t-tests when appropriate, and multiple comparisons use One-factor ANOVA followed by the least significant difference (LSD) test. All graphs were generated using GraphPad Prism 7 software (San Diego, CA).

Table 2. Demographic and disease characteristics in the female sexual arousal disorder and normal control groups

Items	NC group (n = 9)	FSAD group (n = 9)
Age	45.50±3.62	45.67±5.79
Post-menopause	0 (0%)	1 (11%)
Desire	3.70±1.16	2.90±0.59
Arousal	4.15±0.74	2.45±0.72**
Lubrication	5.25±0.65	3.70±0.73**
Orgasm	4.93±1.00	3.67±0.39**
Satisfaction	5.27±0.78	4.20±0.55*
Pain	5.33±0.33	3.53±0.93**

*P-value<0.05
**P-value<0.01

RESULTS

MiR-122-5p is Upregulated while VIPR1 Protein Levels are Downregulated in Vaginal Tissue of FSAD Patients

As shown in Table 2, the average ages of subjects were 45.67±5.79 and 45.5±3.62 for the FSAD and NC groups (P>0.05), respectively. And only one post-menopausal woman was included in the study, who was in the FSAD group (P >0.05). The sexual arousal disorder scores of patients between two groups were 2.45±0.72 and 4.15±0.74 (P <0.01), respectively. And statistical significance was also found for differences in lubrication, orgasm, satisfaction, and pain scores between the two groups.

Then, by searching miRbase, we found that miR-122-5p is conserved in mice, rats, humans and other species, and that VIPR1 is its target (Figures 1A and 1B). qRT-PCR analysis was performed to validate miR-122-5p expression levels in 12 human vaginal tissues from the two groups, and Bulge-loop miRNA qRT-PCR Primer Sets (one RT primer and a pair of qPCR primers for each set) specific for miR-122-5p were designed by RiboBio (Guangzhou, China). As shown in Figure 1C, the miR-122-5p expression level in the tissue of the FSAD group was significantly higher (P <0.01) than that of the NC group. Moreover, to validate the VIPR1 protein expression level, we used tissue from another 3 participants in each group for western blot analysis and found that VIPR1 protein in the FSAD group decreased significantly (P <0.05) in contrast to that in the NC group (Figure 1D).

MiR-122-5p Overexpression Attenuates the Relaxation Function of Vaginal Smooth Muscle Cells

miR-122-5p function in smooth muscle relaxation was examined in this experiment using rat vaginal SMCs. Under the inverted phase-contrast microscope, we found that when miR-122-5p was overexpressed, the rat vaginal SMCs formed a contraction shape (Figure 2A), and their length was significantly shorter than that in the NC group (P <0.05) (Figure 2B), which indicates that the overexpression of miR-122-5p has a contraction effect on vaginal SMCs. Then, we detected the content of intracellular free calcium ions. As shown in Figure 2C, the fluorescence intensity of intracellular free Ca^{2+} was higher in the miR-122-5p overexpression group than in the NC group (P <0.05). The effect of miR-122-5p on rat vaginal SMC proliferation was examined with a Cell counting kit-8 (CCK8) assay. At the same time, flow cytometry and TUNEL assay were exploited to simultaneously detect the role of miR-122-5p on rat vaginal SMCs apoptosis. The results showed that the proliferation and apoptosis of rat SMCs were not affected by miR-122-5p, and there was no notable difference between the two groups

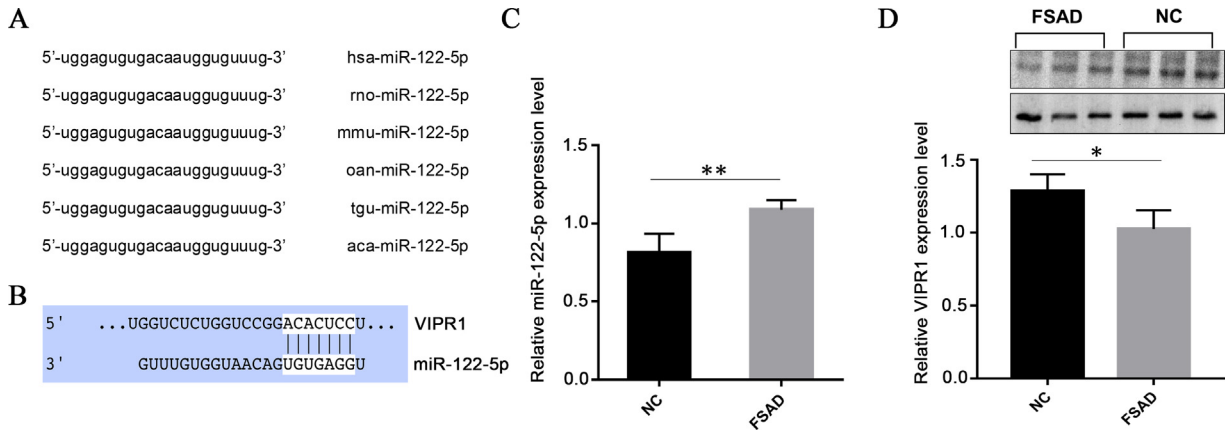

Figure 1. Expression levels of miR-122-5p and vasoactive intestinal peptide receptor 1 (VIPR1) protein in vaginal tissue of FSAD patients. (A) Conservation of the binding region of VIPR1 3'UTR with miR-122-5p. (B) Prediction of miR-122-5p and VIPR1 targeting points. (C) The relative miR-122-5p expression level was analyzed in 6 pairs of vaginal tissues from FSAD and the normal control group by quantitative real-time PCR (qRT-PCR). (D) The expression level of VIPR1 protein was evaluated by western blot (WB) analysis with another 3 pairs of tissues in 2 groups. All indicators after being extracted from 18 samples between 2 groups were detected 3 times. The results are the mean ± standard error of the mean (SEM) and the differences were analyzed by t-tests. *: P-value<0.05; **: P value<0.01.

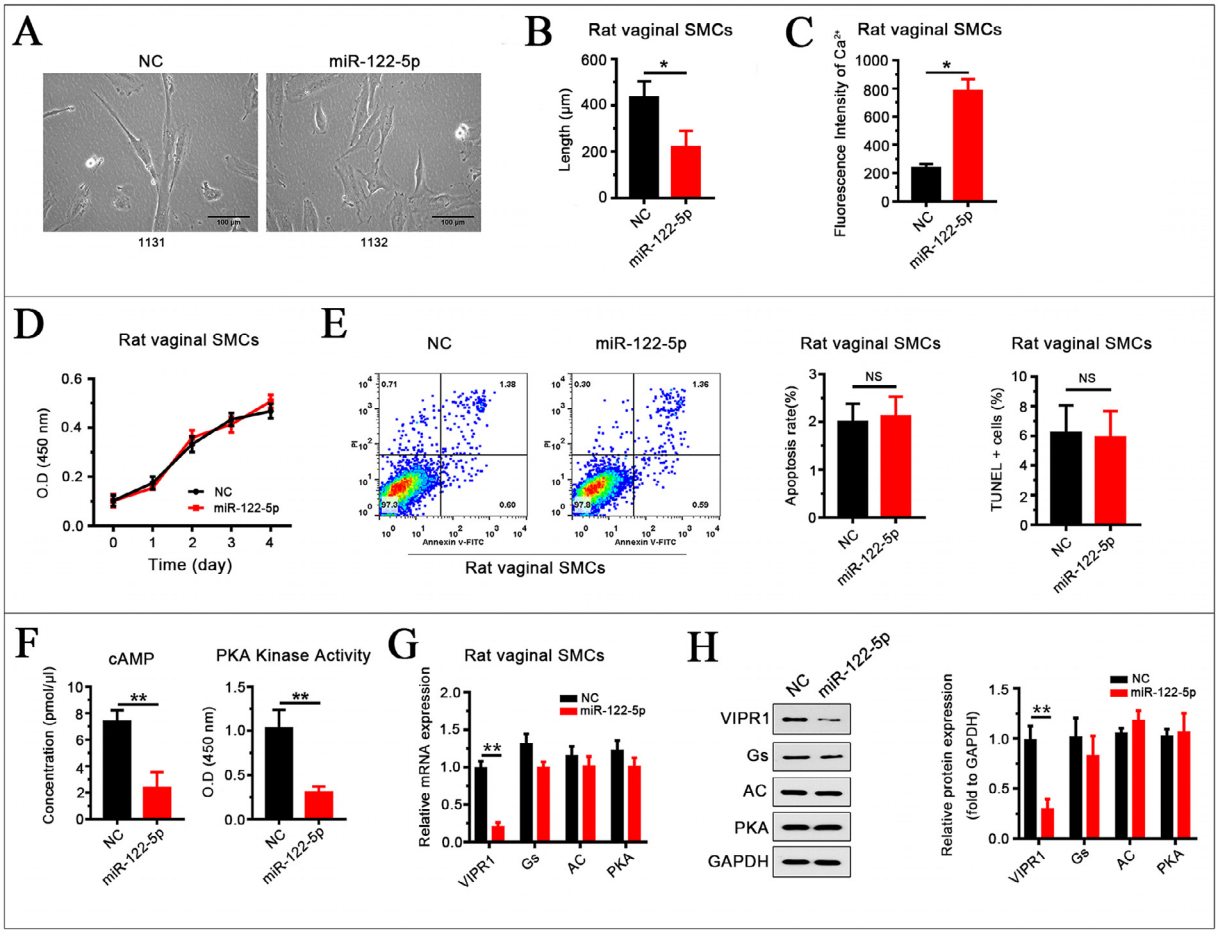

Figure 2. MiR-122-5p overexpression attenuates the relaxation function of vaginal smooth muscle cells (SMCs). (A) Morphology of rat vaginal SMCs under an inverted microscope. (B) Length of rat SMCs. (C) Free Ca^{2+} content in rat vaginal SMCs. (D) The proliferation rate of rat vaginal SMCs measured with a Cell counting kit-8 (CCK8) assay. (E) The apoptosis rate of rat vaginal SMCs with flow cytometry and terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay. (F) Cyclic adenosine monophosphate (cAMP) concentration and protein kinase A (PKA) activity. (G) mRNA expression levels of vasoactive intestinal peptide receptor 1 (VIPR1), stimulatory G protein (Gs), adenylate cyclase (AC), and PKA. (H) VIPR1, Gs, AC, and PKA protein expression determined by western blotting. Vaginal SMCs were isolated from one rat. All indicators extracted from SMCs were detected three times except for cellular morphology. The results are the mean ± standard error of the mean (SEM) and were analyzed by t-tests. *: *P*-value<0.05; **: *P*-value<0.01.

(Figures 2D and 2E). cAMP concentration and PKA activity were detected by a cAMP Assay Kit (ab234585, Abcam, Cambridge, UK) and a PKA enzyme activity kit (ab139435, Abcam, Cambridge, UK), severally. In contrast to the NC group, they were significantly decreased in the miR-122-5p overexpression group (P <0.01) (Figure 2F).

At the molecular level, the expression level of VIPR1 mRNA was detected after miR-122-5p overexpression. The consequences exhibit that the miR-122-5p overexpression group expressed less VIPR1 mRNA than the NC group (P <0.01). The mRNA expression levels of Gs, AC, and PKA were also lower in the miR-122-5p overexpression group than in the NC group, yet their difference did not achieve statistical significance (Figure 2G). Western blotting was subsequently utilized to detect the expression levels of VIPR1, Gs, AC, PKA protein, and the internal reference GAPDH. Significant differences in the VIPR1 protein level were observed between the miR-122-5p overexpression group and the NC group (P <0.01), but the expression levels of the other proteins were equal (Figure 2H).

MiR-122-5p Silencing Results in Increased Relaxation Function of Rat Vaginal Smooth Muscle Cells

To further observe the effect of miR-122-5p on the relaxation function of rat SMCs, we silenced its expression. As shown in Figures 3A and 3B, rat SMCs showed a more relaxed morphology together with a longer length (P <0.05) when miR-122-5p was inhibited, illustrating that the relaxation function of vaginal SMCs in the anti-miR-122-5p group was strengthened. Figure 3C demonstrates that the content

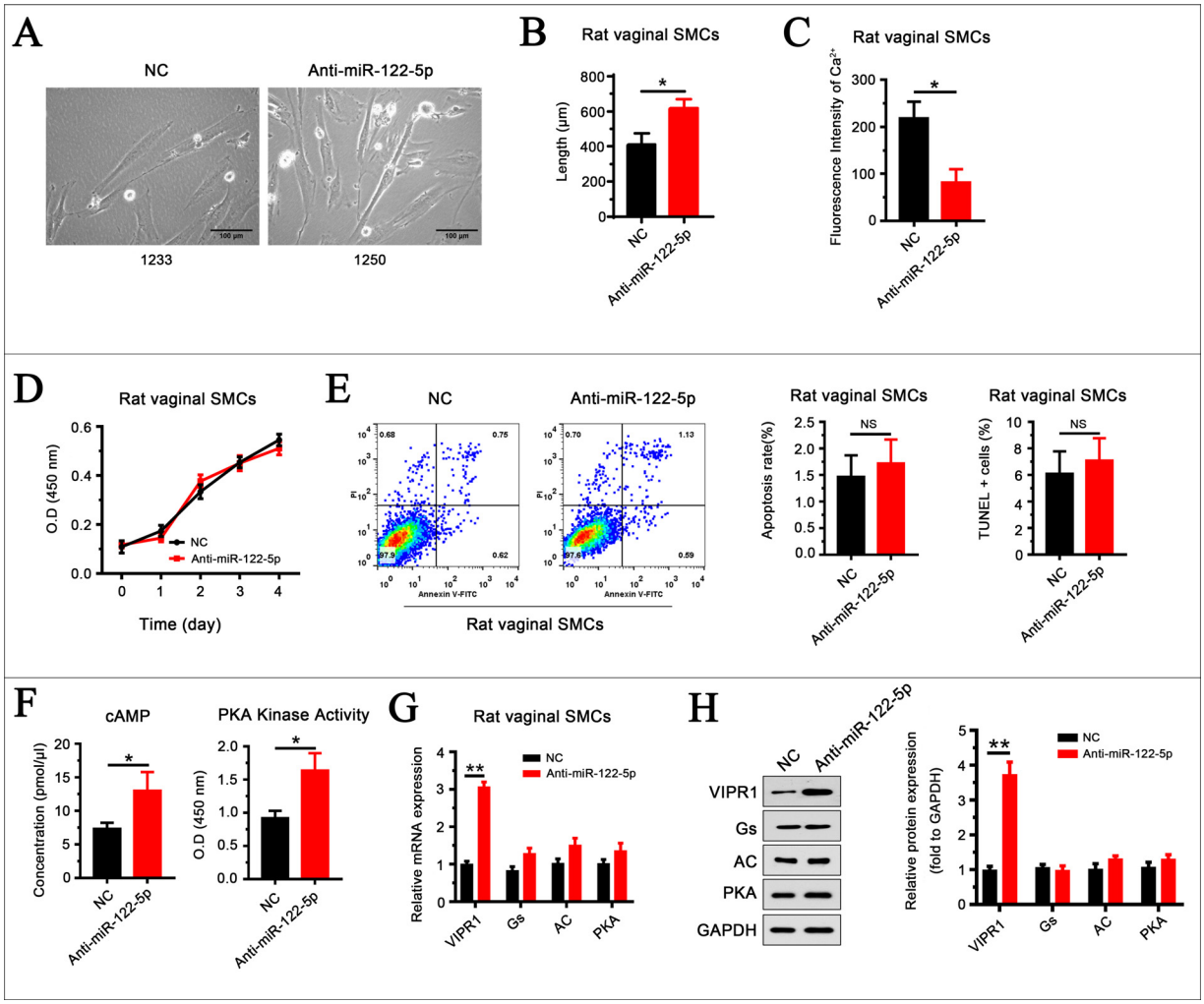

Figure 3. MiR-122-5p silencing results in increased relaxation function of rat vaginal smooth muscle cells (SMCs). (A) Morphology of rat vaginal SMCs under an inverted microscope. (B) Length of rat SMCs. (C) Free Ca^{2+} content in rat vaginal SMCs. (D) The proliferation rate of rat vaginal SMCs detected with a Cell counting kit-8 (CCK8) assay. (E) The apoptosis rate of rat vaginal SMCs with flow cytometry and terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay. (F) Cyclic adenosine monophosphate (cAMP) concentration and protein kinase A (PKA) activity. (G) mRNA expression levels of vasoactive intestinal peptide receptor 1 (VIPR1), stimulatory G protein (Gs), adenylate cyclase (AC), and PKA. (H) VIPR1, Gs, AC, and PKA protein expression determined by western blotting. Vaginal SMCs were isolated from one rat. All indicators extracted from SMCs were detected three times except for cellular morphology. The results are the mean ± standard error of the mean (SEM) and were analyzed by t-tests. *: *P*-value<0.05; **: *P*-value<0.01.

of intracellular free Ca^{2+} was decreased when miR-122-5p was silent (P <0.05), while the proliferation and apoptosis rates of the rat vaginal SMCs were almost equal between the anti-miR-122-5p group and the NC group (P >0.05), which can be seen in Figures 3D and 3E. In addition, we also found that miR-122-5p inhibition gives rise to an increase in cAMP concentration (P <0.05) and PKA activity (P <0.05) (Figure 3F). Versus the NC group, VIPR1 mRNA was significantly upregulated in the anti-miR-122-5p group (P <0.01), while the mRNA levels of Gs, AC, and PKA also increased, but there was no significant difference (Figure 3G). Similarly, after inhibiting miR-122-5p, the VIPR1 protein had a higher expression level (P <0.01), and the protein expression levels of Gs, AC, and PKA changed but not significantly (Figure 3H).

Overexpression of VIPR1 Alleviated the Repressive Effect of MiR-122-5p Overexpression on Rat Vaginal Smooth Muscle Cell Relaxation

We showed that miR-122-5p is related to the relaxation of vaginal SMCs, but the specific mechanism is unclear. We overexpressed miR-122-5p and VIPR1 concurrently, and each indicator in the maternal group (refers to the blank control group) and the NC group (refers to the unloaded group) was equal (P >0.05). At the same time, overexpression of miR-122-5p causes the rat vaginal SMCs to contract (P <0.01), and when miR-122-5p and VIPR1 are overexpressed concurrently, the cells show more relaxation along with a longer length (P <0.01) (Figures 4A and 4B). Ca^{2+} presented lower fluorescence intensity when simultaneously overexpressing miR-122-5p and VIPR1

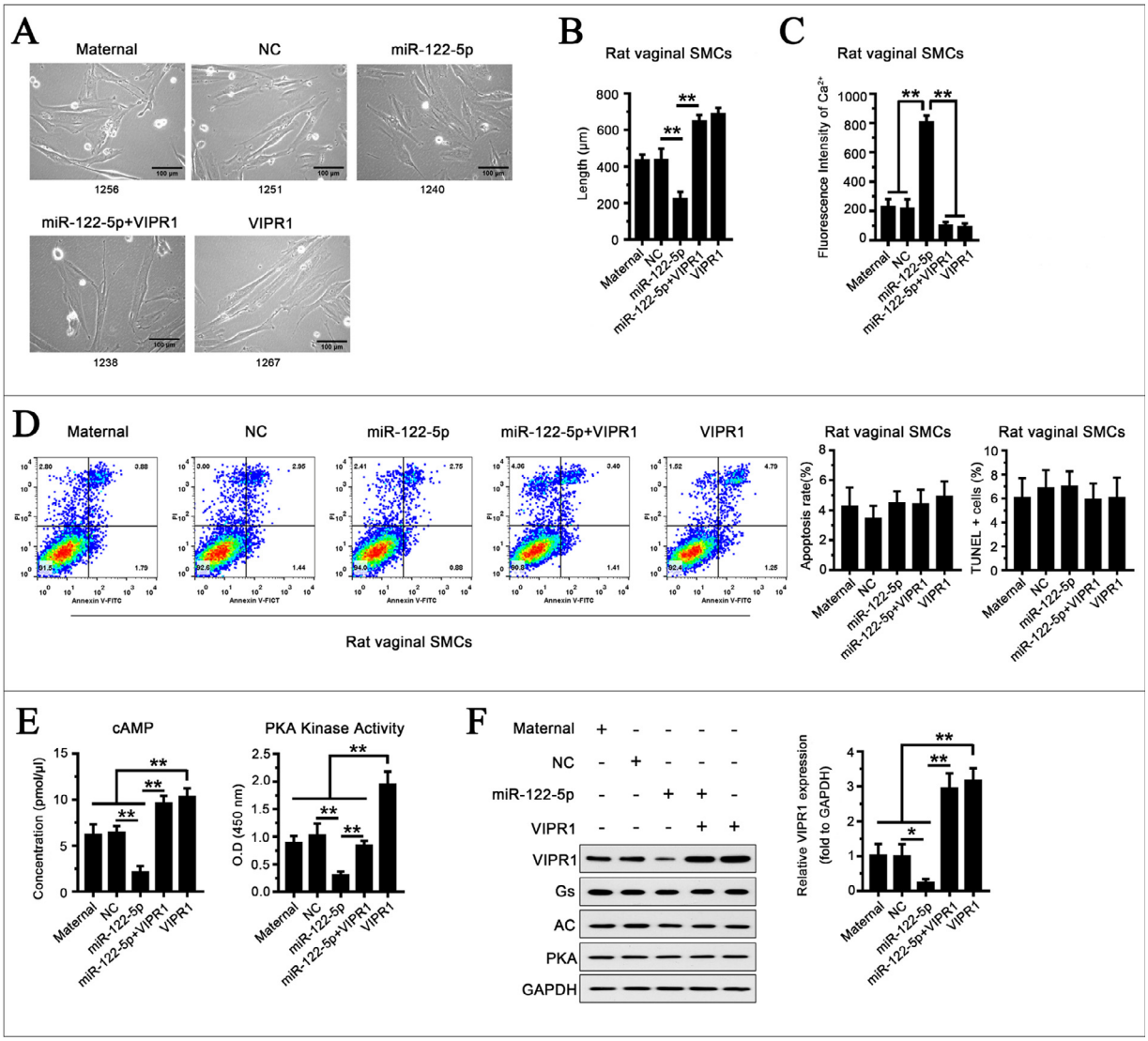

Figure 4. Overexpression of vasoactive intestinal peptide receptor 1 (VIPR1) redressed the inhibitory effect of miR-122-5p overexpression on rat vaginal smooth muscle cells (SMCs) relaxation. (A) Morphology of rat vaginal SMCs under an inverted microscope. (B) Length of rat SMCs. (C) Free Ca^{2+} content in rat vaginal SMCs. (D) The apoptosis rate of rat vaginal SMCs with flow cytometry and terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay. (E) Cyclic adenosine monophosphate (cAMP) concentration and protein kinase A (PKA) activity. (F) VIPR1, stimulatory G protein (Gs), adenylate cyclase (AC), and PKA protein expression determined by western blotting. Vaginal SMCs were isolated from one rat. All indicators extracted from SMCs were detected three times except for cellular morphology. The results are the mean ± standard error of the mean (SEM) and were analyzed by One-factor ANOVA and the least significant difference (LSD) test. *: *P*-value<0.05; **: *P*-value<0.01; maternal group: the blank control group; NC group: the unloaded group.

compared to overexpressing miR-122-5p alone (P <0.01) (Figure 4C). There was no significant difference in the apoptosis rate among the maternal, NC, miR-122-5p, miR-122-5p +VIPR1, and VIPR1 groups (P >0.05) (Figure 4D). Additionally, versus the maternal, NC and miR-122-5p+VIPR1 overexpression groups, the concentration of cAMP and the activity of PKA were significantly lower in the miR-122-5p overexpression group (P <0.01). And PKA activity in the VIPR1 overexpression group was higher than that in the miR-122-5p+VIPR1 group (P <0.01). (Figure 4E). At the molecular level, overexpression of miR-122-5p notably repressed the VIPR1 protein level (P <0.05), which was redressed by VIPR1 overexpression (P <0.01), while other protein expression levels showed no significant change among the five groups (Figure 4F).

Simultaneous Silencing of MiR-122-5p and VIPR1 Leads to a Decrease in the Relaxation Function of Rat Vaginal Smooth Muscle Cells

Subsequently, we silenced the expression of miR-122-5p and VIPR1 at the same time for further verification. There is no difference between the maternal group (refers to the blank control group) and the NC group (refers to the unloaded group) (P >0.05). It was found that repression of miR-122-5p caused rat vaginal SMCs to

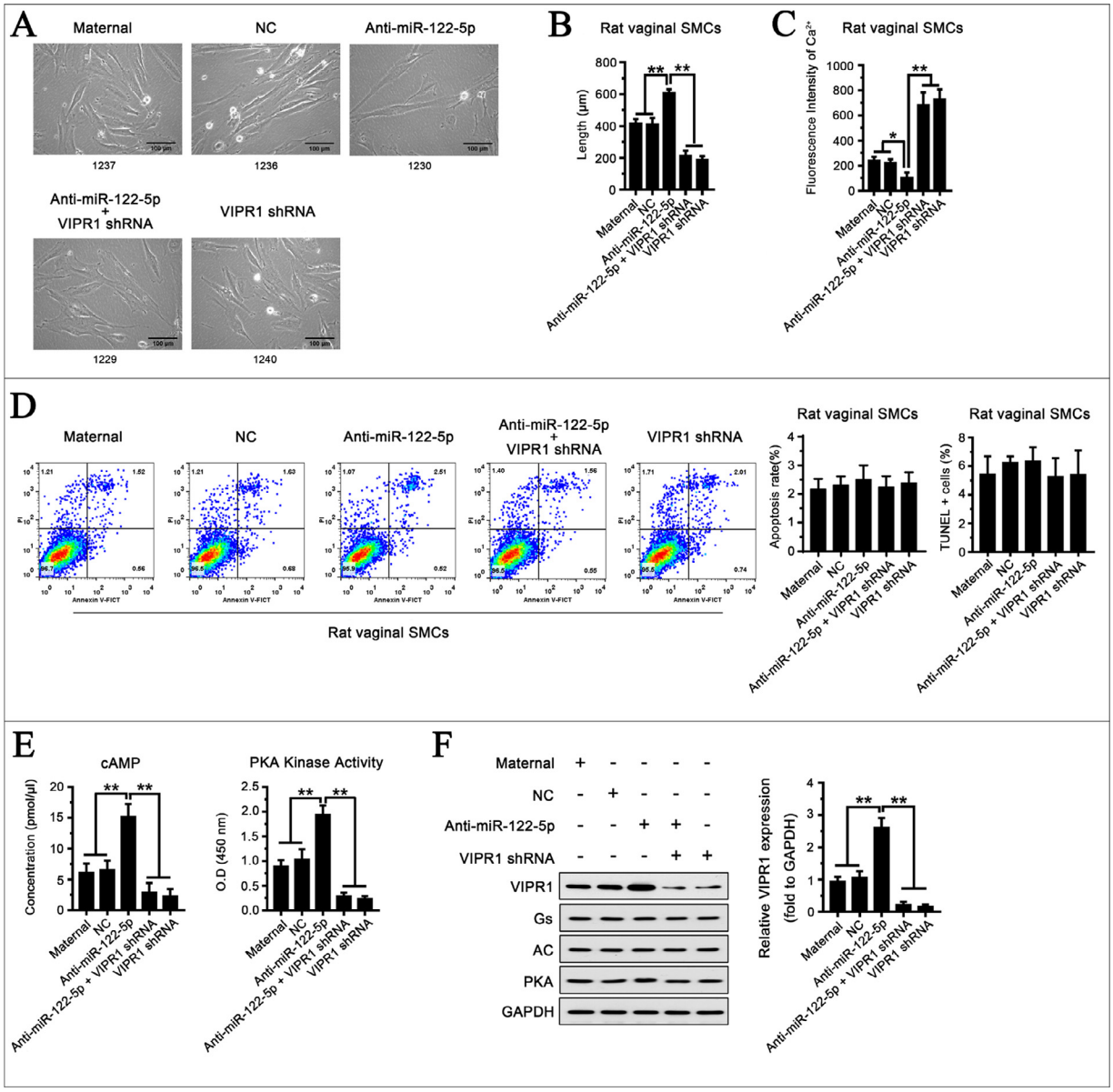

Figure 5. Simultaneous silencing of miR-122-5p and vasoactive intestinal peptide receptor 1 (VIPR1) leads to a decrease in the relaxation function of rat vaginal smooth muscle cells (SMCs). (A) Morphology of rat vaginal SMCs under an inverted microscope. (B) Length of rat SMCs. (C) Free Ca^{2+} content in rat vaginal SMCs. (D) The apoptosis rate of rat vaginal SMCs with flow cytometry and terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay. (E) Cyclic adenosine monophosphate (cAMP) concentration and protein kinase A (PKA) activity. (F) VIPR1, stimulatory G protein (Gs), adenylate cyclase (AC), and PKA protein expression determined by western blotting. Vaginal SMCs were isolated from one rat. All indicators extracted from SMCs were detected 3 times except for cellular morphology. The results are the mean ± standard error of the mean (SEM) and were analyzed by One-factor ANOVA and the least significant difference (LSD) test. *: *P*-value<0.05; **: *P*-value<0.01; maternal group: the blank control group; NC group: the unloaded group.

relax (P <0.01), and when miR-122-5p and VIPR1 were inhibited at the same time, the cells showed more contraction, and their length was shorter (P <0.01) (Figures 5A and 5B). Simultaneously, the fluorescence intensity of Ca^{2+} in anti-miR-122-5p+VIPR1 shRNA group was also significantly higher than that in the anti-miR-122-5p group (P <0.01) (Figure 5C). Flow cytometry and TUNEL assay (Figure 5D) demonstrated no difference in the apoptosis rate of rat SMCs among the maternal, NC, anti-miR-122-5p, anti-miR-122-5p+VIPR1 shRNA and VIPR1 shRNA groups (P >0.05). The cAMP concentration and PKA activity were enhanced in the anti-miR-122-5p group, relative to the anti-miR-122-5p+VIPR1 shRNA, VIPR1 shRNA, maternal and NC groups (all P <0.01) (Figure 5E). At the molecular level, the protein expression level of VIPR1 in the anti-miR-122-5p group increased (P <0.01) and that in the anti-miR-122-5p+VIPR1 shRNA group was notably lower than that in the anti-miR-122-5p group (P <0.01), while the other proteins (Gs, AC, PKA, cAMP) were not different among the five groups (Figure 5F).

DISCUSSION

Female sexual arousal is an indispensable physiological feature for a satisfactory sexual life, and its disorder may lead to obvious pain and decreased sexual life satisfaction.[37] An increasing number of women suffering from FSAD are seeking medical help. Nevertheless, little is known about FSAD, and its treatment needs to be further explored. For example, sildenafil, prostaglandin E1 and Chinese traditional medicine for the treatment of FSAD, which are derived from treatments for male sexual dysfunction, are still in the research stage, and their efficacy is not yet conclusive.[38] Therefore, there is a need to study the pathogenesis of FSAD and find efficient treatments for it.

Currently, the pathophysiological mechanisms of FSAD that we know about include the contraction or relaxation function of vascular smooth muscles,[39] cell proliferation, apoptosis and fibrosis,[40] and a lack of vaginal estrogen,[41] etc. Among these, vaginal smooth muscle could be an ideal target for gene therapy of FSAD, although the causal relationship between them is not clear. According to limited study reports, both inadequate vaginal lubrication and diminished blood flow are linked to smooth muscle relaxation dysfunction, which may play a crucial role in the pathogenesis of FSAD.[10,11] Furthermore, researchers recently found that miRNAs are an underlying therapeutic target for many diseases, such as lubrication disorder.[42] Therefore, exploring the role of miRNAs in the relaxation function of vaginal smooth muscle is essential and may help to discover potential molecular targets for the diagnosis and treatment of FSAD.

In this study, upregulated miR-122-5p was verified in the FSAD population. Then, we validated the role of miR-122-5p in the relaxation function of vaginal smooth muscle through rat vaginal SMCs in cell experiments. Here, through an inverted microscope, we observed that the overexpression of miR-122-5p causes rat SMCs to assume a contracted morphology and decreases the length of rat SMCs, while the opposite is true when miR-122-5p is silenced, which indicates that miR-122-5p has a contraction effect on vaginal SMCs. These results were further confirmed by the intracellular free calcium ion (Ca^{2+}) content assay, which showed that the intracellular free Ca^{2+} content increased in the miR-122-5p overexpression group and conversely decreased in the miR-122-5p inhibition group. As is well-known, increased intracellular Ca^{2+} content can lead to the contraction of SMCs.[43] It is therefore clear that miR-122-5p can regulate the relaxation function of vaginal SMCs. Nevertheless, miR-122-5p does not affect the proliferation and apoptosis of vaginal SMCs.

Furthermore, we also found that miR-122-5p can affect the expression of the VIPR1 protein. VIPR1 is one of the receptors for VIP, which is a non-cholinergic neurotransmitter.[44] VIP can act on the VIP receptors of vaginal smooth muscle and activate AC by binding to Gs protein, which leads to an increase in intracellular cAMP content. Increased cAMP activates cAMP-dependent protein kinase A, causing smooth muscle cell hyperpolarization and a decrease in Ca^{2+} influx, thus relaxing smooth muscle.[30] In this study, based on qRT-PCR and western blot analysis, we found that overexpression of miR-122-5p downregulated VIPR1 mRNA and protein expression levels. The opposite was true when miR-122-5p is silenced. Therefore, we speculated that miR-122-5p regulated VIPR1 by degrading VIPR1 mRNA. This is compatible with previous studies confirming that miRNA can regulate gene expression by degrading mRNA or inhibiting the translation process.[16-19]

Of particular interest, the effect of miR-122-5p on the relaxation function of vaginal SMCs may be achieved by acting on VIPR1. By simultaneously overexpressing miR-122-5p and VIPR1, it was observed that the overexpression of miR-122-5p diminished the protein expression of VIPR1, and this effect was rescued by overexpressing VIPR1. At the same time, the relaxation function of rat vaginal SMCs was elevated, and the intracellular Ca^{2+} content was decreased. The preceding effect can be ignored that from the vector on account of no difference between the NC and maternal groups. When miR-122-5p and VIPR1 were simultaneously silenced, the protein expression level of VIPR1 and rat vaginal smooth muscle function were opposite to those of the simultaneous overexpression group. These data illustrate that miR-122-5p can suppress the relaxation function of vaginal SMCs through the inhibition of VIPR1. However, the difference in the expression levels of downstream proteins of VIPR1, such as AC and PKA, was not statistically significant. Therefore, we performed further experiments to observe their concentration and activity. In agreement with the previous studies, our data show that VIPR1 causes the cAMP concentration and PKA activity to increase,[30] but the overexpression of miR-122-5p suppressed them, while simultaneously overexpressing VIPR1 rescued them. Concurrently, the cAMP concentration and PKA activity in the anti-miR-122-5p +VIPR1 shRNA group were lower than those in the group with silenced miR-122-5p alone, which further proved our inference that miR-122-5p regulates the relaxation function of vaginal smooth muscle by regulating VIPR1 and further affecting its downstream proteins. However, we found a contradiction that the group of overexpressing miR-122-5p and VIPR1 had equal VIPR1 expression to the group of overexpressing VIPR1 alone but less PKA activity, which may be due to other unclear factors affecting the activity of PKA, causing the PKA activity in the miR-122-5p+VIPR1 group to fail to reach the VIPR1 group. It needs to be further explored.

In summary, all of these data show that miR-122-5p can restrain the relaxation of vaginal SMCs by down regulating the expression of VIPR1, which is one of the key proteins involved in vaginal smooth muscle relaxation.

However, our study still has some limits. First, the latest study by Parish SJ et al[45]. suggested that FSAD includes 2 subtypes of arousal diseases: female genital arousal disorder (FGAD) and female cognitive arousal disorder (FCAD). The most rigorous assessment of FGAD uses the FSFI lubrication domain, while the FSFI arousal domain reflects FCAD.[46] However, our study started in

2017, so we did not consider the difference between the two categories, and the population we included in the FSAD group was screened by arousal disorder score≤3.15 and lubrication disorder score≤4.05 according to the cut-off value reported by Ma et al[2]. Therefore, it is necessary to strictly distinguish the two concepts in the future. Second, the length of rat SMCs was compared with the NC group, which was comparable to rat SMCs at baseline. Nevertheless, it is more adequate to compare the variations in the length of the same cell at two states (after treatment/at baseline) between two groups. Third, our work only explored the regulatory mechanism of miR-122-5p on the relaxation function of vaginal SMCs at the cellular level, and we will continue to conduct animal experiments to verify our results later.

CONCLUSIONS

In conclusion, our study revealed that miR-122-5p could act as an inhibitor and suppress the relaxation function of vaginal smooth muscle by downregulating VIPR1 expression, which may be a potential mechanism of FSAD. This finding may provide a promising therapeutic and diagnostic target for patients with FSAD.

Corresponding Authors: Aixia Zhang, MD, School of Nursing, Nanjing Medical University; Women's Hospital of Nanjing Medical University (Nanjing Maternity and Child Health Care Hospital), #123 Tianfei Alley, Qianhuai District, Nanjing 210004, China. Tel: Þ86 025 52226311. E-mail: majiehua@126.com; Jiehua Ma, PhD, Women's Hospital of Nanjing Medical University (Nanjing Maternity and Child Health Care Hospital), #123 Tianfei Alley, Qianhuai District, Nanjing 210004, China. Tel: Þ86 025 52226162. E-mail: zhangaixia@njmu.edu.cn

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STATEMENT OF AUTHORSHIP

Category 1

(a) Conception and Design: Aixia Zhang and Jiehua Ma
(b) Acquisition of Data: Shengnan Cong; Tao Gui; Jingyi Feng; Qinchuan Shi
(c) Analysis and Interpretation of Data: Jiehua Ma; Lianjun Pan; Jingjing Zhang; Shengnan Cong

Category 2

(a) Drafting the Article: Shengnan Cong; Tao Gui; Jinlong Li
(b) Revising It for Intellectual Content: Shengnan Cong; Jingjing Zhang; Jingyi Feng; Jiehua Ma; Aixia Zhang; Lianjun Pan; Qinchuan Shi

Category 3

(a) Final Approval of the Completed Article

Shengnan Cong; Tao Gui; Qinchuan Shi; Jingjing Zhang; Jingyi Feng; Lianjun Pan; Aixia Zhang; Jiehua Ma

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