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ORIGINAL ARTICLE

Erectile Dysfunction

Combination of stromal vascular fraction and *Ad-COMP-Ang1* gene therapy improves long-term therapeutic efficacy for diabetes-induced erectile dysfunction

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Men with diabetic erectile dysfunction (ED) respond poorly to the currently available oral phosphodiesterase-5 inhibitors. Therefore, functional therapies for diabetic ED are needed. Stromal vascular fraction (SVF) and the adenovirus-mediated cartilage oligomeric matrix angiopoietin-1 (*Ad-COMP-Ang1*) gene are known to play critical roles in penile erection. We previously reported that SVF and *Ad-COMP-Ang1* have only a short-term effect in restoring erectile function. Further improvements to ED therapy are needed for long-lasting effects. In the present study, we aimed to test if the combination of SVF and *Ad-COMP-Ang1* could extend the erection effect in diabetic ED. We found that the combination therapy showed a long-term effect in restoring erectile function through enhanced penile endothelial and neural cell regeneration. Combination therapy with SVF and *Ad-COMP-Ang1* notably restored cavernous endothelial cell numbers, pericyte numbers, endothelial cell–cell junctions, decreased cavernous endothelial cell permeability, and promoted neural regeneration for at least 4 weeks in diabetic mice. In summary, this is an initial description of the long-term effect of combination therapy with SVF and *Ad-COMP-Ang1* in restoring erectile function through a dual effect on endothelial and neural cell regeneration. Such combination therapy may have therapeutic potential for the treatment of diabetic ED.

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INTRODUCTION

Erectile dysfunction (ED) is predominantly a vascular disease that affects up to 75% of all men with diabetes.[1–4] In addition, men with diabetic ED respond poorly to the currently available oral phosphodiesterase-5 (PDE5) inhibitors.[5–7] The reduced responsiveness to PDE5 inhibitors in men with diabetes may be related to the severity of neuropathy and angiopathy.[8,9] Therefore, functional therapies for diabetic ED are needed.

During the past decade, a variety of studies have been performed on neurovascular regeneration from damaged erectile tissue. These studies have included the delivery of adipose tissue-derived stem cells (ADSCs),[10,11] adipose tissue-derived stromal vascular fraction (SVF),[12] vascular endothelial growth factor (VEGF),[13] angiopoietin-1 (Ang1),[14,15] and hepatocyte growth factor (HGF).[16] However, ED in humans is multifactorial in etiology, and single-candidate protein or cell therapy is still unable to completely resolve both the angiogenesis and neuroregeneration in diabetic ED.[17] Therefore, combined therapy may be more effective and ideal in the treatment of this refractory ED.

Recently, SVF was introduced as an ideal source of stem cells that can be easily obtained in abundance without the culturing step.[15,18,19] SVF can differentiate into vascular cells and neurons *in vitro*,[18,19] and it secretes several angiogenic growth factors, such as VEGF and Ang1.[16,20,21] In a rat model of cavernous nerve injury, SVF restores erectile function by promoting neural regeneration.[22] In addition, adenovirus-mediated cartilage oligomeric matrix Ang1 (*Ad-COMP-Ang1*) has also shown a potential effect for the treatment of ED by preventing vascular leakage.[14] However, single therapy with either SVF or *Ad-COMP-Ang1* cannot resolve both angiogenesis and the neuroregeneration effect. Therefore, these studies prompted us to hypothesize that combination therapy with SVF and *Ad-COMP-Ang1* may enhance therapeutic efficacy for both neural regeneration and vascular stability in diabetic ED penises.

To our knowledge, this is the first study to investigate the effectiveness of combination therapy with freshly isolated SVF and

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Ad-COMP-Ang1 on angiopathy and neuropathy, which extends the therapy effect on erection, in a mouse model of diabetic ED.

MATERIALS AND METHODS

Isolation of SVF and generation of COMP-Ang1 adenovirus

SVF was isolated as previously reported.[12] Briefly, we isolated SVF from epididymal adipose tissues of 10-week-old C57BL/6J mice. The adipose tissue was incubated in Hanks' balanced salt solution containing 0.2% collagenase type 2 (Sigma-Aldrich, St. Louis, MO, USA) for 60 min at 37°C. After incubation, digestion enzyme activity was neutralized with Dulbecco's modified Eagle's medium (GIBCO, Carlsbad, CA, USA) containing 10% fetal bovine serum. The cell suspension was filtered through 70-μm and 40-μm nylon meshes (Becton Dickinson, Mountain View, CA, USA) and then centrifuged (Thermo Fisher Scientific, Waltham, MA, USA) at 580 *g* for 4 min at 4°C. The adipose tissue-derived stromal vascular fraction (AD-SVF) pellet was resuspended in sterile phosphate-buffered saline (PBS, GIBCO). Recombinant adenovirus expressing COMP-Ang1 or LacZ was provided by Professor Ji-Kan Ryu at Inha University, and the amplification was performed as previously reported.[14,23] Briefly, HEK293T cells (purchased from the cell bank of the Chinese Academy of Sciences) were used for *COMP-Ang1* gene or bacterial *β-galactosidase* (hereafter LacZ) gene infection.

Animals and treatment

Male 8-week-old C57BL/6J mice were purchased from Pengyue Experimental Animal Breeding Center (Jinan, China) and were randomly grouped in this study. Diabetes was induced in 8-week-old mice by intraperitoneal injections of streptozotocin (50 mg kg^{-1}, Sigma-Aldrich, St. Louis, MO, USA) for 5 consecutive days as previously described.[14] Eight weeks after diabetes was induced, animals were anesthetized with chloral hydrate (300 mg kg^{-1}, Sangon Biotech, Shanghai, China) intramuscularly, and the penis was exposed by the use of sterile technique. A 30G insulin syringe (Becton Dickinson) was used to administer repeated injections of PBS (days 3 and 0; 20 μl), Ad-LacZ (1×10^8 particles per 20 μl), AD-SVF pellet (1×10^5 cells per 20 μl of PBS), *Ad-COMP-Ang1* (1×10^8 particles per 20 μl), or AD-SVF plus *Ad-COMP-Ang1* combination into the midportion of the corpus cavernosum. We evaluated erectile function by electrical stimulation of the cavernous nerve 2 and 4 weeks after treatment, and the penis was then harvested for histological examination. Fasting and postprandial blood glucose levels were determined with an Accu-Check blood glucose meter (ACCU-CHEK Performa; Roche Diagnostics, Shanghai, China) before the mice were sacrificed. All mice were treated in accordance with the guidelines for animal care and use of the National Institutes of Health, and ethical approval was granted by the Binzhou Medical University Animal Ethics Committee, Yantai, China. All other methods were performed in accordance with the relevant guidelines and regulations.

Measurement of erectile function

The mice in each group (n = 5) were anesthetized with chloral hydrate (300 mg kg^{-1}, Sangon Biotech) intramuscularly. Bipolar platinum wire electrodes (BIOPAC Systems Inc., Goleta, CA, USA) were placed around the cavernous nerve. The stimulation parameters were as follows: 5 V at a frequency of 12 Hz, a pulse width of 1 ms, and a duration of 1 min. The maximal intracavernous pressure (ICP) was recorded during tumescence. The total ICP was determined by the area under the curve from the beginning of cavernous nerve stimulation to a point 20 s after stimulus termination. Systemic blood pressure was measured using a noninvasive tail-cuff system (Visitech Systems, Apex, NC, USA) before performing ICP determination. The ratios of maximal ICP and total ICP (area under the curve) to mean systolic blood pressure (MSBP) were calculated to adjust for variations in systemic blood pressure as previously described.[24]

Mouse major pelvic ganglion neurite-sprouting assay

Mouse major pelvic ganglion (MPG) tissues were prepared and maintained as described previously.[25,26] Briefly, the MPG tissues were isolated from mice in each group using a microscope (Olympus Stereo Microscope, Tokyo, Japan), transferred into sterile vials containing Hank's balanced salt solution (GIBCO), and then rinsed and washed twice in PBS. The MPG tissues were cut into small pieces, and the samples were plated on poly-D-lysine hydrobromide (Sigma-Aldrich)-coated 12-well plates. The MPG tissues were completely covered with Matrigel matrix (Corning), and the culture plate was placed on ice for 5 min and then incubated at 37°C for 10–15 min in 5% CO_2 atmosphere. Complete Neurobasal™ medium (1 ml, GIBCO) supplemented with 2% serum-free B-27 (GIBCO) and 0.5 nmol l^{-1} GlutaMAX-I (GIBCO) was then added. The dishes were then incubated at 37°C in 5% CO_2 atmosphere. After 3 days of incubation, we evaluated neurite outgrowth against neurofilament (Sigma-Aldrich; 1:100) and mouse tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibodies (Zymed Laboratories, San Francisco, CA, USA; 1:200).

Western blot

Equal amounts of protein (50 μg per lane) were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels (8%–12%, Sigma-Aldrich), transferred to nitrocellulose membranes, and probed with antibodies against phospho-endothelial nitric oxide synthase (eNOS) (Ser1177, Cell Signaling; 1:500), eNOS (Becton Dickinson; 1:500), cleaved caspase-3 (Asp175, Cell Signaling, Beverly, MA, USA; 1:500), claudin-5 (Thermo Fisher Scientific; 1:500), or β-actin (Abcam, Cambridge, UK; 1:6000). The results were quantified by densitometry at n = 4 per group (ImageJ 1.34, National Institutes of Health, Bethesda, MD, USA).

Histological examinations

For fluorescence microscopy, the penis tissue sections (12-μm thick) were fixed in 4% paraformaldehyde for 24 h at 4°C, and frozen tissue sections were incubated with antibodies against CD31 (Chemicon, Temecula, CA, USA; 1:50), PDGFR-β (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:100), phosphohistone H3 (Abcam; 1:50), cleaved caspase-3 (Asp175, Cell Signaling; 1:50), phospho-eNOS (Ser1177, Cell Signaling; 1:1000), claudin-5 (Thermo Fisher Scientific; 1:100), oxidized low density lipoprotein (LDL; Abcam; 1:400), neurofilament (Sigma-Aldrich; 1:100), or nNOS (Santa Cruz Biotechnology; 1:100) at 4°C overnight. After several washes with PBS, the sections were incubated with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated (Zymed Laboratories) or FITC-conjugated secondary antibodies (Molecular Probes, Inc., Waltham, MA, USA) for 2 h at room temperature. Samples were mounted in a solution containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., CA, USA) for nuclei staining. Signals were visualized, and digital images were obtained with a confocal microscope using standard and high magnification (×100, ×200, ×400, ×800; Leica DMI4000B; Leica Microsystems, Heidelberg, Germany).

All digital image quantitative analyses and western blot band densitometry analyses were done with an image analyzer system (ImageJ 1.34, National Institutes of Health).

Statistical analysis

The results are expressed as the mean ± standard deviation (s.d.). Statistical analysis was performed using one-way ANOVA

followed by Newman–Keuls *post hoc* test. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using SigmaStat 3.11 software (Systat Software Inc., San Jose, CA, USA).

RESULTS

Metabolic variables

Fasting and postprandial blood glucose concentrations were higher in the STZ-induced diabetic mice than in the control mice. In addition, body weight was lower in the STZ-induced diabetic mice than in the controls. The body weight and blood glucose levels of the diabetic mice did not differ regardless of the treatment given (**Supplementary Table 1** and **2**).

Combination therapy with SVF and Ad-COMP-Ang1 gene restores more durable erectile function in STZ-induced diabetic mice

A representative intracavernous tracing after stimulation of the cavernous nerve (5 V, 12 Hz, and 1 ms) for 1 min in each group 2 and 4 weeks after treatment is shown in **Figure 1a** and **1b**, respectively. During electrical stimulation of the cavernous nerve, the ratios of maximal ICP and total ICP to MSBP were lower in STZ-induced diabetic mice treated with PBS or ad-LacZ (2×10^8 particles per 20 μl) than that in age-matched controls. At 2 weeks after treatment, a single intracavernous injection of SVF (1×10^5 cells per 20 μl), *Ad-COMP-Ang1* gene (2×10^8 particles per 20 μl), or the combination treatment restored erection parameters, which reached up to 86%–90% (maximal ICP) or 94%–99% (total ICP) of control values (**Figure 1c** and **1d**). At 4 weeks after the administration of SVF, *Ad-COMP-Ang1* gene, or the combination, the recovery of erectile function was not as great as that observed at 2 weeks for the SVF and *Ad-COMP-Ang1* gene groups. However, the combination treatment group maintained high erection parameters, which reached up to 88% (maximal ICP) or 86% (total ICP) of control values (all $P < 0.05$; **Figure 1e** and **1f**)). No detectable differences were found in MSBP among the experimental groups (**Supplementary Table 1** and **2**).

Cavernous endothelial cell and pericyte numbers are improved by combination therapy in STZ-induced diabetic mice

Immunohistochemical staining of cavernous tissue with antibodies against CD31 and PDGFR-β was performed in age-matched control and diabetic mice 4 weeks after treatment. Notably, lower cavernous endothelial cell and pericyte numbers were found in the PBS or ad-LacZ-treated diabetic mice than in the control mice. Intracavernous injection of SVF (1×10^5 cells per 20 μl) or *Ad-COMP-Ang1* gene (2×10^8 particles per 20 μl) partially restored cavernous endothelial cell and pericyte numbers. Moreover, the combination treatment notably restored cavernous endothelial cell and pericyte numbers, but not to the level of the control group (all $P < 0.05$; **Figure 2**).

Combination therapy restores cavernous endothelial cell numbers through an increase in proliferation and a decrease in apoptosis

To determine if the increase in cavernous endothelial cell number was the result of endothelial cell proliferation, we assessed the number of endothelial cells and pericytes staining positive for phosphohistone H3 (a nuclear protein indicative of cell proliferation). Significant decreases were found in phosphohistone H3-positive endothelial cells in the PBS- and ad-LacZ-treated diabetic mice compared to the control mice. Intracavernous injection of SVF (1×10^5 cells per 20 μl) or *Ad-COMP-Ang1* gene (2×10^8 particles per 20 μl) partially increased cavernous endothelial cell

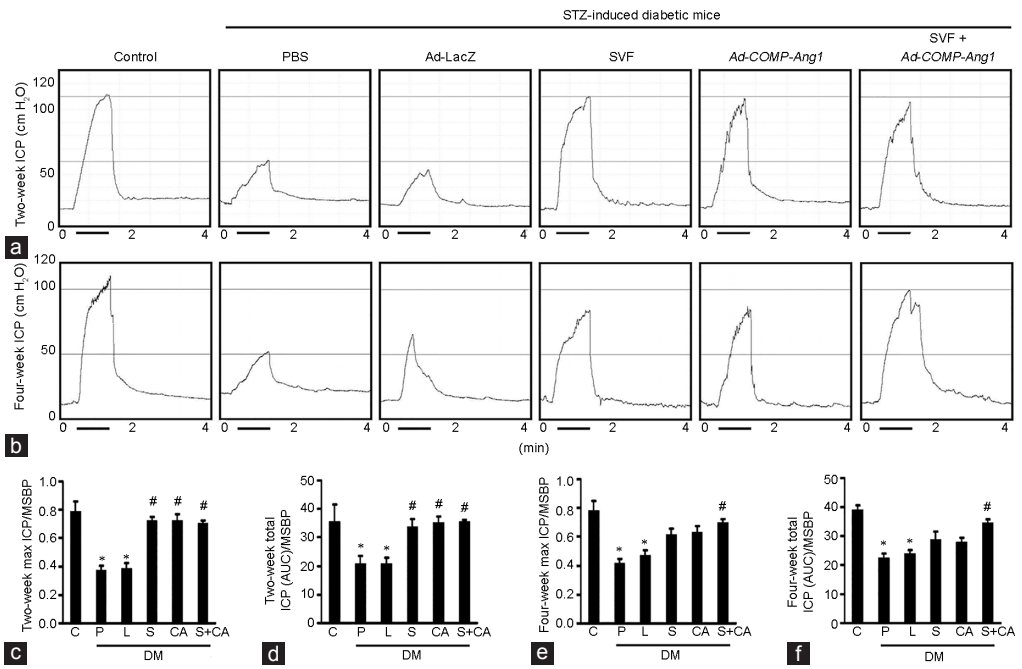

Figure 1: Combination therapy with SVF and *Ad-COMP-Ang1* gene restores erectile function. (**a**) Representative ICP responses for age-matched control mice (C) group and STZ-induced diabetic mice stimulated at 2 weeks after intracavernous injection of PBS (P, 20 μl), ad-LacZ (L, 2×10^8 particles per 20 μl), SVF (S, 1×10^5 cells per 20 μl), *Ad-COMP-Ang1* (CA, 2×10^8 particles per 20 μl), or combination (S + CA). The stimulus interval is indicated by a solid bar (1 min). (**b**) Representative ICP responses at 4 weeks. Ratios of (**c**) mean maximal ICP and (**d**) total ICP (area under the curve) to MSBP for each group in two weeks. Ratios of (**e**) mean maximal ICP and (**f**) total ICP (area under the curve) to MSBP for each group in four weeks. Each bar depicts the mean value ± standard deviation from $n = 5$ animals per group. $^*P < 0.001$, P or L versus C group. $^\#P < 0.05$, S or CA or S + CA versus P or L groups. Each bar depicts the mean value ± standard deviation from $n = 5$ animals per group. The P values were determined by one-way ANOVA. *Ad-COMP-Ang1*: adenovirus-mediated cartilage oligomeric matrix angiopoietin-1; DM: diabetes mellitus; ICP: intracavernous pressure; MSBP: mean systolic blood pressure; PBS: phosphate-buffered saline; STZ: streptozotocin; SVF: stromal vascular fraction.

proliferation, but the combination treatment increased cavernous endothelial cell proliferation to a level higher than that in the control group (all $P < 0.01$; **Figure 3a** and **3b**). Furthermore, combination therapy with SVF (1×10^5 cells per 20 μl) and *Ad-COMP-Ang1* gene (2×10^8 particles per 20 μl) decreased apoptosis to the level of the control group (**Supplementary Figure 1**).

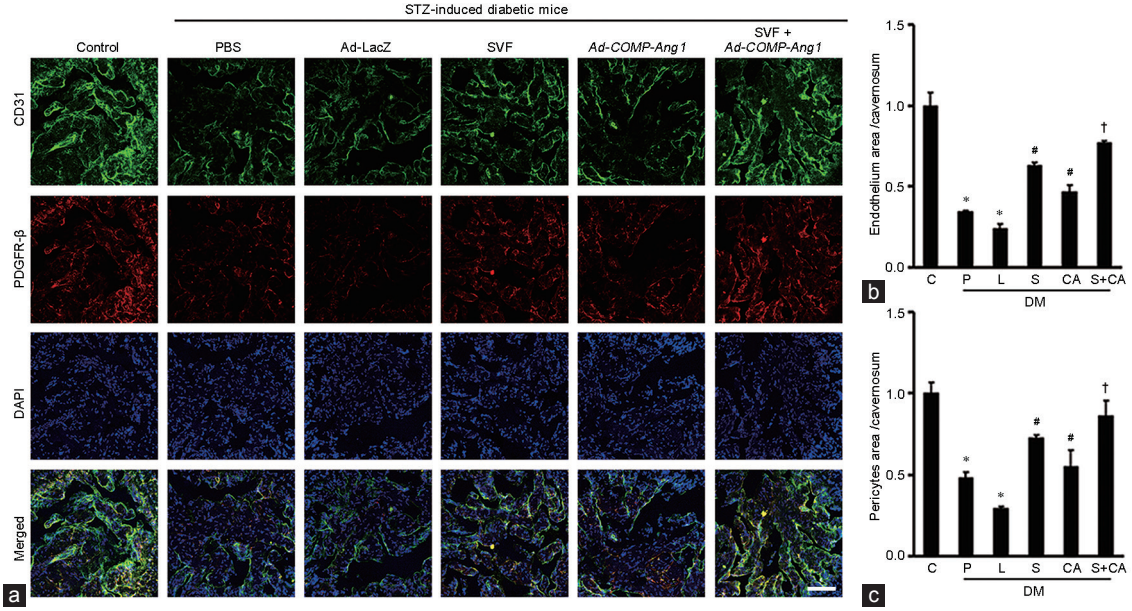

Figure 2: The cavernous endothelial cell and pericyte numbers are improved by combination therapy with SVF and *Ad-COMP-Ang1* gene in STZ-induced diabetic mice. (**a**) CD31 (green) and PDGFR-β (red) staining in cavernous tissue from age-matched control mice (C) group and STZ-induced diabetic mice stimulated at 4 weeks after intracavernous injection of PBS (P, 20 μl), ad-LacZ (L, 2×10^8 particles per 20 μl), SVF (S, 1×10^5 cells per 20 μl), *Ad-COMP-Ang1* (CA, 2×10^8 particles per 20 μl), or combination (S + CA). Nuclei were labeled with DAPI (blue). Scale bars = 100 μm. Quantification of (**b**) cavernous endothelial cell and (**c**) pericyte numbers by ImageJ. Each bar depicts the mean values ± standard deviations from $n = 5$ animals per group. $^*P < 0.001$, P or L versus C group. $^\#P < 0.05$, S or CA versus P or L groups. $^\dagger P < 0.05$, S + CA versus S or CA groups. The *P* values were determined by one-way ANOVA. *Ad-COMP-Ang1*: adenovirus-mediated cartilage oligomeric matrix angiopoietin-1; DAPI: 4',6-diamidino-2-phenylindole; DM: diabetes mellitus; PBS: phosphate-buffered saline; STZ: streptozotocin; SVF: stromal vascular fraction; PDGFR-β: platelet-derived growth factor receptor-beta.

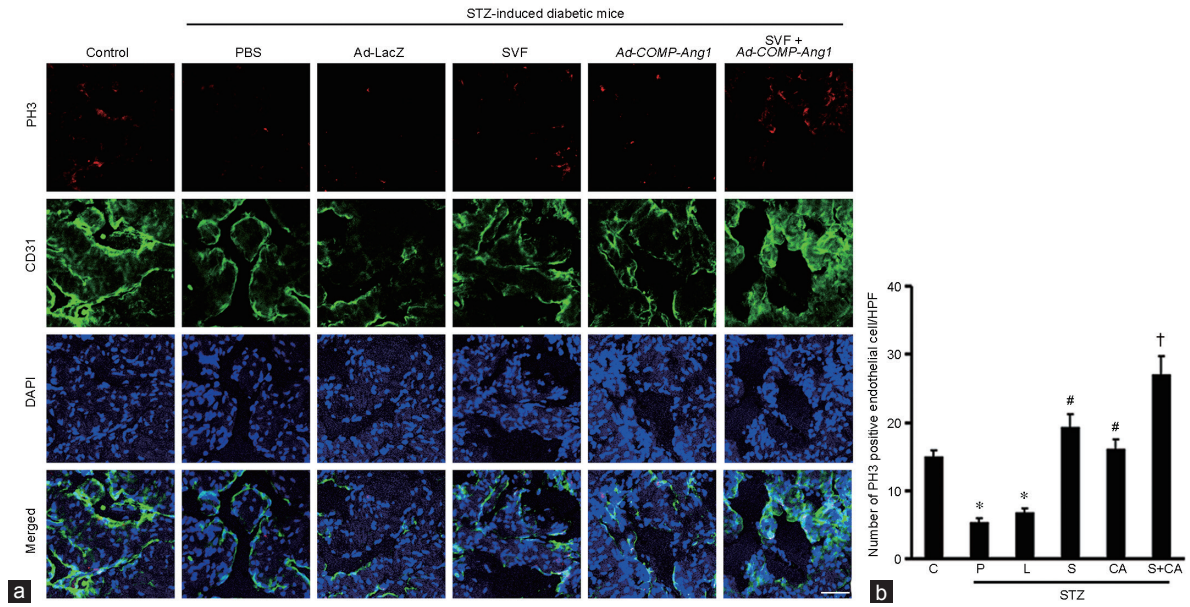

Figure 3: Combination therapy with SVF and *Ad-COMP-Ang1* gene induces cavernous endothelial cell proliferation in STZ-induced diabetic mice. (**a**) CD31 (green) and phosphohistone H3 (PH3; red) staining in cavernous tissue from age-matched control mice (C) group and STZ-induced diabetic mice stimulated at 4 weeks after intracavernous injection of PBS (P, 20 μl), ad-LacZ (L, 2×10^8 particles per 20 μl), SVF (S, 1×10^5 cells per 20 μl), *Ad-COMP-Ang1* (CA, 2×10^8 particles per 20 μl), or combination (S + CA). Nuclei were labeled with DAPI (blue). Scale bars = 50 μm. (**b**) Number of PH 3-immunopositive endothelial cells per HPF. Each bar depicts the mean value ± standard deviation from $n = 5$ animals per group. $^*P < 0.01$, P or L versus C group. $^\#P < 0.01$, S or CA versus P or L groups. $^\dagger P < 0.01$, S + CA versus S or CA groups. The *P* values were determined by one-way ANOVA. *Ad-COMP-Ang1*: adenovirus-mediated cartilage oligomeric matrix angiopoietin-1; DAPI: 4',6-diamidino-2-phenylindole; HPF: high-power field; PBS: phosphate-buffered saline; STZ: streptozotocin; SVF: stromal vascular fraction.

Cavernous eNOS phosphorylation is induced by combination therapy in STZ-induced diabetic mice

Immunohistochemical staining of cavernous tissue with antibodies against phospho-eNOS (Ser1177) and CD31 was performed in age-matched control and diabetic mice 4 weeks after treatment. Phospho-eNOS expression was lower in the PBS- and ad-LacZ-treated diabetic mice than in the control mice. A partial effect of induced endogenous cavernous eNOS phosphorylation was detected in SVF (1×10^5 cells per 20 μl) and *Ad-COMP-Ang1* gene (2×10^8 particles per 20 μl) single treatments, whereas the combination treatment induced endogenous cavernous eNOS phosphorylation to the level of the control group (all $P < 0.05$; **Figure 4**).

Combination therapy restores Claudin-5 and decreases cavernous endothelial cell extravasation of oxidized LDL in STZ-induced diabetic mice

We performed immunohistochemical staining to evaluate the cavernous tissue expression of tight junction protein (Claudin-5) in age-matched control and diabetic mice 4 weeks after treatment. The cavernous expression of Claudin-5 protein was markedly lower in the PBS- and ad-LacZ-treated diabetic mice than in the control mice. Single treatment with SVF (1×10^5 cells per 20 μl) or *Ad-COMP-Ang1* gene (2×10^8 particles per 20 μl) partially restored Claudin-5 protein expression, whereas the combination treatment restored Claudin-5 protein expression to a level higher than that in the control group (all $P < 0.05$; **Figure 5**). In addition, combination therapy with SVF (1×10^5 cells per 20 μl) and *Ad-COMP-Ang1* gene (2×10^8 particles per 20 μl) decreased the extravasation of oxidized LDL to the level of the control group (**Supplementary Figure 2a** and **2b**).

Cavernous nNOS and neurofilament content are induced by combination therapy in STZ-induced diabetic mice

The expression of nNOS-containing nerve fiber (cavernosum) and axonal contents (neurofilament in dorsal nerve bundle) was evaluated by immunohistochemical staining in age-matched control and diabetic mice 4 weeks after treatment. The expression of nNOS and neurofilament was lower in the PBS- and ad-LacZ-treated diabetic mice than in the control mice. Intracavernous injection of SVF (1×10^5 cells per 20 μl) or *Ad-COMP-Ang1* gene (2×10^8 particles per 20 μl) as single agents only partially increased nNOS expression, whereas the combination treatment completely restored nNOS (**Figure 6a** and **6d**) expression to the level of the control group. However, only a partial effect was observed in neurofilament (**Figure 6b** and **6e**) regeneration ($P < 0.05$).

Combination therapy enhances neurite sprouting in an ex vivo major pelvic ganglion tissue culture in STZ-induced diabetic mice

To test the neurotrophic effects of SVF and *Ad-COMP-Ang1* gene combination therapy, we isolated and cultivated mouse major pelvic ganglion (MPG) in Matrigel matrix from age-matched control and diabetic mice 4 weeks after treatment. Immunohistochemical staining of MPG with antibodies against neurofilament showed that the neurite sprouting from MPG tissue was markedly lower in the PBS- or ad-LacZ-treated diabetic group than in the control group. Intracavernous injection of SVF (1×10^5 cells per 20 μl) or *Ad-COMP-Ang1* gene (2×10^8 particles per 20 μl) as single agents partially enhanced neurite sprouting from MPG tissue, but the combination treatment notably enhanced neurite sprouting to a level higher than that in the control group ($P < 0.05$; **Figure 6c** and **6f**).

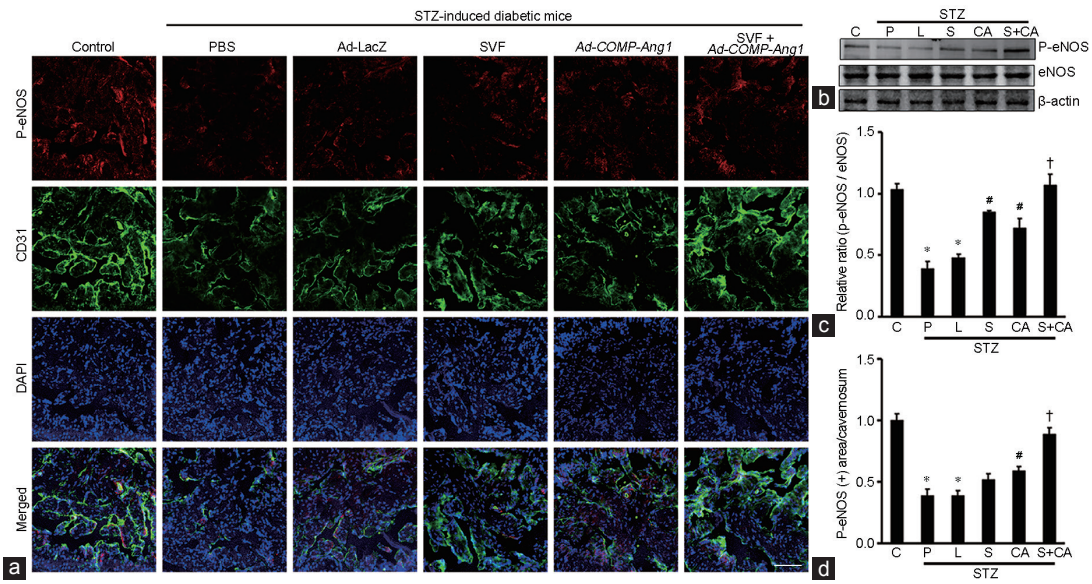

Figure 4: Cavernous eNOS phosphorylation is induced by combination therapy with SVF and *Ad-COMP-Ang1* gene in STZ-induced diabetic mice. (**a**) CD31 (green) and phospho-eNOS (p-eNOS, red) staining in cavernous tissue from age-matched control mice (C) group and STZ-induced diabetic mice stimulated at 4 weeks after intracavernous injection of PBS (P, 20 μl), ad-LacZ (L, 2×10^8 particles per 20 μl), SVF (S, 1×10^5 cells per 20 μl), *Ad-COMP-Ang1* (CA, 2×10^8 particles per 20 μl), or combination (S + CA). Nuclei were labeled with DAPI (blue). Scale bars = 100 μm. (**b**) Representative western blots for (**c**) relative ratio phospho-eNOS (and eNOS in the mouse penis compared with that of β-actin. Each bar depicts the mean value ± standard deviation from $n = 4$ animals per group. $^*P < 0.001$, P or L versus C group. $^\#P < 0.05$, S or CA versus P or L groups. $^\dagger P < 0.01$, S + CA versus S or CA groups. (**d**) Quantification of the phospho-eNOS immunopositive area in cavernous tissue by ImageJ. Each bar depicts the mean values ± standard deviations from $n = 5$ animals per group. $^*P < 0.001$, P or L versus C group. $^\#P < 0.05$, CA versus P or L groups. $^\dagger P < 0.001$, S + CA versus S or CA groups. The P values were determined by one-way ANOVA. *Ad-COMP-Ang1*: adenovirus-mediated cartilage oligomeric matrix angiopoietin-1; DAPI: 4',6-diamidino-2-phenylindole; PBS: phosphate-buffered saline; STZ: streptozotocin; SVF: stromal vascular fraction; eNOS: endothelial nitric oxide synthase.

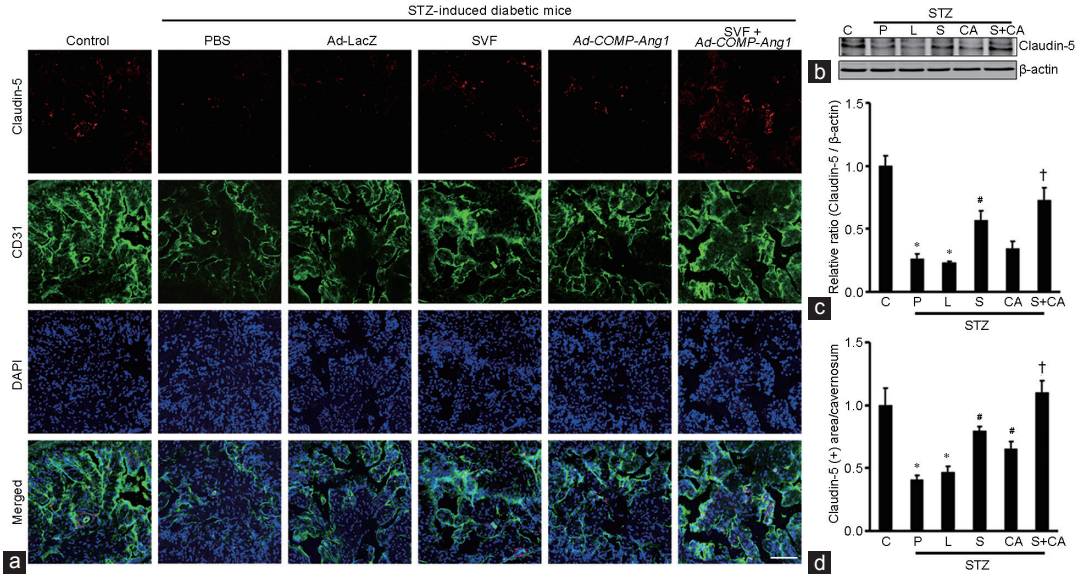

Figure 5: Combination therapy with SVF and *Ad-COMP-Ang1* gene restores claudin-5 expression in STZ-induced diabetic mice. (**a**) CD31 (green) and Claudin-5 (red) staining in cavernous tissue from age-matched control mice (C) group and STZ-induced diabetic mice stimulated at 4 weeks after intracavernous injection of PBS (P, 20 μl), ad-LacZ (L, 2×10^8 particles per 20 μl), SVF (S, 1×10^5 cells per 20 μl), *Ad-COMP-Ang1* (CA, 2×10^8 particles per 20 μl), or combination (S + CA). Nuclei were labeled with DAPI (blue). Scale bars=100 μm. (**b**) Representative western blots and (**c**) relative ratio for claudin-5 in the mouse penis compared with that of β-actin. Each bar depicts the mean value ± standard deviation from $n = 4$ animals per group. $^*P < 0.001$, P or L versus C group. $^\#P < 0.05$, S or CA versus P or L groups. $^\dagger P < 0.05$, S + CA versus S or CA groups. (**d**) Quantification of the claudin-5 immunopositive area in cavernous tissue by ImageJ. Each bar depicts the mean values ± standard deviations from $n = 5$ animals per group. $^*P < 0.001$, *P* or L versus C group. $^\#P < 0.05$, S or CA versus P or L groups. $^\dagger P < 0.01$, S + CA versus S or CA groups. The *P* values were determined by one-way ANOVA. *Ad-COMP-Ang1*: adenovirus-mediated cartilage oligomeric matrix angiopoietin-1; DAPI: 4',6-diamidino-2-phenylindole; PBS: phosphate-buffered saline; STZ: streptozotocin; SVF: stromal vascular fraction.

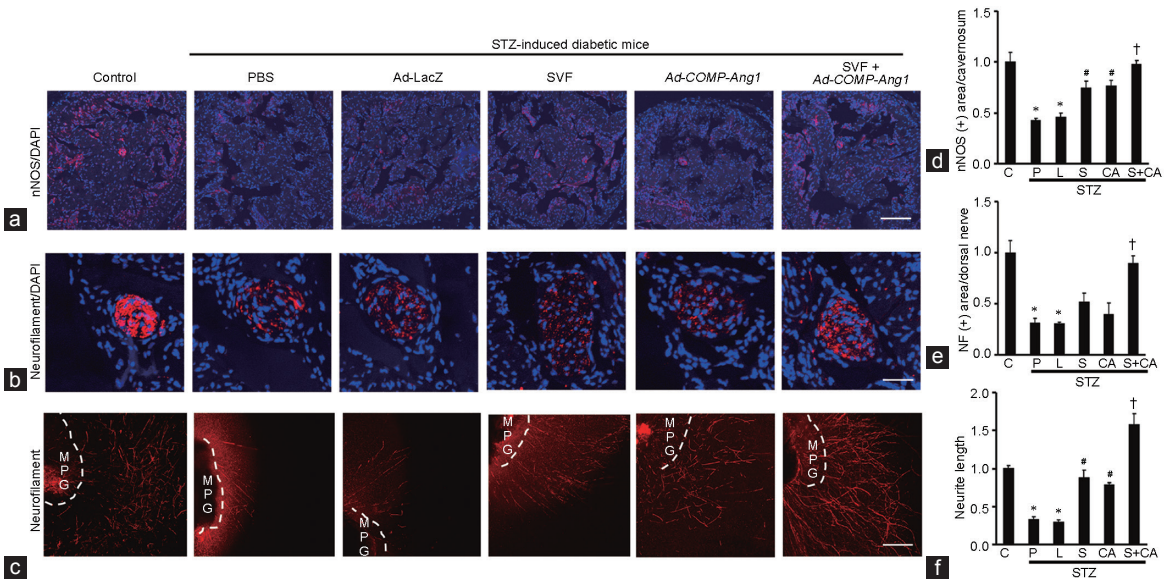

Figure 6: Combination therapy with SVF and *Ad-COMP-Ang1* gene increases penile nNOS and neurofilament content in STZ-induced diabetic mice and enhances neurite sprouting in an *ex vivo* MPG tissue culture. (**a**) Anti-nNOS (red) and (**b**) anti-neurofilament (NF; red) staining in cavernous tissue from age-matched control mice (C) group and STZ-induced diabetic mice stimulated at 4 weeks after intracavernous injection of PBS (P, 20 μl), ad-LacZ (L, 2×10^8 particles per 20 μl), SVF (S, 1×10^5 cells per 20 μl), *Ad-COMP-Ang1* (CA, 2×10^8 particles per 20 μl), or combination (S + CA). Nuclei were labeled with DAPI (blue). Scale bars = 50 μm (cavernosum) or 25 μm (dorsal nerve). (**c**) Neurofilament (red) staining in MPG-cultured tissue. Scale bars = 200 μm. Quantification of the (**d**) nNOS and (**e**) NF immunopositive areas in cavernous tissue by ImageJ. Each bar depicts the mean value ± standard deviation from $n = 5$ animals per group. $^*P < 0.001$, P or L versus C group. $^\#P < 0.001$, S or CA versus P or L groups. $^\dagger P < 0.05$, S + CA versus S or CA groups. (**f**) Quantification of neurite length by ImageJ. Each bar depicts the mean values ±standard deviations from $n = 4$ animals per group. $^*P < 0.001$, P or L versus C group. $^\#P < 0.001$, S or CA versus P or L groups. $^\dagger P < 0.001$, S + CA versus S or CA groups. The *P* values were determined by one-way ANOVA. *Ad-COMP-Ang1*: adenovirus-mediated cartilage oligomeric matrix angiopoietin-1; DAPI: 4',6-diamidino-2-phenylindole; PBS: phosphate-buffered saline; nNOS: neuronal nitric oxide synthase; STZ: streptozotocin; SVF: stromal vascular fraction; MPG: major pelvic ganglion.

DISCUSSION

Our previous study showed that freshly isolated SVF is also used for the treatment of radical prostatectomy-induced ED and diabetic ED.[12,27] In addition, the *Ad-COMP-Ang1* gene successfully restores the endogenous NO-cGMP pathway and inhibition of endothelial cell apoptosis. These changes restore erectile function for 2 weeks (90%) and 4 weeks (79%) in mice with STZ-induced diabetes.[14,15] Furthermore, the *COMP-Ang1* gene rescues erectile function and cavernous blood flow up to 8 weeks after treatment in a hypercholesterolemic mouse model, but cavernous endothelial cell number recovery is only detected at 2 weeks.[28] However, both SVF and *COMP-Ang1* gene can restore erectile function at 2 weeks in a severe endothelial dysfunction and neurodegeneration model, such as STZ-induced diabetic mice. Based on these observations, we hypothesized that combination therapy with SVF and *Ad-COMP-Ang1* gene may significantly improve the dual angiogenic and neurotrophic effects, which may restore more durable erectile function.

Our study showed that SVF or *Ad-COMP-Ang1* gene alone restored erectile function up to 80% (maximal ICP) after 4 weeks of treatment, whereas combination therapy restored erectile function to more than 88% (maximal ICP) of control values. Our previous studies have shown that treatment with SVF and *COMP-Ang1* gene induces cavernous endothelial cell numbers, eNOS phosphorylation, and endothelial cell–cell junctions but decreases cavernous endothelial cell permeability in STZ-induced diabetic mice.[12,14] The combination therapy of these agents further enhanced these factors in STZ-induced diabetic mice for at least 4 weeks. Immunohistochemical examination of phosphohistone H3 and cleaved caspase-3 expression revealed that the increased cavernous endothelial cell and pericyte numbers resulted from endothelial cell and pericyte survival. Our previous studies have also shown that pericyte restoration induces recovery of erectile function[2] by tightly regulating vascular endothelial permeability. These findings suggest that pericyte survival may be a key mechanism in the long-term effect of an erection. However, the exact mechanisms of SVF and *Ad-COMP-Ang1* gene combination in the regulation of the expression of pericytes remain unclear.

Previous studies have shown that the amount of intact neurofilament-positive axonal and nNOS-containing nerve fibers is critical for maintaining erectile function.[29,30] In agreement with this finding, we found that combination therapy notably restored neurofilament-positive axonal and nNOS-containing nerve fibers and induced eNOS phosphorylation in the penises of STZ-induced diabetic mice. We further isolated mouse MPG and performed an *ex vivo* culture from conditioned mice. A severe impairment in neurite outgrowth in MPG tissue from diabetic mice was found. However, MPG neurite sprouting from the combination therapy group was profoundly enhanced compared with the groups treated with a single intracavernous injection of SVF or *Ad-COMP-Ang1* gene. Previous studies have shown that SVF is a source of various angiogenic factors, such as Ang1, VEGF-A, and HGF.[16,20,21] Moreover, the Ang1-Tie2 signaling pathway regulates the expression of neurotrophic factors.[9] Thus, these findings suggest that the SVF and *Ad-COMP-Ang1* gene combination may recover dual angiogenic and neurotrophic effects, which may induce long-term recovery of erectile function.

Although it has been reported that a single intracavernous injection of SVF or *Ad-COMP-Ang1* gene may restore erectile function for 2 weeks, this is the first report showing that combination therapy with SVF and *Ad-COMP-Ang1* gene may be useful for restoring more lasting erectile function up to 88% of control values for at least 4 weeks in STZ-induced diabetic mice. The dual angiogenic and neurotrophic effects of combination therapy may afford these changes.

Our study had some limitations. This study did not elucidate the detailed mechanisms by which the combination therapy regulates the dual angiogenic and neurotrophic effects. Second, a longer duration of recovery of erectile function should be tested in the combination therapy group, such as 6 or 8 weeks after treatment of STZ-induced diabetic mice.

AUTHOR CONTRIBUTIONS

GNY performed the molecular genetic studies and drafted the manuscript. LW participated in the manuscript revision. XNL performed the intracavernous pressure study and cared for the animals. LS participated in the study design. ZLG conceived of the study. FCH generated the COMP-Ang1 or LacZ adenovirus. PL assisted with material support and performed the statistical analysis. YCJ performed the immunoassays. JKS and JKR participated in the study design and provided technical support. XW and HRJ conceived of the study, participated in its design and coordination, and helped draft the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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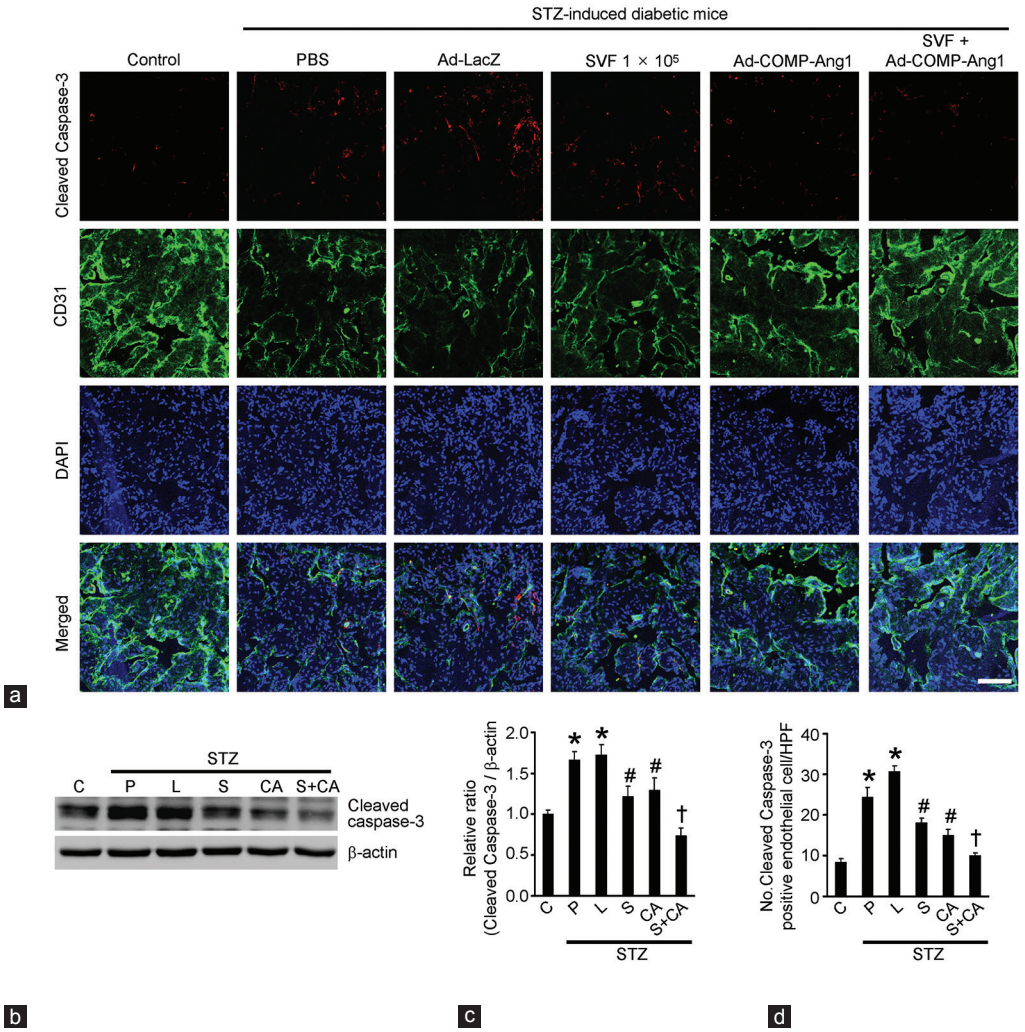
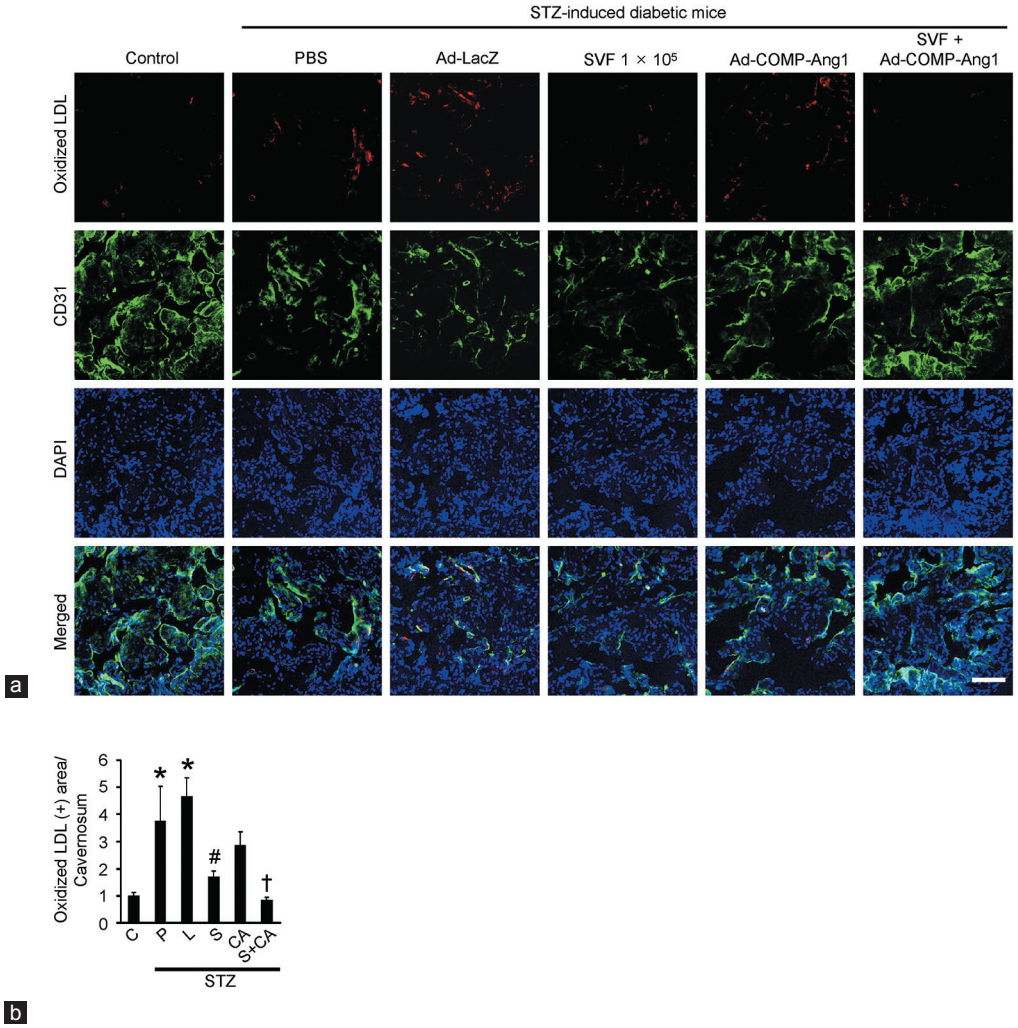


Supplementary Figure 1: Combination therapy with SVF and *Ad-COMP-Ang1* gene decreases cavernous endothelial cell apoptosis in STZ-induced diabetic mice. (**a**) CD31 (green) and cleaved caspase-3 (red) staining in cavernous tissue from age-matched control mice (C) group and STZ-induced diabetic mice stimulated at 4 weeks after intracavernous injection of PBS (P; 20 μl), ad-LacZ (L; 2×10^8 parts per 20 μl), SVF (S; 1×10^5 cells per 20 μl), *Ad-COMP-Ang1* (CA; 2×10^8 parts per 20 μl), or combination (S + CA). Nuclei were labeled with DAPI (blue). Scale bars = 100 μm. (**b**) Representative western blots and (**c**) relative ratio for cleaved caspase-3 in mouse penis compared with that of β-actin. Each bar depicts the mean values ± standard deviations from $n = 4$ animals per group. $^{*}P < 0.01$ compared with C group. $^{\#}P < 0.05$ compared with the P and L groups. $^{\dagger}P < 0.05$ compared with the S and CA groups. (**d**) Number of cleaved caspase-3-immunopositive endothelial cells per HPF. Each bar depicts the mean values ± (standard deviations) from $n = 5$ animals per group. $^{*}P < 0.001$ compared with C group. $^{\#}P < 0.01$ compared with the P and L groups. $^{\dagger}P < 0.05$ compared with the S and CA groups. The *P* values were determined by one-way ANOVA. *Ad-COMP-Ang1*: adenovirus-mediated cartilage oligomeric matrix angiopoietin-1; DAPI: 4',6-diamidino-2-phenylindole; HPF: high-power field; PBS, phosphate-buffered saline; STZ: streptozotocin; SVF: stromal vascular fraction.



Supplementary Figure 2: The extravasation of oxidized LDL is decreased by combination therapy with SVF and *Ad-COMP-Ang1* gene in STZ-induced diabetic mice. (**a**) CD31 (green) and oxidized LDL (red) staining in cavernous tissue from age-matched control mice (C) group and STZ-induced diabetic mice stimulated at 4 weeks after intracavernous injection of PBS (P; 20 μl), ad-LacZ (L; 2×10^8 parts per 20 μl), SVF (S; 1×10^5 cells per 20 μl), *Ad-COMP-Ang1* (CA; 2×10^8 parts per 20 μl), or combination (S + CA). Nuclei were labeled with DAPI (blue). Scale bars = 100 μm. (**b**) quantification of the oxidized LDL immunopositive area in cavernous tissue by ImageJ. Each bar depicts the mean values ± standard deviations from $n = 5$ animals per group. $^{*}P < 0.05$ compared with C group. $^{\#}P < 0.05$ compared with the P and L groups. $^{\dagger}P < 0.05$ compared with the S and CA groups. The P values were determined by one-way ANOVA. *Ad-COMP-Ang1*: adenovirus-mediated cartilage oligomeric matrix angiopoietin-1; DAPI: 4',6-diamidino-2-phenylindole; PBS: phosphate-buffered saline; STZ: streptozotocin; SVF: stromal vascular fraction.

Supplementary Table 1: Physiologic and metabolic parameters in mice: 2 weeks after treatment

	Control	*STZ-induced diabetic mice*				
		PBS	*Ad-LacZ*	*SVF (1×10⁵)*	*Ad-COMP-Ang1*	*SVF + Ad-COMP-Ang1*
Body weight (g)	30.7±1.3	25.6±2.2*	21.3±3.9*	22.6±1.6*	24.0±1.3*	22.5±2.1*
Fasting glucose (mg dl^{-1})	110.4±7.4	462.5±67.3*	459.0±98.4*	335.2±71.2*	306.4±52.0*	393±33.9*
Postprandial glucose (mg dl^{-1})	148.8±12.5	587.8±18.4*	567.0±41.5*	537.0±60.3*	576.4±42.7*	540.2±53.2*
MSBP (mmHg)	134±4.1	134±10.3	128±3.3	128±1.5	133±6.4	135±2.5

Values are the mean±s.d. for n=5 animals per group. *P<0.05 versus control group. *Ad-COMP-Ang1*: adenovirus-mediated cartilage oligomeric matrix angiopoietin-1; PBS: phosphate-buffered saline; MSBP: mean systolic blood pressure; STZ: streptozotocin; SVF: stromal vascular fraction; s.d.: standard deviation

Supplementary Table 2: Physiologic and metabolic parameters in mice: 4 weeks after treatment

	Control	*STZ-induced diabetic mice*				
		PBS	*Ad-LacZ*	*SVF (1×10^5)*	*Ad-COMP-Ang1*	*SVF + Ad-COMP-Ang1*
Body weight (g)	29.8±1.5	21.9±1.0*	21.8±0.9*	22.9±1.9*	21.6±1.7*	22.0±0.9*
Fasting glucose (mg dl^{-1})	109.8±5.8	270.2±19.1*	342.8±69.8*	296.8±57.2*	407.2±27.9*	398.4±40.1*
Postprandial glucose (mg dl^{-1})	137.6±4.0	498.4±37.1*	508.8±15.6*	498±25.9*	566±42.6*	561±37.1*
MSBP (mmHg)	135±4.6	133±7.6	128±3.3	130±2.1	132±3.5	133±3.8

Values are the mean±s.d. for n=5 animals per group. *P<0.05 versus control group. *Ad-COMP-Ang1*: adenovirus-mediated cartilage oligomeric matrix angiopoietin-1; PBS: phosphate-buffered saline; MSBP: mean systolic blood pressure; STZ: streptozotocin; SVF: stromal vascular fraction; s.d.: standard deviation