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.

Asian Journal of Andrology (2018) 20, 465-472; doi: 10.4103/aja.aja_16_18; published online: 17 April 2018

Keywords: angiopoietin-1; erectile dysfunction; neural cell regeneration; stromal vascular fraction

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).15 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.18-20 SVF can differentiate into vascular cells and neurons in vitro18,19 and it secretes several angiogenic growth factors, such as VEGF and Ang1.18,20 Therefore, functional therapies for diabetic ED are needed. Functional studies in a rat model of cavernous nerve injury, SVF restores erectile function by promoting neural regeneration.21 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 Ad-COMP-Ang1.
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. 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. 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. 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 × 10⁵ particles per 20 µl), AD-SVF pellet (1 × 10⁷ cells per 20 µl of PBS), Ad-COMP-Ang1 (1 × 10⁷ 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 systolic blood pressure (MSBP) were calculated to adjust for variations in systemic blood pressure as previously described.

**Mouse major pelvic ganglion neurite-sprouting assay**

Mouse major pelvic ganglion (MPG) tissues were prepared and maintained as described previously. 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% CO₂ 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% CO₂ 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 × 10^7 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 × 10^7 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 × 10^7 cells per 20 µl) or Ad-COMP-Ang1 gene (2 × 10^8 particles per 20 µl) partially increased cavernous endothelial cell numbers in the STZ-induced diabetic mice (all P < 0.05; Figure 3).

**Figure 1:** Combination therapy with SVF and Ad-COMP-Ang1 gene restores erectile function. (a) Representative ICP responses for age-matched control mice (C) 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 × 10^7 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 \((1 \times 10^5 \text{ cells per } 20 \mu l)\) and Ad-COMP-Ang1 gene \((2 \times 10^8 \text{ particles per } 20 \mu l)\) 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-\(\beta\) (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 \(\mu l\)), ad-LacZ (L, \(2 \times 10^8\) particles per 20 \(\mu l\)), SVF (S, \(1 \times 10^5\) cells per 20 \(\mu l\)), Ad-COMP-Ang1 (CA, \(2 \times 10^8\) particles per 20 \(\mu l\)), or combination (S + CA). Nuclei were labeled with DAPI (blue). Scale bars = 100 \(\mu m\). Quantification of (b) cavernous endothelial cell and (c) pericyte numbers by ImageJ. Each bar depicts the mean values \(\pm\) 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. † † \( P < 0.05\), 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-\(\beta\): 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 \(\mu l\)), ad-LacZ (L, \(2 \times 10^8\) particles per 20 \(\mu l\)), SVF (S, \(1 \times 10^5\) cells per 20 \(\mu l\)), Ad-COMP-Ang1 (CA, \(2 \times 10^8\) particles per 20 \(\mu l\)), or combination (S + CA). Nuclei were labeled with DAPI (blue). Scale bars = 50 \(\mu m\). Quantification of (b) number of PH3-positive endothelial cells and (c) pericytes per high-power field. Each bar depicts the mean values \(\pm\) standard deviations 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. † † \( P < 0.01\), 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 x 10^5 cells per 20 µl) and Ad-COMP-Ang1 gene (2 x 10^4 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 x 10^5 cells per 20 µl) or Ad-COMP-Ang1 gene (2 x 10^4 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 x 10^5 cells per 20 µl) and Ad-COMP-Ang1 gene (2 x 10^4 particles per 20 µl) decreased the extravasation of oxidized LDL to the level of the control group (Supplementary Figure 2a and 2b).

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 x 10^6 particles per 20 µl), SVF (S, 1 x 10^5 cells per 20 µl), Ad-COMP-Ang1 (CA, 2 x 10^5 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, ‡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, ‡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; eNOS: endothelial nitric oxide synthase.

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 x 10^5 cells per 20 µl) or Ad-COMP-Ang1 gene (2 x 10^4 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 x 10^5 cells per 20 µl) or Ad-COMP-Ang1 gene (2 x 10^4 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 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 x 10^6 particles per 20 µl), SVF (S, 1 x 10^5 cells per 20 µl), Ad-COMP-Ang1 (CA, 2 x 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. †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. †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 x 10^6 particles per 20 µl), SVF (S, 1 x 10^5 cells per 20 µl), Ad-COMP-Ang1 (CA, 2 x 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. †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; 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.\textsuperscript{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-diabetes.\textsuperscript{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.\textsuperscript{23} 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.\textsuperscript{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\textsuperscript{22} 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.\textsuperscript{20,30} 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.\textsuperscript{16,20,21} Moreover, the Ang1-Tie2 signaling pathway regulates the expression of neurotrophic factors.\textsuperscript{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 HR 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.

ACKNOWLEDGMENTS
This study was supported by grants from the National Natural Science Foundation of China (81471452) and Shandong Province Young and Middle-Aged Scientists Research Awards Fund (BS2013SF008) to HR.

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

REFERENCES
1. Andersson KE. Mechanisms of penile erection and basis for pharmacological treatment of ED. Pharmacol Rev 2011; 63: 81–99.
2. Yin GN, Das ND, Choi MJ, Song KM, Kwon MH, et al. The pericyte as a cellular regulator of penile erection and a novel therapeutic target for ED. Sci Rep 2015; 5: 10891.
3. Hakim LS, Goldstein I. Diabetic sexual dysfunction. Endocrinol Metab Clin North Am 1996; 25: 379–400.
4. Malavige LS, Levy JC. ED in diabetes mellitus. J Sex Med 2009; 6: 1232–47.
5. Carson CC, Burnett AL, Levine LA, Nehra A. The efficacy of sildenafil citrate (Viagra) in clinical populations: an update. Urology 2002; 60: 12–27.
6. Martinez-Jabaloyas JM, Gil-Salom M, Villamon-Fort R, Pastor-Hernandez F, Martinez-Garcia R, et al. Prognostic factors for response to sildenafil in patients with ED. Eur Urol 2001; 40: 641–6.
7. Rendell MS, Rajfer J, Wicker PA, Smith MD. Sildenafil for treatment of ED in men with diabetes: a randomized controlled trial. Sildenafil Diabetes Study Group. JAMA 1999; 281: 421–6.
8. Musicki B, Burnett AL. Endothelial dysfunction in diabetic ED. Int J Impot Res 2007; 19: 129–38.
9. Yin GN, Choi MJ, Kim WJ, Kwon MH, Song KM, et al. Inhibition of Ninjurin 1 restores erectile function through dual angiogenic and neurotrophic effects in the diabetic mouse. Proc Natl Acad Sci U S A 2014; 111: E2731–40.
10. Lin CS, Xin ZC, Wang Z, Deng C, Huang YC, et al. Stem cell therapy for ED: a critical review. Stem Cells Dev 2012; 21: 343–51.
11. Lindroos B, Suuronen R, Miettinen S. The potential of adipose stem cells in regenerative medicine. Stem Cell Rev 2011; 7: 269–91.
12. Ryu JK, Tumurbaatar M, Jin HR, Kim WJ, Kwon MH, et al. Intracavernous delivery of freshly isolated stromal vascular fraction rescues erectile function by enhancing endothelial regeneration in the streptozotocin-induced diabetic mouse. J Sex Med 2012; 9: 3051–65.
13. Dall’Era JE, Meacham RB, Mills JJ, Koul S, Carlsen SN, et al. Vascular endothelial growth factor (VEGF) gene therapy using a nonviral gene delivery system improves erectile function in a diabetic rat model. Int J Impot Res 2008; 20: 307–14.
14. Jin HR, Kim WJ, Song JS, Piao S, Choi MJ, et al. Intracavernous delivery of a designed angiopoietin-1 variant rescues erectile function by enhancing endothelial regeneration in the streptozotocin-induced diabetic mouse. Diabetes 2011; 60: 969–80.
15. Jin HR, Kim WJ, Song JS, Piao S, Tumurbaatar M, et al. Intracavernous delivery of synthetic angiopoietin-1 protein as a novel therapeutic strategy for ED in the type II
SVF and Ad-COMP-Ang1 combined therapy in DM-ED

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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 × 10⁸ parts per 20 µl), SVF (S; 1 × 10⁵ cells per 20 µl), Ad-COMP-Ang1 (CA; 2 × 10⁸ 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. ‡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. ‡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 Table 1: Physiologic and metabolic parameters in mice: 2 weeks after treatment

|                      | Control         | STZ-induced diabetic mice |
|----------------------|-----------------|---------------------------|
|                      | PBS             | Ad-LacZ                   | SVF (1x10^5) | 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 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 x 10⁸ parts per 20 µl), SVF (S; 1 x 10⁵ cells per 20 µl), Ad-COMP-Ang1 (CA; 2 x 10⁸ 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. †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 2: Physiologic and metabolic parameters in mice: 4 weeks after treatment

| Parameter                | 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.