ORIGINAL RESEARCH

Serum-Derived Small Extracellular Vesicles From Diabetic Mice Impair Angiogenic Property of Microvascular Endothelial Cells: Role of EZH2

Zhongjian Cheng 🆔, PhD; Venkata Naga Srikanth Garikipati, PhD; May M. Truongcao, BS; Maria Cimini, PhD; Grace Huang, MS; Chunlin Wang, BS; Cindy Benedict, PhD; Carolina Gonzalez, MS; Vandana Mallaredy 🆔, PhD; David A. Goukassian, MD, PhD; Suresh K. Verma, PhD; Raj Kishore, PhD

BACKGROUND: Impaired angiogenic abilities of the microvascular endothelial cell (MVEC) play a crucial role in diabetes mellitus–impaired ischemic tissue repair. However, the underlying mechanisms of diabetes mellitus–impaired MVEC function remain unclear. We studied the role of serum-derived small extracellular vesicles (ssEVs) in diabetes mellitus–impaired MVEC function.

METHODS AND RESULTS: ssEVs were isolated from 8-week-old male db/db and db/+ mice by ultracentrifugation and size/number were determined by the Nano-sight tracking system. Diabetic ssEVs significantly impaired tube formation and migration abilities of human MVECs. Furthermore, local transplantation of diabetic ssEVs strikingly reduced blood perfusion and capillary/arteriole density in ischemic hind limb of wildtype C57BL/6J mice. Diabetic ssEVs decreased secretion/expression of several pro-angiogenic factors in human MVECs. Mechanistically, expression of enhancer of zest homolog 2 (EZH2), the major methyltransferase responsible for catalyzing H3K27me3 (a transcription repressive maker), and H3K27me3 was increased in MVECs from db/db mice. Diabetic ssEVs increased EZH2 and H3K27me3 expression/activity in human MVECs. Expression of EZH2 mRNA was increased in diabetic ssEVs. EZH2-specific inhibitor significantly reversed diabetic ssEVs-enhanced expression of EZH2 and H3K27me3, impaired expression of angiogenic factors, and improved blood perfusion and vessel density in ischemic hind limb of C57BL/6J mice. Finally, EZH2 inactivation repressed diabetic ssEVs-induced H3K27me3 expression at promoter of pro-angiogenic genes.

CONCLUSIONS: Diabetic ssEVs impair the angiogenic property of MVECs via, at least partially, transferring EZH2 mRNA to MVECs, thus inducing the epigenetic mechanism involving EZH2-enhanced expression of H3K27me3 and consequent silencing of pro-angiogenic genes. Our findings unravel the cellular mechanism and expand the scope of bloodborne substances that impair MVEC function in diabetes mellitus.

Key Words: angiogenesis ■ diabetes ■ enhancer of zest homolog 2 ■ pro-angiogenic factor ■ serum-derived small extracellular vesicles

D iabetes mellitus is one of the most prevalent metabolic disorders. There are 34.2 million Americans who have diabetes mellitus and 88 million American adults who have prediabetes mellitus (National Diabetes Statistics Report, 2020). A patient with diabetes mellitus has a 2- to 4-fold increased risk of developing cardiovascular diseases compared with a patient without diabetes mellitus.[1] Ischemic tissue injury, such as myocardial infarction, sudden cardiac death, stroke,

CLINICAL PERSPECTIVE

What is New?

- Diabetic serum-derived small extracellular vesicles impair tube formation/migration of microvascular endothelial cells in vitro and blood flow/capillary density in ischemic hind limb of wildtype C57BL/6J mice that are rescued by inactivation of enhancer of zest homolog 2.
- This study unravels a cellular mechanism by which serum-derived small extracellular vesicles from diabetic mouse independently impair angiogenic property of microvascular endothelial cells via, at least partially, epigenetic mechanism involving enhancer of zest homolog 2–enhanced expression of H3K27me3 and consequent silencing of pro-angiogenic genes.

What Are the Clinical Implications?

- Serum-derived small extracellular vesicles may become a valuable diagnostic tool for diabetes-impaired microvascular endothelial cell function/biology.
- EZH2 in serum-derived small extracellular vesicles may be a novel therapeutic target for ischemic tissue injury in diabetes mellitus.

Nonstandard Abbreviations and Acronyms

Ang1	angiopoietin 1
Ang2	angiopoietin 2
EC	endothelial cell
EVs	extracellular vesicles
EZH2	enhancer of zest homolog 2
FGF2	fibroblast growth factor 2
H3K27me3	tri-methylation at the 27th lysine residue of the histone H3 protein
HMVEC	human microvascular endothelial cell
IHL	ischemic hind limb
MVEC	microvascular endothelial cell
sEVs	small extracellular vesicles
ssEVs	serum-derived small extracellular vesicles

refractory wound and critical ischemic limb, where oxygen supplement is insufficient because of impaired growth of blood vessels (angiogenesis/neovascularization), is one of the major cardiovascular complications in patients with diabetes mellitus.[2] As angiogenesis in vivo is initiated in microvessels, loss of microvascular endothelial cell (MVEC) function after ischemic injury will impair and delay the growth of blood vessels.[3] We have provided strong evidence that MVEC function and angiogenesis are critical for ischemic heart and limb repair.[4-7] We and others also reported that MVEC dysfunction is involved in the pathogenesis of ischemic hind limb (IHL) in db/db mice.[5,8] Despite these observations, the underlying mechanisms of diabetes mellitus–impaired MVEC function remain incompletely understood.

Recent studies indicate that small membrane-derived extracellular vehicles ranging 30 to 120 nm (sEVs, exosomes) facilitate intercellular communication under various disease processes.[9,10] sEVs derived from different conditions contain distinct functional factors with either detrimental or beneficial properties. Under physiological condition, sEVs promote angiogenesis and tissue regeneration by transferring functional cargo (ie, proteins, mRNAs, and lipids), thereby regulating the bioactivity of the recipient cells.[11] We have reported that sEVs derived from embryonic stem cells and endothelial progenitor cells of healthy mice and human CD34+ stem cells significantly improved neovascularization and cardiac function following myocardial infarction,[6,12-14] whereas sEVs derived from interleukin-10 knockout endothelial progenitor cells impaired MVEC and heart function post–myocardial infarction.[6,13] Altered level, cargo, and function of sEVs have been implied in the pathogenesis of cardiovascular disease in patients with diabetes mellitus and animals with diabetes mellitus.[11,15,16] Under diabetic/hyperglycemic conditions, sEVs released by various type of cells, such as peripheral blood mononuclear cells,[17] cardiomyocytes,[18] adipocytes,[19] and pancreatic β-cells,[20] yield a deleterious impact on angiogenesis and cell survival.

Since ECs locate in the inner layer of vessels and are constantly in contact with the contents in blood, there is a great potential that EC function is affected upon uptake of the circulating sEVs. Indeed, studies reported that serum/plasma sEVs from healthy donors improved angiogenic property of ECs and blood flow in IHL of C57BL/6J mice.[21] Recent studies reported that, under diabetic condition, circulating sEVs have detrimental effects on EC function/biology in macrovasculature, such as aorta.[22,23] Circulating sEVs from patients with diabetes mellitus induced alterations of morphology and migratory activity of human aortic ECs (macrovascular ECs).[22] Serum-derived sEVs (ssEVs) from db/db mice attenuated acetylcholine-induced endothelium-dependent relaxation in the macrovascular aorta from db/+ mice because of modulation of contents in ssEVs.[23] Whether modulated contents in diabetic ssEVs impair the angiogenic property of MVECs and the underlying mechanisms remain unclear. We hypothesized that diabetic ssEVs impair MVEC function via delivery of the modulated contents.

The present study demonstrates that ssEVs from db/db mice impair angiogenic properties of MVECs, blood flow, and vessel density in IHL of nondiabetic C57BL/6J mice. Further investigation unravels a cellular mechanism by which ssEVs from diabetic mouse impair MVEC function via, at least partially, epigenetic mechanism upregulation/activation of involving enhancer of zest homolog 2 (EZH2), the major methyltransferase responsible for catalyzing tri-methylation at the 27th lysine residue of the histone H3 protein (H3K27me3), –induced expression of H3K27me3 and consequent silencing of pro-angiogenic genes. Our findings expand the scope of bloodborne substances that impair MVEC function in diabetes mellitus.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request. Studies did not use any human subjects or materials and were exempted from Institutional Review Board approval.

Animal Groups

Male wildtype C57BL/6J db/db mice, and their control littermate db/+ mice (nondiabetic) at 8-10-weeks-old, were obtained from Jackson Laboratories. The phenotypes of severe obesity, hyperglycemia, hyperinsulinemia, and hyperlipidemia in db/db mice are because of a spontaneous homozygous mutation of leptin receptor (Lep$^{db/db}$). All animal experiments were approved by the Institutional Animal Care and Use Committee of Temple University. Induction of IHL in C57BL/6J was performed by ligation of the left femoral artery as described previously.[5] ssEVs were collected, ultrapurified, and quantified by Nano-sight (NS300) as described previously.[14] We had 2 sets of in vivo experiments. In Set 1, we examined the effects of diabetic ssEVs on IHL recovery in C57BL/6J mice. ssEVs from db/+ or db/db mice were injected intramuscularly into wildtype C57BL/6J mice immediately after induction of IHL (3×10^9 particles/limb) as described previously.[24] In Set 2, we examined effects of inactivation of EZH2 in diabetic ssEVs-impaired IHL recovery in C57BL/6J mice. ssEVs from db/db mice were injected into wildtype C57BL/6J mice intramuscularly immediately after induction of IHL (3×10^9 particles/limb). The EZH2-specific inhibitor 3-deazaneplanocin (DZNep), an S-adenosylhomocysteine hydrolase inhibitor that depletes the intracellular level of EZH2 and has been extensively used in in vivo experimental studies, was administered by intraperitoneal injection starting from 1 day before IHL surgery and continuing to the end of experiments as described previously (1.5 mg/kg per day, every 2 days).[25,26] DZNep was diluted with 100% dimethyl sulfoxide (DMSO) and then further diluted by saline (final concentration of DMSO was 1%).[25,26] Mice administered with vehicle (Vehi., 1% DMSO) severed as controls. Blood flow was measured by laser Doppler perfusion imager before (pre-) and immediately after surgery (day 0), and on postligation days 3, 7, 14, and 21 as described previously.[5] Blood perfusion in the IHL was presented as ratio of blood flow of ischemic to nonischemic limb.[5] At the end of experiments, thigh muscles from ischemic hind limbs were dissected and stored in −80°C for immunostaining.

Isolation of ssEVs

Blood was collected from the carotid aorta from male db/+ or db/db mice at the age of 8-to-10 weeks with the animals under anesthesia, as described previously.[5] Serum was collected from the blood after 15 minutes centrifugation (1008g, 4°C) and stored in −80°C after passed through a 0.2-µm syringe filter. ssEVs were collected and ultrapurified as we described previously.[12,14] Briefly, the serum was clarified by centrifugation (14 000g for 20 minutes) and the sEVs were collected by ultracentrifugation (100 000g for 1 hour) on a 30% sucrose-D2O solution (density ≈1.127 g/cm³), washed with 1× phosphate-buffered saline, and pelleted. Then the purified sEVs fraction was resuspended in 1× phosphate-buffered saline for use. Quality/quantity of sEVs was evaluated using a Nanosight instrument (NS300) and by Western blot with antibody against polyclonal rabbit flotillin-1 (1:1000, Cat. #: 3253, Cell Signaling) or monoclonal mouse tsg 101 (1:200, Cat. #: SC-7964, Santa Cruz).

Cell Culture and Treatment

Human microvascular endothelial cells (HMVECs) and mouse primary MVECs were cultured as we described previously.[5,8,27] HMVECs were treated with or without ssEVs (10^8 particles/mL, 48 hours) in exosome-free FBS (10%) culture medium (EBM-2, CC-3156 plus CC-4147 excluded FBS, Lonza) for 48 hours after 6 hours starvation (0% FBS). To explore the role of EZH2, HMVECs were treated with EZH2-specific inhibitor GSK343 (0.1 µM) for 48 hours. HMVECs treated with the standard culture medium (EBM-2, CC-3156 plus CC-4147, Lonza) in the presence of 10% exosome-free FBS with or without vehicle (vehicle 0.1% DMSO) served as respective controls.

Tube Formation Assays

Tube formation of HMVECs was performed as we described previously.[5-7] Briefly, 4×10^4 HMVECs were

plated on 120 µL matrigel (BD Falcon) in a 48-well plate. After incubation at 37°C in an atmosphere of 5% CO_2 for 16 hours, the tubes were observed and photographed using a high-power phase contrast microscope (Nikon, TS100). The tube formation was counted by number of branches in 5 random/high-power vision fields using ImageJ.

Migration Assays

Migratory activity of HMVECs was performed using a modified Boyden chamber (Coning, Cat. #: 3422) as we described previously.[5,6] Briefly, we placed 100 µL basal medium (EBM-2, Cat. #: CC-3156, Lonza) in the upper chamber and 750 µL EBM-2 medium containing EGM-2mv bullet kit (Cat. #: CC-4147, Lonza) in the lower chamber, respectively. HMVECs were suspended in 200 µL basal medium and then placed in the upper chamber. After 16 hours incubation, the cells on the upper surface were removed and the cells on the underside were fixed with 3.7% formaldehyde and stained with Giemsa. The magnitude of HMVEC migration was evaluated by counting the migrated cells in 5 random/high-power vision fields by ImageJ.

Immunohistochemistry

Muscle sections (5-µm thickness) were heated with citrate buffer at 95°C for 40 minutes for antigen retrieval. After blocking with 5% BSA in phosphate-buffered saline,,muscle sections were co-incubated with polyclonal goat antibody against CD31 (1:30, Cat. #: AF3628, R&D) and monoclonal mouse antibody against α-smooth muscle actin (1:1000, Cat. #: A2547, Sigma) overnight at 4°C. For every section, a negative control without primary antibody was processed simultaneously. After 15 minutes of washing in 1x phosphate-buffered saline, secondary antibodies were added for 1 hour at room temperature (1:200). Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Molecular Probes). CD31 and α-smooth muscle actin were stained with fluorescent secondary antibody against mouse (red) and rabbit (green), repetitively. The capillary and arteriole density were then determined by counting the number of capillaries and arterioles in each section of muscle. Images were taken by a fluorescent microscope (Nikon TIE3000 or NIE) and counted by ImageJ.

To study whether EZH2 expression was enhanced in diabetic mouse MVECs, we examined EZH2 and its downstream target H3K27me3 level in MVECs from lungs of db/+ and db/db mice. The MVECs from db/+ and db/db mice were co-incubated with mouse monoclonal antibody against EZH2 (1:200, Invitrogen, Cat. #: MA5-15101) and rabbit polyclonal antibody against H3K27me3 (1:200, Cell Signaling, Cat. #: 9733) overnight. EZH2 and H3K27me3 were stained with fluorescent secondary antibody against mouse (green) and rabbit (red) for 1 hour, respectively. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Molecular Probes). Images were taken by confocal microscopy (Zeiss LSM 710 confocal). EZH2 and H3K27me3 expression was counted by ImageJ.

Angiogenic Protein Profile Array

To explore whether diabetic ssEVs impair secretion of pro-angiogenic factors in MVECs, we examined angiogenic factors in conditioned culture medium in HMVECs using Proteome Profiler Human Angiogenesis Array Kit (Cat. #: ARY007, R&D System) according to the manufacturer's protocol. The HMVECs were treated with ssEVs from either db/+ or db/db mice for 48 hours. To explore the role of EZH2 in diabetic ssEVs-impaired secretion of pro-angiogenic factors, HMVECs were treated with diabetic ssEVs plus EZH2 specific inhibitor GSK343 (0.1 µM) for 48 hours. Quantification of selected angiogenic factors in culture medium of HMVEC was carried out by densitometry using ImageJ.

Isolation of Primary MVECs From Mice

Primary MVECs were isolated from mouse lungs as we described previously.[8,27]

ssEVs Uptake by HMVECs

Uptake of ssEVs by HMVECs was examined by treating the HMVECs with Cy3-tagged ssEVs. The ssEVs were tagged with Cy3 by loading Cy3 siRNA using Exo-Fect siRNA/miRNA Transfection Kit (Cat. #: EXFT200A-1, System Biosciences) according to the manufacturer's protocol. Then HMVECs were treated with Cy3-tagged ssEVs (10^8 particles/mL) for 48 hours. Uptake of Cy3-tagged ssEVs was evaluated using a fluorescent microscope (Nikon TE3000).

Epigenetic Chromatin Remodeling Factor Polymerase Chain Reaction Array

Total RNA of MVECs from db/+ and db/db mice was purified and then qualified/quantified by Agilent. Epigenetic chromatin remodeling factors in MVECs of db/+ and db/db mice were examined using RT^2 profiler TM polymerase chain reaction (PCR) array mouse epigenetic chromatin remodeling factor kit (PAMM-086ZC-6, Qiagen) according to the manufacturer's protocol.

EZH2 Activity

EZH2 activity was measured in the nuclear fraction of HMVECs following the manufacturer's instruction with slight modification (P-3005, EPIGENTEK, Farmingdale,

NY). Briefly, HMVECs were cultured in a 10-cm dish and treated with ssEVs from either db/+ or db/db mice in the presence or absence of EZH2 inhibitor GSK343 (0.1 µM) for 48 hours. The nuclear fraction (4.5 µg) was incubated in a H3 substrate-coated microplate with a methyl donor that methylated the H3 at lysine 27 for 1 hour in the presence or absence of EZH2 inhibitor GSK343 (0.1 µM). Formation of H3K27me3 was detected using specific antibody against H3K27me3 provided in the kit. The EZH2 activity was counted by subtracting the histone methyltransferase activity in the absence of GSK343 by that in the presence of GSK343.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was used for exploring whether recruitment of H3K27me3 at promoter of pro-angiogenic factors was affected by diabetic ssEVs in HMVECs followed the manufacturer's protocol (Cat. #: 9002, Cell Signaling). HMVECs were treated with ssEVs from db/+ or db/db mice (10^8 particles/mL) in the presence or absence of GSK343 (0.1 µM) for 48 hours. Binding of H3K27me3 (Cat. #: 9733S, Cell Signaling Technologies) to specific pro-angiogenic factor angiopoietin 1 (Ang1), artemin, and fibroblast growth factor 2 (FGF2) was examined by real-time polymerase chain reaction (RT-PCR) with 4 promoter primers per target (Table 1). The promoter primers were synthesized by Integrated DNA Technologies (IDT). Statistical analysis of RT-PCR results was processed either with each primer or with a combination of the 4 primers as counted with area under the curve.

Quantitative RT-PCR

To examine the effects of diabetic ssEVs on pro-angiogenic gene expression in HMVECs, transcripts of Ang1, artemin, and FGF2 were quantified in HMVECs treated with nondiabetic or diabetic ssEVs

(10^8 particles/mL, 48 hours) by RT-PCR as described previously.[28] To examine the role of EZH2 in diabetic ssEVs-regulated pro-angiogenic gene expression, the HMVECs were treated with diabetic ssEVs plus GSK343 (0.1 µM) for 48 hours. HMVECs treated with diabetic ssEVs plus DMSO (0.1%) served as controls. EZH2 mRNA level was also examined in ssEVs from db/+ and db/db mice by RT-PCR. RNA was collected with Trizol RNA isolation kit then reverse transcribed with iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Amplification was performed using a SYBR Green (Applied Biosystems, Foster City, CA). The primer sequences were synthesized by IDT (Table 2). Relative mRNA expression of target genes was normalized to the endogenous GAPDH or RSP13 gene (Applied Biosystems, Foster City, CA) and represented as % versus respective controls.

Western Blot

Protein extracted from the ECs (10–20 µg) or ssEVs (5–6 µg) was used for separated by 9% SDS-PAGE and transferred to nitrocellulose blot. Selected protein level was detected by using primary antibodies: polyclonal rabbit flotillin-1 (1:1000, Cat. #: 3253, Cell Signaling); monoclonal mouse tsg 101 (1:200, Cat. #: SC-7964, Santa Cruz); polyclonal rabbit H3K27me3 (1:1000, Cat. #: 9733, Cell Signaling); polyclonal rabbit H3, (1:1000, Cat. #: 4499T, Cell Signaling); polyclonal rabbit EZH2 (1:1000, Cat. #: 4, Cell Signaling or Cat. #. 191080, Abcam); and respective second antibodies. Signals were detected and quantified using Odyssey Fc Imaging System (LI-COR Biosciences, model number 2800).

Chemicals

GSK343 was diluted by DMSO (0.1 µM, final concentration of DMSO was 0.1%). ECs treated with

Table 1. Primer Sequences for RT-PCR

Primer Name	ID	Forward Primer (5'-3')	Reverse Primer (5'-3')
ANG1	1	AAGCCATCAGCAATCCTTAGT	AGACCCTTTCCTCTACCCTATC
ANG1	2	TCTTAGGGTCCCTCGACTAAA	TGTGCATGCTGACCTACTAAA
ANG1	3	GGAACTTCCACACCAGTGTTA	CAGAATGCAACCCTTCATGTTC
ANG1	4	GGCTGTTAGCACACCTGATA	GGTGGCTATGGCTGTCTTTA
ARTN	1	CTACCTACCTCCTCACCCTATC	TGGATGACTCTGGCTGTAGTA
ARTN	2	TGATTTGGGTCTGGGTTGAG	CGCTGGCAAAGGCAATTT
ARTN	3	CACCTGCCAAATTGCCATAAT	AGGATCTGACTCTGCTCTAGTC
ARTN	4	CAGCCAGAGTCATCCACAAA	GGGTGAGTAACATGGATGGAAA
FGF2	1	CTCATCTTTCTGCGTCGTCTAA	GCTGAAGTCGTCTACCAACAA
FGF2	2	CTCTGACACAGACTCTTCCTTG	CCAAACCACGTTTAGGCTTTC
FGF2	3	ATCTCCCACACACTCAACATTAT	TAACTCGGACCTGGCATTTG
FGF2	4	GGCACCTGCTATATCCTACTGA	GGAGCAGATTGGGTTGCTAAA

ANG1 indicates angiopoietin; ARTN, artemin; FGF, fibroblast growth factor 2; and RT-PCR, real-time polymerase chain reaction.

Table 2. Primer Sequences for RT-PCR

Primer Name	Forward Primer (5'-3')	Reverse Primer (5'-3')
ANG1	CAGAAAACAGTGGGAGAAGATATAACC	TGCCATCGTGTTCTGGAAGA
ARTN	TTTGCAGACTGGACCCTTAC	TCCTTCAAATGCTGTCCCTAC
FGF2	CTGCTGGGGGTCTACCAAG	CTGCGCCTACCACTGTTCC
EZH2	AGTGACTTGGATTTTCCAGCAC	AATTCTGTTGTAAGGGCGACC

ANG1 indicates angiopoietin; ARTN, artemin; EZH2, enhancer of zest homolog 2; FGF2, fibroblast growth factor 2; and RT-PCR, real-time polymerase chain reaction.

0.1% DMSO (vehicle) served as control. If not otherwise specified, all chemicals were purchased from Sigma-Aldrich.

Statistical Analysis

Results are expressed as the mean±SEM. In vitro studies were repeated at least 3 times with triplicates/group per experiment. Statistical significance between 2 groups was examined using unpaired Student t test. Nonparametric Mann-Whitney tests were used when normality test was not passed and/or sample size was <4. Comparison of ≥3 groups was performed by 1-way ANOVA followed by Newman-Keuls post hoc multiple comparisons tests. Comparison of different groups on blood perfusion measured by laser Doppler imaging at different time points post-IHL was performed by 2-way ANOVA followed by Bonferroni post hoc tests. Statistical analysis was performed using Graph Pad Prism v 7.0 software. A probability value $P<0.05$ was considered to be significant.

RESULTS

Diabetic ssEVs Impaired Angiogenic Property of ECs

Nano-sight data revealed small EVs based on their size (average size ≈126.8 nm, Figure 1A, left panel), therefore we labeled them as small EVs (sEVs). In addition, Western blotting, using sEV surface marker flotillin-1 and tsg101 antibodies, was performed for examining the presence and quality of ssEVs. The Western blot showed that, compared with cellular extracts, both flotillin-1 and tsg101, but not GAPDH, were strongly expressed in the ssEVs but not cells that confirmed the presence and quality of ssEVs (Figure 1A, right panel). We also noticed that ssEVs concentration in male db/db mice was similar to the age- and sex-matched db/+ mice (Figure 1B). Furthermore, we examined the uptake of ssEVs by HMVECs by treating the cells with Cy3-labeled ssEVs (10^8 particles/mL) for 48 hours. Cy3-labeled ssEVs were properly taken by HMVECs (Figure 1C), suggesting ssEVs were able to enter into MVECs.

We examined the effects of diabetic ssEVs on MVEC function by tube formation and migration assay 48 hours posttreatment and observed that ssEVs from db/db mice significantly reduced tube formation and migratory activity of HMVECs (Figure 1D and 1E, $P<0.05$).

Diabetic ssEVs Impaired Blood Flow and Capillary Density in IHL of C57BL/6J Mice

We also examined the effects of diabetic ssEVs on blood flow recovery and capillary density in IHL of wildtype C57BL/6J mice. ssEVs from db/+ or db/db mice were administered by intramuscular injection immediately after ligation of the left femoral artery (3×10^9 particles/limb) as described previously.[24] We noticed that, compared with control ssEVs treatment, diabetic ssEVs significantly impaired blood perfusion and capillary/arteriole formation in IHL of C57BL/6J mice (Figure 2A and 2B, $P<0.05$).

Diabetic ssEVs Decreased Pro-Angiogenic Protein Secretion From HMVECs

A balance between pro- and anti-angiogenic factors secreted from MVECs is critical for controlling angiogenesis/neovascularization in response to ischemic tissue injury.[29] We next examined whether diabetic ssEVs regulate angiogenic factor secretion from HMVECs. Using angiogenic protein profile array, we found that diabetic ssEVs significantly decreased secretion of pro-angiogenic factor Ang1 and 2, artemin, FGF2, and insulin-like growth factor binding protein 1 and 2 (Figure 3, *$P<0.05$).

EZH2 and H3K27me3 Level Was Enhanced in MVECs From db/db Mice

Accumulating evidence suggests a link between epigenetic modification and the diabetes mellitus–impaired angiogenic property of MVECs.[26,30,31] To explore whether epigenetic modification is involved in the diabetic ssEVs-impaired angiogenic property of MVECs, we assessed epigenetic chromatin remodeling factors (enzymes and factors) in primary
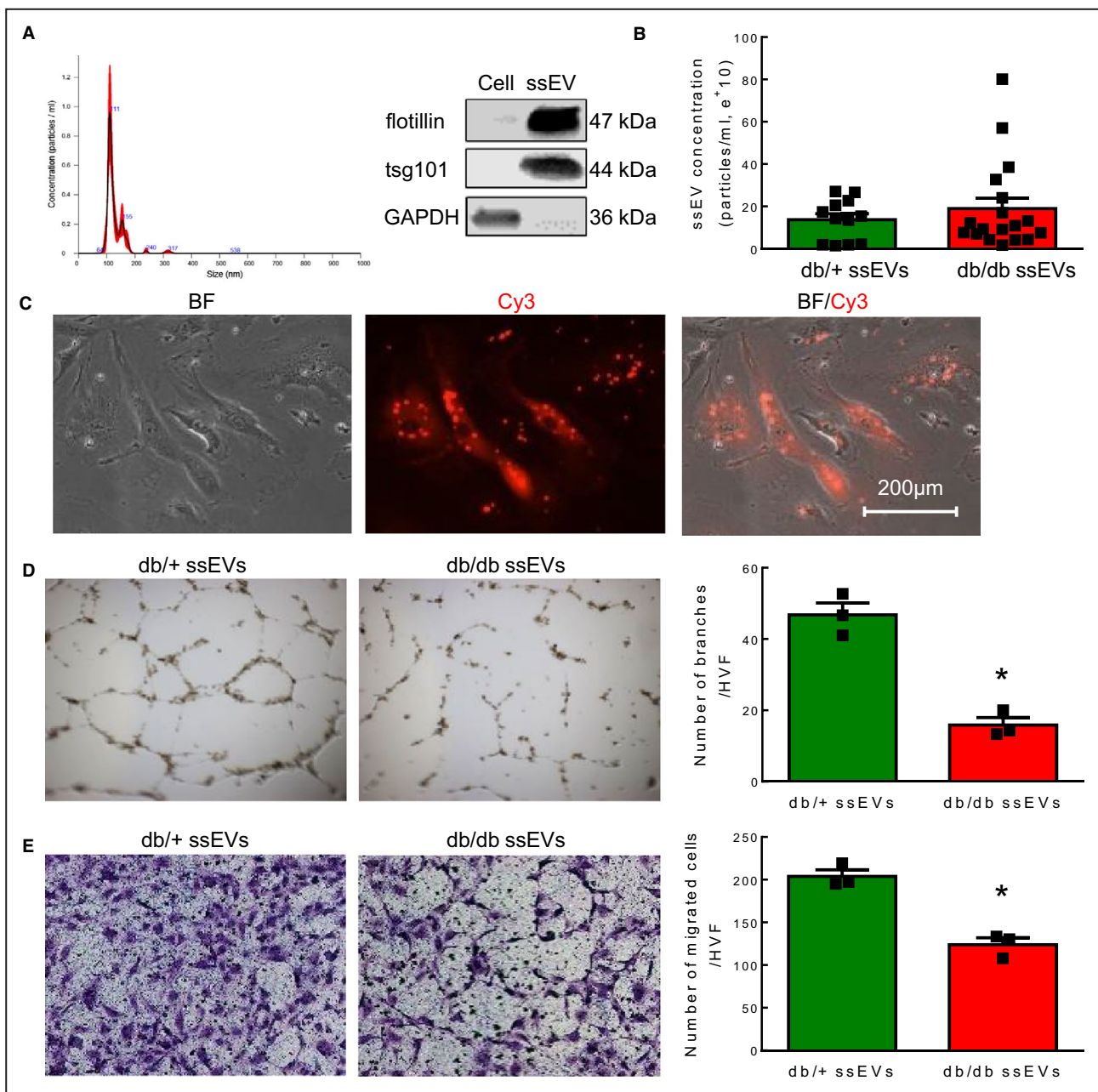
Figure 1. Diabetic ssEVs suppressed angiogenic properties of ECs.
A, Characterization of ssEVs isolated from serum using ultracentrifugation methods. Size and number of ssEVs were determined by Nano-sight instrument (**left panel**). Western blot analysis was performed in EV lysate for Nano-EV protein marker CD81 and flotillin. Cell lysate and GAPDH served as controls. **B,** ssEV concentration in db/db mice was similar to the age- and sex-matched db/+ mice (by Nano-sight). n=12–18. **C,** Image of uptake of ssEVs by HMVECs. The ssEVs were tagged with Cy3 siRNA using Exo-Fect siRNA/miRNA Transfection Kit (EXFT200A-1, System Biosciences). **D,** Diabetic ssEVs impaired tube formation activity of HMVECs. n=3. **E,** Diabetic ssEVs suppressed migratory activity of HMVECs. n=3. HMVECs were treated with ssEVs (10^8 particles/mL) for 48 hours in culture medium that contained 10% Exo-free FBS after 6 hours starving (0% FBS). Images were taken by fluorescent microscope (Nikon TIE3000 or NIE). *P<0.05 vs db/+ ssEVs-treated HMVECs. ECs indicates endothelial cells; FBS, fetal bovine serum; HMVECs, human microvascular endothelial cells; HVF, high-power visual field; and ssEVs, serum-derived small extracellular vesicles.

MVECs from db/+ and db/db mice by PCR arrays. Heat map of the epigenetic chromatin remodeling factor PCR array showed that several chromatin remodeling factors were increased in diabetic MVECs (Figure 4A, Figure S1). Within those factors,

the mRNA level of EZH2, a well-known histone methyltransferase capable of catalyzing histone H3 bi (me2)- and tri (me3)-methylation of lysine 27 (H3K27me2 and H3K27me3) in mammalian cells,[32] was significantly increased (35%) in MVECs from db/
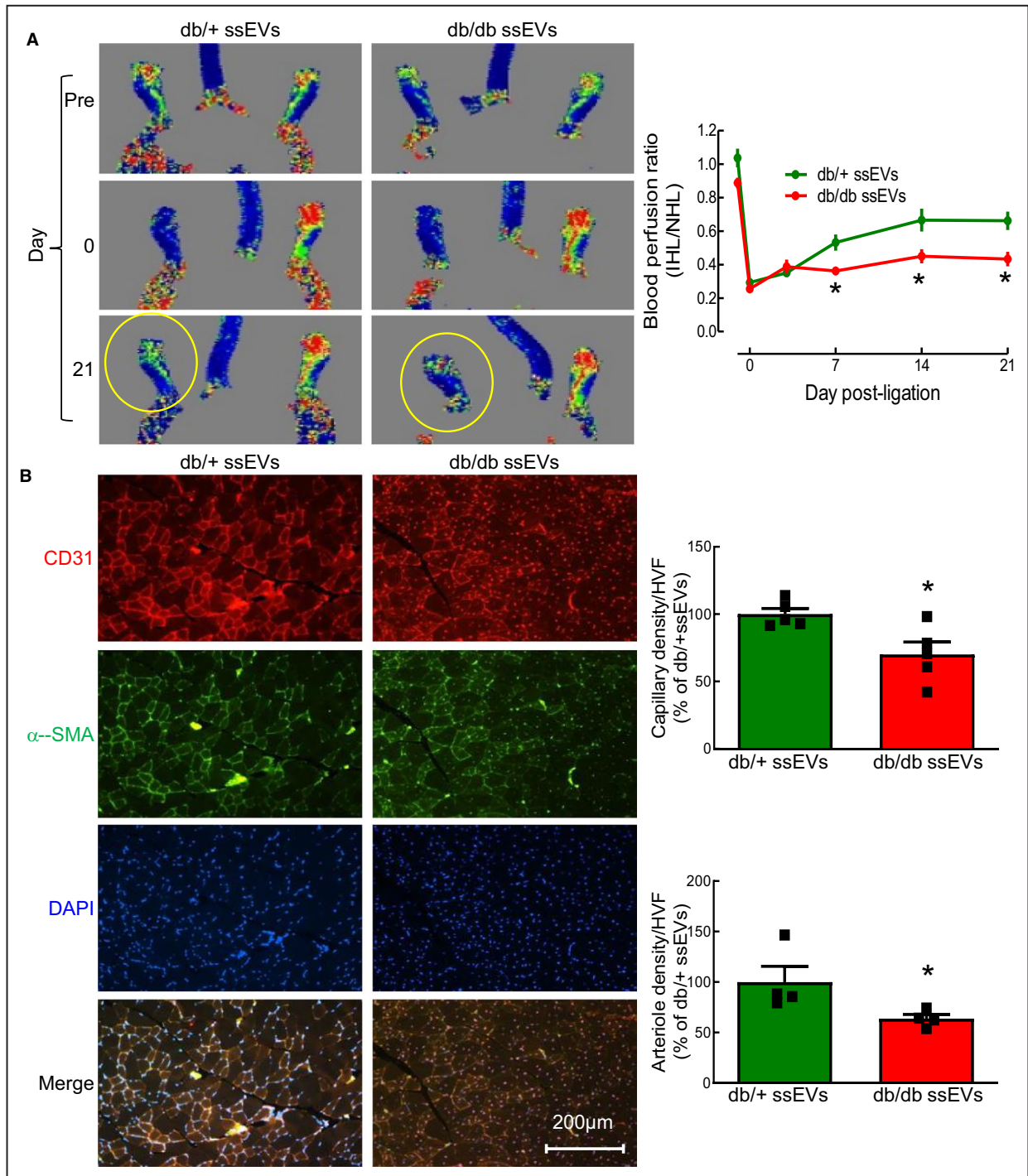
Figure 2. Diabetic ssEVs significantly suppressed blood flow recovery and capillary density in IHL of wildtype C57BL/6J mice.

A, Diabetic ssEVs impaired blood perfusion in IHL of C57BL/6J mice. n=7. **B,** Diabetic ssEVs decreased capillary (**upper right panel**) and arteriole (**lower right panel**) density in IHL of C57BL/6J mice. ssEVs (3×10^9 particles) from db/+ or db/db mice were injected intramuscularly immediately following ligation of left femoral artery ligation in 8-week-old male C57BL/6J mice. Blood perfusion in IHL was measured by Laser Doppler Imager at pre-, 0, 3, 7, 14, and 21 days postligation and expressed as ratio of blood perfusion of ischemic to nonischemic limb. Mice were euthanized and ischemic skeletal muscle was collected at 21 days postligation. **Left panel:** Representative images of blood perfusion at pre-, 0, and 21 days post-IHL. Skeletal muscle sections were co-immunostained with CD31/α-SMA antibodies for measurement of neovascularization in response to IHL injury. n=4–5. *$P<0.05$ vs IHL C57BL/6J mice injected with db/+ ssEVs. HVF indicates high-power visual field; IHL, ischemic hind limb; NHL, nonischemic hind limb; and ssEVs, serum-derived small extracellular vesicles.
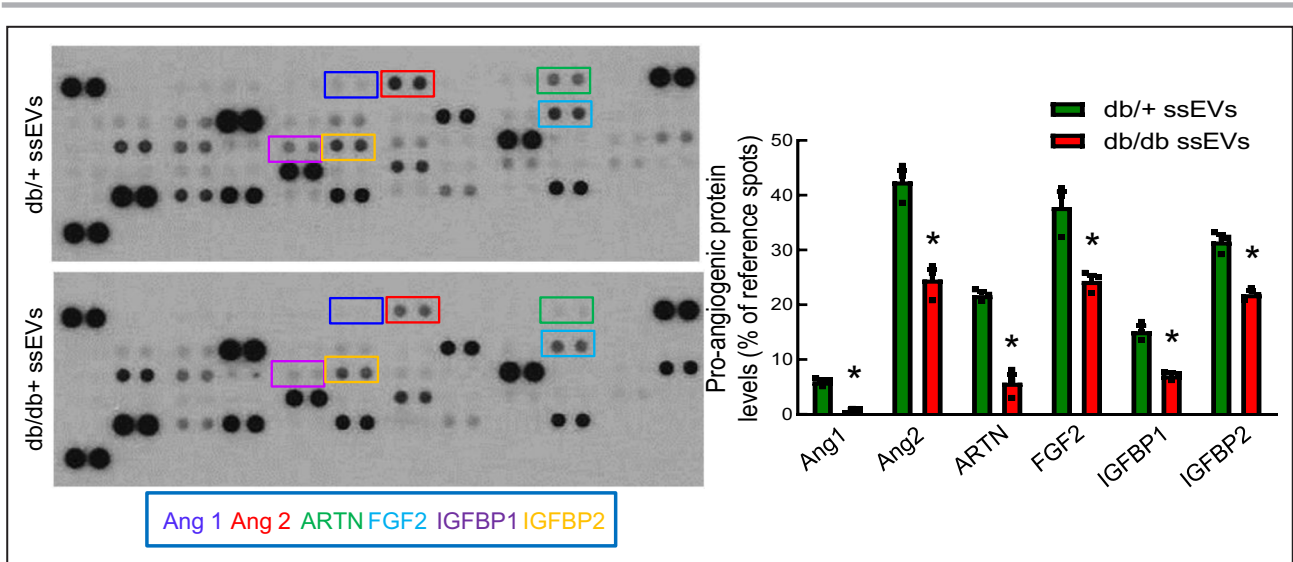
Figure 3. Diabetic ssEVs decreased secretion of pro-angiogenic proteins from HMVECs.
HMVECs were treated with ssEVs (10^8 particles/mL, 48 hours) either from db/+ or from db/db mice after 6 hours starving (0% FBS). The position of selected angiogenic factors in the membranes is marked with color-lined boxes. n=3. *$P<0.05$ vs db/+ ssEVs-treated HMVECs. Ang1 indicates angiopoietin 1; Ang2, angiopoietin 2; ARTN, artemin; FBS, fetal bovine serum; FGF2, fibroblast growth factor 2; HMVECs, human microvascular endothelial cells; IGFBP1, insulin-like growth factor binding protein 1; IGFBP2, insulin-like growth factor binding protein 2; and ssEVs, serum-derived small extracellular vesicles.

db mice compared with db/+ mice (Figure 4A, Figure S1). By immunostaining, we also observed that protein expression of EZH2 and H3K27me3 was significantly increased in the MVECs from db/db mice compared with db/+ mice (Figure 4B, $P<0.05$).

Diabetic ssEVs Increased EZH2 Expression/Activity in HMVECs

We next examined whether diabetes mellitus enhanced EZH2 and H3K27me3 expression and activity in MVECs. HMVECs were treated with either diabetic
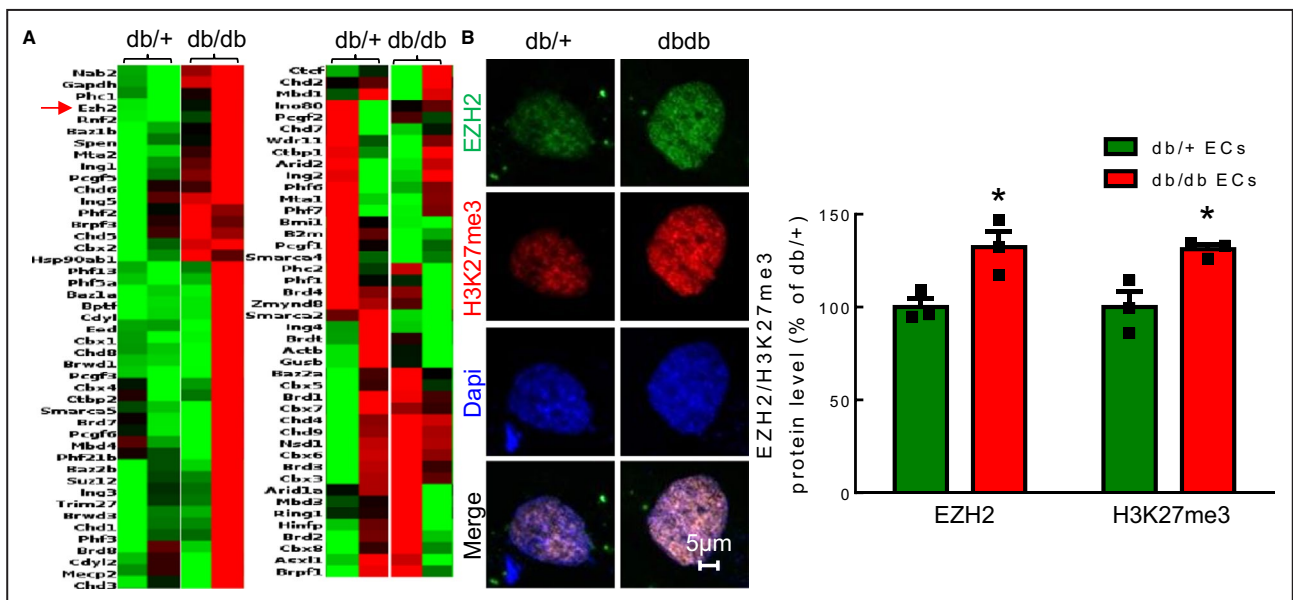

Figure 4. EZH2 and H3K27me3 expression was enhanced in primary MVECs from db/db mice.
A, EZH2 mRNA level was increased in MVECs from db/db mice. Heat map of the epigenetic chromatin remodeling factor PCR array (n=2). **B,** EZH2 and H3K27me3 protein expression was increased in MVECs from db/db mice. n=3. *$P<0.05$ vs db/+ mice. Dapi indicates 4',6-diamidino-phenylindole; EZH2, enhancer of zest homolog 2; H3K27me, tri-methylation at the 27th lysine residue of the histone H3 protein; MVECs, microvascular endothelial cells; and PCR, polymerase chain reaction.
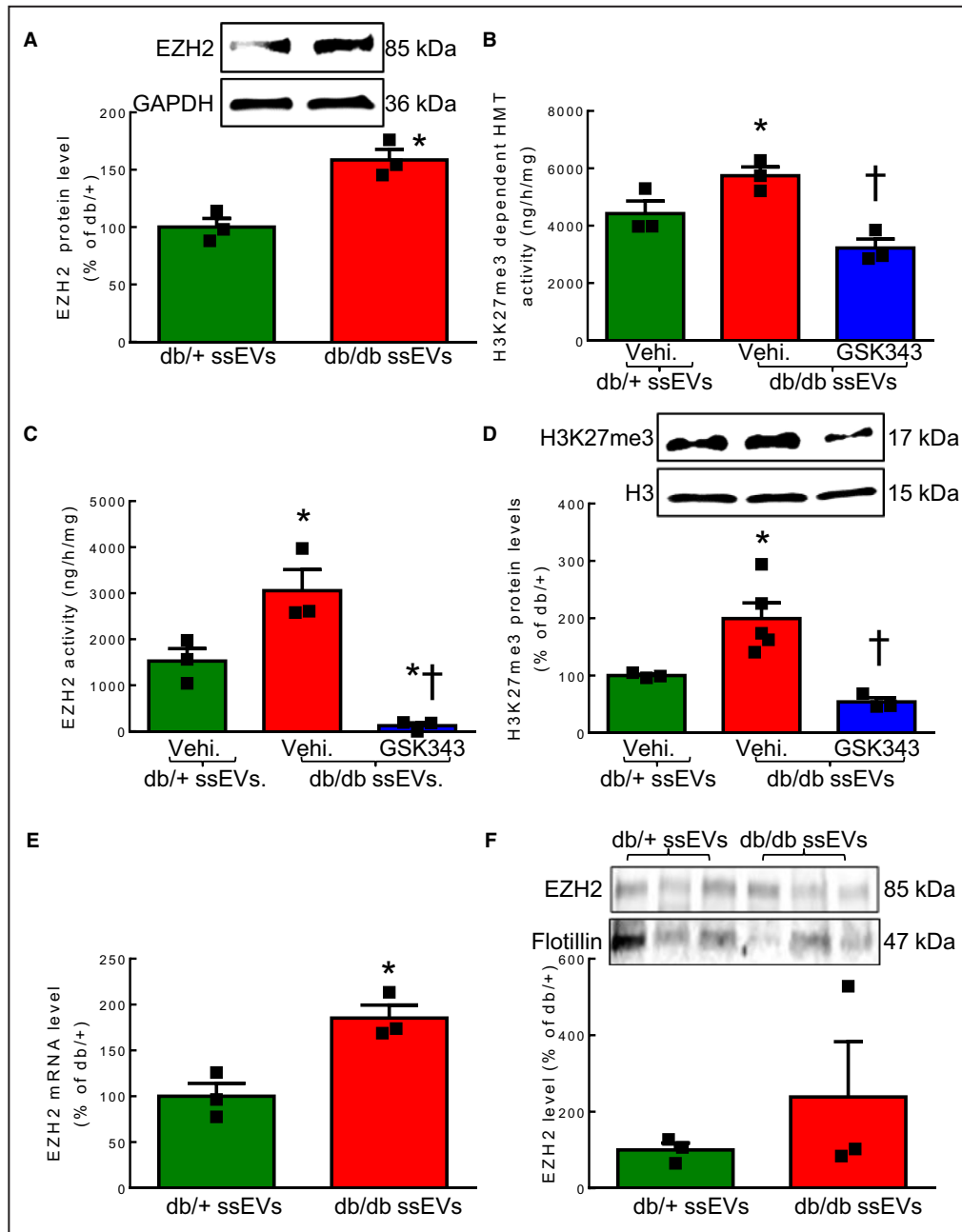
Figure 5. **Diabetic ssEVs increased expression/activity of EZH2 and H3K27me3 in HMVECs, which was reduced by EZH2 specific inhibitor GSK343.**

A, Diabetic ssEVs from db/db mice increased EZH2 protein level in HMVECs. n=3. **B,** GSK343 decreased diabetic ssEVs-enhanced HMT activity responsible for generation of H3K37me3 in HMVECs. n=3. **C,** GSK343 inhibited diabetic ssEVs-enhanced EZH2 activity in HMVECs. n=3. **D,** Diabetic ssEVs from db/db mice increased protein level of H3K27me3, which is mainly regulated by EZH2 in HMVECs. EZH2 inhibitor GSK343 reduced diabetic ssEVs-enhanced H3K27me3 level in HMVECs. HMVECs were treated with ssEVs from either db/+ or db/db mice (10^8 particles/mL in the presence or absence of GSK343 [0.1 µM] for 48 hours after starving for 6 hours [0% FBS]). n=3-5. **E,** EZH2 mRNA level was increased in ssEVs from db/db mice. n=3. **F,** EZH2 protein concentration was not significantly changed in ssEVs from db/db mice compared with db/+ mice. n=3. *$P<0.05$ vs db/+ ssEVs or db/+ ssEVs+Vehi-treated HMVECs; †$P<0.05$ vs db/db ssEVs+Vehi-treated HMVECs. EZH2 indicates enhancer of zest homolog 2; FBS, fetal bovine serum; H3K27me3, tri-methylation at the 27th lysine residue of the histone H3 protein; HMT, histone methyltransferase; HMVECs, human microvascular endothelial cells; ssEVs, serum-derived small extracellular vesicles; and Vehi., Vehicle.

or nondiabetic ssEVs for 48 hours. We observed that diabetic ssEVs significantly increased EZH2 protein expression in HMVECs compared with nondiabetic ssEVs (Figure 5A, $P<0.05$). We also examined the EZH2 methyltransferase activity in HMVECs treated with diabetic or nondiabetic ssEVs by EpiQuik Histone Methyltransferase Activity/Inhibition Assay Kit (H3K27) following the manufacturer's instruction (P-3005, EPIGENTEK, Farmingdale, NY). We found that diabetic ssEVs significantly increased H3K27me3-dependent histone methyltransferase and EZH2 activity in HMVECs compared with nondiabetic ssEVs (Figure 5B and 5C, $P<0.05$). EZH2 inhibitor GSK343 markedly reduced diabetic-enhanced H3K27me3-dependent histone methyltransferase and EZH2 activity in HMVECs (Figure 5B and 5C, $P<0.05$).

EZH2 Inhibitor GSK343 Completely Rescued Diabetic ssEVs-Induced H3K27me3 Expression in HMVECs

EZH2 is the major enzyme known to catalyze transcriptional repression mark H3K27me3.[32] Enhanced EZH2/H3K27me3 signaling suppressed gene expression in EC is involved in cell adhesion and communication responsible for the regulation of angiogenesis.[26,30,31] We examined the effects of diabetic ssEVs on H3K27me3 expression in HMVECs. We found that diabetic ssEVs treatment for 48 hours dramatically increased H3K27me3 expression in HMVECs compared with nondiabetic ssEVs (Figure 5D, $P<0.05$). Inhibition of EZH2 activity by GSK343 significantly blocked diabetic ssEVs-enhanced H3K27me3 expression in HMVECs (Figure 5D, $P<0.05$). Our findings indicate that EZH2 is the major enzyme regulating H3K27me3 level in MVECs.

Diabetic ssEVs Contain a Higher mRNA Level of EZH2

We examined the mRNA and protein level of EZH2 in ssEVs from db/+ and db/db mice. We found that the mRNA level of EZH2 was significantly enhanced in diabetic ssEVs (Figure 5E, $P<0.05$), whereas the protein level of EZH2 in diabetic ssEVs was similar to that in nondiabetic ssEVs (Figure 5F). Our data suggest that diabetic ssEVs increased EZH2 expression in HMVECs and MVECs from db/db mice via, at least partially, transfer of EZH2 mRNA from ssEVs.

Inactivation of EZH2 Rescued Diabetic ssEVs-Impaired Secretion/Production of Pro-Angiogenic Factor in ECs

Next, we tested the role of EZH2 in the causation of diabetic ssEVs-impaired secretion/production of

pro-angiogenic factor in HMVECs by examining the effects of EZH2-specific inhibitor GSK343. HMVECs were treated with either nondiabetic or diabetic ssEVs in the presence or absence of GSK343 (0.1 µM, 48 hours). By Angiogenic Protein Profile Array, we found that GSK343 significantly enhanced or tended to enhance diabetic ssEVs-impaired secretion of pro-angiogenic factor Ang1 ($P<0.05$), artemin ($P<0.05$), and FGF2 ($P=0.0667$) in HMVEVs (Figure 6A). By RT-PCR, we also observed that GSK343 tended to increase Ang1 and significantly increased artemin ($P<0.05$) and FGF2 ($P<0.05$) expression in HMVECs treated with diabetic ssEVs (Figure 6B, $P<0.05$). Our findings suggest that diabetic ssEVs impair MVEC function/biology via EZH2-dependent reduction of pro-angiogenic factor Ang1, artemin, and FGF2.

Inactivation of EZH2 Improved Diabetic ssEVs-Impaired Tube Formation by HMVECs

We also observed that diabetic ssEVs-impaired tube formation by HMVECs was significantly improved by EZH2 specific inhibitor GSK343 (Figure 6C, $P<0.05$). Our findings indicate that enhanced EZH2 activity/expression plays an important role in diabetic ssEVs-impaired angiogenic property of MVECs.

Inactivation of EZH2 Rescued Diabetic ssEVs-Impaired IHL Recovery in Wildtype C57BL/6J Mice

We next examined whether inactivation of EZH2 prevents diabetic ssEVs-impaired IHL recovery. DZNep was administered into C57BL/6J mice by intraperitoneal injection 1 day before induction of IHL and the injection was repeated every other day until the end of the experiment as described previously.[25,26] In good accordance with a previous study,[26] we found that intraperitoneal injection of DZNep with 1.5 mg/kg per day for 3 weeks significantly decreased EZH2 expression in the muscle of IHL of C57BL/6J mice (Figure S2A, $P<0.05$). Administration of DZNep did not affect body weight of IHL C57BL/6J mice, indicating that it was well tolerated as indicated in a previous report (Figure S2B).[26] Noteworthily, we observed that inactivation of EZH2 by DZNep significantly prevented diabetic ssEVs-impaired blood perfusion and capillary/arteriole formation in IHL of C57BL/6J mice (Figure 7A and 7B, $P<0.05$), indicating that diabetic ssEVs impaired IHL recovery via activation of EZH2.

Diabetic ssEVs Increased H3K27me3 Expression at Promoter of Ang1, Artemin, and FGF2 Gene in HMVECs

We next examined whether diabetic ssEVs increase EZH2-mediated gene repressive epigenetic mark
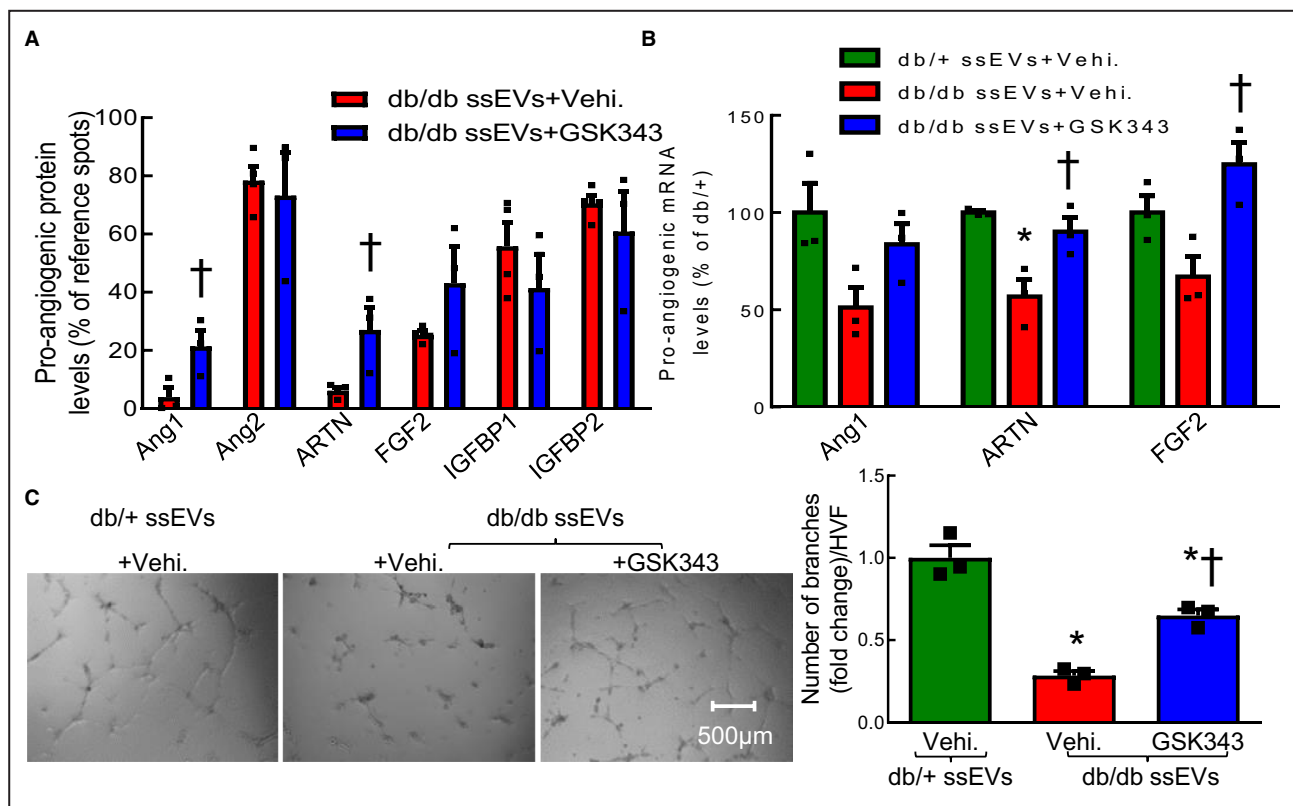
Figure 6. EZH2 inhibitor GSK343 rescued diabetic ssEVs-inhibited pro-angiogenic factor Ang1, ARTN, and FGF2 secretion/expression and tube formation in HMVECs.

A, GSK343 rescued diabetic ssEVs-impaired pro-angiogenic protein secretion including Ang1, ARTN, and FGF2 from HMVECs. n=3. **B,** GSK343 increased diabetic ssEVs-reduced gene expression of Ang1, ARTN, and FGF2 in HMVECs. n=3. **C,** GSK343 rescued diabetic ssEVs-mediated inhibition of tube formation in HMVECs. HMVECs were treated with nondiabetic or diabetic ssEVs in the presence or absence of GSK343 (0.1 μM, 48 hours) after starving for 6 hours (0% FBS). Angiogenic factors secreted from HMVECs were examined in conditioned medium by angiogenic protein profile array. mRNA expression of the angiogenic factors was examined in the cell lysate by RT-PCR. n=3. *$P<0.05$ vs db/+ ssEVs+Vehi-treated HMVECs; †$P<0.05$ vs db/db ssEVs+Vehi-treated HMVECs. Ang1 indicates angiopoietin 1; Ang2, angiopoietin 2; ARTN, artemin; EZH2, enhancer of zest homolog 2; FGF2, fibroblast growth factor 2; HMVECs, human microvascular endothelial cells; HVF, high-power visual field; IGFBP1, insulin-like growth factor binding protein 1; IGFBP2, insulin-like growth factor binding protein 2; RT-PCR, real-time polymerase chain reaction; ssEVs, serum-derived small extracellular vesicles; and Vehi., Vehicle.

H3K27me3 at promoter of Ang1, artemin, and FGF2 in HMVECs by chromatin immunoprecipitation assay. Because the binding efficiency of primer to target gene may be different with different sequences, we examined the H3K27me3 level at promoter of Ang1, artemin, and FGF2 gene with 4 primers. We then quantified the H3K27me3 level at promoter of each gene either by single primer or a combination of the 4 primers as area under the curve. We observed that diabetic ssEVs significantly increased H3K27me3 expression at promoter of Ang1, artemin, and FGF2 gene in HMVECs (Figure 8A to 8C, $P<0.05$). GSK343 significantly rescued H3K27me3 level at promoter of Ang1, artemin, and FGF2 gene (Figure 8A to 8C, $P<0.05$). Our data indicate that diabetic ssEVs-impaired pro-angiogenic factor artemin, and FGF2 production/secretion in MVECs through increasing EZH2-mediated H3K27me3 expression at their promoters.

DISCUSSION

Although there have been extensive studies, the underlying mechanisms of diabetes mellitus–impaired ischemic tissue injury remain incompletely understood. We have reported that MVEC dysfunction plays an important role in diabetes mellitus–impaired IHL repair in db/db mice.[5] In this study, we explored the underlying mechanisms of the diabetes mellitus–impaired angiogenic property of MVECs, specifically focusing on the role of ssEVs. Here, we report 3 major novel findings: (1) Diabetic ssEVs from db/db mice impaired angiogenic properties and secretion/expression of pro-angiogenic factors from HMVECs in vitro, and blood flow/vessel density in IHL of wildtype C57BL/6J mice; (2) Expression and/or activity of EZH2 and H3K27me3 was increased in MVECs from db/db mice and in diabetic ssEVs-treated HMVECs;
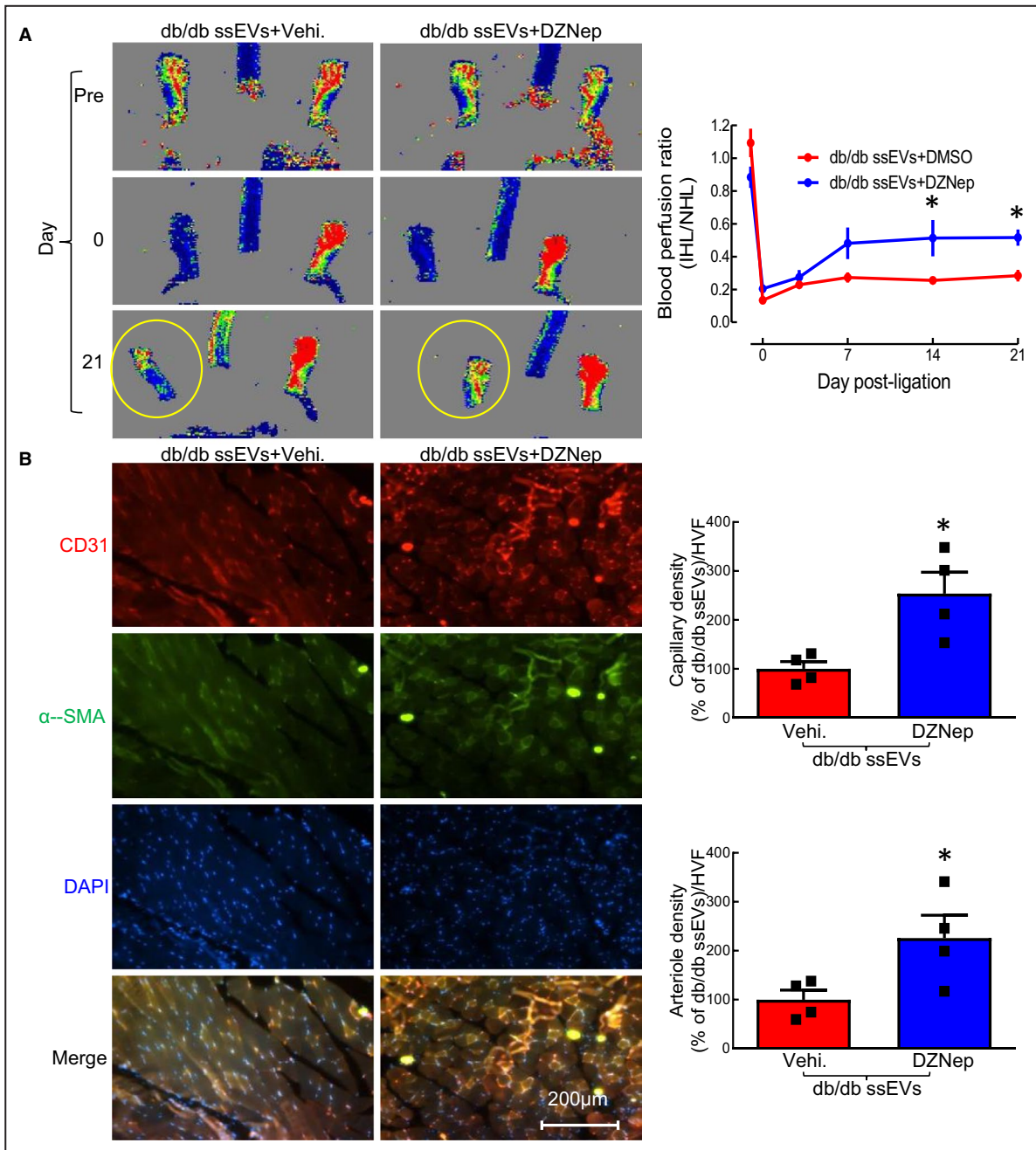
Figure 7. **Inactivation of EZH2 rescued diabetic ssEVs-impaired blood perfusion and capillary/arteriole formation in IHL of wildtype C57BL/6J mice.**

A, Administration of EZH2 inhibitor DZNep rescued diabetic ssEVs-impaired blood perfusion in IHL of C57BL/6J mice. n=6. **B,** DZNep rescued diabetic ssEVs-impaired capillary (right upper panel, CD31), arteriole (right middle panel, α-SMA) density in IHL of C57BL/6J mice. Diabetic ssEVs from db/db mice were injected intramuscularly immediately following ligation of left femoral artery in 8-week-old male C57BL/6J mice (3×10^9 particles/mouse). EZH2 inhibitor DZNep was administered by intraperitoneal injection (ip,1.5 mg/kg/d, every 2 days) starting from 1 day before IHL surgery and continuing to the end of experiments. Mice administered with vehicle (1% DMSO, Vehi.) served as controls. Blood perfusion in IHL was measured by Laser Doppler Imager at pre-, 0, 3, 7, 14, and 21 days postligation and expressed as ratio of blood perfusion of ischemic to nonischemic limb. **Left panel:** Representative images of blood perfusion. Mice were euthanized and ischemic skeletal muscle was collected at 21 days postligation. Skeletal muscle sections were co-immunostained with CD31/α-SMA antibodies for measurement of neovascularization in response to IHL injury. n=4. P*<0.05 vs IHL C57BL/6J mice injected with diabetic ssEVs plus Vehi. DAPI indicates 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; DZNep, 3-deazaneplanocin; EZH2, enhancer of zest homolog 2; HVF, high-power visual field; IHL, ischemic hind limb; NHL, nonischemic hind limb; ssEVs, serum-derived small extracellular vesicles; and Vehi., Vehicle.
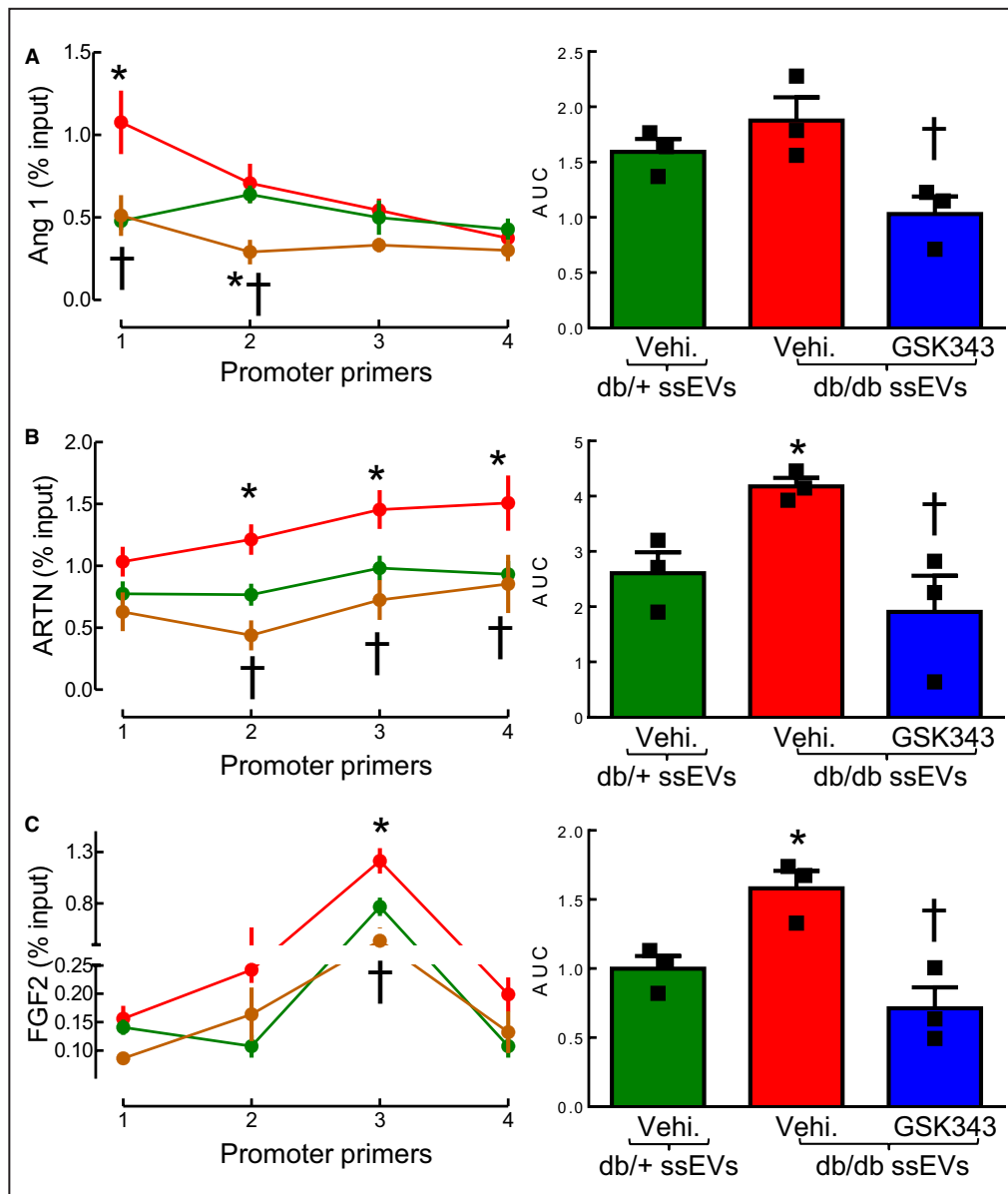
Figure 8. Diabetic ssEVs increased recruitment of H3K27me3 at promoter of Ang1, ARTN, and FGF2 in HMVECs via EZH2.
A, Recruitment of H3K27me3 to promoter of Ang1. **B,** Recruitment of H3K27me3 to promoter of ARTN. **C,** Recruitment of H3K27me3 to promoter of FGF2. HMVECs were treated with either nondiabetic or diabetic ssEVs in the presence or absence of GSK343 (0.1 μM) for 48 hours after starving for 6 hours (0% FBS). ChIP assay for examining recruitment of H3K27me3 on promoter of Ang1, ARTN, and FGF2 was examined by 4 promoter primers and presented either with % of input or AUC. n=3. *$P<0.05$ vs db/+ Exo+Vehi-treated HMVECs; †$P<0.05$ vs db/db ssEVs+Vehi-treated HMVECs. Ang1 indicates angiopoietin 1; ARTN, artemin; AUC, area under curve; ChIP, chromatin immunoprecipitation; FBS, fetal bovine serum; FGF2, fibroblast growth factor 2; H3K27me3, tri-methylation at the 27th lysine residue of the histone H3 protein; HMVECs, human microvascular endothelial cells; ssEVs, serum-derived small extracellular vesicles; and Vehi., Vehicle.

and (3) Inactivation of EZH2 significantly reversed diabetic ssEVs-enhanced expression of EZH2 and H3K27me3, enhanced expression of angiogenic factors, and improved blood perfusion and vessel density in IHL of C57BL/6J mice, and enhanced H3K27me3 expression at promoter of pro-angiogenic genes in HMVECs. This study highlights the role of ssEVs as a bloodborne regulator of angiogenic property of MVECs and pinpoints EZH2 as a critical component of ssEVs that participates in impaired angiogenic property of MVECs in diabetes mellitus via epigenetic mechanisms of H3K27me3-suppressed
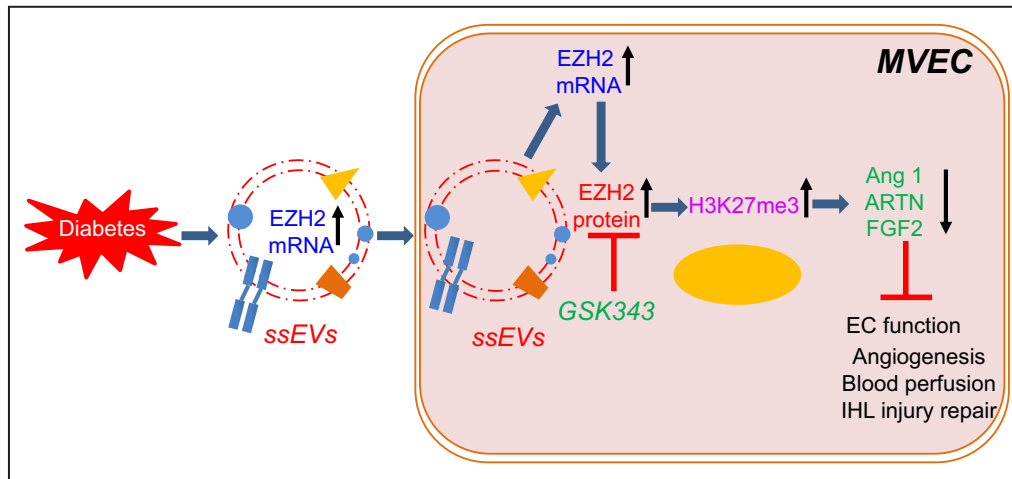
Figure 9. Mechanistic scheme of diabetic ssEVs-impaired angiogenic property of MVECs.
Ang1 indicates angiopoietin 2; ARTN, artemin; EC, endothelial cell; EZH2, enhancer of zest homolog 2; IHL, ischemic hind limb; MVECs, microvascular endothelial cells; and ssEVs, serum-derived small extracellular vesicles.

pro-angiogenic genes by delivery of EZH2 mRNA (Figure 9).

Angiogenesis is essential to the formation of new blood vessels, which allows oxygen and nutrients to reach ischemic tissues, thereby facilitating tissue repair. Enhanced expression of pro-angiogenic genes during hypoxia/ischemia is a primary prerequisite for angiogenesis/neovascularization.[33] Previously, we and others have reported that insufficient pro-angiogenic factors are involved in MVEC dysfunction and impairment of IHL repair in diabetes mellitus.[5,34] However, the underlying mechanisms of diabetes mellitus–impaired pro-angiogenic factor remain incompletely understood.

In recent years, sEVs (exosomes) have been attracting increasing attention as a novel mechanism for regulation of cell function/biology by transferring functional cargo.[6,9,10,12,35] Diabetic sEVs released by various types of cells have a deleterious impact on angiogenesis and cell survival.[17-20] Since ECs are constantly in contact with blood, the potential that ECs are upon uptake of the circulating sEVs is raised. In addition, a very recent study suggested that EVs from blood may be new and improved diagnostic tools and therapeutics for diabetic complications.[36] Therefore, understanding the role of ssEVs on diabetes mellitus–impaired biology/function of MVECs may provide fundamental insights for diagnosis and therapy of impaired angiogenesis in patients with diabetes mellitus. However, the role of ssEVs in the diabetes mellitus–impaired angiogenic property of MVECs has never been studied. In the present study, we demonstrated that ssEVs from db/db mice impaired tube formation and migratory activity of HMVECs. In in vivo experiments, we also showed that intramuscular injection of diabetic ssEVs impaired

blood perfusion and capillary density in IHL of wildtype C57BL/6J mice. Moreover, we provided evidence that diabetic ssEVs impaired secretion/production of pro-angiogenic factors in HMVECs, suggesting that impaired secretion/production of pro-angiogenic factors is involved in the ssEVs-impaired angiogenic property of MVECs in diabetes mellitus.

Accumulating evidence indicates that epigenetic chromatin modifications contribute to the modulation of EC gene expression in response to pathophysiological conditions. Posttranslational modifications in the N-terminal amino acid residues of histone, including acetylation, phosphorylation, ubiquitination, and methylation are suggested to be involved in the chronic diabetic complications.[37] Within different modifications of histone, studies demonstrated that methylation is relatively more stable than others. Methylation of histone lysine is the crucial mechanistic trigger by diabetes mellitus/hyperglycemia and plays an important role in the pathogenesis of diabetes mellitus–induced EC dysfunction.[26,30,38] In the present study, we found that several epigenetic chromatin remodeling factors were modulated in MVECs of db/db mice. Within diabetes mellitus–regulated epigenetic remodeling factors, we noticed that EZH2, the well-known and major methyltransferase responsible for generation of repressive transcription marker H3K27me3 in mammalian cells,[32] was increased to 35% in the primary MVECs from db/db mice. By immunostaining, we also observed that expression of EZH2 and H3K27me3 were significantly increased in the primary MVECs from db/db mice. We and others have reported that sEVs promote angiogenesis by transferring functional cargo

(ie, proteins, noncoding RNAs, and lipids) to the recipient cells.[6,9,39] Whether diabetic ssEVs contain enriched EZH2 that can be transferred to MVECs and thus impair their function/biology has never been studied. In the present study, we found that the level of EZH2 mRNA, but not protein, was significantly increased in ssEVs from db/db mice. We also provided the evidence that ssEVs were taken up by HMVECs in vitro. Moreover, treatment with diabetic ssEVs for 48 hours significantly increased EZH2/H3K27me3 expression in HMVECs. Our data suggest that diabetic ssEVs-enhanced EZH2 expression in MVECs is because of, at least partially, transfer of EZH2 mRNA. We demonstrated that ssEVs plays an important role in enhanced EZH2/H3K27me3 expression in MVECs under diabetic condition.

Enhanced EZH2/H3K27me3 signaling suppressed gene expression in ECs is suggested to be involved in cell adhesion and communication responsible for the regulation of EC function and angiogenesis.[26,30,38,40] Hypoxia/ischemia–enhanced EZH2 expression impaired angiogenesis in IHL of C57BL/6J mice via H3K27me3-induced repression of gene transcription of eNOS and brain-derived neurotrophic factor in ECs.[26] Silencing of EZH2 in human umbilical vein ECs induced overexpression of angiogenic genes.[30] High glucose elevated H3K27me3 levels and EZH2 activity, and the recruitment of EZH2 at the matrix metalloproteinase-9 promoter in human retinal ECs.[41] Inhibition of EZH2 prevented hyperglycemia-induced matrix metalloproteinase-9 transcription and mitochondria damage in human retinal ECs.[41] High glucose decreased vascular endothelial growth factor levels in human retinal ECs via upregulation of EZH2 in human retinal ECs.[42] Together, EZH2/H3K27me3 plays an important role in diabetes mellitus–impaired EC function. However, the underlying mechanisms of diabetes mellitus–enhanced EZH2/H3K27me3 expression/activity in MVECs remain unclear.

Our studies demonstrated a novel cellular mechanism that ssEVs-enhanced EZH2/H3K27me3 expression is involved in diabetes mellitus–impaired MVEC function. We also provided evidence that inhibition of EZH2 activity rescues diabetic ssEVs-impaired tube formation and endothelial pro-angiogenic gene secretion/expression in MVECs and prevents diabetic ssEVs-impaired IHL recovery in wildtype C57BL/6J mice. Moreover, chromatin immunoprecipitation assay showed enhanced expression of repressive transcription marker H3K27me3 at the promoter of several pro-angiogenic genes, which was completely rescued by inactivation of EZH2. Our findings strongly support the ssEVs-induced EZH2/H3K27me3-mediated epigenetic mechanism in diabetic MVEC function. ssEVs-enhanced EZH2 expression/activity in MVECs may be a novel target for therapeutic treatment of ischemic tissue injury in patients with diabetes mellitus.

Previous studies reported that the level of plasma/cell–derived sEV is increased in diabetic patients/animals and suggested that enriched plasma/cell–derived sEVs play an important role in the pathogenesis of diabetes mellitus.[11,15,43] For example, the concentration of plasma-derived sEVs was significantly increased in streptozocin-induced diabetic mice (3 months onset of diabetes mellitus) compared with that from control mice.[15] Comparing with normal pregnant women, the concentration of sEV in the plasma of pregnant women with gestational diabetes mellitus was increased by ≈2-fold.[43] A very recent study also reported that the concentration of ssEV (<100 nm) was significantly increased in male db/db mice at the age of 12 weeks.[23] In the present study, the similar concentration of ssEVs in db/+ and db/db mice may be because of the severity of diabetes mellitus, mouse strains, and the quality and size of isolated ssEVs. Our findings suggest that enhanced EZH2 mRNA expression in ssEVs, but not increased concentration of ssEV, plays a critical role in diabetes mellitus–impaired MVEC dysfunction.

Limitations in the present study are the following: (1) Because of technical limitations, we cannot exclude the possible involvement of a non-ssEVs source of EZH2 mRNA although to a lesser degree; (2) Since EZH2 regulates the transcription of many different genes, the rescue effects by silencing of EZH2 in diabetic ssEVs may be influenced by the inhibition of healthy genes. Effects of silencing of EZH2 in diabetic ssEVs were not examined in vivo; (3) Studies to explore the detailed mechanism underlying the origin of EZH-enriched ssEVs, and secretion and uptake of ssEVs were not performed; (4) Clinical relevance of ssEVs in diabetes mellitus–impaired angiogenic property of MVECs was not elucidated.

In summary, here we report a detailed study revealing that diabetic ssEVs impair angiogenic property of MVECs, at least partially, by transferring EZH2 mRNA. ssEVs from db/db mice induced upregulation and/or activation of EZH2/H3K27me3 and increased EZH2-mediated H3K27me3 enrichment at the promoters of pro-angiogenic genes, thereby silencing pro-angiogenic genes in MVECs. We demonstrate that ssEVs-derived EZH2 is critically involved in the impaired angiogenic property of MVECs in diabetic mice. The present study unravels, with potential clinical relevance, the previously undefined importance of ssEVs in the diabetes mellitus–impaired angiogenic property of MVECs. ssEVs may become a valuable diagnostic tool and therapeutic target for impaired MVEC function/biology in patients with diabetes mellitus.

ARTICLE INFORMATION

Affiliations

Center for Translational Medicine, Lewis Katz School of Medicine, Temple University, Philadelphia, PA (Z.C., M.M.T., M.C., G.H., C.W., C.B., C.G., V.M., R.K.); Department of Emergency Medicine, Dorothy M. Davis Heart Lung and Research Institute, The Ohio State University Wexner Medical Center, Columbus, OH (V.N.S.); Cardiovascular Research Center, Icahn School of Medicine at Mount Sinai, New York, NY (D.A.G.); Department of Medicine-Cardiovascular Disease, The University of Alabama at Birmingham, Birmingham, AL (S.K.V.); and Department of Pharmacology, Lewis Katz School of Medicine, Temple University, Philadelphia, PA (R.K.).

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Disclosures

Supplementary Material

Figures S1–S2

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Supplemental Material

A

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	Arid1a/ -1.04	Arid2/ -1.01	Asxl1/ -1.02	Baz1a/ 1.16	Baz16/ 1.18	Baz2a/ 1.03	Baz2b/ 1.1	Bmi1/ -1.07	Bptf/ 1.13	Brd1/ 1.05	Brd2/ 1.03	Brd3/ 1.05
B	Brd4/ -1.05	Brd7/ 1.08	Brd8/ 1.04	Brdt/ -4.34	Brtf1/ 1.05	Brpf3/ 1.24	Brwd1/ 1.14	Brwd3/ 1.16	Cbx1/ 1.07	Cbx2/ 1.33	Cbx3/ 1.38	Cbx4/ 1.3
C	Cbx5/ 1.14	Cbx6/ 1.1	Cbx7/ 1.05	Cbx8/ 1.22	Cdyl/ 1.04	Cdyl1/ 1.01	Chd1/ 1.2	Chd2/ -1.06	Chd3/ 1.07	Chd4/ 1.41	Chd5/ 2.52	Chd6/ 1.24
D	Chd7/ -1.1	Chd8/ 1.08	Chd9/ 1.27	Ctbp1/ -1.02	Ctbp2/ 1.05	Ctcf/ 1.02	Eed/ 1.08	Ezh2/ 1.35	Hinfp/ 1.03	Ing1/ 1.43	Ing2/ 1.04	Ing/ 1.23
E	Ing4/ -1.1	Ing5/ 1.19	Ino80/ 1.02	Mbd1/ -1.03	Mbd3/ 1.0	Mbd4/ -1.01	Mecp2/ 1.02	Mta1/ -1.04	Mta2/ 1.22	Nab2/ 1.29	Nsd1/ 1.15	Pcfg1/ -1.07
F	Pcgf2/ 1.0	Pcgf3/ 1.06	Pcgf5/ 1.12	Pcgf6/ 1.15	Phc1/ 1.34	Phc2/ -1.05	Phf1/ -1.14	Phfl3/ 1.36	Phf2/ 1.17	Phf21b/ -1.0	Phf3/ 1.17	Phf5a/ 1.5
G	Phf6/ -1.0	Phf7/ -1.05	Ring1/ -1.02	Rnf2/ 1.25	Smarca2/ -1.78	Smarco4/ -1.07	Smarco5/ 1.1	Spen/ 1.18	Suz1/ 1.11	Trim27/ 1.14	Wdr11/ -1.06	Zmynd8/ -1.08

B
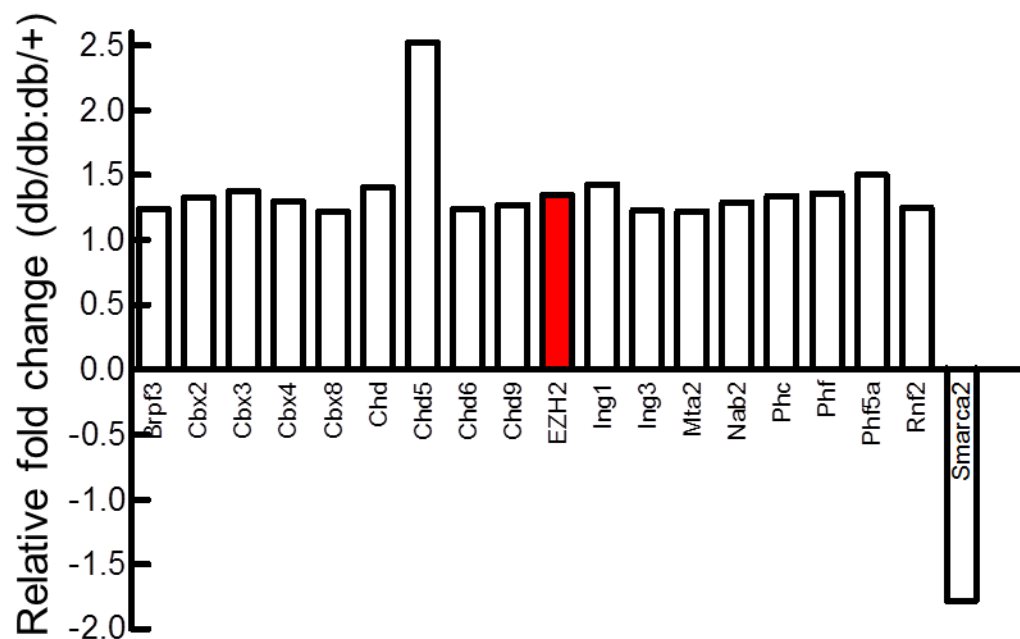
Supplementary Figure I. Modulation of epigenetic chromatin remodeling factors in MVECs of db/db mice. (A). Layout of ratios of epigenetic chromatin remodeling factor from MMVECs of db/db mice compared to db/+ mice by PCR array. **(B).** Epigenetic chromatin remodeling factors (>1.2-fold) in MMVECs of db/db mice compared to db/+ mice by PCR array. n=2.
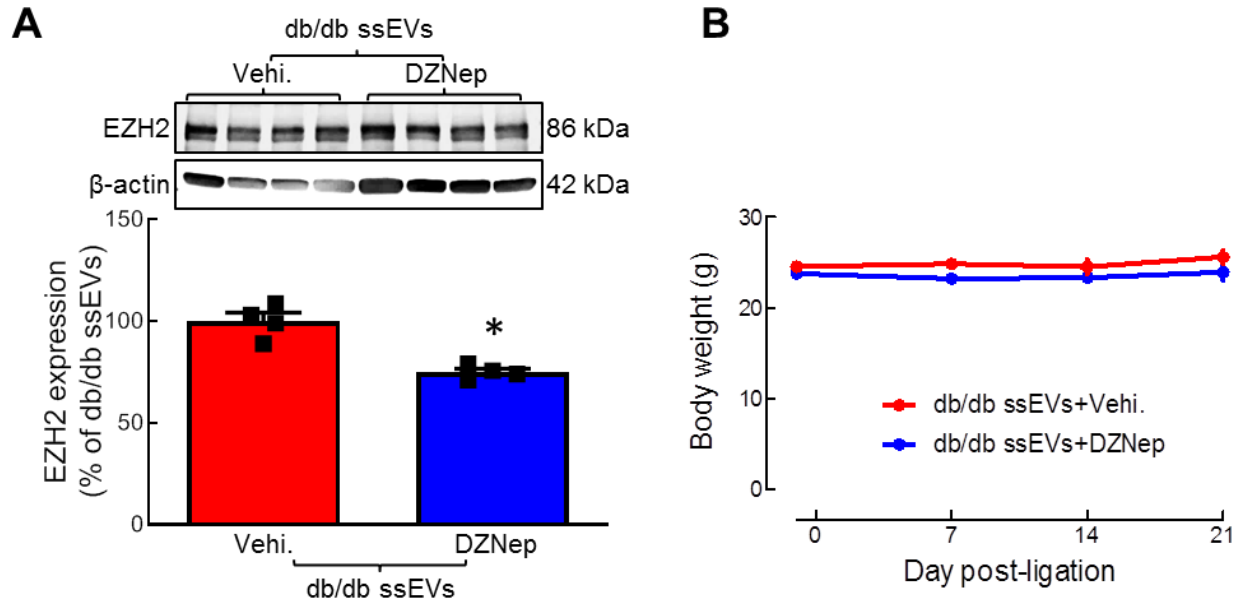
Supplementary Figure II. Administration of EZH2 inhibitor DZNep for 3 weeks reduced EZH2 expression in skeletal muscle of IHL of C57BL/6J mice. (A). EZH2 expression in skeletal muscle of IHL of C57BL/6J mice. n=4. p*<0.05 vs. IHL C57BL/6J mice injected with diabetic ssEVs plus Vehi. **(B).** Body weight was not affected by administration of DZNep. Diabetic ssEVs ($3x10^9$ particles) from db/db mice were injected intramuscularly immediately following ligation of left femoral artery in 8-week-old male C57BL/6J mice. EZH2 inhibitor DZNep was administered by intraperitoneal injection (i.p.,1.5 mg/kg/day, every 2 days) started from one day before IHL surgery and continued to the end of experiments. Mice administered with vehicle (1% DMSO, Vehi.) severed as controls. Body weight was measured 1 day pre-IHL, and 3, 7, 14 and 21 days post-IHL. n=7.