Up-regulation of VEGF by Small Activator RNA in Human Corpus Cavernosum Smooth Muscle Cells

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ABSTRACT

Introduction. Functional failure of smooth muscle cells and endothelial cells in corpus cavernosum contributes to erectile dysfunction (ED) in aging men. Given that vascular endothelial growth factor (VEGF) may improve the function of smooth muscle cells and endothelial cells through different mechanisms, it is thus expected that increasing the expression of VEGF may have beneficial effects on erectile function.

Aim. The aim of this article is to explore the possibility that VEGF can be induced by ribonucleic acid activation (RNAa) technology, and VEGF induction by RNAa has the potential of treating ED.

Methods. Primary human corpus cavernosum smooth muscle cells (CCSMCs) were isolated and cultured in vitro. The expression of α-smooth muscle actin was detected by immunohistochemistry to identify CCSMCs. A previously identified VEGF promoter-targeted small activator RNA (saRNA, double-stranded [ds]VEGF-706) and a negative control dsRNA were chemically synthesized. Cultured human CCSMCs were transfected with the saRNAs. The expression of VEGF messenger RNA (mRNA) and protein in transfected CCSMCs was evaluated by real-time polymerase chain reaction (RT-PCR) and Western blotting assay, respectively. Immunofluorescent staining was also used to confirm VEGF protein expression in cultured CCSMCs.

Main Outcome Measure. The expression of VEGF was assessed by RT quantitative PCR, Western blotting, and immunofluorescence assays.

Results. After transfection, RT quantitative PCR analysis showed that the expression of VEGF mRNA was significantly induced in dsVEGF-706 transfected cells compared with cells receiving control treatments ($P < 0.05$). Consistent with mRNA induction, Western blotting and immunofluorescence analysis showed that VEGF protein expression was also induced by dsVEGF-706.

Conclusion. VEGF expression can be activated by RNAa in primary human CCSMCs, suggesting a potential application of RNAa-mediated VEGF activation for the treatment of ED. **Chen R, Wang T, Rao K, Yang J, Zhang S, Wang S, Liu J, and Ye Z. Up-regulation of VEGF by small activator RNA in human corpus cavernosum smooth muscle cells. J Sex Med 2011;8:2773–2780.**

Key Words. Erectile Dysfunction; Corpus Cavernosum Smooth Muscle Cells; VEGF; RNAa

Introduction

Small double-stranded ribonucleic acids (dsRNAs) are known to be the trigger of RNA interference (RNAi), a phenomenon in which these dsRNAs mediate sequence-specific suppression of gene expression. Surprisingly, Li et al. further found that dsRNAs could induce sequence-specific gene expression by targeting gene promoter regions, and termed this phenomenon RNA activation or RNAa and such dsRNA as small activator RNA (saRNA) [1]. They demonstrated the activation of several genes including vascular endothelial growth factor (VEGF) in human cells [1]. Other groups have reported similar findings in human cells [2] and in other mammalian species as well [3].

RNAi is being actively pursued as therapeutics for many human diseases caused by overexpression of a particular gene product, such as an oncogene in cancer. However, many diseases, especially diseases related to aging, are often resulted from inadequate secretion of or decreased expression of certain gene product involved in certain crucial physiological function [4]. These defects are difficult to correct by RNAi. To increase the expression of specific genes in a cell, the common method is the transgenic approach using viral vectors, a therapy also known as gene therapy. Gene therapy can lead to robustly restore gene expression or replace a mutated or deleted gene, but its clinical application is limited by its adverse effects associated with the use of viral vector, such as insertional mutagenesis. Therefore, a clinically safe method of restoring gene function is urgently needed. Small RNA-based drugs, because of its relative small molecular weight and high specificity, hold promise of acting as a new vehicle for delivering gene therapy by exploiting the RNAa mechanism.

Erectile dysfunction (ED) is a major health problem that has profound effects upon the quality of life of both patients and their partners [5]. Phosphodiesterase type 5 inhibitors are the first-line treatment for men with ED; however, troublesome adverse effects, such as headache, flushing, dyspepsia, and so on, may limit its wider use. Other treatments, such as intracavernosal injection, prosthesis implantation, and so on, may be options for some men; however, the tolerability of these agents is variable [6]. Gene therapy is expected to replace these methods of treatment. In many previous studies, increasing expression of related genes, such as endothelial nitric oxide synthase, calcitonin gene-related peptide, superoxide dismutase, and so on, through transfection of corresponding viral vectors improved erectile function in animal experiments [7]. RNAa may provide us new strategies that increase the expression of targeting genes to treat ED in a virus-free manner.

VEGF has shown to improve overall endothelial and smooth muscle cell dysfunction in models of ED [8]. Animal experiments confirmed that VEGF could improve erectile function through various pathways [9,10]. It is reasonable to speculate that VEGF is a potential treatment for ED. Given the advantages of RNAa mentioned above, it is worthwhile to explore whether the dsRNAs could facilitate VEGF expression in human corpus cavernosum smooth muscle cells (CCSMCs).

Methods

Human CCSMC Culture

The study protocol was approved by the ethic committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. Informed consent was obtained from the patients without the history of ED before the study [11]. Penile corpus cavernosal tissues were obtained from patients undergoing penectomy for penile cancer [12]. Homogeneous explant cell cultures of human CCSMCs were prepared as previously described [13]. Sections of approximately $3 \times 3 \times 4$ mm^3 in size were excised from the removed penile tissue of each patient. These specimens consisted exclusively of smooth muscle, endothelium, and connective tissue, with occasional nerve fibers. The tissue was washed, cut into approximately $1 \times 1 \times 0.5$ mm^3 pieces, and placed in tissue culture flasks with a minimal volume of Dulbecco's Modified Eagle Medium (Gibco, Carlsbad, CA, USA) with 20% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, Zhejiang, China). Then, tissue culture flasks were upturned and put into a cell incubator at standard conditions of 37°C with 5% carbon dioxide and 95% humidity, and tissue culture flasks were upturned again slowly to normal after the tissues attached to the plate about 0.5–1 hour later. Additional medium was added in 1–2 days later. Smooth muscle cells migrated from the explant and underwent proliferation. Primary culture cells were subsequently detached using 0.25% trypsin (Gibco) plus 0.02% ethylenediaminetetraacetic acid (Shantou Xilong Chemical Factory Co., Ltd., Shantou, Guangdong, China) to establish secondary cultures. Medium was replaced again in 4–6 hours after passage, and explant was removed because of not attaching to the plate. Cultures were maintained for no more than four passages. Image was obtained under an Olympus IX70 photomicroscope (Olympus Corporation, Tokyo, Japan).

Immunocytochemistry

Immunocytochemistry of human CCSMCs was prepared as previously described [5,14]. Human CCSMCs were plated at a density of 10,000 cells/cm^2 in culture slides and cultured overnight. Cells were rinsed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 30 minutes. Cells were then immunostained with mouse anti-α-smooth muscle actin (α-SMA, Boster Biotechnology Company, Wuhan, Hubei,
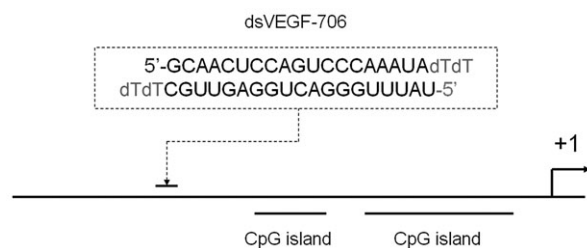
Figure 1 A schematic representation of the VEGF promoter and the location of the small activator ribonucleic acid target. CpG = -C-phosphate-G-, dsVEGF = double-stranded vascular endothelial growth factor.

China). Secondary antibody was goat antimouse IgG (Boster Biotechnology Company) added at a dilution of 1:200. After washing, sections were immunostained with Strepto-Avidin-Biotin Complex (Boster Biotechnology Company) and diaminobenzidine followed by counterstaining with hematoxylin.

Transfection

Human CCSMCs were cultured in six well plates at 1×10^6/well in 2-mL medium [15]. When 60–70% cells grew into monolayer, they were transfected with dsRNA molecules according to the manual for Entranster-R Transfection Reagent kit (Engreen Biosystem Co., Ltd., Beijing, China). Fifty-nanomolar dsRNA, 5-µL Entranster-R transfection reagent, and 2-mL medium were used for each well. For mock transfection, dsRNA was replaced by deoxyribonuclease-free dH$_2$O. A previously identified saRNA targeting the VEGF gene promoter at position −706 relative to the transcription start site (dsVEGF-706) was used to activate VEGF expression in human primary CCSMCs (Figure 1). The dsVEGF-706 sequences used in this study were according to the previous report [1]. After 4–6 hours, the medium with transfection reagent was replaced with normal medium. The CCSMCs were transfected for 72 hours before analysis. The sequences of dsRNAs used are presented in Table 1.

Quantification of RNA Expression by Real-Time (RT) Quantitative Polymerase Chain Reaction (PCR)

Total cellular RNA from human CCSMCs was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA), as described previously [15,16]. Complementary deoxyribonucleic acid (cDNA) was synthesized from 400 ng of RNA according to the manual for Takara RT-PCR kit (Takara bio, Dalian, Liaoning, China). The reaction was incu-

bated at 42°C for 30 minutes and terminated by heat inactivation at 99°C for 5 minutes. RT quantitative PCR was performed on the Mx3000P system (Stratagene, La Jolla, CA, USA) using SYBR Green RT-PCR Master Mix (Toyobo, Tokyo, Japan) according to the manufacturer's recommendations. The sequences of the PCR primers used are presented in Table 1. Amplification was performed with the following cycling conditions: an initial step at 95°C for 1 minute, then 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 15 seconds. Fluorescent readings were completed after each cycle. The threshold cycle was automatically calculated by the Mx3000P system, and a melt curve was generated from 55 to 95°C with fluorescent readings every 0.2°C. Specificity of primers was verified by melt curve analysis. Results are reported as the relative expression level compared with the calibrator cDNA after normalization of the transcript amount to the endogenous control (glyceraldehide-3-phosphate dehydrogenase [GAPDH]).

Protein Isolation and Western Blotting Analysis

Western blot analysis was performed using samples obtained from different groups of human CCSMCs as described in [17] and [18]. Samples were suspended in sodium dodecyl sulfate polyacrylamide gel electrophoresis sample buffer. Protein samples (20 µg per lane) were separated on a Tris-HCl Ready Gel (12% resolving, 5% stacking, Bio-rad) and transferred to a nitrocellulose membrane. Membranes were blocked in 5% (w/v) nonfat dried milk. After several washes with washing buffer (PBS Tween, 0.1%), the membranes were incubated with the primary antibodies for 2 hours at room temperature. The primary antibodies (monoclonal antibodies) were as follows: (i) VEGF (1/500) (Santa Cruz Biotechnol-

Table 1 Sequences for dsRNAs and real-time quantitative polymerase chain reaction primers used

	dsRNAs
dsVEGF-706 S	5′-GCAACUCCAGUCCCAAAUAdTdT-3′
dsVEGF-706 AS	5′-UAUUUGGGACUGGAGUUGCdTdT-3′
dsControl S	5′-UUCUCCGAACGUGUCACGUdTdT-3′
dsControl AS	5′-ACGUGACACGUUCGGAGAAdTdT-3′
	Primers
GAPDH-F	5′-GAAGGTGAAGGTCGGAGTC-3′
GAPDH-R	5′-GAAGATGGTGATGGGATTTC-3′
VEGF-F	5′-ATGACGAGGGCCTGGAGTGTG-3′
VEGF-R	5′-CCTATGTGCTGGCCTTGGTGAG-3′

dsRNA = double-stranded ribonucleic acid; dsVEGF = double-stranded vascular endothelial growth factor; GAPDH = glyceraldehide-3-phosphate dehydrogenase.

ogy, Santa Cruz, CA, USA) and (ii) GAPDH (1/8,000) (Promab, Changsha, Hunan, China). The washed membranes were incubated for 1 hour at room temperature with a secondary antibody of 1/80,000 dilution, linked to horseradish peroxidase. After several washes, bands were revealed by enhanced chemiluminescence detection system.

Immunofluorescence

Immunofluorescence techniques were used to detect the expression of VEGF as described in [19] and [20]. Cultured human CCSMCs of different groups were fixed by 4% paraformaldehyde for 30 minutes at room temperature. The cells were incubated with mouse anti-VEGF monoclonal antibodies (1/500) (Santa Cruz Biotechnology) at 4°C overnight. After rinsed by PBS, the cells were then incubated with rabbit antimouse IgG CY3 fluorescent antibodies (Sigma-Aldrich Corp., St. Louis, MO, USA) at 37°C for 1 hour. Cell nuclei were stained with 5-ng/mL 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich Corp.) in immunofluorescence assay. Finally, the slides were analyzed under an Olympus BX51 fluorescence microscope (Olympus Corporation).

Statistical Analysis

All values are presented as means ± standard deviation. Statistical significances were derived by comparisons among intervention groups, control groups, and mock groups using analysis of variance. $P < 0.05$ was considered statistically significant [2].

Results

Establishment of Primary Human CCSMC Culture and Cell Transfection

We were able to successfully establish primary human CCSMC culture. After two to three passages, the isolated cells were mainly spindle cells, and cobblestone-like growth of endothelial cells was rare (Figure 2A). Almost all of the cultured cells were positive for α-SMA expression (Figure 2B), which confirmed that the cultured cells were human CCSMCs. After human CCSMCs were transfected with 5′-carboxyfluorescein labeled dsRNA, green fluorescent could be seen in 85.4 ± 3.84% of the CCSMCs (Figure 2C) judged by counting fluorescence positive cells, indicating a high dsRNA transfection efficiency in primary cells.
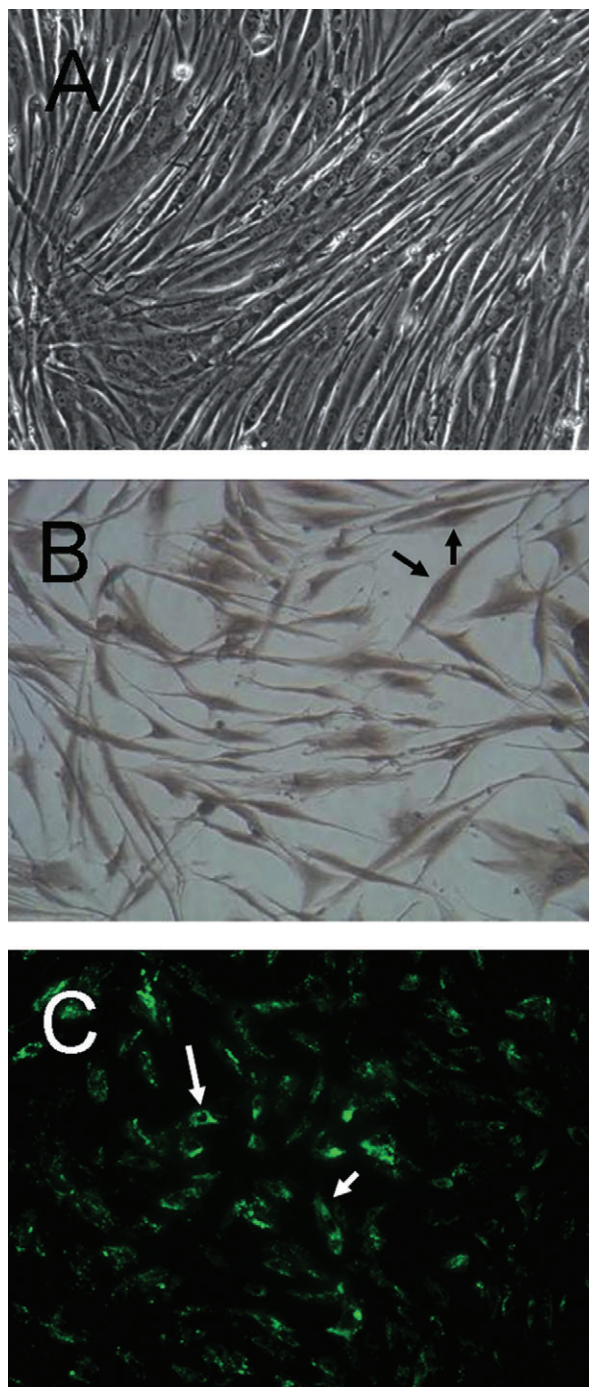

Figure 2 Establishment of primary human CCSMC culture. (A) Spindle smooth muscle cells derived from human corpus cavernosum. Image was obtained using phase-contrast microscopy; (B) α-SMA staining of cultured human CCSMCs and photomicrograph showing positive immunoreactivity for α-SMA in most of the cells; and (C) majority of human CCSMCs showed green fluorescence after transfected with 5′-carboxyfluorescein labeled double-stranded ribonucleic acid. Image was obtained using fluorescence microscopy. The magnification is ×200. CCSMC = corpus cavernosum smooth muscle cell, α-SMA = α-smooth muscle actin.
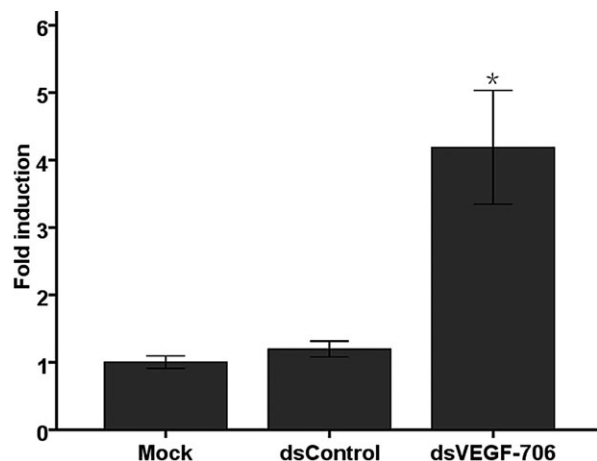
Figure 3 Induction of VEGF mRNA expression by dsVEGF-706. Corpus cavernosum smooth muscle cells were transfected with 50 nM of the indicated small activator RNA for 72 hours. RNA was isolated from the transfected cells. VEGF mRNA expression was detected by real-time quantitative polymerase chain reaction, and the results were normalized to glyceraldehide-3-phosphate dehydrogenase and presented as means ± standard deviation of three independent experiments; *$P < 0.05$ vs. dsControl transfected group and mock group. dsVEGF = double-stranded vascular endothelial growth factor, mRNA = messenger RNA.

VEGF Activation in Human CCSMCs by Promoter-Targeted saRNA

CCSMCs were mock transfected or transfected with 50 nM of dsVEGF-706 or a control dsRNA. Seventy-two hours later, the expression of VEGF was detected by RT quantitative PCR, Western blotting analysis, and immunofluorescence. The expression of VEGF that showed dsVEGF-706 transfected cells were induced at the mRNA level by 4.19-fold compared with mock transfection ($P < 0.05$) (Figure 3). The expression of VEGF protein was also significantly induced by dsVEGF-706 transfection as assessed by Western blotting assay ($P < 0.05$) (Figure 4). Consistently, the expression of VEGF protein was induced by dsVEGF-706 transfection as assessed by immunofluorescent staining (Figure 5). Immunofluorescent staining showed that VEGF was localized in the cytoplasm.

Discussion

Erectile function depends on the development of an appropriate penile vasculature and maintenance of an effective blood supply to the erectile tissues of the penis [21]. VEGF is one of the most important vascular growth factors, which was originally discovered and identified as an endothelial-specific growth factor involved in the formation of new blood vessels (angiogenesis) [22]. Now, it is clear that VEGF regulates not only endothelial cells but also smooth muscle cells and nerve cells [23–25]. The endothelium, smooth muscle, and nerve tissue cover almost all of the functional organization of the penis. VEGF also cross talks with other angiogenic growth factors controlling the angiogenic processes in vascularized tissues [26]. These observations indicate that VEGF plays an important role in erection physiology.

Many animal experiments had confirmed that VEGF could improve erectile function. Park et al.
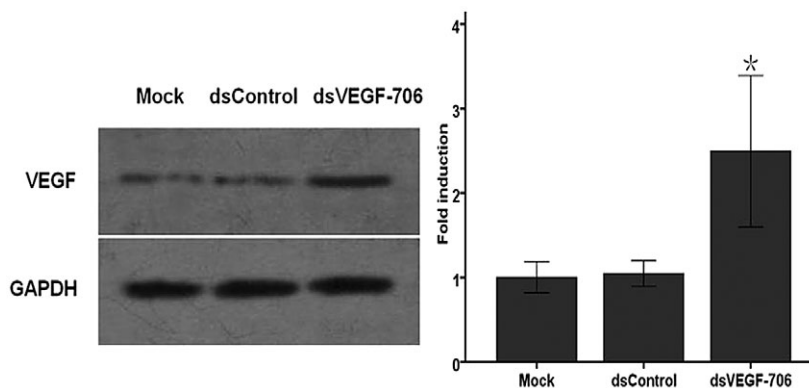

Figure 4 VEGF activation by the protein level by dsVEGF-706. Corpus cavernosum smooth muscle cells were transfected with 50 nM of the indicated small activator ribonucleic acid for 72 hours. The transfected cells were harvested for protein isolating using radioimmunoprecipitation assay. VEGF protein expression was detected by Western blot analysis, and the results were normalized to GAPDH and presented as means ± standard deviation of three independent experiments; *$P < 0.05$ vs. dsControl transfected group and mock group. dsVEGF = double-stranded vascular endothelial growth factor, GAPDH = glyceraldehide-3-phosphate dehydrogenase.
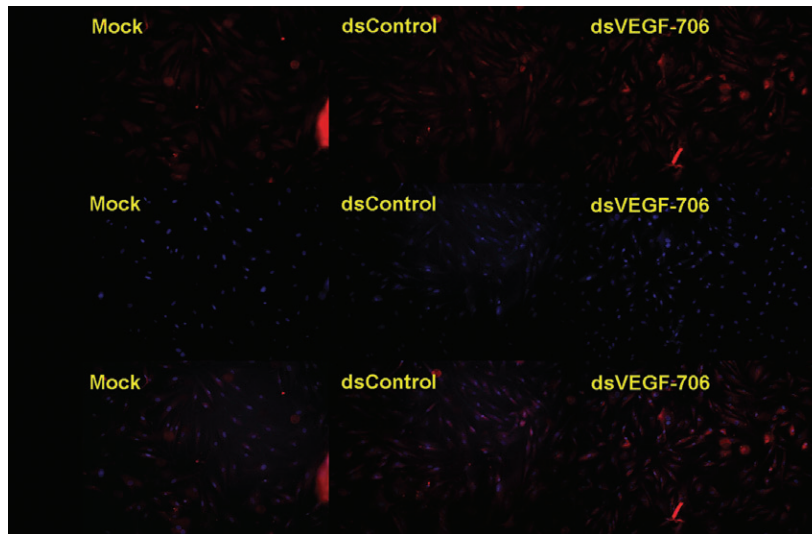
Figure 5 Induction of VEGF protein expression detected by immunofluorescence in human CCSMCs. CCSMCs were transfected on coverslips with 50 nM of the indicated small activator ribonucleic acid for 72 hours. The transfected cells were fixed and stained with a VEGF antibody. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole. Image was obtained using fluorescence microscopy. The magnification is ×200. CCSMC = corpus cavernosum smooth muscle cell, VEGF = vascular endothelial growth factor.

[27] found that intracavernosal injection of VEGF could restore smooth muscle integrity and improve erectile function in aged rats. Another study showed that intracavernosal injection with VEGF alone could enhance nerve regeneration and promote neural and erectile recovery. Combined VEGF and virus-mediated brain-derived neurotrophic factor treatments could lead to even better results [28]. After VEGF gene was constructed into virus vector and formation of adeno-associated virus-mediated VEGF (AAV-VEGF), intracavernous AAV-VEGF prevented the veno-occlusive dysfunction in castrated animals [29]. These studies have further confirmed the effect of VEGF in the treatment of ED in vivo.

RNAi triggered by dsRNAs, such as siRNA and micro-RNA, is associated with the silencing of target mRNA sequences. Several groups had also reported that targeting promoter regions with dsRNAs induced transcriptional silencing by triggering histone modification and/or DNA methylation [1]. It is indicated that dsRNA cannot modulate gene expression not only on mRNA level but also on DNA level. Li et al. further confirmed that RNAa was a molecular phenomenon different from RNAi by a series of experiments [1,30,31].

RNAa is a new method of up-regulating gene expression. Classic RNAi by siRNA transfection is thought to be relatively short-lived lasting only 5–7 days, while gene activation by RNAa lasts much longer. For example, E-cadherin induction remains detectable 13 days after a signal saRNA transfection [1]. Currently, RNA-based drugs for RNAi have entered the stage of commercial development [32]. The dsRNA used in RNAi and RNAa

is identified in chemistry and structure. Treatment of ED through RNAa has less ethical controversies than transgene through virus vector.

Previously Li's group identified a saRNA target on the VEGF promoter and demonstrated the activation of VEGF by this saRNA (dsVEGF-706) in HeLa cells on a 72-hour transfection. In a subsequent study, they further showed that the same saRNA could also activate VEGF expression in nonhuman primate cells that have the identical target sequence and identified an additional saRNA on the VEGF promoter [33]. However, another group failed to induce VEGF expression using saRNA dsVEGF-706 in HeLa cells on a 48-hour transfection [34]. It is likely that the discrepancy was caused by difference in transfection duration. It has been shown that gene activation by RNAa does not appear until 48 hours following transfection [1,35]. Therefore, RNAa transfection should be carried out at least for 72 hours. Our study demonstrated, for the first time, that VEGF could be activated by RNAa in primary human cells. Thus, RNAa-mediated VEGF activation may have the potential for treating ED in patients by enhancing VEGF function in smooth muscle cells and endothelial cells. In this regard, Turunen et al. [3] found that RNAa-mediated VEGF activation improves vascularity and blood flow in an ischemic mouse hindlimb model.

VEGF is one of the intriguing determinants of erectile biology. It is the critical mediator of endothelial and smooth muscle physiology, and its reduction is associated with a number of pathophysiological changes in the penis. In various models of ED, VEGF was down-regulated [36]. As

mentioned above, it had been confirmed by a large number of animal experiments that increasing VEGF levels in the penis could improve erectile function. As the sequence dependent of RNAa, up-regulated VEGF expression in human CCSMCs has confirmed the potentiality of VEGF for future treatment of ED in another important aspect besides animal experiments. Although treatment of ED through RNAa to up-regulated VEGF expression has yet to be confirmed by further animal experiments and many functional studies need to be further verified in vivo, we have reason to believe that treatment of ED through RNAa could become a new choice.

Conclusion

Although the exact mechanisms of RNAa remain unclear, our findings suggested that dsVEGF-706 could induce expression of VEGF in primary human CCSMCs. As the sequence dependent of RNAa, up-regulated VEGF expression in human CCSMCs has revealed the potentiality of VEGF for future treatment of ED in another important aspect besides animal experiments. It would approve a new gene-based approach to the treatment of ED.

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Conflict of Interest: None declared.

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