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Original Article

Decreased expression of *LncRNA CRYM-AS1* promotes apoptosis through the Hippo-YAP1 signaling pathway leading to diabetic erectile dysfunction

Ze-Bo Zhang[1], Ying Chen[1,2], Zhan-Sen Huang[1], Zi-Jun Du[1], Wei Wang[1], Bo-Wen Tang[1], Su-Shun Yuan[1], Di-Ling Chen[3], Shu-Guang Zhu[4], Jun Chen[1]

[1]Department of Infertility and Sexual Medicine, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou 510630, Guangdong, P. R. China; [2]Department of Urology, Sun Yat-sen University Cancer Center, Guangzhou 510060, Guangdong, P. R. China; [3]Guangzhou Laboratory, No. 9 XingDaoHuanBei Road, Guangzhou International Bio Island, Guangzhou 510005, Guangdong, P. R. China; [4]Department of Hepatic Surgery and Liver Transplantation Center, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou 510630, Guangdong, P. R. China

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Abstract: Long non-coding RNAs (lncRNAs) primarily engage with mRNA, DNA, proteins, and microRNAs (miRNAs), thereby regulating gene expression; however, its specific role in diabetic erectile dysfunction (DED) has not been studied. This study aims to investigate the effects and mechanisms of *LncRNA CRYM-AS1* in DED. The differential target gene *LncRNA CRYM-AS1* was identified in the penile tissues of rats with DED through bioinformatics analyses. A KEGG signaling pathway enrichment analysis suggested a potential association between *LncRNA CRYM-AS1* and the Hippo-YAP1 pathway. Real-time fluorescent quantitative PCR (RT-qPCR) results indicated a significantly lower expression of *LncRNA CRYM-AS1* in the penile tissue of DED rats compared to the control group. Western Blot and immunohistochemistry (IHC) staining results demonstrated significantly elevated protein expression levels of YAP1, Caspase3, BAX, and Bcl-2, with a decreased Bcl-2/BAX ratio. CCK8 cell viability results showed a significant decrease in cell viability in the high glucose group at 4 days of modeling, and compared with the normal glucose group, RT-qPCR results showed that the expression of *LncRNA CRYM-AS1* in the high glucose group in human umbilical vein endothelial cells (HUVECs) was significantly reduced; Western Blot results showed that the protein expression of YAP1, Cleaved-caspase3 and BAX was significantly up-regulated, and the protein expression of Bcl-2 was significantly down-regulated in the high glucose group. Compared with the empty vector group, RT-qPCR results after transfection of si*LncRNA CRYM-AS1* showed that the expression of *LncRNA CRYM-AS1* was down-regulated, the mRNA and protein expression of YAP1, Caspase3, Cleaved-caspase3, BAX, and Bcl-2 were significantly up-regulated, and the Bcl-2/BAX ratio decreased. Flow cytometry results showed that the apoptosis rate of HUVECs increased after interference. Low expression of *LncRNA CRYM-AS1* may activate the Hippo-YAP1 signaling pathway to regulate apoptosis in HUVECs, leading to ED development, and the discovery of new target genes may provide new therapeutic targets to regulate diabetic erectile disfunction.

Keywords: Diabetic erectile dysfunction, *LncRNA CRYM-AS1*, Hippo-YAP1 signaling pathway, umbilical vein endothelial cells, apoptosis

Introduction

Erectile dysfunction is the inability to achieve or maintain an erection sufficient to accomplish satisfactory sexual intercourse, causing some distress to the patients and their sexual partners [1]. Diabetes is an important risk factor of ED, which is inextricably linked with the development of ED. As per epidemiologic studies [2-4]. The prevalence of ED is high among diabetic patients and is thrice higher than the prevalence among non-diabetic patients [5]. However, standard treatment regimens such as phosphodiesterase 5 (PDE5) inhibitors show poor efficacy in diabetic patients with ED [6]. Therefore, it is necessary to identify new potential targets for treating diabetes patients with DED.

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Long non-coding RNAs (lncRNAs) are regulatory RNAs that do not encode proteins with a length greater than 200 nucleotides [7-10]. An increasing number of non-coding RNAs have been found to be involved in the regulation of gene expression at the transcriptional and epigenetic levels, influencing processes such as apoptosis, proliferation, invasion, and metastasis. Previous studies have reported that lncRNAs may be related to erectile function. In the rat model of erectile dysfunction, *LncRNA MIAT* promoted the differentiation of bone marrow mesenchymal stem cells into endothelial cells by targeting miR-200a, thereby improving erectile dysfunction [11], and Downregulation of *LncRNA MEG3* promoted endothelial differentiation and repair of erectile dysfunction in bone marrow mesenchymal stem cells [12]. However, studies on the association of lncRNAs with diabetic ED are rare, some of which are still in their preliminary stages. Whether lncRNAs affect erectile function in other ways remains to be investigated.

In this research, the above research background was combined, the differential target gene *LncRNA-CRYM-AS1* related to DED was screened by bioinformatics principles and methods, a type 1 diabetic ED rat model was constructed, and an umbilical vein endothelial cell *in vitro* model of high glucose-treated cells was constructed. The target genes, were verified and interfered with the target genes of the further potential target gene *LncRNA-CRYM-AS1* to explore the related mechanism of *LncRNA CRYM-AS1* regulating erectile function and shed light on other treatment avenues to cure diabetic erectile dysfunction.

Material and methods

Laboratory animals

Fifty 8-week-old SPF male SD rats with a body mass of 200±20 g were placed in an SPF sterile animal rearing room with a room temperature of 23±2°C, air humidity of 45%±5%, natural light and sterile water. Certificate of Conformity: 44827200000058. License: SYXK(GD)2016-0165 provided by the Institute of Animal Health, Guangdong Academy of Agricultural Sciences. The animals were officially included in the experiment at the Institute of Animal Health, Guangdong Academy of Agricultural Sciences, after one week of routine feeding and acclimatization. This experiment was approved by the Animal Ethics Committee of the Institute of Animal Health, Guangdong Academy of Agricultural Sciences (SPF2021-020).

Construction of animal model of type 1 diabetes mellitus in SD rats

Fifty 8-week-old SPF-grade male SD rats were randomly divided into a control group (n=15) and an experimental group (n=35). All rats were fed a normal diet and were given free access to water and food. They were maintained with 12 hours of light daily. After one week of acclimatization feeding, the rats were fasted for 8 h. Streptozotocin (STZ) was intraperitoneally injected into the rats in the experimental group at a dose of 60 mg/kg [13-16], while the rats in the control group were injected with the same dose of sodium citrate-citrate buffer simultaneously. Random blood glucose was measured 3 days after intraperitoneal injection using the tail clipping method, whereby a ≥ 16.7 mmol/L was considered successful in modeling diabetes. Penile intracavernous pressure (ICP) and mean carotid artery blood pressure (MAP) were measured after 8 weeks of normal chow feeding. We anesthetized the rats with 3% sodium pentobarbital (40 mg/kg). Referring to the experiment of La Favor et al [17], the changes of ICP and MAP were recorded with a BL-420S physiological recorder, and the parameters of electrical stimulation were set as follows: voltage of 5.0 V, frequency of 20 Hz, and wave width of 0.2 ms, and the ICP/MAP ratio was used to evaluate the erectile function. Modeling of diabetic ED rats was considered successful when ICP/MAP was ≤0.3. At the end of the experiment, we euthanized the rats using the cervical dislocation method. Penile tissue specimens were harvested from the two groups of rats after the erectile function test was completed. The rats were divided into the control group (Control) and the type I diabetic ED group (DED).

Cell culture

HUVECs cell lines were obtained from the Laboratory of Liver Diseases, The Third Affiliated Hospital of Sun Yat-sen University. HUVECs were resuscitated and cultured in a 37°C 5% CO_2 incubator and were divided into four groups: the normal glucose group (NG

group), the high-glucose group (HG group), the empty vector group (Si-NC group), the siRNA interference group (Si-*LncRNA CRYM-AS1* group). The NG group was cultured with conventional RPMI-1640 complete medium (glucose concentration of 2 g/L) and the HG group was cultured with RPMI-1640 complete medium (glucose concentration of 7.4 g/L) for 1, 2, 3 and 4 days [17, 18], respectively.

Cell counting kit-8 (CCK-8) cell viability assay

Cell viability was assayed as per the instructions stipulated in the CCK-8 kit (Beyotime, C0038). 96-well plates were inoculated with 1×10^3 suspended cells per well and repeated for 3 replicate wells. Cells were cultured in regular RPMI-1640 complete medium (2 g/L) for the normal glucose group and RPMI-1640 complete medium (7.4 g/L) for the high glucose group after wall attachment. The 96-well plates were incubated in a 5% CO_2 at 37°C incubator for 0, 1, 2, 3, and 4 d. After incubation, 10 μL of CCK-8 solution was added to each well. After 1 h of incubation, the absorbance (OD) at 450 nm was measured by Thermo Varioskan Flash Multifunctional Enzyme Labeler (Thermo Scinetific, USA).

Western blotting

The total protein of penile tissue was extracted by protein extraction kit and protein concentration was determined by BCA reagent, samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 80 V and then 110 V, transferred to the membrane at 110 V for 110 min, and blocked with 5% skimmed milk powder for 90 min, anti-YAP1 (1:1000) (Servicebio, GB113975-100), BAX (1:800) (Proteintech, 50599-2-Ig), Bcl-2 (1:1000) (Proteintech, 26593-1-AP) and Caspase3 (1:1000) (Beyotime, AC030), Tubulin (1:5000) (Proteintech, 66031-1-Ig), GAPDH (1:1000) (Cell Signaling Technology, 5174S). Primary antibodies were added for overnight incubation at 4°C, and washed with membrane-washing buffer (TBST). The membrane was washed with TBST (20 min × 4), and then Goat Anti-Rabbit IgG (H+L) antibody (Affinity, S001-100 μL) or Goat Anti-Mouse IgG (H+L) antibody (Affinity, S002-100 μL) was added, and incubated for 60 min, and then washed with TBST (20 min × 4). After color development and image acquisition using BIO-RAD ChemiDoc XRS Chemiluminescence Gel Imaging System (BIO-RAD), the grayscale values of the target proteins and the internal reference proteins were analyzed and the ratio of the two was calculated using Image Lab software.

Immunohistochemical staining (IHC)

Penile tissue paraffin deparaffinization was followed sequentially by microwave antigen repair, blocking of tissue endogenous peroxidase, serum closure, and primary antibody incubation. Anti-YAP1 (1:200) (Proteintech, 13584-1-AP), BAX (1:2000) (Proteintech, 50599-2-Ig), Bcl-2 (1:400) (Proteintech, 26593-1-AP) and Caspase3 (1:200) (Proteintech, 19677-1-AP) antibodies were diluted in PBS and refrigerated overnight at 4°C. The secondary antibody Goat Anti-Mouse IgG (H+L) HRP (1:100) was diluted in PBS and incubated at room temperature for 1 h. Finally, the positive staining signals were illuminated with 3,3'-Diaminobenzidine DAB and the nuclei of tissue hematoxylin cells were stained, dehydrated and sealed.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Cellular genes were extracted with SteadyPure Universal RNA Extraction Kit (AG accurate biology, AG21017), and RNA concentration was measured by NanoDrop 2000 spectrophotometer. Total RNA from each group of penile tissues was extracted using the TransSeript TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen Biotech, AT311-02), and total RNA was reverse transcribed to cDNA according to the reverse transcription kit instructions. The amplification reaction was performed as per the instructions stipulated on the PerfetStart Green qPCR SuperMix kit (TransGen Biotech, AQ601-02). Relative expression was calculated by the $2^{-\Delta\Delta}$Ct method. The primer sequences used are listed in **Table 1**.

Interfering LncRNA CRYM-AS1 in HUVECs

In this study, siRNA was designed according to the gene, and Sangon Biotech (Shanghai) was selected for siRNA and negative control synthesis (**Table 2**). HUVECs were cultured in 6-well plates, and the medium containing streptomycin and penicillin was replaced when the cells grew to 30-40% confluence. HUVECs were divided into two groups: an empty group (Si-

Table 1. Primer sequences for genes involved in RT-qPCR experiments

Primer	Sequence (5' to 3')
LncRNA CRYM-AS1	Forward: TTGTGGAAATGGTCCTCACA
	Reverse: CTTCTGGAAGGCACGTAAGC
YAP1 (human)	Reverse: CTTCTGGAAGGCACGTAAGC
	Reverse: GGTTCATGGCAAAACGAGGG
Caspase3 (human)	Forward: GGCGGTTGTAGAAGAGTTTCG
	Reverse: TCACGGCCTGGGATTTCAAG
GAPDH (rat)	Forward: AATGGGCAGCCGTTAGGAAA
	Reverse: GCCCAATACGACCAAATCAGAG
GAPDH (human)	Reverse: GCCCAATACGACCAAATCAGAG
	Reverse: GCTGATGATCTTGAGGCTGTTGTC

Table 2. *LncRNA CRYM-AS1* siRNA sequences

Interference genes	siRNA sequence (5'-3')
siNC-sense	UUCUCCGAACGUGUCACGUTT
siNC-antisense	ACGUGACACGUUCGGAGAATT
si*LncRNA CRYM-AS1*-sense	CCAGAAGUACUGUGGUUCA
si*LncRNA CRYM-AS1*-antisense	UGAACCACAGUACUUCUGG

NC group) and a siRNA interference group (Si-*LncRNA CRYM-AS1* group), and transfected according to the instructions of the Lipofetamin RNAiMAX kit. 48 h after cell transfection, cell RNA was collected, and the interference efficiency of siRNA and the mRNA expression of YAP1 and Caspase3 in HUVECs were detected by RT-qPCR.

Flow cytometry

The apoptosis rate was detected using the Fluorescein Isothiocyanate (FITC)-coupled Annexin-V Apoptosis Detection Kit (BD Biosciences Pharmingen; San Diego, USA) as directed by the manufacturer. After HUVECs were transfected with siRNA for 48 h, the cells were collected, washed with PBS, added with 2 μL Annexin V antibody and 100 μL Annexin V binding buffer (1×) (1:50), and incubated for 20 minutes on ice kept in light-deprived conditions. 300 μL Annexin V binding buffer (1×) was subsequently added and was mixed and centrifuged. The supernatant was removed and resuspended in 400 μL of 2% serum contained 0.5 μL of propidium iodide PI (Invitrogen, 320999-000). The apoptosis rate of HUVECs by BD (BD LSRFortessa X-20) was subsequently detected. The results were analyzed using Flowjo software for data analysis.

Statistical analysis

Image Lab software was used to analyze the grayscale values of the target and internal reference proteins in the Western Blot experiments and the ratio of the two numerical data was calculated, and the data were expressed as the mean ± standard deviation; the relative expression of the genes was calculated by the $2^{-\Delta\Delta}$Ct method, and the relative expression of the genes was calculated by using GraphPadPrism (version 7.0) and Adobe Illustrator CC 2017 software for plotting and statistical analysis. FlowJo software was used to analyze the flow cytometry results; finally, GraphPad Prism version 7.0 software was used to conduct statistical analyses of the data of each group; unpaired t-tests were used between the two groups of data for cell viability, and paired t-tests were used to analyze the data between the two groups for the other groups; $P < 0.05$ was considered to be statistically significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Results

Bioinformatics analysis

The dataset GSE2457 was obtained from the GEO database. The open database T2DKP (Type 2 Diabetes Knowledge Portal), and GeneSifter (https://login.genesifter.net.) online analysis software was used to analyze the gene microarray data of penile tissues of normal rats and diabetic erectile dysfunction. The KEGG pathway analysis tool that comes with the gene chip data analysis software was used to perform the pathway enrichment analysis of differentially expressed genes. The heat map results showed that *LncRNA CRYM-AS1* was the most significantly down-regulated among the differentially expressed lncRNAs associated with erection (**Figure 1A**). Each dot in the volcano atlas represents a specific lncRNA, and its position reflects the degree of difference and statistical significance of its expression in the two rat tissues, with green dots indicating down-regulated genes and red dots indicating up-regulated genes (**Figure 1B**). The top 10 pathways with the smallest Q value were selected for display

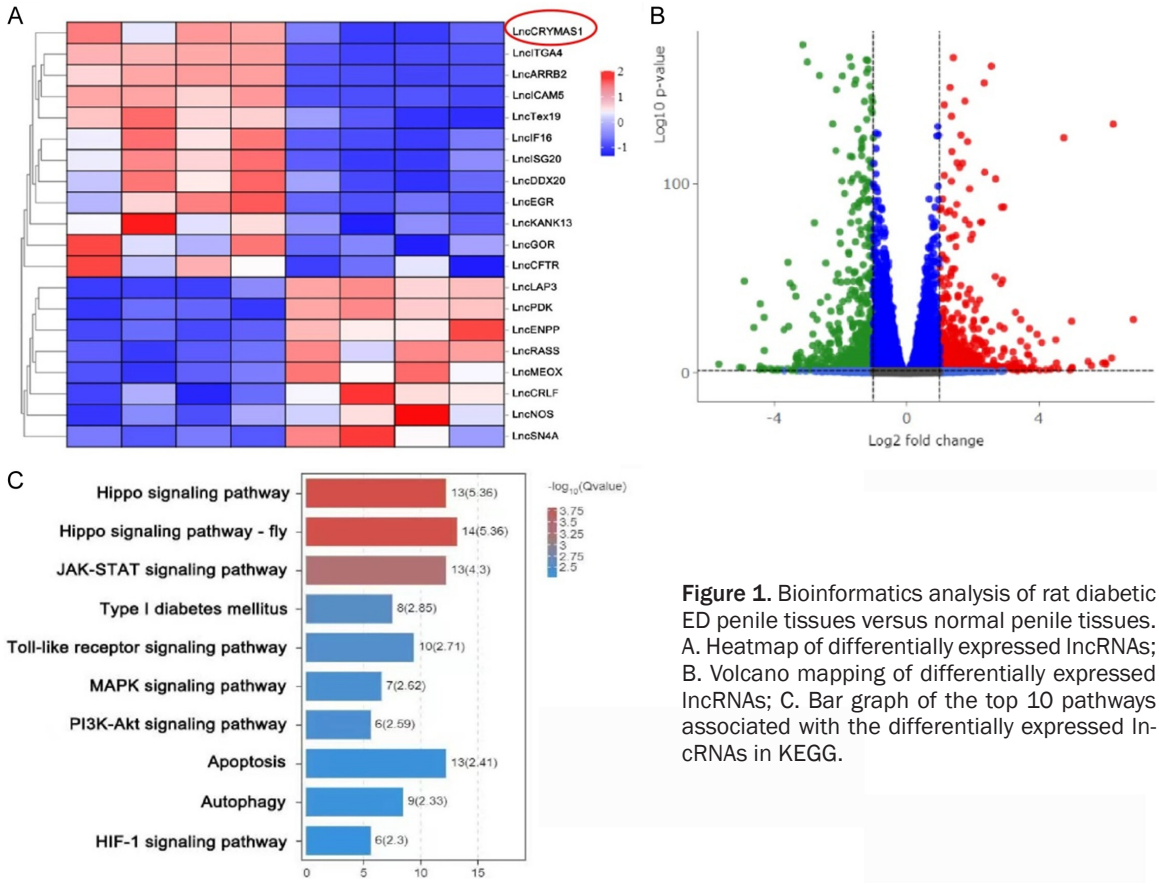

Figure 1. Bioinformatics analysis of rat diabetic ED penile tissues versus normal penile tissues. A. Heatmap of differentially expressed lncRNAs; B. Volcano mapping of differentially expressed lncRNAs; C. Bar graph of the top 10 pathways associated with the differentially expressed lncRNAs in KEGG.

(**Figure 1C**), and the color of the bar represents the Q value. The Q value was inversely proportional to the reliability of the enrichment of differentially expressed genes in the pathway. The horizontal axis of the bar indicates the number of differentially expressed genes enriched in the pathway. The KEGG pathway enrichment analysis showed that the Hippo pathway is the main signaling pathway for the enrichment of differentially expressed lncRNAs and regulates apoptosis.

Measurement of erectile function in diabetic rats

Changes in MAP and ICP after electrical stimulation of the cavernous nerve in both groups of rats were investigated and recorded. The ICP/MAP of the diabetic group was significantly lower than that of the control group, with a statistically significant difference ($P < 0.001$). The results indicated that diabetes could lead to decreased erectile function in SD rats (**Figure 2**).

RT-qPCR analysis of LncRNA CRYM-AS1 in penile tissue of diabetic ED rats

The expression level of *LncRNA CRYM-AS1* was significantly reduced in SD rats with diabetic ED compared to normal SD rats, with a statistically significant difference ($P < 0.001$) (**Figure 3**), which was identical to the bioinformatics analysis results.

Expression levels of YAP1 and apoptosis-related proteins in penile tissues of DED rats

Compared with normal SD rats, SD rats with diabetic ED had significantly higher YAP1 protein expression levels, with statistically significant differences ($P < 0.001$); BAX protein expression levels were significantly higher, with statistically significant differences ($P < 0.001$); Bcl-2 protein expression levels were higher, with statistically significant differences ($P < 0.01$); Bcl-2/BAX ratio decreased, with a statistically significant difference ($P < 0.001$); apoptotic cleavage protein Cleaved-caspase3 pro-

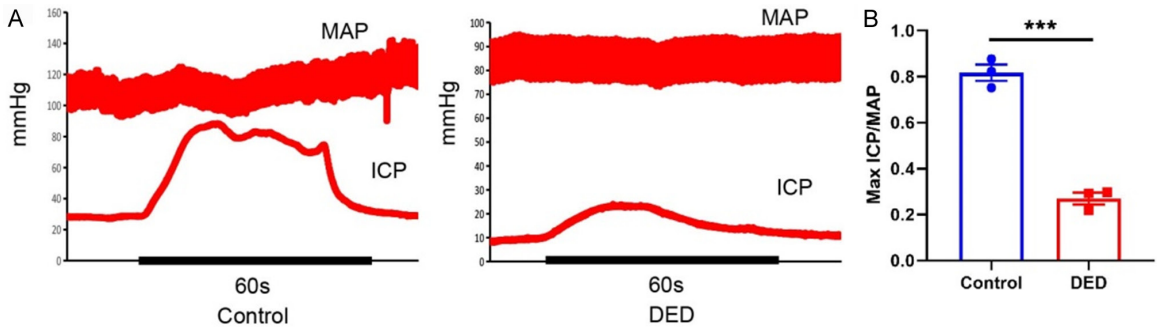

Figure 2. Measurement of erectile function. A. Representative graph of erectile function measurements in SD rats; ICP: maximal intracavernous pressure; MAP: mean arterial pressure. B. Maximal intracavernous pressure/mean arterial pressure was significantly lower in DED group (n=3) than in control group (n=3). ***$P < 0.001$.

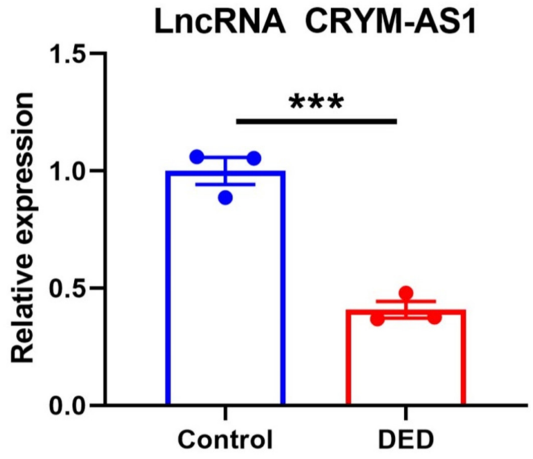

Figure 3. RT-qPCR results of *LncRNA CRYM-AS1* in rat penile tissues. Control group consisted of normal SD rats, and DED group consisted of diabetic ED rats. ***$P < 0.001$.

tein expression level increased, with a statistically significant difference ($P < 0.05$) (**Figure 4**).

Immunohistochemistry of YAP1 and apoptosis-related proteins in penile tissues of SD rats

Immunohistochemistry results showed that the vascular endothelial cells were monolayered and flattened, with a weak positive expression of YAP1, Caspase3, BAX, and Bcl-2 in penile cavernous vascular endothelial cells of the Control group (shown by red arrows), and strongly positive expression of YAP1, Caspase3, BAX, and Bcl-2 in penile cavernous vascular endothelial cells of the DED rats (shown by red arrows). It is contended that the endothelial cells of the penile corpus cavernosum of diabetic rats underwent apoptosis after activation of YAP1 (**Figure 5**).

Inhibition of growth viability of umbilical vein endothelial cells by high glucose

CCK8 results showed that the growth vigor of HUVECs stimulated by high glucose for 1, 2, 3 and 4 days was lower than that of the NG group, and the differences were all statistically significant ($P < 0.05$) (**Figure 6**). The difference was significant on day 4 of modeling and this time point was chosen for the protein experiment.

RT-qPCR

RT-qPCR results of high glucose-treated vascular endothelial cells: The expression level of *LncRNA CRYM-AS1* was detected by RT-qPCR in HUVECs of HG and NC groups cultured for 0, 1, 2, 3 and 4 days, respectively. The results showed that the expression level of *LncRNA CRYM-AS1* in HUVECs of the HG group stimulated for 1, 2, 3 and 4 days was significantly lower than that of the NG group, which was consistent with the results of the bioinformatics analysis, and the difference between the two was statistically significant ($P < 0.05$) (**Figure 7A**).

Detection of effective siRNA interference efficiency of LncRNA CRYM-AS1 in HUVECs and effect on YAP1 and Caspase3 expression: The siRNA was transfected into HUVECs at 30%-40% cell density, and after 48 h of cell growth, RT-qPCR was used to detect the interference effect of siRNA and the expression of apoptosis-related genes. The results showed that siRNA had a significant interference effect, with a statistically significant difference ($P < 0.001$) (**Figure 7B**); compared with the control group, the expression of YAP1 and Caspase3 was

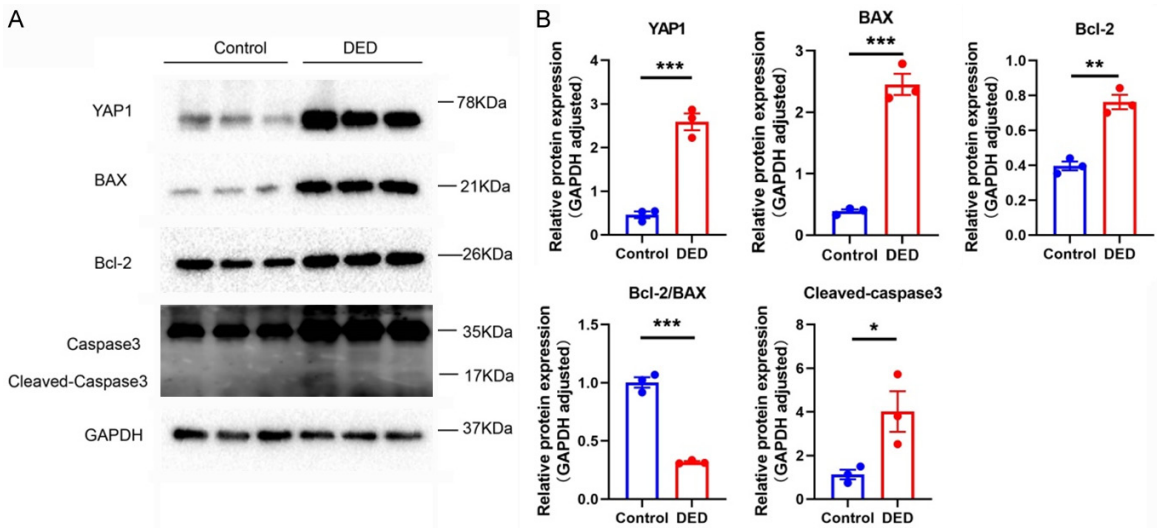

Figure 4. Western blot results of YAP1 and apoptosis-related parameters in rat penile tissues. A, B. Protein level expression of YAP1, BAX, Bcl-2, Caspase3 and cleaved-Caspase3. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

increased after the interference of *LncRNA CRYM-AS1*, and the difference was statistically significant ($P < 0.001$) (**Figure 7C** and **7D**), suggesting that *LncRNA CRYM-AS1* knockdown may lead to activation of the Hippo-YAP1 signaling pathway and increased expression of intracellular Caspase3, which promotes apoptosis and leads to decreased erectile function.

Intracellular YAP1 and apoptosis-related protein expression levels

In vitro high glucose treatment of HUVECs may promote apoptosis through the Hippo signaling pathway: The results of this study showed that after 4 days of high glucose action, the expression levels of YAP1 and Cleaved-caspase3 in umbilical vein endothelial cells in the high glucose group were significantly higher than those in the NG group, with statistically significant differences ($P < 0.001$); the expression levels of BAX were higher than those in the NG group, with statistically significant differences ($P < 0.05$); and the expression levels of Bcl-2 were lower than those in the NG group, with statistical significance ($P < 0.05$), indicating that *in vitro* high glucose treatment of HUVECs may activate the Hippo signaling pathway to promote apoptosis (**Figure 8**).

Knockdown of LncRNA CRYM-AS1 may activate the Hippo signaling pathway to induce HUVECs apoptosis: The results of this study showed that after *LncRNA CRYM-AS1* interference, the expression levels of YAP1 and Cleaved-caspase3 in HUVECs were significantly higher than those in the Si-NC group, with a statistically significant difference ($P < 0.001$); the expression levels of BAX and Bcl-2 were higher than those in the Si-NC group, with a statistically significant difference ($P < 0.01$); the Bcl-2/BAX ratio was decreased, and the difference was statistically significant ($P < 0.001$). It indicated that *LncRNA CRYM-AS1* knockdown could activate the Hippo signaling pathway to promote apoptosis (**Figure 9**).

Apoptosis of HUVECs after LncRNA CRYM-AS1 interference by flow cytometry

The apoptosis of HUVECs after *LncRNA CRYM-AS1* interference was detected by flow cytometry. The apoptosis rate of HUVECs in the Si-*LncRNA CRYM-AS1* group was significantly increased compared with the Si-NC group, and the difference was statistically significant ($P < 0.01$) (**Figure 10**).

Discussion

Endothelial cell injury, nerve endings injury, smooth muscle cell proliferation and phenotype transformation, as well as the synergistic effect of interstitial proliferation, lead to erectile dysfunction, and endothelial cell injury in the corpus cavernosum of the penis is the main factor in erectile dysfunction progression [19-23]. Continuous hyperglycemia can lead to

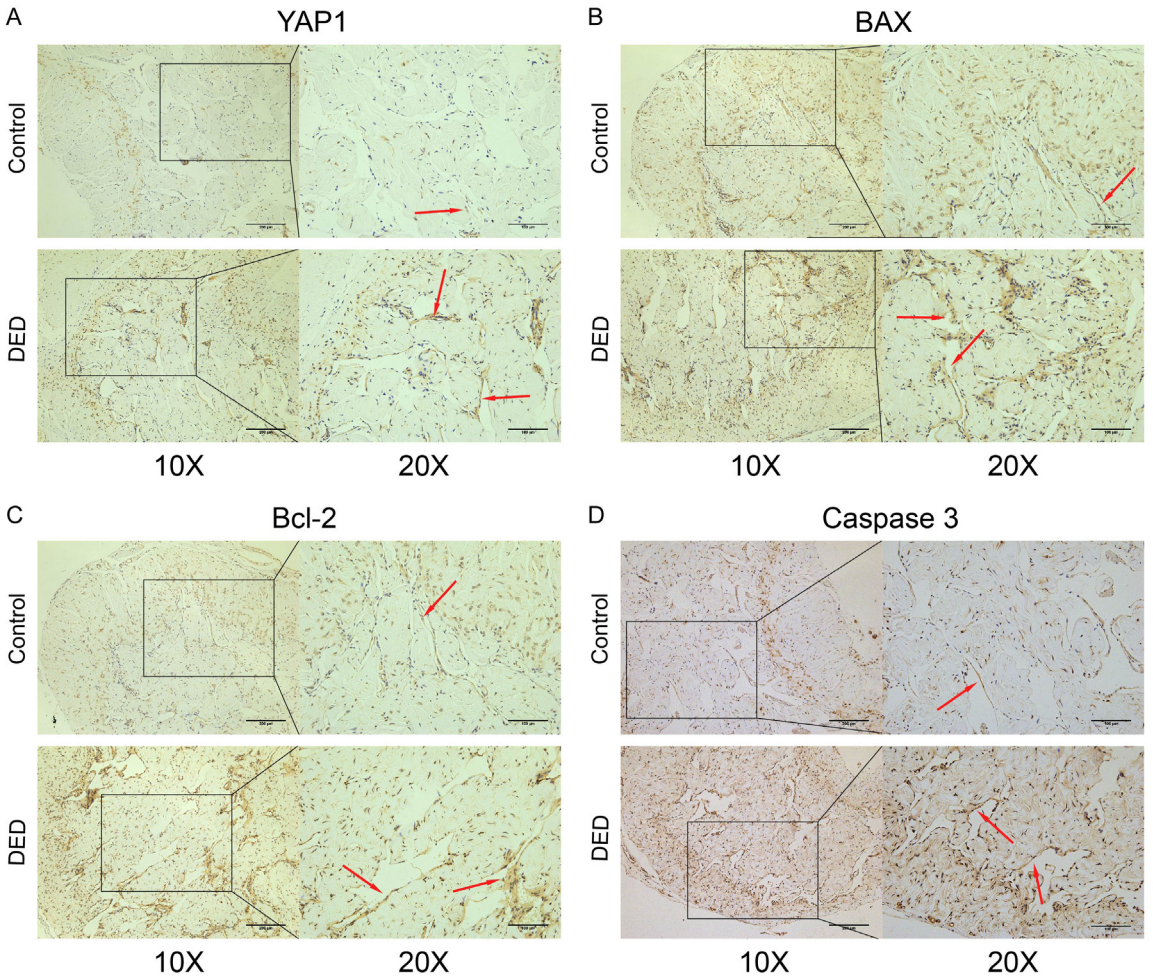

Figure 5. IHC results of YAP1, BAX, Bcl-2 and Caspase3 in rat penile tissues. A-D. Red arrows marked focal positive expression in penile cavernous vascular endothelial cells, indicating increased positive expression of YAP1, BAX, Bcl-2 and Caspase3 in the DED group. The left scale bar is 200 μm, the right scale bar is 100 μm.

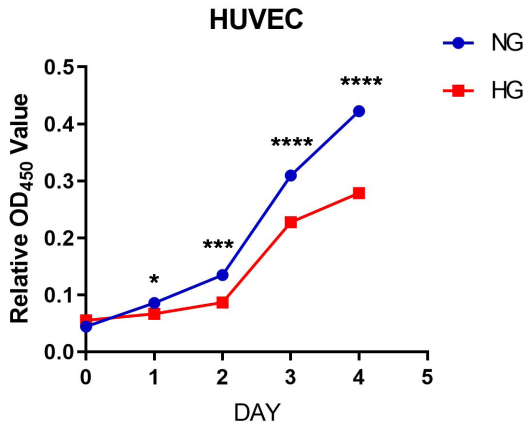

Figure 6. CCK8 cell viability assay. Cell viability in NG and HG groups after 0, 1, 2, 3, and 4 days of culture in conventional RPMI-1640 complete medium (2 g/L) and high glucose RPMI-1640 complete medium (7.4 g/L), respectively. *$P < 0.05$, ***$P < 0.001$.

endothelial dysfunction, which in turn can cause a decrease in nitric oxide function and reduced vasoconstriction [24-26]. This decrease will synergistically impair erectile function [27]. Damage to endothelial cells can lead to a decrease in endogenous nitric oxide and synthase (eNOS) synthesis, leading to a decrease in NO production, which ultimately causes the onset and progression of ED [28, 29].

LncRNAs are RNAs of more than 200 nucleotides in length, with tissue-specific and developmental specificity. In this study, the analysis of the GSE2457 dataset and the open database T2DKP showed that the expression level of *LncRNA CRYM-AS1* was reduced in diabetic ED tissues; related studies have shown that the *LncRNA CRYM-AS1* has an inhibitory effect on aerobic glycolysis and cell proliferation in

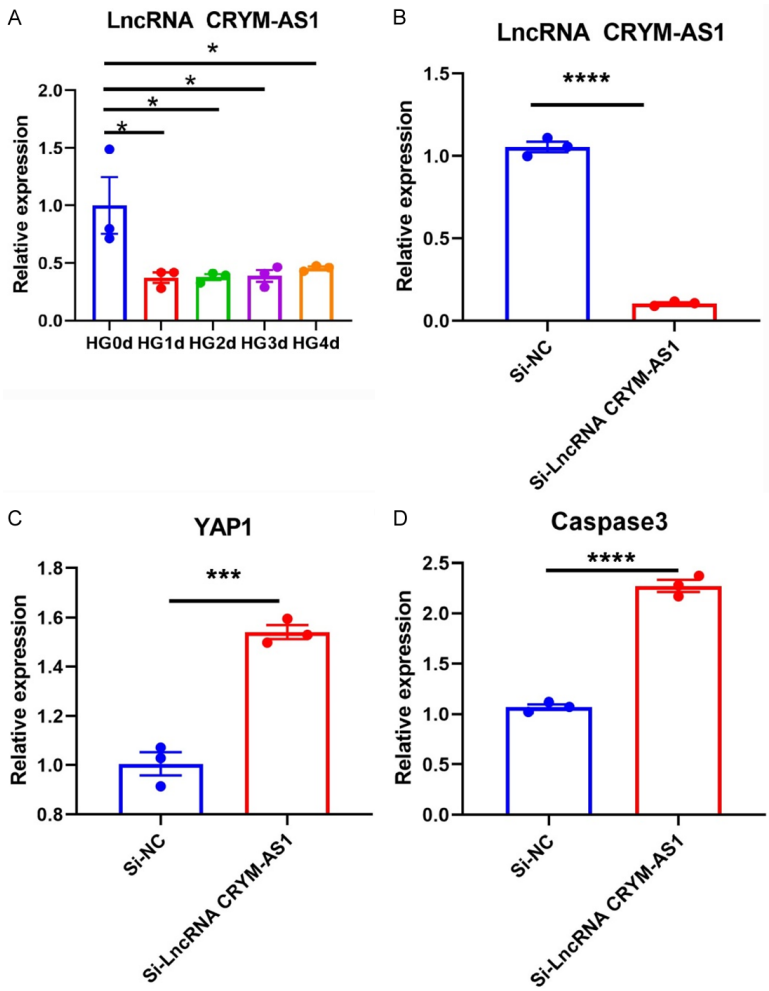

Figure 7. Detection of effective siRNA interference efficiency of *LncRNA CRYM-AS1* in HUVECs and effects on the expression of YAP1 and Caspase 3. A. Real-time quantitative polymerase chain reaction (RT-qPCR) of *LncRNA CRYM-AS1* in HUVECs after 0, 1, 2, 3, and 4 days of incubation in high glucose RPMI-1640 complete medium (7.4 g/L); B-D. RT-qPCR results of *LncRNA CRYM-AS1*, YAP1 and Caspase 3 in HUVECs after interfering with the *LncRNA CRYM-AS1*.

human gastric cancer cells [30], but it has not been reported in the literature in the context of erectile dysfunction; the analysis of KEGG enrichment analysis of the differentially expressed genes suggested that the signaling pathway in which the differentially expressed genes were mainly enriched was the Hippo pathway which was associated with apoptosis. The Hippo signaling pathway is a kinase cascade reaction consisting of a series of protein kinases and transcription factors [31, 32], which controls organ size primarily by regulating cell survival, proliferation and apoptosis [33-36]. Apoptosis has been demonstrated in conditions of high levels of glucose that can lead to cellular dysfunction [37], inducing apoptosis in HUVECs [38-41]. Relevant literature has shown that ox-LDL can inhibit the normal expression of Hippo-YAP/ZAP pathway proteins through miR-496 expression and through epigenetic modification. It can induce apoptosis and dysfunction in vascular endothelial cells [42]. Moreover, naringenin was found to reverse ox-LDL-triggered HUVECs apoptosis by inhibiting the YAP pathway [43].

Recently, an increasing body of evidence suggests that lncRNAs play an important role in regulating the biological functions of the Hippo signaling pathway. For example, *LncRNA-BCAR4* small molecule inhibitor in concert with Hippo-Yap metabolic pathway inhibitor can inhibit tumorigenesis [44]; *LncRNA-NEAT1* can regulate the self-renewal of the liver cancer stem cells through the Hippo signaling pathway [45]. A recent article in Cell Research found that *LncRNA SNHG9* promotes breast tumor development by promoting LATS1 phase separation and inhibiting the Hippo pathway [46]. Therefore, through the previous study, the hypothesis that *LncRNA CRYM-AS1* could promote apoptosis in HUVECs by activating the Hippo signaling pathway, which could lead to the development of DED was proposed.

This study was carried out to explore it more deeply by constructing a type 1 diabetic ED rat model and performing differential gene validation on penile tissues in order to confirm our hypothesis. RT-qPCR results showed that the expression of *LncRNA CRYM-AS1* was significantly down-regulated in the penile cavernous tissues of SD rats with the DED group, which

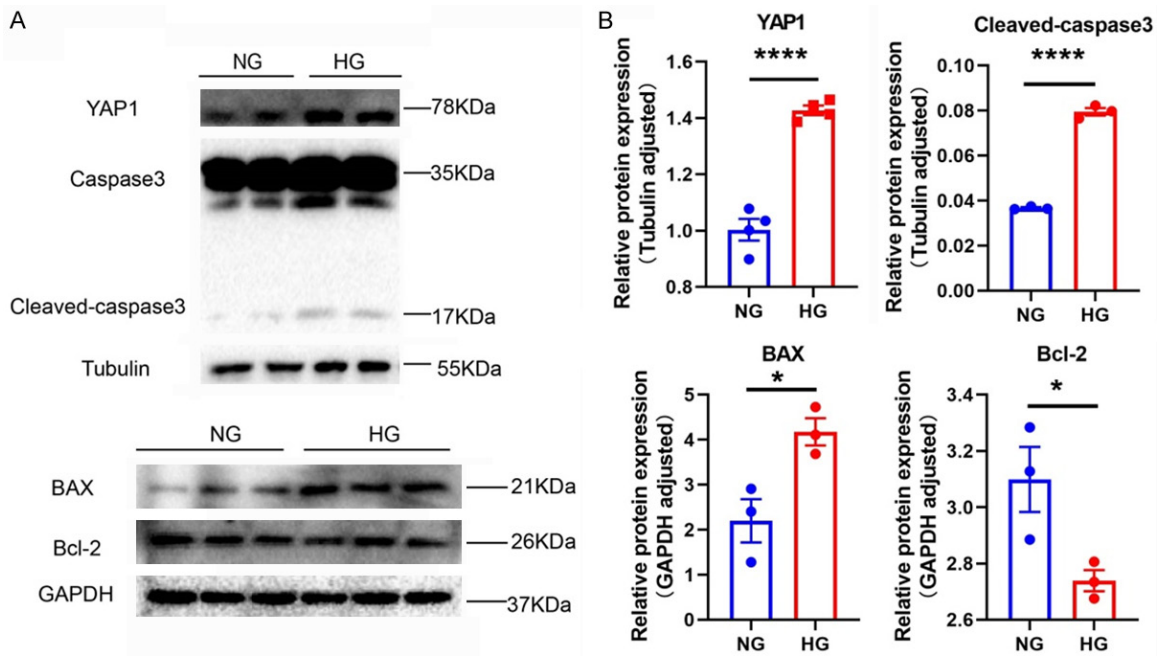

Figure 8. Expression of YAP1 and apoptosis-related proteins at protein levels in normoglycemia and hyperglycemia. A, B. Changes in expression levels of YAP1 and apoptosis-related proteins in HUVECs in normoglycemia and hyperglycemia. *$P < 0.05$.

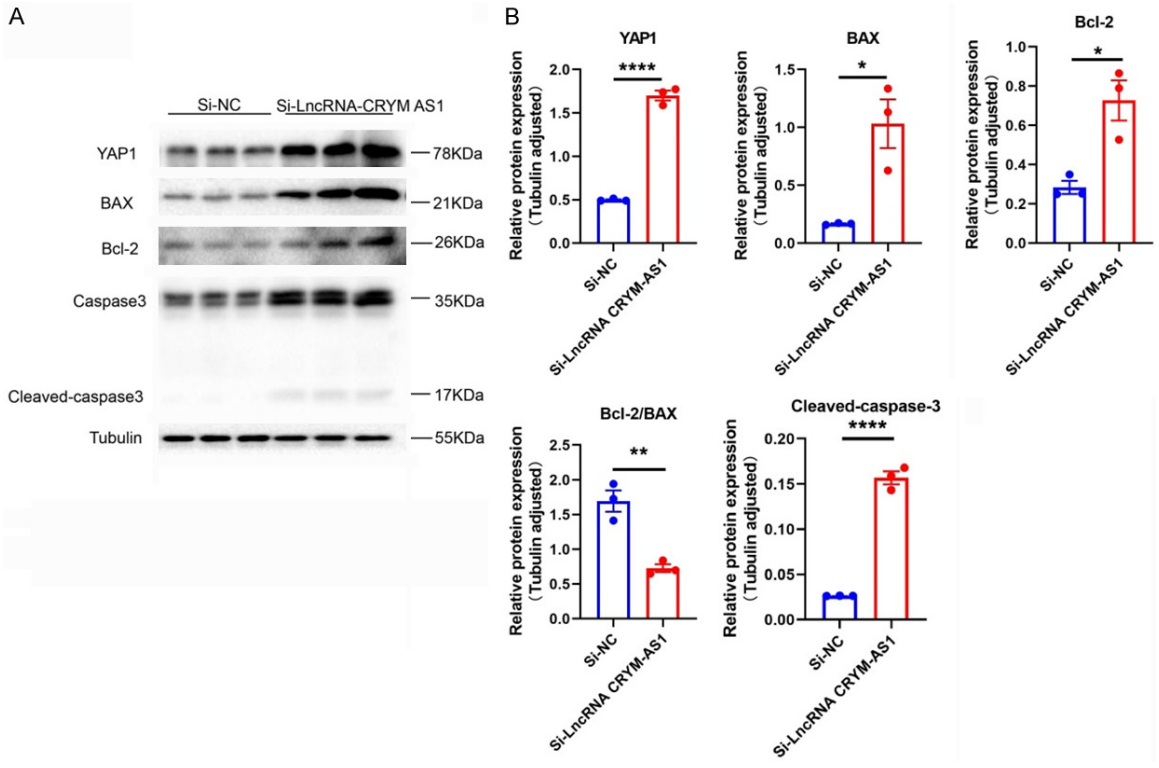

Figure 9. Changes in the expression levels of YAP1 and apoptosis-related proteins in HUVEC after interfering with *LncRNA CRYM-AS1*. A, B. Expression of YAP1, BAX, Bcl-2, Caspase3, Cleaved-Caspase3 proteins in Si-NC group and Si-*LncRNA CRYM-AS1* group. *$P < 0.05$, **$P < 0.01$.

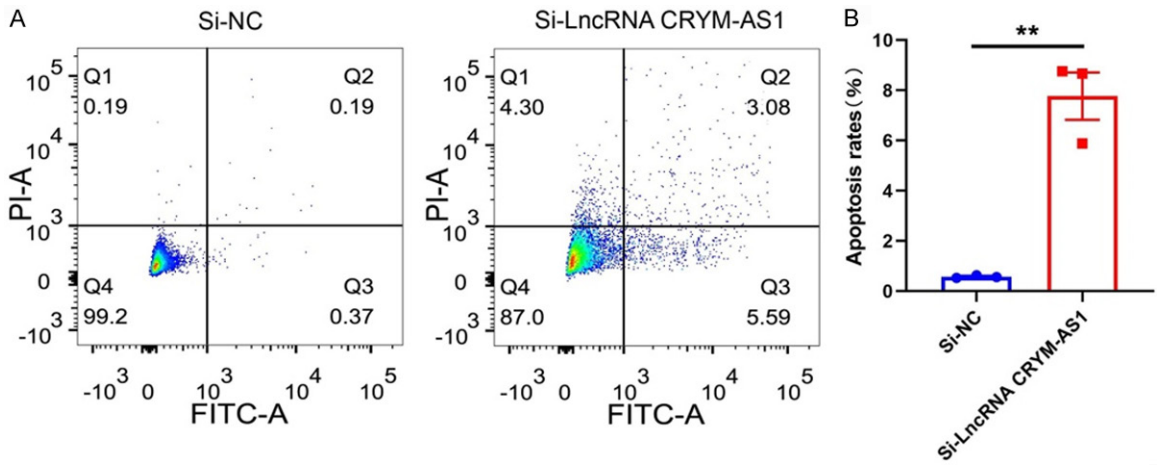

Figure 10. Apoptosis level of HUVECs after interfering with *LncRNA CRYM-AS1*. A, B. The apoptosis level of HUVECs increased after interfering with *LncRNA CRYM-AS1*. ***P < 0.01*.

was identical to that of our bioinformatic analysis. Western Blot and IHC results showed that the protein expression of YAP1, Caspase3, BAX, and Bcl-2 was significantly elevated, and the Bcl-2/BAX ratio was decreased, which suggests that the down-regulation of *LncRNA CRYM-AS1* in the tissues of type 1 diabetic ED rats may promote penile cavernous endothelial cell apoptosis through activation of Hippo signaling pathway and thus induce ED. An *in vitro* study also simulated the similar results. The results of the study showed that the protein expression of YAP1, Cleaved-caspase3, and BAX was significantly up-regulated, and the protein expression of Bcl-2 was significantly down-regulated, suggesting that the *LncRNA CRYM-AS1* may promote HUVECs apoptosis by activating the Hippo signaling pathway, and participate in the pathophysiological process of erectile dysfunction.

LncRNA CRYM-AS1 was interfered with in HUVECs to explore the mechanism by which *LncRNA CRYM-AS1* activates the Hippo signaling pathway to regulate HUVECs apoptosis. RT qPCR results showed the downregulation of *LncRNA CRYM-AS1* expression, indicating significant interference. Western blot results showed upregulation of YAP1, BAX, Bcl-2, and Caspase3 protein expression levels, indicating that downregulation of *LncRNA CRYM-AS1* can activate the upregulation of effector YAP1 in the hippocampal signaling pathway, thereby activating the production of caspase3 protein cleavage by caspase [47]. Cutting cysteine 3 induces cell apoptosis and leads to decreased erectile function. Flow cytometry results also showed that the apoptosis rate of HUVECs increased after interference. These results suggest that inhibiting the expression of *LncRNA CRYM-AS1* may activate the Hippo signaling pathway and promote endothelial cell apoptosis, leading to HUVECs damage and inducing decreased erectile function. The low expression of *LncRNA CRYM-AS1* could promote HUVECs apoptosis through the Hippo-YAP1 signaling pathway, leading to DED, thereby providing a new potential target for preventing and treating erectile function in diabetes. The target gene *LncRNA CRYM-AS1* has not yet been studied at the animal level, and there are still some shortcomings in current research that need further improvement. Researchers seldom study the relationship between lncRNAs and diabetes ED. They mainly focus on repairing erectile function by influencing the differentiation of stem cells into endothelial cells. However, in recent years, researchers have paid more attention to the role of lncRNAs in diseases, and believe that more diabetes ED-related lncRNAs will be explored in the near future.

Conclusion

It was concluded that lncRNA CRYM-AS1 was significantly down-regulated in the penile corpus cavernosum of DM rats, and the down-regulation of its expression may affect ED development by regulating the Hippo-YAP1 pathway to cause endothelial cell apoptosis. The results of this study are expected to be a new target for

the treatment of erectile dysfunction. However, whether lncRNA CRYM-AS1 acts directly or indirectly with the Hippo pathway cannot be fully accounted for and the exact mechanism needs further exploration.

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Disclosure of conflict of interest

None.

Address correspondence to: Jun Chen, Department of Infertility and Sexual Medicine, The Third Affiliated Hospital of Sun Yat-sen University, Tianhe Road, Guangzhou 510630, Guangdong, P. R. China. Tel: +86-18902336333; Fax: +86-20-85253151; ORCID: 0000-0002-7180-2129; E-mail: chenjn27@mail.sysu.edu.cn; Shu-Guang Zhu, Department of Hepatic Surgery and Liver Transplantation Center, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou 510630, Guangdong, P. R. China. E-mail: za200909@163.com

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