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Heat Shock Protein 70 in Penile Neurovascular Regeneration Requires Cystathionine Gamma-Lyase

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Purpose: Diabetes mellitus, one of the major causes of erectile dysfunction, leads to a poor response to phosphodiesterase-5 inhibitors. Heat shock protein 70 (Hsp70), a ubiquitous molecular chaperone, is known to play a role in cell survival and neuroprotection. Here, we aimed to assess whether and how Hsp70 improves erectile function in diabetic mice.

Materials and Methods: Eight-week-old male C57BL/6 mice and Hsp70-Tg mice were used in this study. We injected Hsp70 protein into the penis of streptozotocin (STZ)-induced diabetic mice. Detailed mechanisms were evaluated in WT or Hsp70-Tg mice under normal and diabetic conditions. Primary MCECs, and MPG and DRG tissues were cultivated under normal-glucose and high-glucose conditions.

Results: Using Hsp70-Tg mice or Hsp70 protein administration, we demonstrate that elevated levels of Hsp70 restores erectile function in diabetic mice. We found that cystathionine gamma-lyase (Cse) is a novel target of Hsp70 in this process, showing that Hsp70-Cse acts through the SDF1/HO-1/PI3K/Akt/eNOS/NF-κB p65 pathway to exert its neurovascular regeneration-promoting effects. Coimmunoprecipitation and pull-down assays using mouse cavernous endothelial cells treated with Hsp70 demonstrated physical interactions between Hsp70 and Cse with a dissociation constant of 1.8 nmol/L.

Conclusions: Our findings provide novel and solid evidence that Hsp70 acts through a Cse-dependent mechanism to mediate neurovascular regeneration and restoration of erectile function under diabetic conditions.

Keywords: Cystathionine gamma-lyase; Diabetes mellitus; Erectile dysfunction; Heat shock protein 70; Neovascularization; Nerve regeneration

INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder whose long-term high-blood glucose levels can lead to erectile dysfunction (ED) [1]. Approximately 50% to 75% of diabetic patients suffer from ED, which is

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mainly caused by severe angiopathy and peripheral neuropathy [2,3]. Long-term devascularization of erectile tissue in patients with DM can cause hypoxia and structural changes in erectile tissue, resulting endothelial dysfunction, smooth muscle cell apoptosis and peripheral neuropathy—effects that can lead to poor responses to oral phosphodiesterase-5 inhibitors [4]. A number of angiogenic or neurotrophic factors, such as vascular endothelial growth factor (VEGF), angiopoietins and brain-derived neurotrophic factor (BDNF) [5-7], have recently been tested as treatment options for ED at the preclinical level. However, inadequate efficacies, side effects such as inflammation, as well as difficulties in protein engineering of medicines, have proven to be major hurdles for their clinical development. Therefore, new treatment strategies are needed to solve the complicated angiopathy and neuropathy pathologies in diabetic ED.

Heat shock protein 70 (Hsp70 or HSPA1A) is a ubiquitous molecular chaperone and a fundamental component in cellular systems, playing a role in cell survival, proliferation, and regulation of polypeptide folding, protein degradation, protein-protein interactions, and transmembrane translocation [8]. The second messenger cAMP is involved in penile erection, and Hsp70 is known to increase intracellular cAMP levels both *in vivo* and *in vitro* [9]. In addition, Hsp70 is required for interleukin (IL)-5–induced angiogenic responses by activating endothelial nitric oxide synthase (eNOS) [10]; it also prevents neuropathy in neurodegenerative disease by improving neuronal survival [11]. Recent studies have further shown that Hsp70 has anti-inflammatory and neuroprotective functions in heart muscle, skeletal muscle, adipose tissue, and liver under hyperglycemia conditions [12,13]. Expression of Hsp70 at transcriptional and translational levels is reduced in patients with insulin resistance and hyperglycemia [12]. Moreover, induction of heat shock factor 1/Hsp70 signaling is currently considered an exciting strategy for the treatment of diabetes and metabolic disorders [14]. However, little is known about the underlying cellular and molecular mechanisms of Hsp70 in penile neurovascular regeneration under diabetic conditions. Therefore, the aim of the present study was to determine whether exogenous delivery of Hsp70 protein could improve erectile function in diabetic mice and the detailed mechanism of Hsp70 in this process.

In the present study, we found that Hsp70 is highly expressed in blood vessels and nerves of the mouse penis, but its expression was significantly reduced under hyperglycemic conditions. In addition, we demonstrated that exogenous delivery of Hsp70 protein improved erectile function by enhancing neurovascular regeneration in cavernosum tissues. Moreover, RNA sequencing and cytokine antibody array analyses showed that these effects of Hsp70 in penile neurovascular regeneration are cystathionine gamma-lyase (Cse)-dependent. Furthermore, Hsp70-Cse act through a signaling pathway involving stromal cell-derived factor 1 (SDF1), heme oxygenase-1 (HO-1) phosphoinositide 3-kinase (PI3K), Akt, eNOS and nuclear factor (NF)-κB p65 (SDF1/HO-1/PI3K/Akt/eNOS/NF-κB p65 pathway). These results provide important molecular mechanistic insights into Hsp70-mediated neurovascular regeneration in diabetes-induced ED.

MATERIALS AND METHODS

1. Study design

The primary purpose of the current study was to elucidate the mechanisms by which intracavernous injection of Hsp70 improves erectile function under hyperglycemia conditions. To this end, we injected Hsp70 protein into the penis of streptozotocin (STZ)-induced diabetic mice. Detailed mechanisms were evaluated in wild-type (WT) or Hsp70-transgenic (Tg) mice under normal and diabetic conditions. Primary MCECs, and major pelvic ganglion (MPG) and dorsal root ganglion (DRG) tissues were cultivated under normal-glucose and high-glucose conditions.

2. Animals and treatment

Eight-week-old male C57BL/6 (Orient Bio, Seongnam, Korea) and Hsp70-Tg (Macrogen, Seoul, Korea) mice were used in this study. Hsp70-Tg mice were genotyped by polymerase chain reaction (PCR) using the primer pair, 5'-CGT GGA TAG CGG TTT GAC TCA-3' (forward) and 5'-GCG ATC TCC TTC ATC TTC GTC-3' (reverse). All parameters of diabetic mice and genetically modified mice were compared with those of littermate controls. All animal experiments were approved by the Institutional Animal Care and Use Committee of Inha University (approval number: 180801-583-1 and 180420-559-1). Diabetes was induced by intraperitoneal injection of STZ (50 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) for five consecutive days as described

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previously [15]. Eight weeks after diabetes induction, mice were anesthetized with intramuscular injections of ketamine (100 mg/kg) and xylazine (5 mg/kg).

To evaluate the effectiveness of Hsp70 protein, we first randomly divided age-matched mice into three groups (n=5–6 mice/group): age-matched controls, STZ-induced diabetic mice receiving repeated (days -3 and 0) intracavernous injections of phosphate-buffered saline (PBS) (20 μL; Gibco, Carlsbad, CA, USA) or Hsp70 protein (5 μg/20 μL; R&D Systems Inc., Minneapolis, MN, USA). Erectile function was evaluated during electrical stimulation of the cavernous nerve 2 weeks after the second intracavernous injection (day 0) or PBS or Hsp70 protein, and the penis was harvested for histologic examination.

For the Hsp70-Tg study, Hsp70-Tg mice were distributed into three groups (n=5 mice/group): Hsp70-Tg mice, Hsp70-Tg mice+STZ+scrambled control shRNA (shCon; 5×10^4 transforming units per mouse) lentivirus particles, and Hsp70-Tg mice+SMART vector lentivirus expressing shRNA targeting Cse (shCse; 5×10^4 transforming units per mouse). Eight weeks after the induction of diabetes, erectile function during electrical stimulation of the cavernous nerve was measured, after which the penis was harvested for histologic examination. Fasting and postprandial blood glucose levels were measured using an Accu-check blood glucose meter (Roche Diagnostics, Risch-Rotkreuz, Switzerland) before mice were sacrificed.

3. Cell culture

MCECs were prepared and maintained as described previously [16-18]. Penis tissue was harvested and transferred into sterile vials containing Hank's balanced salt solution (HBSS; Gibco) and washed twice in PBS. The glans penis, urethra, and dorsal neurovascular bundle were removed from the penis, and only corpus cavernosum tissue was used for culture of MCECs. After cells had sprouted and spread on the bottom of the dish (after ~12–14 days in culture), they were subcultured onto dishes coated with 0.2% gelatin (Sigma-Aldrich). Cells from passages 2 to 4 were used for all experiments.

Diabetes-induced angiopathy conditions were mimicked by first serum-starving cells overnight and then exposing them to normal-glucose (5 mM, Sigma-Aldrich) or high-glucose (30 mM, Sigma-Aldrich) conditions for 5 days at 37℃ in a humidified 5% CO_2 atmosphere [19].

4. Measurement of erectile function

Mice from each group were anesthetized with an intramuscular injection of ketamine (100 mg/kg) and xylazine (5 mg/kg). The cavernous nerve was exposed and then electrically stimulated (5 V; frequency, 12 Hz; pulse width of 1 ms; duration, 1 minute) using bipolar platinum wire electrodes placed around the cavernous nerve. Each electrical stimulus was replicated 2 times at least at 10-minute intervals. Maximal intracavernous pressure (ICP) and total ICP were recorded during tumescence. Total ICP was determined as the area under the curve (AUC) from the beginning of cavernous nerve stimulation to a point 20 seconds after stimulus termination. mean systolic blood pressure (MSBP) was measured using a noninvasive computerized tail-cuff system (BP-2000; Visitech Systems, Apex, NC, USA) prior to performing ICP determinations. The validity of this system was demonstrated previously [15]. To adjust for variations in systemic blood pressure, we calculated ratios of maximal ICP and total ICP (AUC) to MSBP as previously described [15,20]. All mice treatment and ICP assessments were performed by one researcher, however, other researchers changed the mice's labels after treatment. Therefore, the functional tests were performed by researchers blinded to the grouping of mice after treatment.

5. Tube formation assay

Tube-formation assays were performed as described previously [17]. Briefly, MCECs were pre-cultured in normal-glucose (5 mM) or high-glucose (30 mM) complete Media 199 (M199; Gibco) for 5 days, with or without Hsp70 (500 ng/mL). Thereafter, Matrigel-coated 96-well tissue culture plates were prepared by dispensing ~50 μL of growth factor-reduced Matrigel (Becton Dickinson, Mountain View, CA, USA) per well and allowing it to gel at 4℃. After allowing to stand at 37℃ for at least 10 minutes, Matrigel-coated plates were seeded with MCECs (4×10^4 cells/well in 200 μL of M199 medium). Cells were treated immediately with 500 ng/mL of Hsp70. Tube formation was monitored at 12 to 16 hours under a phase-contrast microscope and quantified by counting the number of master junctions in six separate experiments in a blinded manner using Image J software (ver. 1.34; National Institutes of Health, Bethesda, MD, USA; https://rsbweb.nih.gov/ij/).

6. Cell migration assay

For migration assays, MCECs were cultured in complete M199 medium for 5 days in normal-glucose (5 mM) or high-glucose (30 mM) medium, with or without Hsp70 (500 ng/mL). To create uniform scratches, we seeded MCECs in the SPLScar Block system (SPL Life Sciences, Pocheon, Korea) at >95% confluence on 60-mm culture dishes. The blocks were removed 5 hours after seeding, and the cells were further incubated in different conditioned M199 medium containing 2% fetal bovine serum and thymidine (2 mM, Sigma-Aldrich) for 24 hours. Images were acquired with a phase-contrast microscope, and migrated cells were analyzed by measuring the ratio of cells that moved into the frame line from six separate block systems in a blinded manner using Image J software.

7. Aortic ring assay

Aortas harvested from 8-week-old C57BL/6 mice were positioned in the 8-well Nunc Lab-Tek Chamber Slide System (Sigma-Aldrich) and kept in place with an overlay of 50 μL Matrigel. Aortic rings were cultured in complete M199 for 5 days in normal-glucose (5 mM) or high-glucose (30 mM) medium, with or without Hsp70 (500 ng/mL). Images of aortic segments and sprouting cells were acquired using a phase-contrast microscope, and the results were analyzed by measuring the area of sprouting microvessels in six separate experiments in a blinded manner using Image J software.

8. *Ex vivo* endothelial cell sprouting assay

Mouse corpus cavernosum tissue was cut into small pieces and kept in place with an overlay of 50 μL Matrigel. Corpus cavernosum tissues were cultured in complete M199 for 5 days in normal-glucose (5 mM) or high-glucose (30 mM) medium, with or without Hsp70 (500 ng/mL). Images of tissue segments and sprouting cells were acquired with a phase-contrast microscope, and the results were analyzed by measuring the area of sprouting cells in six separate experiments in a blinded manner using Image J software.

9. TUNEL assay

TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assays were performed using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA, USA) according to the manufacturer's instructions. Frozen tissue section samples were mounted in a solution containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA, USA), included to stain nuclei. Digital images and numbers of apoptotic cells were obtained using a confocal fluorescence microscope. Values are expressed per high-power field. The percentage of TUNEL-positive cells in the *in vitro* study and the number of TUNEL-positive cells were evaluated.

10. *Ex vivo* neurite sprouting assay

Mouse MPG and DRG tissues were harvested and maintained as described previously [21,22]. Briefly, MPG and DRG tissues were isolated from male mice under a dissecting microscope and transferred into sterile vials containing HBSS. After rinsing and washing twice with PBS, MPG and DRG tissues were cut into small pieces and plated onto a poly-D-lysine hydrobromide (0.1 mg/mL; Sigma-Aldrich)-coated 8-well Nunc Lab-Tek Chamber Slide System. MPG and DRG tissues were entirely covered with Matrigel, and plates were incubated for 10 to 15 minutes at 37°C in a 5% CO_2 atmosphere. Then, 500 μL of complete Neurobasal medium (Gibco) Supplemented with 2% serum-free B-27 (Gibco) and 0.5 nM GlutaMAX-I (Gibco) were added and plates with MPG and DRG tissues were incubated in normal-glucose (5 mM) or high-glucose (30 mM) medium, with or without Hsp70 (500 ng/mL) for 5 days. Neurite outgrowth segments were then fixed in 4% paraformaldehyde for at least 30 minutes and immunostained with an anti-neurofilament antibody (Sigma-Aldrich; 1:50).

11. RNA-sequencing analysis

For RNA-sequencing analyses, MCECs were treated with Hsp70 (500 ng/mL) under normal-glucose (5 mM) or high-glucose (30 mM) conditions. RNA-sequencing assay was performed as a custom service by E-Biogen Inc. (Ebiogen Inc., Seoul, Korea) as described previously [23]. Briefly, total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) 96 hours after exposure to different glucose conditions and Hsp70 treatment. RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands), and RNA was quantified using an ND-2000 Spectrophotometer (Thermo Inc., Wilmington, DE, USA).

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12. Library preparation, sequencing, and data analysis

Libraries were prepared from total RNA using the SMARTer Stranded RNA-Seq Kit (Clontech Laboratories, Inc., San Jose, CA, USA). mRNA for cDNA synthesis and shearing was isolated using a Poly (A) RNA Selection Kit (Lexogen, Inc., Vienna, Austria). Indexing was performed using Illumina indices 1–12, and the enrichment step was performed using PCR. Libraries were subsequently evaluated for mean fragment size using the Agilent 2100 Bioanalyzer (DNA High Sensitivity Kit) and were quantified with an Agilent qPCR NGS Library Quantification Kit using a StepOne Real-Time PCR System (Life Technologies, Inc., Carlsbad, CA, USA). High-throughput sequencing was performed using the Illumina GA Paired-End 100 sequencing technique using a HiSeq 2500 system (Illumina, Inc., San Diego, CA, USA). Quality control of raw sequencing data was performed using FastQC (Available at https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapter and low-quality reads (<Q20) were removed using FASTX_Trimmer (available at http://hannonlab.cshl.edu/fastx_toolkit/) and BBMap (available at https://sourceforge.net/projects/bbmap/). Trimmed reads were then mapped to the reference genome using TopHat [24]. Gene expression levels were estimated from RC (read count) and FPKM (fragments per kb per million reads) values using BEDTools and Cufflinks [25]. Expression values were normalized with the Quantile normalization method using EdgeR within R (available at https://www.r-project.org). Data mining and graphic visualization were performed using ExDEGA (E-Biogen, Inc.). RNA-sequencing data have been deposited in the Gene Expression Omnibus database (available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE180916).

13. Lentiviral-mediated shRNA delivery

For lentiviral infection, scrambled control shRNA (shCon; Santa Cruz Biotechnology, Dallas, TX, USA) and SMART vector mouse lentivirus containing shCse (Dharmacon, Lafayette, CO, USA) were used for *in vitro* (1×10^4 TU/mL lentivirus) and *in vivo* (5×10^4 TU/mL lentivirus) knockdown studies. The sequence of the shCse used was GGAATTCTCGTGCCGTGGT. All knockdown experiments were performed 3 days after lentivirus infection.

14. Pull-down assay

His-tag pull-down assays were performed according to instructions provided with the MagneHis Protein Purification System (Promega, Madison, WI, USA), with minor modifications. MCECs were treated with His-tagged recombinant Hsp70 protein (Stressmarq Biosciences Inc., Victoria, Canada; 25 ng/μL) for 3 hours, after which cells were lysed and cell lysates were incubated with Ni-particles as instructed. After washing, Cse binding to Hsp70 was assessed by immunoblotting the eluate with antibodies against Cse (ThermoFisher Scientific, Waltham, MA, USA; 1:100).

15. Immunoprecipitation

Immunoprecipitation were performed using MCEC cell lysates at an approximate protein concentration of 1 μg/μL. Hsp70 antibody (Abcam, Cambridge, UK; 1:100) or normal mouse IgG antibody (Santa Cruz Biotechnology) was added separately to 500 μL lysate and incubated at 4℃ overnight, after which the antibody-antigen complex was pulled out using protein G-coupled agarose beads (Santa Cruz Biotechnology). Samples were then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblotting for Cse.

16. Solid-phase binding assays

Solid-phase binding assays were performed as described previously [26]. Briefly, recombinant Hsp70 (10 nM) was added to 96-well plates and incubated for 1 hour at room temperature; bovine serum albumen (BSA)-coated wells were used as negative controls. Wells were washed twice with PBS and then blocked by incubating with 1% BSA for 2 hours. Plates were then incubated with different amounts of Cse protein (0, 0.625, 1.25, 2.5, 5, 10 nM; Abnova, Taipei, Taiwan) for 1 hour at room temperature. Cse bound to Hsp70 protein was detected by ELISA using a Cse antibody (ThermoFisher Scientific; 1:1,000) and anti-rabbit secondary antibody (Abcam). The K_d for binding affinity was calculated by non-linear regression analysis of the curve using Prism 8.0.

17. Proteome profiler mouse cytokine array analysis

Secreted cytokines in MCEC- conditioned medium were detected using a proteome profiler mouse cytokine array kit, panel A (R&D Systems Inc.), as described by

the manufacturer. This array measures 40 different mouse cytokines, chemokines, and acute phase proteins. The intensity of dot blots was analyzed using Image J software.

18. Histological examinations

Penis tissues, MPGs, DRGs, and MCECs were fixed in 4% paraformaldehyde at 4℃ for 30 minutes. Frozen tissue sections (12 μm thick), *ex vivo* samples, and MCECs were washed and incubated at 4℃ overnight with antibodies against Hsp70 (Abcam; 1:100), Cse (1:100), PECAM-1 (Millipore, Burlington, MA, USA; 1:50), NG2 (Millipore; 1:50), α-SMA (Sigma-Aldrich; 1:100), phospho-eNOS (Invitrogen; 1:50), neurofilament (Sigma-Aldrich; 1:50), βIII tubulin (Abcam; 1:100), neuronal nitric oxide synthase (nNOS; Santa Cruz Biotechnology; 1:50), phospho-hHistone3 (Millipore; 1:50), and/or claudin-5 (Invitrogen; 1:100), as described in the text. After several washes with PBS, the samples were incubated with species-appropriate tetramethyl rhodamine isothiocyanate- or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Zymed Laboratories, South San Francisco, CA, USA; 1:100) for 2 hours at room temperature. Samples were mounted in a solution containing DAPI, included to stain nuclei, and fluorescence signals were visualized using a confocal microscope (K1-Fluo; Nanoscope Systems, Inc., Daejeon, Korea). Quantitative histologic examinations were performed using Image J software.

19. *In situ* detection of superoxide anion

The oxidative fluorescent dye, hydroethidine (Molecular Probes, ThermoFisher Scientific; 1:5,000), was used to evaluate levels of superoxide anion *in situ* as previously described [27]. After immunofluorescence staining with anti-PECAM-1 antibody and FITC-conjugated secondary antibodies, hydroethidine was applied to each tissue section and the samples were coverslip mounted. Slides were then incubated in a light-protected humidified chamber at 37℃ for 30 minutes. The number of ethidium bromide fluorescence-positive endothelial cells was counted at a screen magnification of ×400 in five different regions. Values were expressed per high-power field.

20. Western blotting

Penis tissue and MCECs were lysed in RIPA buffer (Sigma-Aldrich) Supplemented with protease inhibitors (GenDEPOT, Katy, TX, USA) and phosphatase inhibitors (GenDEPOT). Equal amounts of protein (40 μg/lane) were resolved by SDS-PAGE on 8% to 15% gels, and then transferred to polyvinylidene difluoride membranes. After blocking with 5% nonfat dry milk for 1 hour at room temperature, membranes were probed with antibodies against Hsp70 (Abcam; 1:1,000), neurotrophin-3 (NT-3; Santa Cruz Biotechnology; 1:1,000), nerve growth factor (NGF; Santa Cruz Biotechnology; 1:1,000), BDNF (Santa Cruz Biotechnology; 1:1,000), pro and cleaved caspase-3 (Novus Biologicals, Centennial, CO, USA; 1:1,000), phosphorylated-eNOS (p-eNOS; Invitrogen; 1:500), total eNOS (BD Biosciences, Franklin Lakes, NJ, USA; 1:500), phosphorylated-Akt (p-Akt; Cell Signaling; 1:1,000), total Akt (Cell Signaling; 1:1,000) p-PI3K (Cell Signaling; 1:1,000), total PI3K (Cell Signaling; 1:1,000) phosphorylatedp-ERK (p-ERK; Cell Signaling; 1:1,000), total ERK (Cell Signaling; 1:1,000), p-NF-κB (Cell Signaling; 1:500), total NF-κB (Cell Signaling; 1:500), Cse (Thermo Fisher Scientific; 1:1,000), SDF1 (Abcam; 1:1,000), HO-1 (Santa Cruz Biotechnology; 1:500), and/or β-actin (Abcam; 1:5,000), as described in the text. The results were quantified densitometrically using Image J software.

21. RT-PCR

Total RNA was extracted from cultured cells using TRIzol (Thermo Fisher Scientific) following the manufacturer's protocols. Reverse transcription was performed using 1 μg of total RNA in 20 μL of reaction buffer containing oligo dT primers and AccuPower RT Premix (Bioneer Inc., Daejeon, Korea). Relative changes in the indicated mRNAs were measured using the following primer pairs: mouse *Cth*, 5'-TCG TTT CCT GGA GAC CAA TC-3' (forward) and 5'-CTG CGT ATG CTC CGT AAT GA-3'(reverse); *GAPDH*, 5'-CCA CTG GCG TCT TCA CCA C-3' (forward) and 5'- CCT GCT TCA CCA CCT TCT TG-3' (reverse). PCR was performed on a DNA Engine Tetrad Peltier Thermal Cycler using an AccuPower PCR Premix kit (Bioneer Inc.), with denaturation at 94℃ for 30 seconds and annealing at 60℃ for 30 seconds. PCR products were analyzed by electrophoresing 10-μL aliquots on 1% agarose gels, followed by detection with ultraviolet light. GAPDH was used as an internal control.

22. Statistical analysis

Data were expressed as mean±SEM of values from

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at least three independent experiments. Student's t-test was used to determine statistically significant differences between two groups using Prism 8 software (Graph Pad Software, San Diego, CA, USA). All p-values <0.05 were considered statistically significant; individual p-values are indicated by asterisks in figure legends (*p<0.05, **p<0.01, ***p<0.001).

RESULTS

1. Metabolic variables

The body weights of STZ-treated WT and Hsp70-Tg diabetic mice were significantly lower than those in age-matched control mice. Fasting and postprandial blood glucose concentrations in diabetic mice were

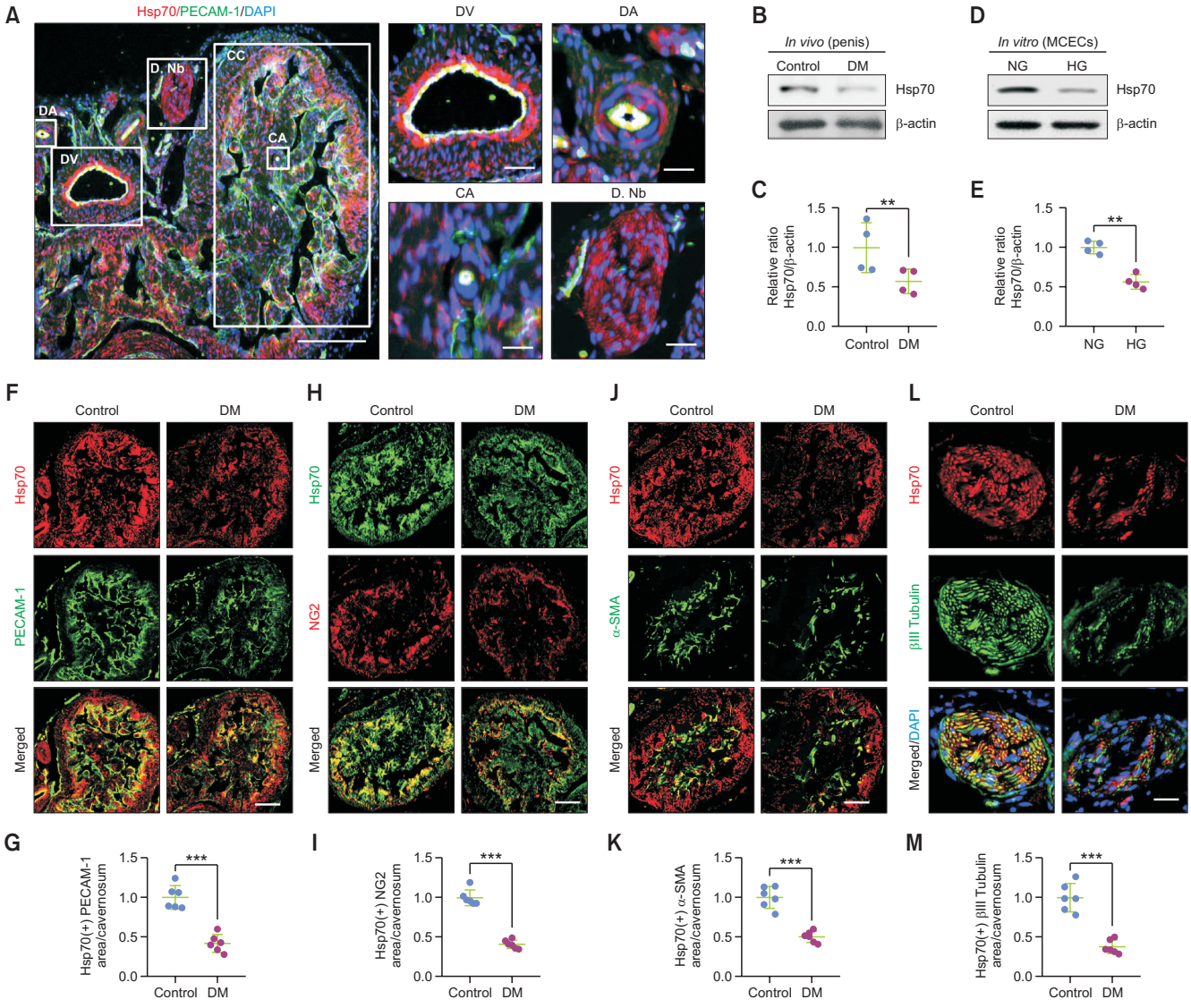

Fig. 1. Heat shock protein 70 (Hsp70) expression under normal and diabetic conditions. (A) Left: Representative images of immunofluorescence staining of normal mouse corpus cavernosum (CC) tissue with antibodies to Hsp70 (red) and PECAM-1 (green); nuclei were labeled by counterstaining with DAPI (blue). Scale bar, 200 μm. Right: Higher-magnification images of Hsp70 and PECAM-1 staining in the dorsal vein (DV; scale bars, 50 μm), dorsal artery (DA; scale bars, 25 μm), cavernous artery (CA; scale bars, 25 μm), and dorsal nerve bundel (D. Nb; scale bars, 25 μm). Nuclei were stained with DAPI (blue). (B-E) Representative western blots for Hsp70 in age-matched control and diabetic mouse penis tissue *in vivo* and MCECs exposed to normal-glucose (NG) or high-glucose (HG) conditions *in vitro*. Hsp70 expression was quantified using Image J, and the results are presented as mean±SEM (n=4). (F-M) Penis cavernous tissue was double-immunostained for Hsp70 (red) and the endothelial cell marker PECAM-1 (green; F and G; scale bars, 100 μm), Hsp70 (green) and the pericyte marker NG2 (red; H and I; scale bars, 100 μm), Hsp70 (red) and the smooth muscle cell marker α-SMA (J and K; green; scale bars, 100 μm), and Hsp70 (green) and the neuronal marker βIII-tubulin (L and M; green; scale bars, 25 μm). PECAM-1–, NG2–, α-SMA– and βIII-tubulin–immunopositive areas in cavernosum tissue were quantified using Image J, and the results are presented as mean±SEM (n=6; **p<0.01, ***p<0.001; Student's t-test). Nuclei were labeled by counterstaining with DAPI (blue). Relative ratios in control and NG groups were set to 1. DM: diabetes mellitus.

significantly increased compared with those in age-matched control mice. However, body weights and blood glucose levels were unchanged with Hsp70 protein treatment or in Hsp70-Tg mice. No significant difference in MSBP was found among experimental groups (Supplement Table 1-4).

2. Hsp70 expression is decreased under diabetic conditions

Given that expression of the Hsp70 gene and protein is low individuals with insulin resistance and hyperglycemia [28], we hypothesized that Hsp70 expression would also be decreased in penile cavernous tissues of STZ-induced diabetic mice. Double immunostaining of mouse cavernous tissues for Hsp70 and the endothelial cell marker PECAM-1 (platelet and endothelial cell adhesion molecule 1) revealed high expression of Hsp70 in blood vessels, including the dorsal vein, dorsal artery, cavernous artery and corpus cavernosum, as well as in dorsal nerve bundles (DNBs) (Fig. 1A). Western blot analyses showed that expression of Hsp70 *in vivo* (mouse penis tissues; Fig. 1B, 1C) and *in vitro* (MCECs; Fig. 1D, 1E) was significantly reduced under diabetic conditions compared with that in the control group. In agreement with these findings, double immunostaining for Hsp70 and PECAM-1, NG2 (pericyte marker), α-SMA (smooth muscle cell maker), or βIII-tubulin (neuronal cell marker) also revealed a decrease in Hsp70 in the corpus cavernosum of STZ-induced diabetic mice (Fig. 1F, 1M). These results demonstrate that Hsp70 expression is decreased under diabetic conditions *in vivo* and *in vitro*.

3. Hsp70 improves erectile function under diabetic conditions through enhanced angiogenesis

It was previously shown that Hsp70 possesses angiogenic activity towards human umbilical vein endothe-

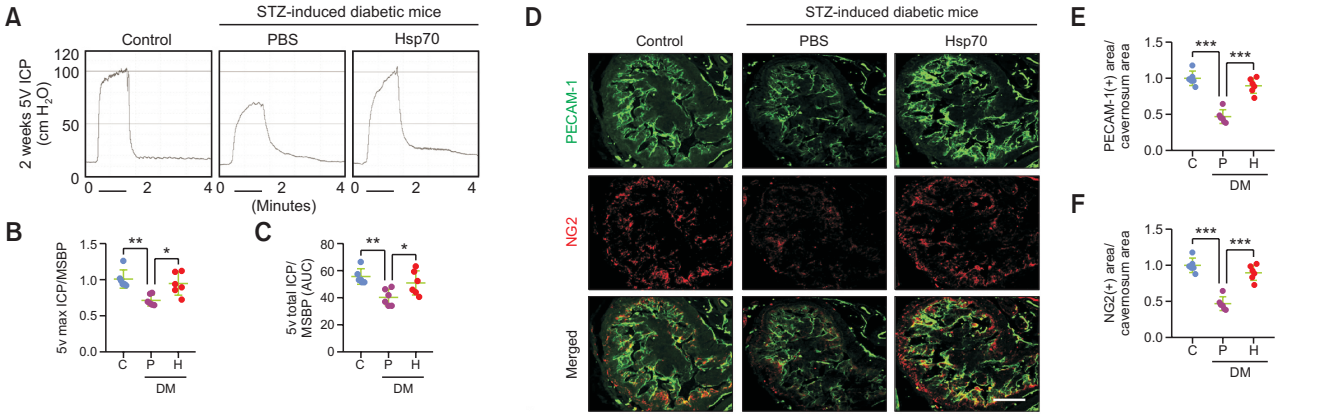

Fig. 2. Heat shock protein 70 (Hsp70) improves erectile function under diabetic conditions through induction of angiogenesis. (A) Representative intracavernous pressure (ICP) responses in cavernous tissue from age-matched control and streptozotocin (STZ)-induced diabetic mice at 2 weeks after repeated (days -3 and 0;) intracavernous injections of phosphate-buffered saline (PBS; 20 μL) or Hsp70 protein (5 μg/20 μL). Solid bar indicates the stimulus interval. Ratios of mean maximal ICP (B) and total ICP (C) (area under the curve) to mean systolic blood pressure (MSBP) were calculated for each group (n=6). (D-F) PECAM-1 (green) and NG2 (red) immunostaining in cavernous tissue from age-matched control and STZ-induced diabetic mice at 2 weeks after repeated (days -3 and 0;) intracavernous injections of PBS (20 μL) or Hsp70 protein (5 μg/20 μL). Quantification of PECAM-1 (E) and NG2 (F) expression using Image J; results are presented as means±SEM (n=6). Scale bars, 100 μm. (G, H) Immunostaining of cavernous tissue from age-matched control or STZ-induced diabetic mice with antibodies to PECAM-1 (green) and PH3 (proliferation marker; red) at 2 weeks after repeated (days -3 and 0;) intracavernous injections of PBS (20 μL) or Hsp70 protein (5 μg/20 μL). Nuclei were labeled by costaining with DAPI (blue). Scale bar, 50 μm. Quantification of the number of PH3-positive endothelial cells per high-power field using ImageJ; results are presented as mean±SEM (n=6). Migration assays (I), tube-formation assays (K), aortic ring assays (M), and *ex vivo* MCEC sprouting assays (O) were performed under normal-glucose (NG) or high-glucose (HG) conditions for 5 days with PBS or Hsp70 protein (500 ng/mL) treatment. Magnification, 40×. Number of migrated endothelial cells (J) or master junctions (L), intensity of the area of microvessels sprouting from aortic rings (N), and endothelial cell sprouting from cavernous tissue (P) were quantified using ImageJ; results are presented as mean±SEM (n=6). (Q-W) Representative western blot showing p-Akt/total Akt, p-ERK/total ERK and p-eNOS/total eNOS in penis tissue from age-matched control and STZ-induced diabetic mice at 2 weeks after repeated (days -3 and 0;) intracavernous injections of PBS (20 μL) or Hsp70 protein (5 μg/20 μL) *in vivo* (Q, left), or MCECs exposed to NG or HG conditions for 5 days and then treated with PBS or Hsp70 protein (500 ng/mL) *in vitro* (Q, right). Expression of the indicated proteins was quantified by assessing the density of the corresponding protein bands using Image J (R-W); results are presented as mean±SEM (n=4; *p<0.05, **p<0.01, ***p<0.001, Student's t-test). Relative ratios in control and NG groups were set to 1. DM: diabetes mellitus, C: control, P: PBS, H: Hsp70 .

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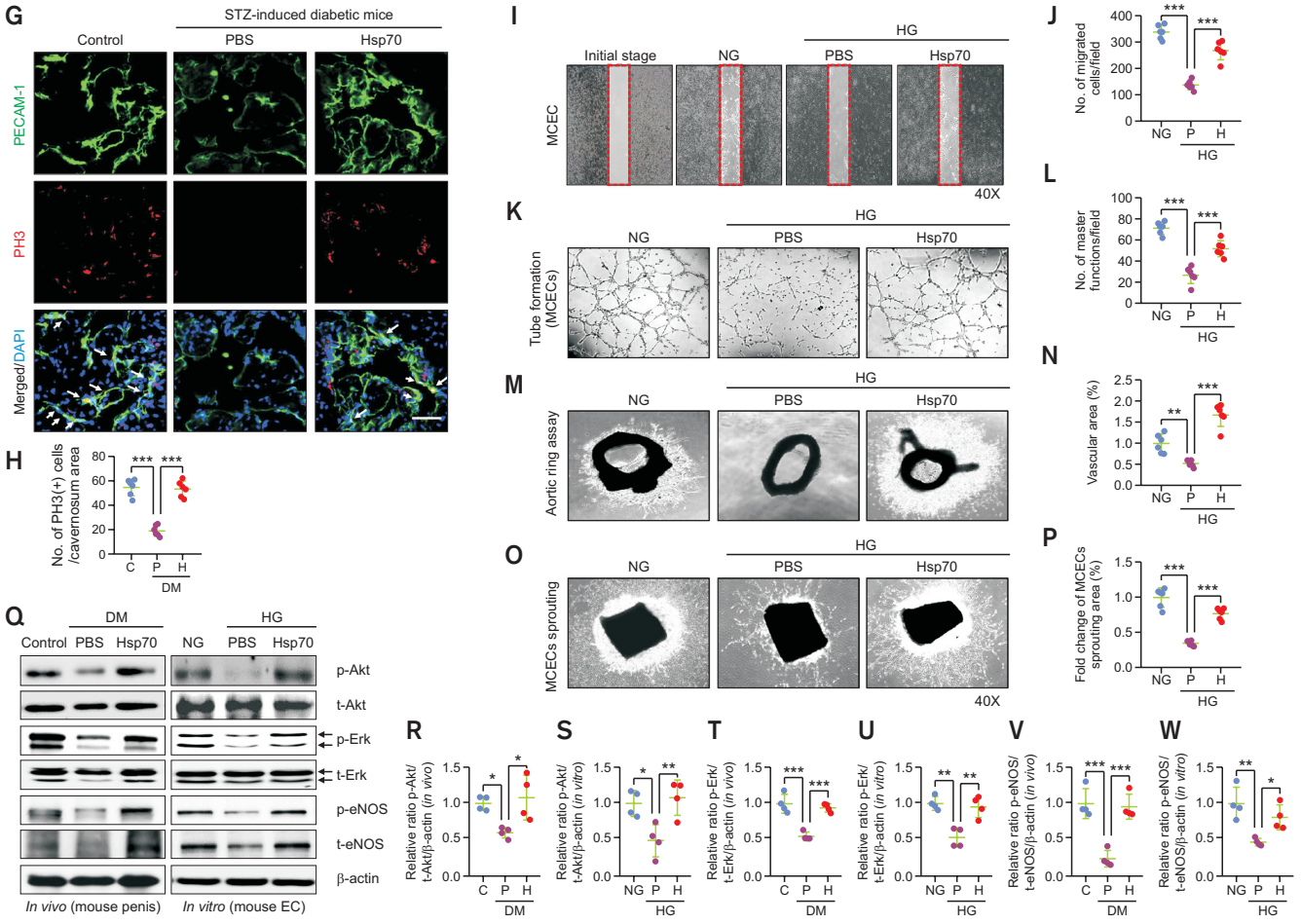

Fig. 2. Continued.

lial cells (HUVECs) *in vitro* and in Matrigel plug assays *in vivo* [10,29]. To determine whether Hsp70 has beneficial effects on erectile function under diabetic conditions, we evaluated erectile function in diabetic mice and age-matched controls during electrical stimulation of the cavernous nerve (5 V, 12 Hz, 1 m/s) 2 weeks after the last of two intracavernous injections (administered on days –3 and 0) of PBS or different concentrations of Hsp70 protein (0.5–10 μg in 20 μL PBS). During electrical stimulation, the ratios of maximal and total ICP to MSBP were significantly decreased in PBS-treated diabetic mice compared with age-matched controls. Notably, repeated intracavernous injections of Hsp70 in diabetic mice significantly improved this erection parameter, which reached up to 94% of control values at concentrations of 5 and 10 μg/20 μL, whereas no significant improvement in erectile function was noted at concentrations of 0.5 or 1 μg/20 μL (Fig. 2A-2C and Supplement Fig. 1). No detectable differences in MSBP were found between experimental groups.

Immunofluorescence staining of cavernous tissue with antibodies to the endothelial cell and pericyte markers, PECAM-1 and NG2, respectively, demonstrated that Hsp70 protein significantly restored cavernous endothelial cell and pericyte content in diabetic mice (Fig. 2D-2F). Similar to erectile function assessments, Hsp70 increased levels of p-eNOS (Supplement Fig. 2A, 2B) and expression of the endothelial cell-cell junction protein, claudin-5, in diabetic mice (Supplement Fig. 3A, 3B), while reducing the production of reactive oxygen species (hydroethidine staining; Supplement Fig. 3C, 3D) and apoptosis (JNK/caspase-3 and TUNEL assay; Supplement Fig. 4). In addition, Hsp70 induced endothelial cell proliferation in diabetic mice *in vivo* (PH3 staining; Supplement Fig. 2G, 2H) and migration and tube formation by MCECs *in vitro* (Fig. 2I-2L and Supplement Fig. 2C, 2D); it also enhanced microvessel sprouting from aortic rings and corpus cavernosum *ex vivo* (Fig. 2M-2P) under high-glucose conditions. Furthermore, a western blot analysis demonstrated

downregulation of PI3K, mitogen-activated protein kinase and nitric oxide (NO) pathways involved in cell survival and proliferation, and penile erection [17,30] in diabetic mice *in vivo* and MCECs cultivated *in vitro* under high-glucose conditions compared with normal controls, as evidenced by significantly decreased levels of p-Akt, p-ERK, and p-eNOS. Treatment with Hsp70 protein rescued the phosphorylation of these factors under diabetic conditions, restoring their phosphorylation to baseline levels (Fig. 2Q-2W). Collectively, these results indicate that Hsp70 promotes the survival and proliferation of cavernous endothelial cells under diabetic conditions, thereby improving erectile function in diabetic mice.

4. Hsp70 induces neural regeneration under diabetic conditions

Hsp70 is known to exert neuroprotective effects in animal models of cerebral ischemia, circulatory shock, and neuropathic pain after peripheral nerve injury [31-34]. Therefore, we hypothesized that Hsp70 could also influence peripheral nerves in penile tissue of STZ-induced diabetic mice. To test this, we immunostained the corpus cavernosum and DNB of control and STZ-induced diabetic mice for βIII-tubulin and nNOS using fluorescently conjugated antibodies. Interestingly, βIII-tubulin and nNOS expression were significantly decreased in the cavernosum and DNB of control (PBS-treated) diabetic mice, an effect that was significantly reversed by treatment with Hsp70 protein (Fig. 3A, 3B, 3E-3H). Western blot analyses for neurotrophic factors also revealed that neurotrophin-3 (NT-3), BDNF and NGF expression in the corpus cavernosum were downregulated in PBS-treated diabetic mice, and were successfully restored by Hsp70 treatment (Fig. 3C, 3I-3K). We further evaluated the effect of Hsp70 on neurite outgrowth in *ex vivo* MPG and DRG explants exposed to high-glucose conditions. Immunofluorescence staining of MPG and DRG tissue for βIII-tubulin showed a significant reduction in neurite sprouting under high-glucose conditions, whereas Hsp70 treatment profoundly induced neurite outgrowth under these conditions (Fig. 3D, 3L-3M). Overall, these findings indicate that Hsp70 promotes neural regeneration in STZ-induced diabetic mice *in vivo* and in *ex vivo* MPG or DRG explants under high-glucose conditions.

5. Cse is a novel target of Hsp70 in the induction of neurovascular regeneration under diabetic conditions

In addition to eNOS, which acts as the main mediator of penile erection [7], several receptors, including CD14, Toll-like receptors (TLR2), lectin-like oxidized low-density lipoprotein-1 (LOX-1), CD91, CD40 and receptor for advanced glycation end products (RAGE) have been reported to interact with Hsp70 [35]. It was also reported that Hsp70 is required for IL-5–induced angiogenesis in HUVECs through the eNOS pathway [10]. However, the molecular mechanisms by which Hsp70 mediates penile neurovascular regeneration under hyperglycemic conditions have not yet been intensively studied. Therefore, to further clarify how Hsp70 mediates neurovascular regeneration, we performed RNA sequencing analyses of MCECs treated with PBS or Hsp70 under normal-glucose and high-glucose conditions. A total of 23,282 genes were detected in three libraries. Significant differentially expressed genes were selected based on two conditions: fold-change >1.5 and log2 >8. Interestingly, only three identified genes that were differentially regulated between NG/HG+PBS and HG+PBS/HG+Hsp70 met these conditions: 3-phosphoglycerate dehydrogenase (*Phgdh*), phosphoserine aminotransferase 1 (*Psat1*), and *Cse* (Fig. 4A, 4B). On the basis of a literature survey and RT-PCR validation, we selected Cse for further investigation as a target for Hsp70 in promoting neurovascular regeneration (Fig. 4C, 4D). Immunoprecipitation experiments, pull-down assays, *in vitro* MCEC binding assays and solid-phase binding assays revealed co-immunoprecipitation of Hsp70 with Cse, with the latter assay yielding an estimated binding affinity (K_d) of 1.8 nmol/L (Fig. 4E-4G). We also exposed mouse MPG and DRG explants to high-glucose conditions and subsequently performed immunofluorescence staining for βIII-tubulin. This analysis showed a significant reduction in neurite sprouting under high-glucose conditions. In contrast, Hsp70 treatment profoundly induced neurite outgrowth under these conditions, an effect that was abolished by Cse knockdown using lentiviral-delivered shCse (Fig. 4H-4K and Supplement Fig. 5A, 5B). *In vitro* tube-formation assays using MCECs also revealed that the severely impaired tube formation observed under high-glucose conditions was completely rescued by Hsp70. Again, this effect was significantly reduced by Cse knockdown (Fig. 4L-4M and Supplement Fig.

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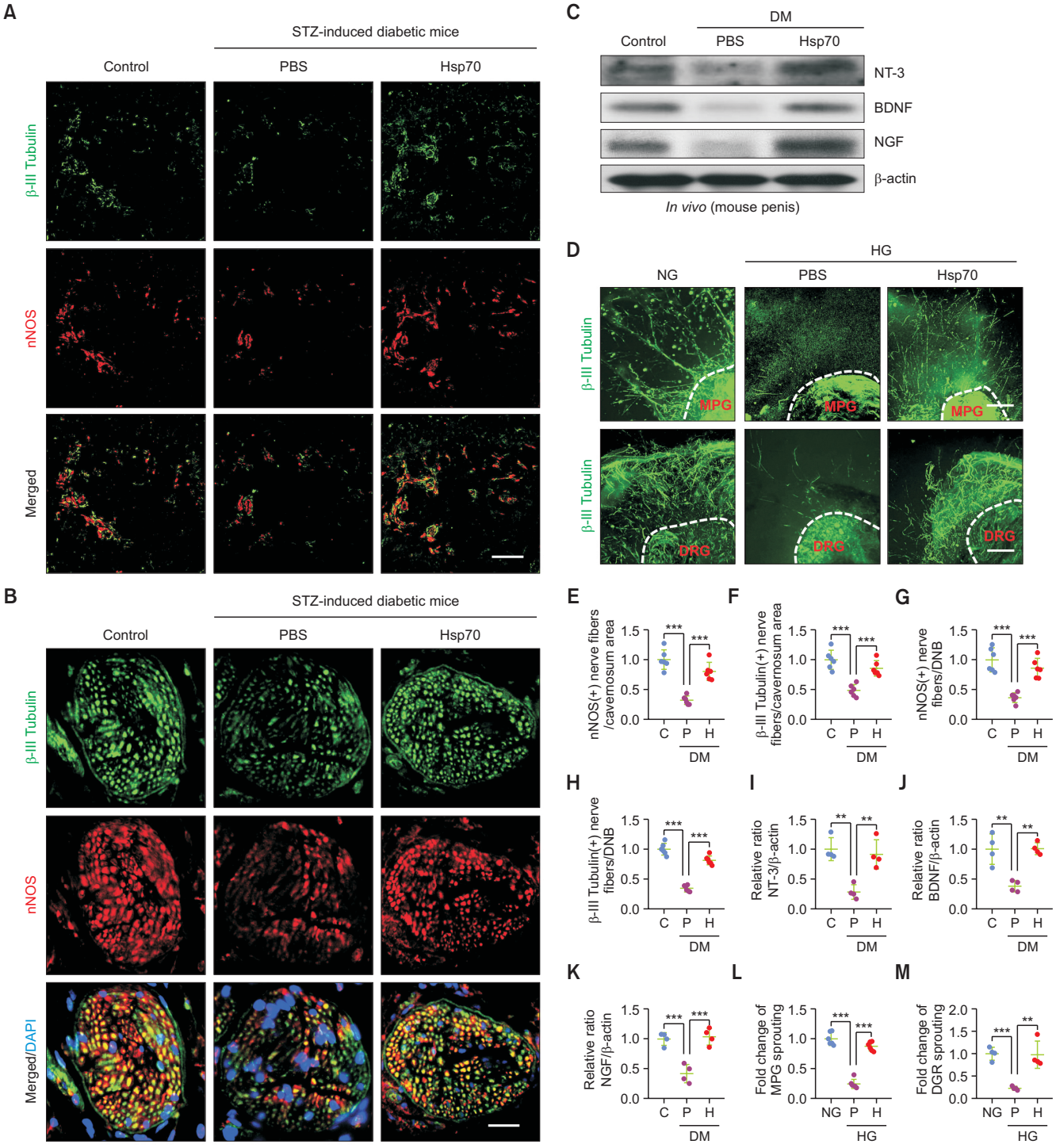

Fig. 3. Heat shock protein 70 (Hsp70) induces nerve regeneration under diabetic conditions. (A, B) βIII-tubulin (green) and nNOS (red) immunostaining in the mouse corpus cavernosum (CC) (A; scale bars, 200 μm) and DNB (B; scale bars, 25 μm) of age-matched control and streptozotocin (STZ)-induced diabetic mice at 2 weeks after repeated (days 3 and 0;) intracavernous injections of phosphate-buffered saline (PBS) (20 μL) or Hsp70 protein (5 μg/20 μL). Nuclei were labeled by counterstaining with DAPI (blue). (C) Representative western blot for neurotrophic factors (NT-3, BDNF, and NGF) in penis tissue from age-matched control or STZ-induced diabetic mice at 2 weeks after repeated (days 3 and 0;) intracavernous injections of PBS (20 μL) or Hsp70 protein (5 μg/20 μL). (D) βIII-tubulin (green) staining in mouse MPG (upper panel) and DRG (lower panel) tissue treated with PBS or Hsp70 protein (500 ng/mL) under normal-glucose (NG) or high-glucose (HG) conditions for 5 days. Scale bar, 100 μm. (E-M) βIII-tubulin- and nNOS-positive axonal areas in the mouse CC (E, F) and DNB (G, H), normalized to neurotrophic factor expression intensity (I-K), and βIII-tubulin–positive neurite length in MPG or DRG tissues (L, M) were quantified using Image J; results are presented as mean±SEM (n=4-6; **p<0.01, ***p<0.001; Student's t-test). Relative ratios in control and NG groups were set to 1. DM: diabetes mellitus, C: control, P: PBS, H: Hsp70.

5A, 5B).

To assess the *in vivo* neurovascular regeneration effects of Hsp70 in a diabetic mouse model, we evaluated erectile function in STZ-induced diabetic mice 2 weeks after shCse lentiviral infection and Hsp70 treatment. The ratio of maximum ICP or total ICP to MSBP observed during electrical stimulation of the cavernous nerve was significantly decreased in STZ-induced diabetic mice compared with that in control mice. Intriguingly, treatment of STZ-induced diabetic mice with Hsp70 significantly improved erectile function; these erectile function-improving effects of Hsp70 were significantly abrogated by shCse lentiviral transfection, but not by control shRNA lentiviral infection (Fig. 4N, 4P-4Q and Supplement Fig. 5C, 5D). Similarly, double immunofluorescence staining of the DNB using antibodies against nNOS and βIII-tubulin or the corpus cavernosum with antibodies against PECAM-1 and NG2 demonstrated that Hsp70 treatment increased neural content in the DNB and endothelial cell and pericyte content in the corpus cavernosum of STZ-induced diabetic mice; in both cases, these effects were abolished by Cse knockdown (Fig. 4O, 4R-4U and Supplement Fig. 5C, 5D). Consistent with this, erectile function as well as penile neurovascular architecture in Hsp70-Tg mice remained intact even after STZ injection, an effect that was eliminated by shRNA-mediated Cse knockdown (Fig. 5). Taken together, these findings indicate that Hsp70-driven penile neurovascular regeneration under diabetic conditions is dependent on Cse.

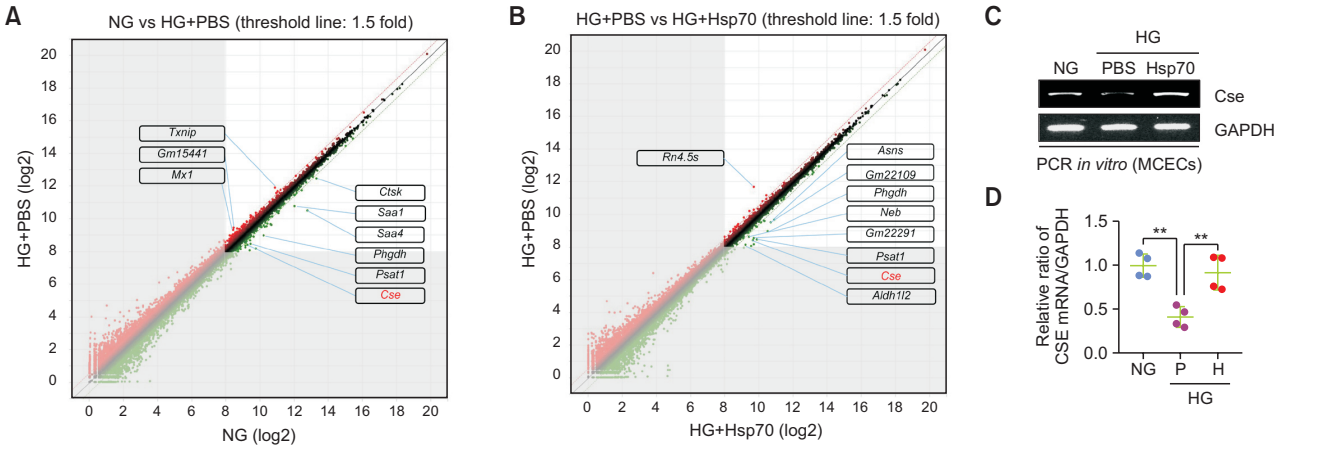

Fig. 4. Cystathionine gamma-lyase (Cse) is a novel target for heat shock protein 70 (Hsp70) in the induction of neurovascular regeneration and improvement of erectile function under diabetic conditions. (A, B) Scatter plot of RNA sequencing data from MCECs treated with phosphate-buffered saline (PBS) or Hsp70 protein (500 ng/mL) under normal-glucose (NG) or high-glucose (HG) conditions for 5 days. Differentially expressed genes between NG/HG+PBS and HG+PBS/ HG+Hsp70 were selected according to the following criteria: fold change >1.5 and log2 >8. (C, D) RT-PCR validation of differentially expressed genes from RNA sequencing analyses. Normalized band intensity was quantified using Image J, and the results are presented as mean±SEM (n=4). (E) Immunoprecipitation (IP) of Cse from whole-cell lysates of MCECs treated with Hsp70 followed by immunoblot detection of Cse (upper panel) and His-tag pull-down assay of Cse (lower panel). (F) Double immunostaining for Hsp70 (red) and Cse (green) in MCECs. Left panel (lower magnification), scale bar=100 μm; right panel (higher magnification), scale bar=25 μm. Nuclei were labeled by costaining with DAPI (blue). (G) Binding affinity of Hsp70 for Cse was determined by solid-phase binding assay, and the binding affinity (K_d) was calculated by non-linear regression analysis of the curve using Prism 8.0. Immunostaining for βIII-tubulin in mouse MPG (H, I) and DRG (J, K) tissues under NG or HG conditions with PBS or Hsp70 protein (500 ng/mL) treatment for 5 days in the presence of scrambled shRNA control (shCon; 1×10^4 TU/mL culture medium) or Cse-knockdown shRNA (shCse, 1×10^4 TU/mL culture medium). Lengths of βIII-tubulin-positive neurites in MPG or DRG tissues were quantified using Image J, and the results are presented as mean±SEM (n=4). Scale bar, 100 μm. (L, M) Tube-formation assays under NG or HG conditions using MCECs treated with PBS or Hsp70 protein (500 ng/mL) for 5 days in the presence of shCon (1×10^4 TU/mL culture medium) or shCse (1×10^4 TU/mL culture medium). Number of master junctions per high-power field were quantified using Image J, and the results are presented as mean±SEM (n=6). (N, P, Q) Representative intracavernous pressure (ICP) responses for age-matched control and streptozotocin (STZ)-induced diabetic mice at 2 weeks after repeated (days 3 and 0;) intracavernous injections of PBS (20 μL) or Hsp70 protein (5 μg/20 μL) in the presence of shCon (5×10^4 TU/mouse) shCse (5×10^4 TU/mouse). Solid bar indicated the stimulus interval. Ratios of mean maximal ICP (P) and total ICP (Q) (area under the curve) to mean systolic blood pressure (MSBP) were calculated for each group (n=6). O, (R-U) Double immunostaining for nNOS (green) and βIII-tubulin (red) in the dorsal nerve part or for PECAM-1 (green) and NG-2 (red) in the corpus cavernosum of age-matched control and STZ-induced diabetic mice at 2 weeks after repeated (days 3 and 0;) intracavernous injections of PBS (20 μL) or Hsp70 protein (5 μg/20 μL) in the presence of shCon (5×10^4 TU/mouse) or shCse (5×10^4 TU/mouse). Quantification of nNOS (R), βIII-tubulin (S), PECAM-1 (T), and NG2 (U) expression using Image J; results are presented as mean±SEM (n=6; **p<0.01, ***p<0.001; Student's t-test). Scale bars, 100 μm. Relative ratios in control and NG groups were set to 1. DM: diabetes mellitus, C: control, P: PBS, H: Hsp70.

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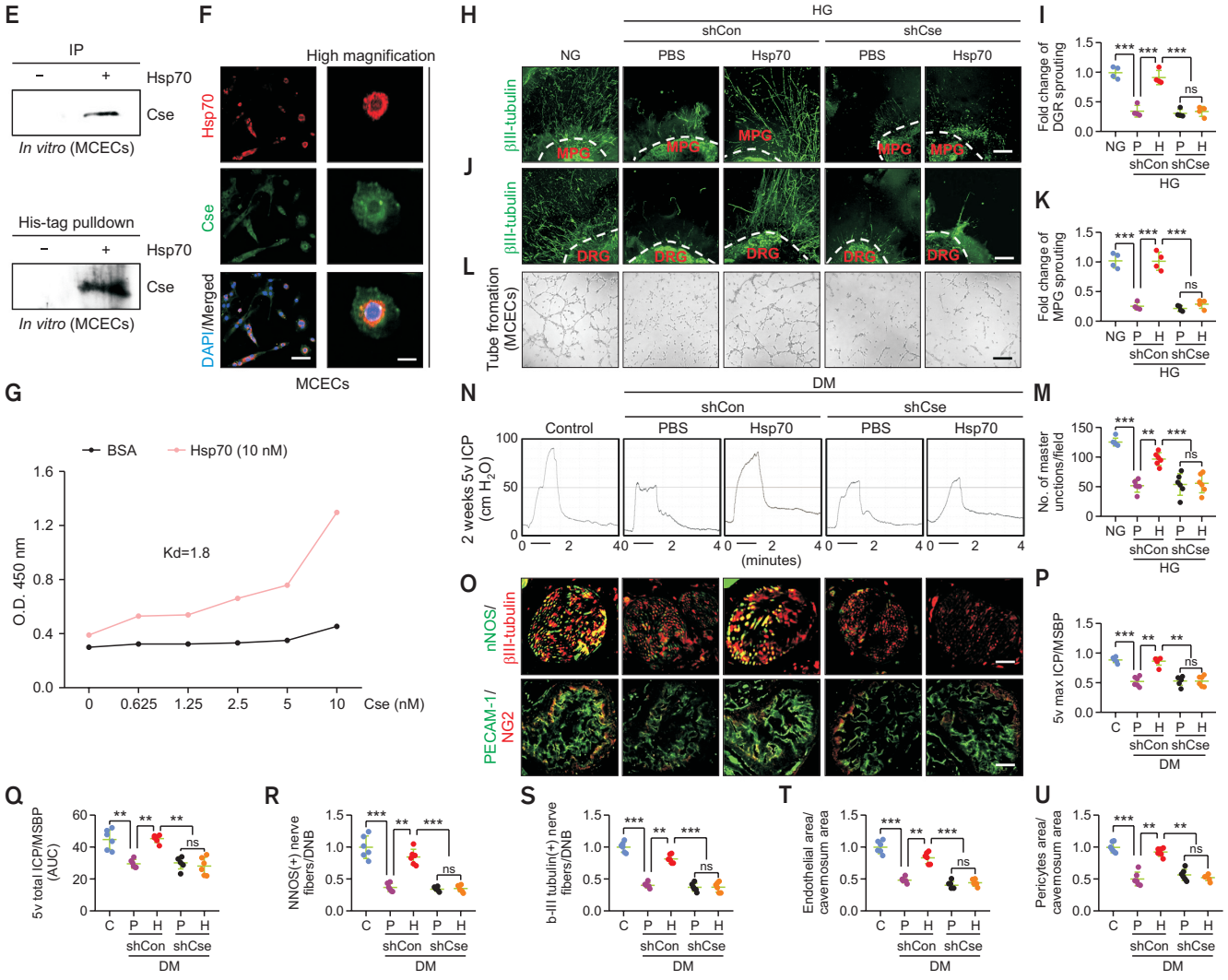

Fig. 4. Continued.

6. Identification of the signaling pathway responsible for Hsp70-Cse–induced neurovascular regeneration under diabetic conditions

Hsp70 promotes production of cytokines or chemokines involved in the angiogenic pathway [36,37]. Therefore, to determine whether Cse has a regulatory effect on Hsp70-induced cytokine secretion, we examined cytokine expression in MCEC-conditioned medium using a cytokine antibody array. Among the 40 cytokines tested, SDF1, also known as C-X-C motif chemokine 12 (CXCL12), was positively regulated by Hsp70 in a Cse-dependent manner in MCEC-conditional medium under high-glucose conditions (Fig. 6A). SDF1 is known to mediate angiogenesis *via* a HO-1-dependent mechanism [38] and induce chemotaxis in T cells through NF-κB *via* PI3K/Akt and NO signaling [39]. Therefore, we further confirmed the activation of PI3K, Akt, eNOS, and NF-κB p65 in response to Hsp70-Cse-SDF-1 signaling by western blot analysis in diabetic penis *in vivo* and MCECs *in vitro* under high-glucose conditions. Intriguingly, SDF1, HO-1, PI3K, Akt, eNOS, and NF-κB p65 phosphorylation were increased after Hsp70 treatment under diabetic conditions, and these effects of Hsp70 were eliminated by shRNA-mediated Cse knockdown (Fig. 6B-6N). These results suggest that SDF1/HO-1/PI3K/Akt/eNOS/NF-κBp65 pathways are involved in Hsp70-Cse–driven angiogenesis and neural regeneration.

DISCUSSION

Diabetic ED is accompanied by severe angiopathy and neuropathy [1]. Moreover, the regeneration po-

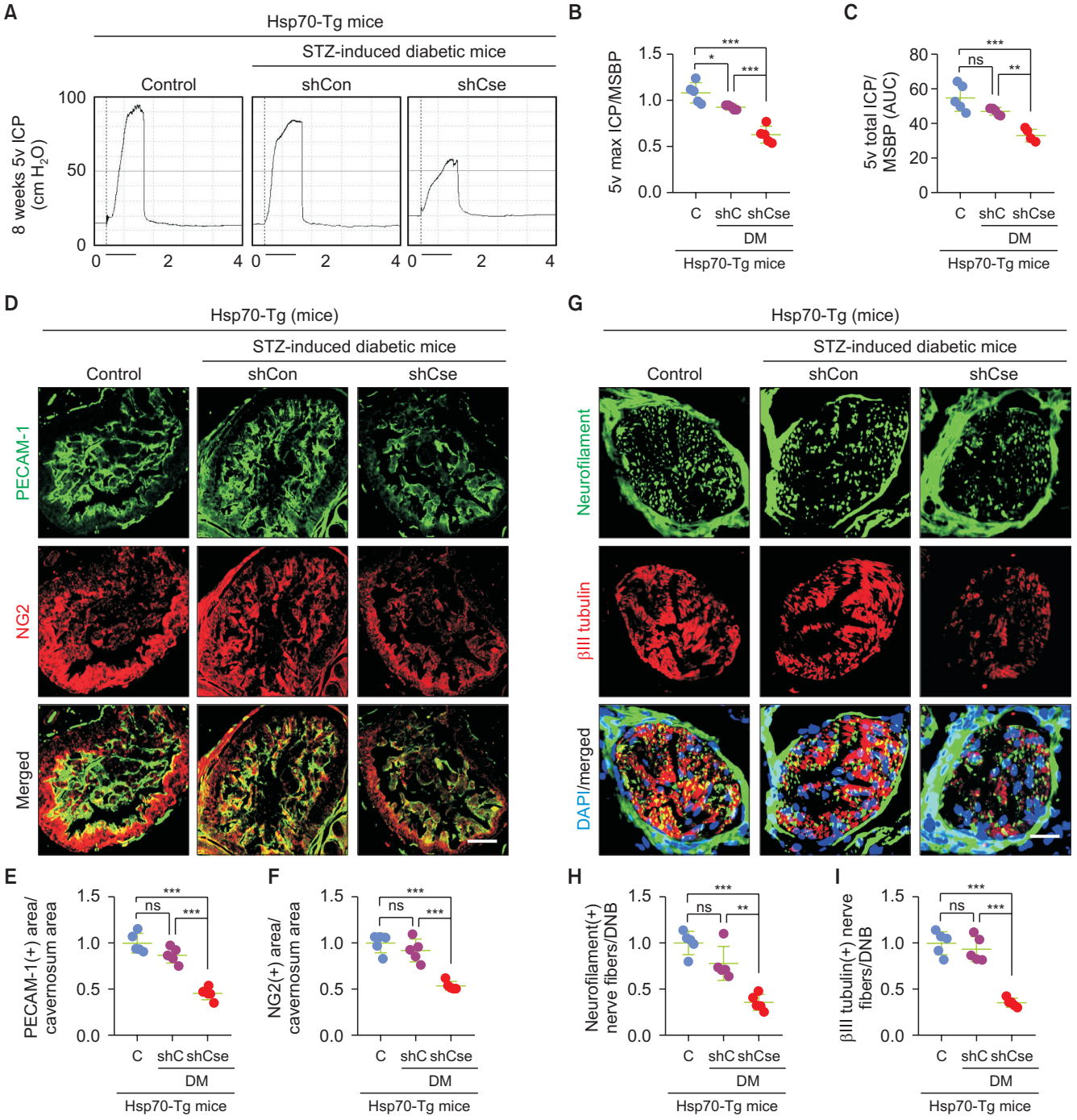

Fig. 5. Heat shock protein 70 (Hsp70) overexpression promotes cystathionine gamma-lyase (Cse)-dependent resistance to diabetes-induced erectile dysfunction. (A) Representative intracavernous pressure (ICP) responses for Hsp70-Tg mice and streptozotocin (STZ)-induced diabetic Hsp70-Tg mice infected with shCon or shCse lentivirus. The cavernous nerve was stimulated at 5 V. The stimulus interval is indicated by a solid bar. ICP was measured 8 weeks after STZ injection. (B, C) Ratios of mean maximal ICP and total ICP (area under the curve) to mean systolic blood pressure (MSBP) were calculated for each group (n=5). (D-F) PECAM-1 (green) and NG2 (red) immunostaining in cavernous tissue from Hsp70-Tg mice and STZ-induced diabetic Hsp70-Tg mice infected with shCon or shCse lentivirus. Quantification of PECAM-1 and NG2 expression using Image J; results are presented as mean±SEM (n=5). Scale bars, 100 μm. (G-I) Neurofilament (green) and βIII-tubulin (red) immunostaining in cavernous DNB tissue from Hsp70-Tg mice and STZ-induced diabetic Hsp70-Tg mice infected with shCon or shCse lentivirus. Quantification of neurofilament and βIII-tubulin expression using Image J; results are presented as mean±SEM (n=5; *p<0.05, **p<0.01, ***p<0.001; Student's t-test). Scale bars, 25 μm. The relative ratio in the control group was set to 1. DM: diabetes mellitus, ns: not significant, C: control.

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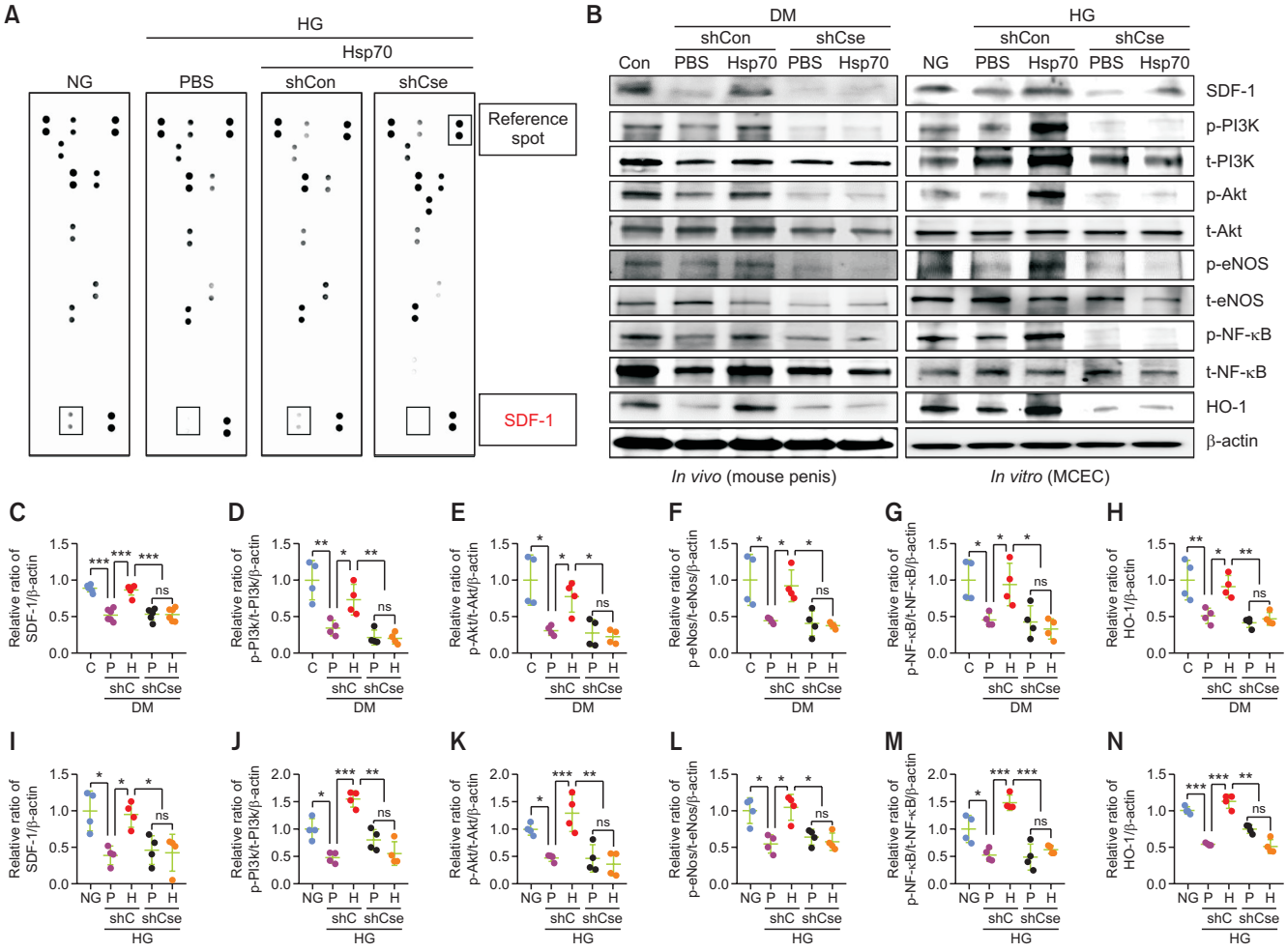

Fig. 6. Identification of the heat shock protein 70 (Hsp70)-cystathionine gamma-lyase (Cse) signaling pathway responsible for inducing neurovascular regeneration under diabetic conditions. (A) Cytokine antibody analysis of conditioned medium from MCECs treated with phosphate-buffered saline (PBS) or Hsp70 protein (500 ng/mL) under normal-glucose (NG) or high-glucose (HG) conditions in the presence of scrambled shRNA control (shCon; 1×10^4 TU/mL culture medium) or Cse-knockdown shRNA (shCse; 1×10^4 TU/mL culture medium) for 5 days. The relative expression of each cytokine was determined by comparing the respective plots to the positive control (reference spot). The frame line indicates positive signals for SDF1 in the Hsp70-Cse signaling pathway. (B) Representative western blots for SDF1, HO-1, p-PI3K/total PI3K, p-Akt/total Akt, p-eNOS/total eNOS, and p-NF-κB/total NF-κB *in vivo* and *in vitro*. Left (*in vivo*): Mouse penis tissue from age-matched control and streptozotocin (STZ)-induced diabetic mice at 2 weeks after repeated (days 3 and 0;) intracavernous injections of PBS (20 μL) or Hsp70 protein (5 μg/20 μL) in the presence of shCon (5×10^4 TU/mouse) or shCse (5×10^4 TU/mouse). Right (*in vitro*): MCECs treated with PBS or Hsp70 protein (500 ng/mL) under NG or HG conditions in the presence of shCon (1×10^4 TU/mL culture medium) or shCse (1×10^4 TU/mL culture medium). (C-N) Expression of the indicated proteins was quantified by assessing the density of the corresponding protein bands using Image J; results are presented as mean±SEM (n=4; *p<0.05, **p<0.01, ***p<0.001; Student's t-test). Relative ratios in control and NG groups were set to 1. DM: diabetes mellitus, ns: not significant, C: control, P: PBS, H: Hsp70.

tential of axon fibers after peripheral nerve injury is known to be impaired in diabetes [40]. The current unmet need for directed therapies for diabetic vascular and neuronal complications has prompted investigations into the development of new treatment options. In the present study, we demonstrated that Hsp70 promotes penile neurovascular regeneration in diabetic mice by binding specifically to Cse, resulting in recovery of erectile function. These conclusions are based on the following observations. First, Hsp70 induced angiogenesis and neural regeneration *in vitro* in MCECs; *ex vivo* in aortic rings, corpus cavernosum, and MPG cultures; and *in vivo* in a mouse model of diabetic ED. Second, Hsp70 physically interacts with Cse, as determined by coimmunoprecipitation, His-Tag pull-down analysis, and specific binding affinity assays. Third, downstream signaling pathways mediated by Hsp70-Cse were identified. Finally, Hsp70-driven penile neurovascular re-

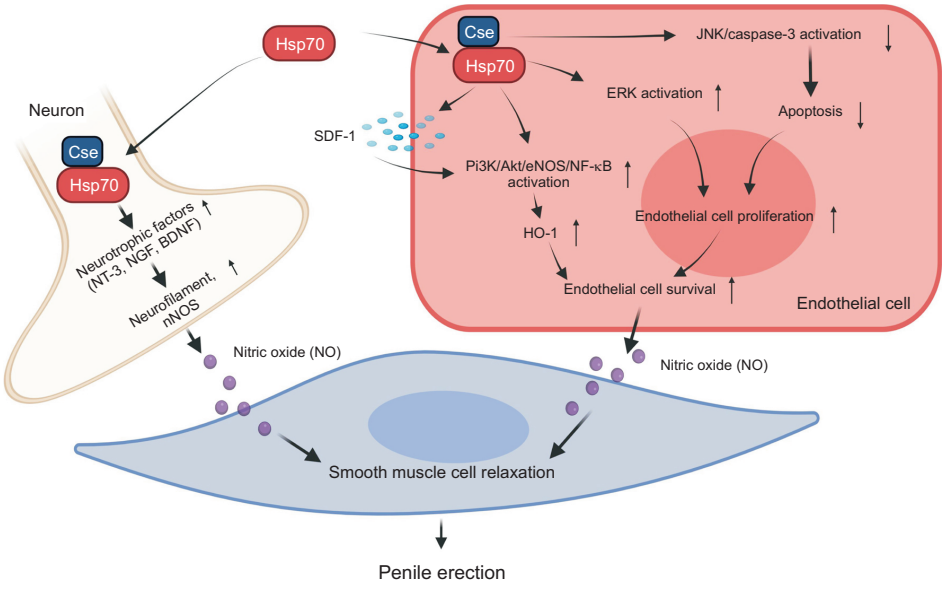

Fig. 7. Schematic depiction of the proposed mechanism for Cse-dependent improvement of diabetic erectile dysfunction by exogenous heat shock protein 70 (Hsp70). NT-3: neurotrophin-3, NGF: nerve growth factor, BDNF: brain-derived neurotrophic factor, nNOS: neuronal nitric oxide synthase, Cse: cystathionine gamma-lyase, SDF1: stromal cell-derived factor 1, HO-1: heme oxygenase-1, PI3K: phosphoinositide 3, Akt: protein kinase B, eNOS: endothelial nitric oxide synthase, NF-κB: nuclear factor kappa B, ERK: extracellular signal-regulated kinases, EC: endothelial cell, JNK: Jun N-terminal kinase.

generation and recovery of erectile function in diabetic mice were abolished by shCse lentiviral-mediated Cse knockdown. Thus, deciphering a novel functional role for Hsp70 and identifying its functional binding target may provide important clues that aid in the development of curative treatment modalities for diabetic ED.

Hsp70 has attracted considerable attention owing to its powerful anti-apoptotic, anti-inflammatory and antioxidant properties, as well as its role in regulation of cell survival [11,41,42]. Several studies have shown that Hsp70 protein and gene expression are reduced in patients with insulin resistance and hyperglycemia [43-45], and overexpression of Hsp70 through heat and pharmacological induction has been shown to inhibit obesity-related insulin resistance in rodents [43]. Recent work suggests that Hsp70 plays important roles in angiogenesis and neuroprotection *via* cell death pathways [35,46]. Our current study demonstrated that Hsp70 is highly expressed in blood vessels and nerves in mouse corpus cavernosum tissues, but its expression is decreased under diabetic conditions. Therefore, we hypothesized that Hsp70 plays a beneficial role in diabetic ED. To test our hypothesis, we exogenously administered Hsp70 protein into the penis of STZ-induced diabetic mice. Subsequent analyses showed that Hsp70 increased phosphorylation of eNOS, expression of endothelial cell-cell junction proteins, and migration and tube formation by MCECs; sprouting of microvessels from aortic rings and corpus cavernosum tissue; and expression of neurotrophic factors (NT-3, BDNF, and NGF) and neurite sprouting in MPG and DRG tissues. These effects synergistically rescued vascular and nerve abnormalities in penile tissues of STZ-induced ED mice and ultimately improved erectile function. These findings indicate that local administration of Hsp70 may be a promising strategy for the treatment of vascular and neuronal complications associated with diabetic ED. In agreement with these findings, overexpression of Hsp70 in Hsp70-Tg mice rescued erectile function through enhanced angiogenesis and neural regeneration under diabetic conditions.

Previous studies have shown that Hsp70 regulates endothelial cell function through PI3K/Akt/eNOS signaling [47] and promotes neuronal survival after central nervous system and peripheral nervous system injury [48,49]. However, detailed mechanisms underlying the role of Hsp70 in the diabetic ED model have not yet been unexplored. Accordingly, we used RNA sequencing technology to analyze the constantly changing cellular transcriptome, identifying Cse as a novel target of Hsp70. Cse is one of the three enzymes that are key for the endogenous production of hydrogen sulfide (H_2S). Numerous studies conducted over the past two decades have shown that H_2S stimulates angiogenesis [50] and relaxes smooth muscle [51], reduces inflammation and mortality after myocardial infarction [52], and protects neurons from oxidative stress [53]. In the current study, we demonstrated that Hsp70 specifically binds to Cse in a concentration-dependent manner. Knockdown of Cse abrogated Hsp70-driven

neurovascular regeneration and subsequent recovery of erectile function under diabetic conditions. In addition, an analysis of MCEC-conditioned medium using a cytokine antibody array demonstrated that secretion of SDF1, also known as CXCL12, is also regulated by Hsp70 in a Cse-dependent manner under high-glucose conditions. Collectively, our results demonstrate that the ubiquitous chaperone protein, Hsp70, exerts its neurovascular regeneration function *via* specific binding to Cse under hyperglycemic conditions.

The structure of blood vessels and nerve fibers show similar patterns and run side by side in certain locations, suggesting that the wiring mechanisms of nerves and blood vessel networks are similar [54]. In addition, many factors, such as BDNF, NT-3, VEGF, angiopoietin, transforming growth factor (TGF)-β1 and hepatocyte growth factor, among others [55], have dual effects on the development of the nervous system and the vascular system. It has been reported that some angioneurins, such as BDNF, NT-3, and VEGF, activate the PI3K/Akt/eNOS/NF-κB pathway p65 to induce vascular endothelial responses and the growth of axons and dendrites [56-58]. Interestingly, our current research showed that Hsp70 is a new type of angioneurin, and that specific binding of Hsp70 to Cse effectively activates SDF1/HO-1/PI3K/Akt/eNOS/NF-κB p65 signaling pathways in the mouse penis *in vivo* and cultured MCECs *in vitro*. Further studies are necessary to confirm that this phenomenon also operates in neuronal cells under hyperglycemic conditions. The detailed mechanism of action of exogenous Hsp70 in improving diabetic ED is summarized in Fig. 7. Our study has some limitations. First, the number of samples in each groups of western blot is too small. Second, we did not directly measure NO levels in mouse penis.

CONCLUSIONS

In summary, our comprehensive study of Hsp70, in which we identified a new Hsp70 target, performed a cell-based functional characterization, elucidated underlying cell signaling networks and examined the therapeutic potential of Hsp70 in *in vivo* and *in vitro* models under hyperglycemic conditions, provides new insights into Hsp70 function. Notably, we demonstrated that exogenous Hsp70 is capable of restoring damaged erectile tissues through the SDF1/HO-1/PI3K/Akt/eNOS/NF-κB p65 signaling pathway *via* binding to Cse, thereby enhancing angiogenesis and neural regeneration. As a new angioneurin molecule, the Hsp70 protein, administered by local injection, may offer a paradigm shift for the development of new therapies, not only for ED, but also for other ischemic vascular and/or neurological diseases.

Conflict of Interest

The authors have nothing to disclose.

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Author Contribution

Conceptualization: KG, GNY, JKS, JKR. Data curation: KG, GNY. Formal analysis: KG, GNY. Funding acquisition: GNY, SSH, JHK, JKR. Investigation: KG, GNY. Methodology: KG, GNY. Project administration: JKS, JKR. Resources: KG, GNY. Software: GNY. Supervision: JKS, JKR. Validation: GNY, JKS, JKR. Visualization: JKS, JKR. Writing – original draft: KG, GNY. Writing – review & editing: JKS, JKR.

Data Sharing Statement

The data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

Supplementary Materials

Supplementary materials can be found *via* https://doi.org/10.5534/wjmh.210249.

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