Supplementary Materials for

SGLT2 inhibitor empagliflozin promotes revascularization in diabetic mouse hindlimb ischemia by inhibiting ferroptosis

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Supplementary methods

Drug treatment

For drug treatment, cells were treated with indicated doses of empagliflozin, dapagliflozin (Macklin Biochemical, Shanghai, China), canagliflozin, ertugliflozin, ipragliflozin, or tofogliflozin (MedChemExpress, Monmouth Junction, NJ) dissolved in 10% DMSO for 24 h. Medium was then changed to FBS-free DMEM with 25 mM glucose (final concentration), and cells were put under hypoxic condition as described above.

For SLC7A11 and GPX4 inhibition, cells were treated with erastin (Macklin Biochemical; final concentration: 500 nM) or RSL3 (MedChemExpress; final concentration: 100 nM) for 24 h, respectively. Cells were then treated with empagliflozin (final concentration: 10 μM) as described above.

For *GPX4* silencing experiment, C2C12 cells were transfected with control vector (shCon) or shRNA expression vectors targeting *GPX4* (shGPX4) using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instruction. Puromycin selection (2 μg/mL) was performed for 36 h started from 24 h after transfection to eliminate untransfected cells. Cells were then treated with indicated drugs as described above.

RNA sequencing and transcriptomic analysis

C2C12 cells were treated with empagliflozin and exposed to hypoxia as described previously. RNA extraction and RNA sequencing analysis were performed by Beijing Novogene Technology Corporation (Beijing, China). Total RNA was extracted using Trizol (Invitrogen), while total amounts and integrity of RNA were assessed using RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA). Total RNA was used as input material for the RNA sample preparations and library construction. Sequencing libraries were generated using the NEBNext UltraTM RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample.

Differential expression analysis of control and empagliflozin-treated group (three biological replicates each) was performed using the DESeq2 R package. The resulting *P*-values were adjusted using the Benjamini and Hochberg's approach for controlling the false

discovery rate. Adjusted *P* value ≤ 0.05 and fold change ≥ 1.5 were set as the threshold for significantly differential expression.

GO and KEGG enrichment analysis of differentially expressed genes was implemented by the cluster Profiler R package, in which gene length bias was corrected. Terms with *P*-value less than 0.05 were considered significantly enriched by differential expressed genes.

Conditioned media

Mature C2C12 cells transfected with shRNA expression vectors or control vector were cultured in hyperglycemic condition as described above and treated with 10% DMSO or empagliflozin for 24 h. Cells were then washed with PBS, starved using FBS-free DMEM and exposed to hypoxia for 24 h. Conditioned media from shCon-transfected C2C12 cells cultured under hyperglycemia and treated with 10% DMSO (CM-shCon) or 10 μM empagliflozin (CM-shCon+Empa), as well as those transfected with shGPX4, cultured under hyperglycemia and treated with 10% DMSO (CM-shGPX4) or 10 μM empagliflozin (CM-shGPX4+Empa) were obtained, centrifuged, and then filtered with 0.22 μm filter to eliminate cell debris.

Enzyme-linked immunosorbent assay (ELISA)

The amount of 4-HNE in C2C12 cells was determined using universal 4-HNE ELISA kit (FineTest, Wuhan, China), according to the manufacturer's guidelines. The amounts of ANG1, PDGF-BB, and VEGF-A in the conditioned media were determined using Mouse ANG1 ELISA kit (FineTest), Mouse PDGF-BB ELISA, and Mouse VEGF-A ELISA kits (Neobioscience, Shenzhen, China), respectively, according to the manufacturer's guidelines.

MDA

The content of MDA in C2C12 cells was determined using Lipid Peroxidation MDA Assay Kit (Beyotime, Shanghai, China), according to the manufacturer's guidelines.

Lipid ROS and lipid peroxidation

Cells were treated as described above and stained using C11-BODIPY (581/591)

(Invitrogen). Lipid ROS was analyzed using flow cytometry. Lipid peroxidation images were obtained using Olympus IX73 (Olympus, Tokyo, Japan), and quantification was accomplished by using ImageJ software. Quantification results were presented as the ratio of oxidated cells (green) to those of non-oxidated cells (red).

EdU incorporation assay and transwell migration assay

HUVECs and MOVAS cells were cultured with indicated conditioned media prior to exposure to hypoxia for 12 h. EdU incorporation assay was performed using BeyoClick™ EdU-488 Cell Proliferation Assay Kit (Beyotime) according to the manufacturer's instruction. Images were obtained using Olympus IX73 (Olympus), and quantification was accomplished by using ImageJ software. The results were presented as the ratio of EdU-positive cells to those of Hoechst-positive cells.

For transwell experiment, HUVECs and MOVAS cells were seeded into the upper compartment of the transwell chamber (Corning, NY), while the indicated conditioned media were placed in the lower compartment of the transwell chamber. Cells were then exposed to hypoxia for 24 h. Cells migrated to the lower compartment were stained using crystal violet (Beyotime) and the number was quantified.

Immunofluorescence, immunohistochemical and hematoxylin and eosin (H&E) staining

Tissues from the gastrocnemius muscle of the left hindlimb of HLI mice were fixed with 4% paraformaldehyde before being embedded in paraffin and sliced at 4 μm thickness using a cryostat. Sections were dewaxed using xylene and then rehydrated.

For immunofluorescence staining of tissue sections, sliced sections were stained with anti-mouse PECAM-1 and monoclonal anti-murine α-SMA antibodies as described previously [29]. Briefly, the tissue sections were incubated with PECAM-1 antibody for 1 h. Afterwards, the tissue was incubated with monoclonal antibody against murine α-SMA conjugated with Cy3 and Alexa Fluor 488 Goat Anti-Rat IgG. Images were obtained with Microsystems-TPS SP8 (Leica, Heidelberg, Germany). For immunostaining of cells, cells were seeded in the glass bottom dish prior to staining, and the nuclei were stained using

DAPI (Beyotime). Images were obtained using Microsystems-TPS SP8 (Leica). Antibodies used are listed in Supplementary Table S2.

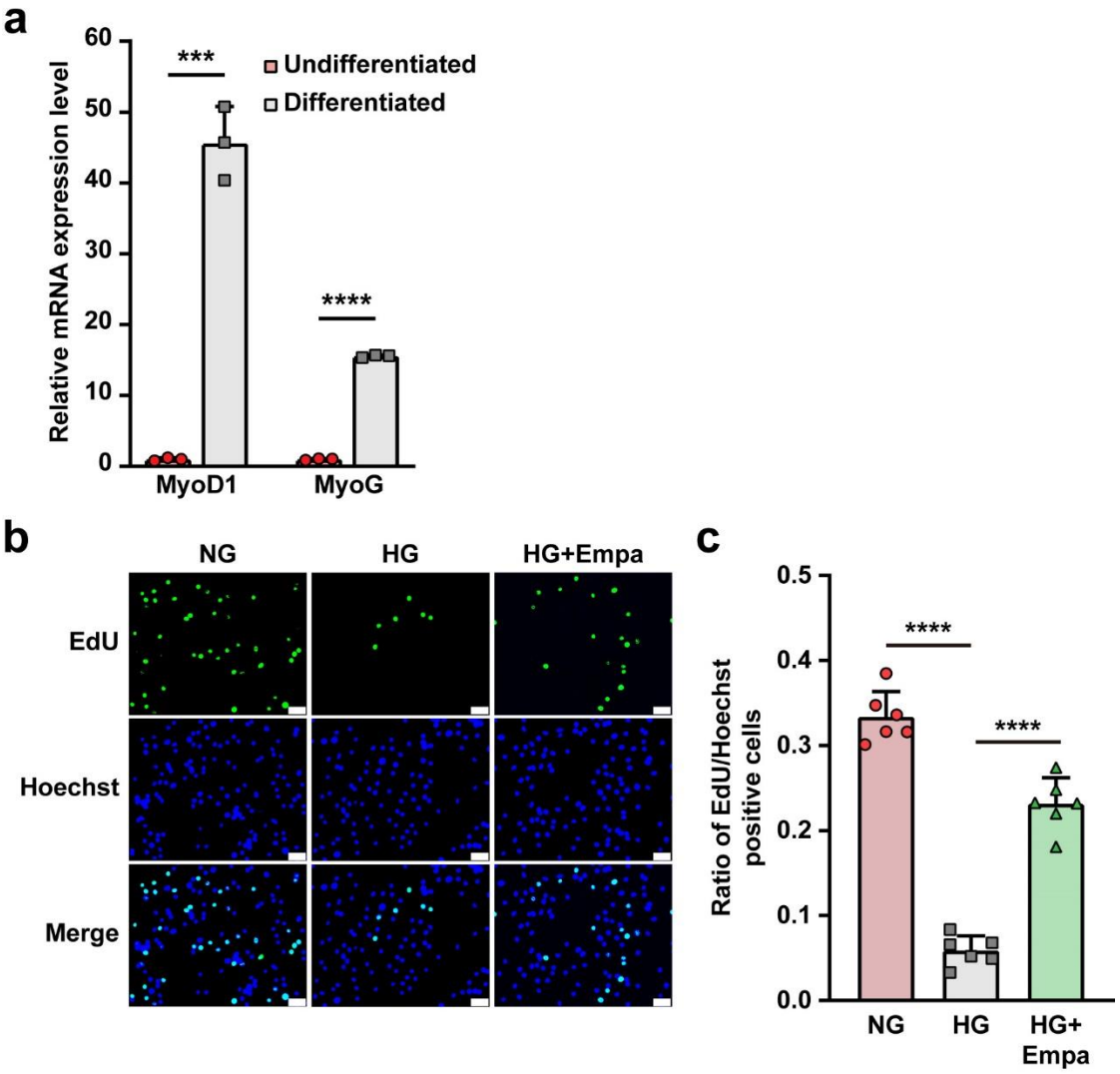
For immunohistochemical staining, sliced sections were incubated overnight at 4 ℃ with first antibody, following by incubation with corresponding secondary antibodies. Images were obtained using Pannoramic MIDI (3DHistech, Budapest, Hungary). Antibodies used are listed in Supplementary Table S2.

For H&E staining, sections were stained with Hematoxylin and Eosin (Beyotime) after being dewaxed and rehydrated as described above. Images were obtained using Olympus IX73 (Olympus).

Cell viability and cell death

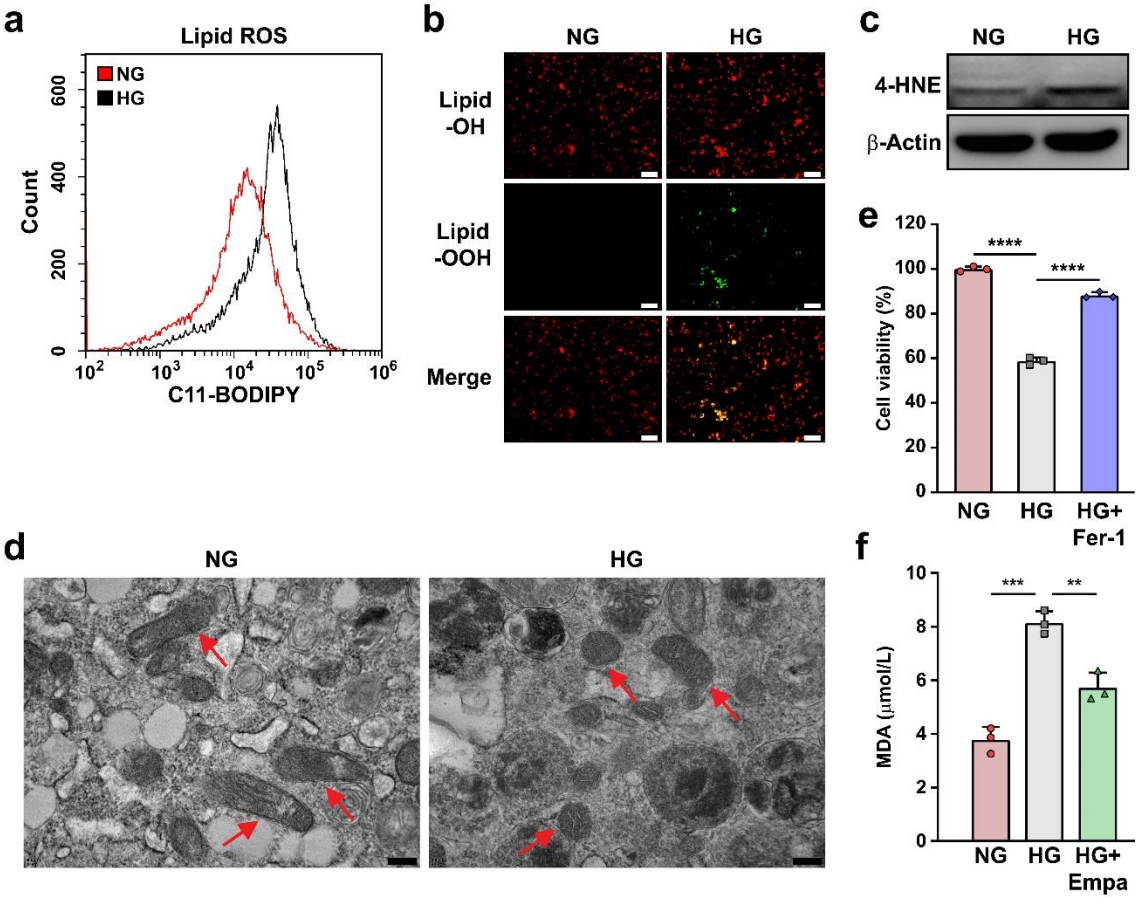
Cells were prepared as described in the manuscript methods. Cell viability was performed by counting the total number of viable cells using MTS (Promega, Madison, WI) according to the manufacturer's protocol. Cell death analysis were performed by propidium iodide (PI; NeoBiosciences, Shanghai, China) staining and flow cytometry.

Supplementary Figure S1



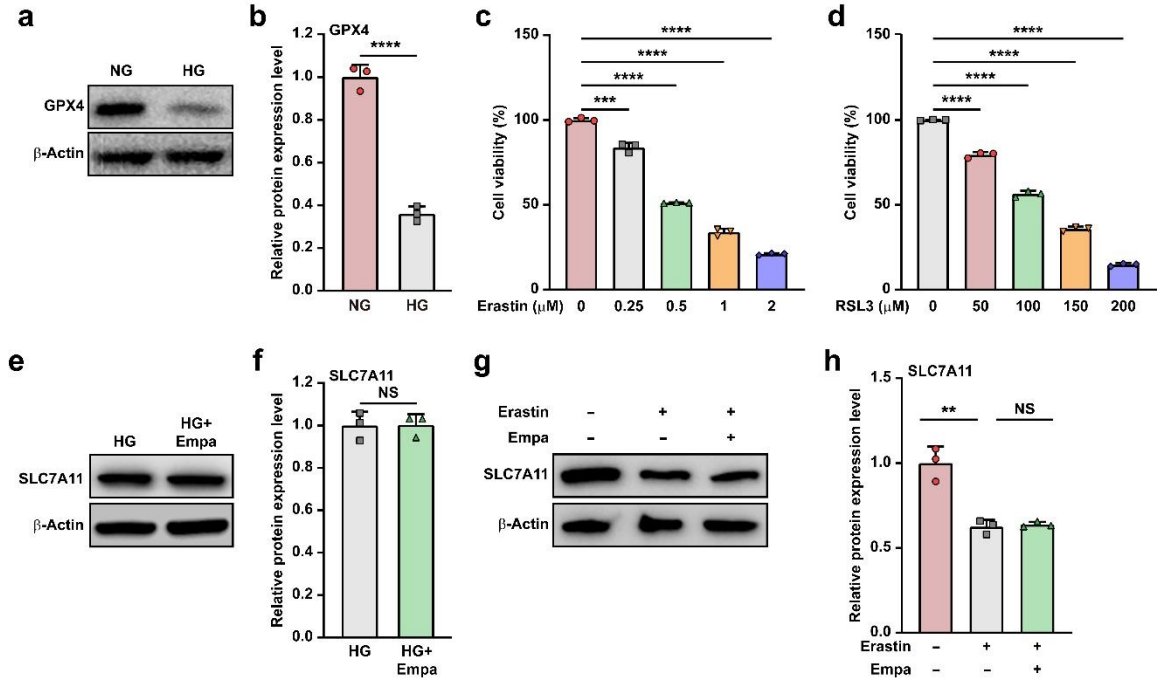
Supplementary Fig. S1. Empagliflozin promotes skeletal muscle cells proliferation. (a) mRNA expression levels of MyoD1 and MyoG in differentiated C2C12 cells, as examined using qRT-PCR (n = 3). β-Actin was used as loading control. **(b–c)** Proliferation potential of empagliflozin-treated C2C12 cells, as examined using EdU incorporation assay. Representative images (b; scale bars: 100 μm) and quantification results (c; n = 6) were shown. All experiments were performed under hyperglycemia unless further indicated. Data were presented as mean ± SD. ***P < 0.001; ****P < 0.0001.

Supplementary Figure S2



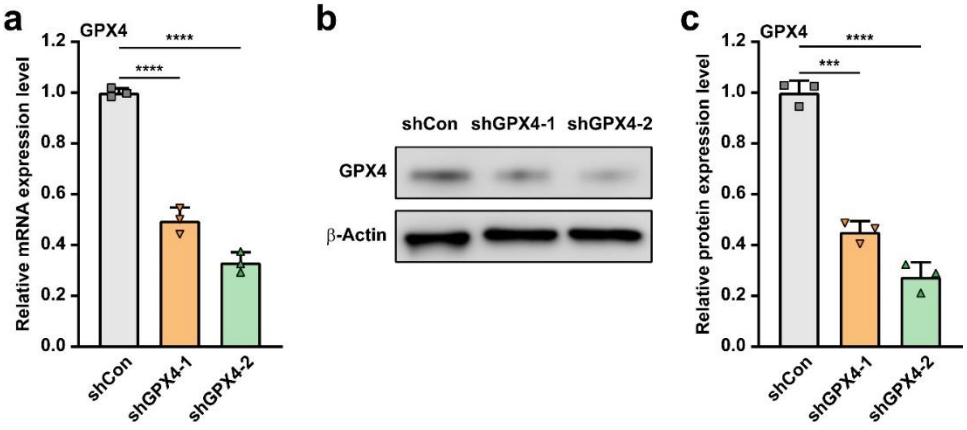
Supplementary Fig. S2. Hyperglycemia induces ferroptosis. (a) Lipid ROS level of C2C12 cells cultured under hyperglycemia, as examined using C11-BODIPY staining and flow cytometry. **(b)** Lipid peroxidation ratio of C2C12 cells cultured under hyperglycemia, as examined using C11-BODIPY staining. **(c)** 4-HNE level of C2C12 cells cultured under hyperglycemia, as examined using western blotting. β-Actin was used as loading control. **(d)** Transmission electron microscopy images of the mitochondria in C2C12 cultured under hyperglycemia. Red arrows: mitochondria; scale bars: 200 nm. **(e)** Cell viability of C2C12 cells treated with 10 μM ferrostatin-1. **(f)** MDA level in C2C12 cells treated with indicated gliflozin, as examined using Lipid Peroxidation MDA Assay Kit. All experiments were performed under hyperglycemia unless further indicated. Data were presented as mean ± SD (n = 3). NG: normoglycemia; HG: hyperglycemia; Fer-1: 10 μM ferrostatin-1; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

Supplementary Figure S3



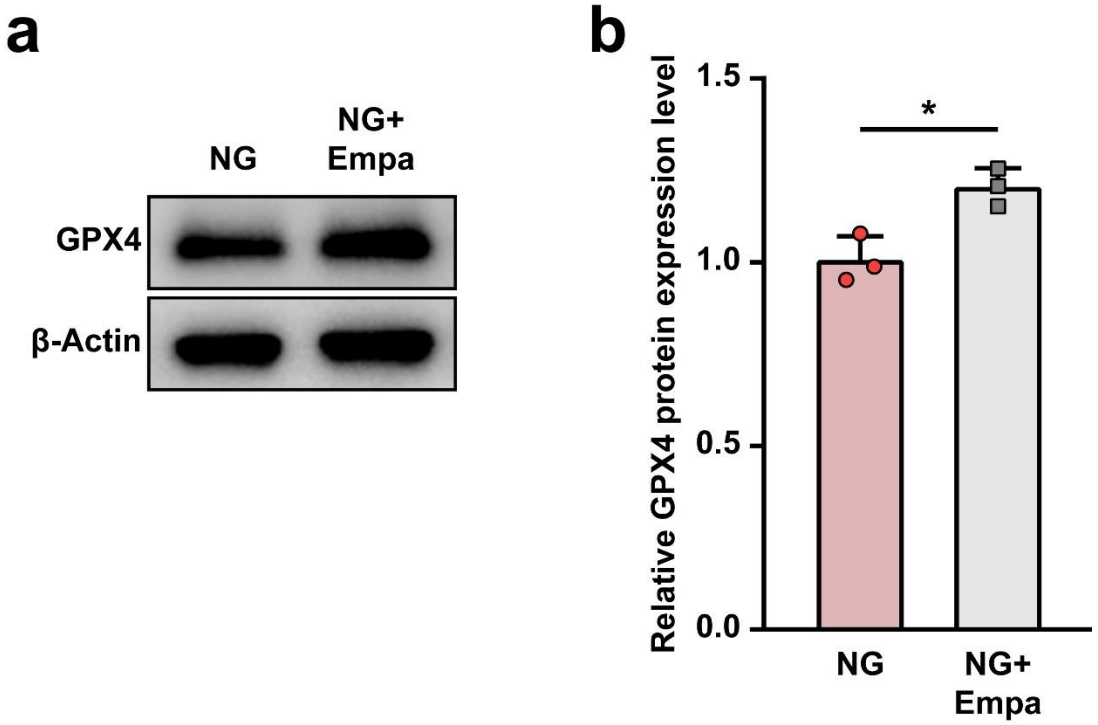
Supplementary Figure S3. The effect of empagliflozin on SLC7A11 expression level. **(a–b)** GPX4 protein expression level in C2C12 cells under hyperglycemia, as examined using western blotting. Representative images (a) and quantification results (b) were shown. **(c–d)** Cell viability of C2C12 cells treated with indicated concentration of erastin (c) or RSL3 (d). **(e–f)** SLC7A11 protein expression level in C2C12 cells treated with 10 μM empagliflozin, as examined using western blotting. Representative images (e) and quantification results (f) were shown. **(g–h)** SLC7A11 protein expression level in C2C12 cells treated with 500 nM erastin and 10 μM empagliflozin, as examined using western blotting. Representative images (g) and quantification results (h) were shown. β-Actin was used as loading control. Data were presented as mean ± SD (n = 3). NG: normoglycemia; HG: hyperglycemia; NS: not significant; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

Supplementary Figure S4



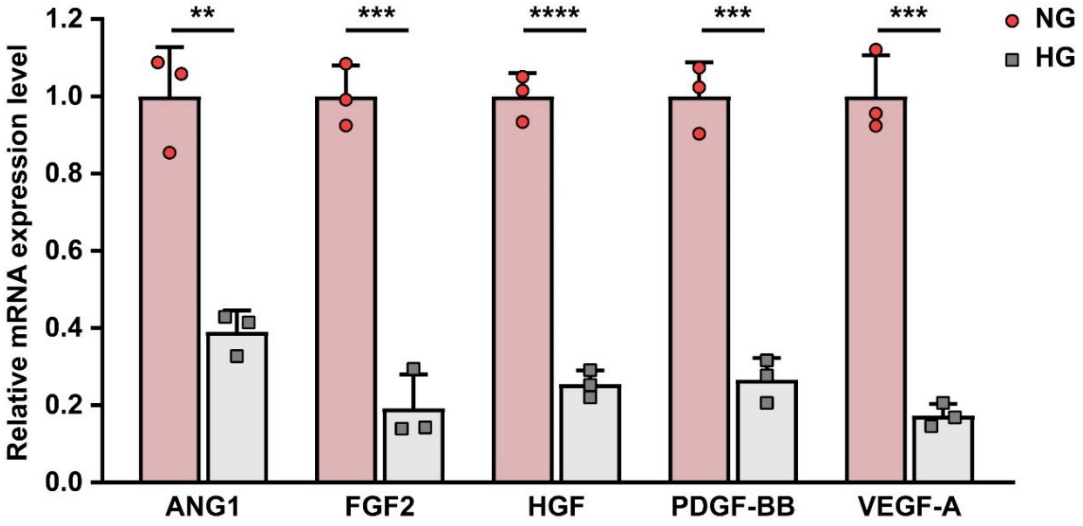
Supplementary Fig. S4. Efficacy of shRNA vectors targeting *GPX4*. (a–c) GPX4 mRNA (a) and protein (b–c) expression levels in C2C12 cells transfected with two shRNA vectors targeting *GPX4*, as examined using qRT-PCR and western blotting, respectively. Representative images (b) and quantification results (c) were shown. β-Actin was used for qRT-PCR normalization and as western blotting loading control. Cells transfected with shCon vectors were used as controls. All experiments were performed under hyperglycemia. Data were shown as relative to control and expressed as mean ± SD ($n = 3$). ***$P < 0.001$; ****$P < 0.0001$.

Supplementary Figure S5



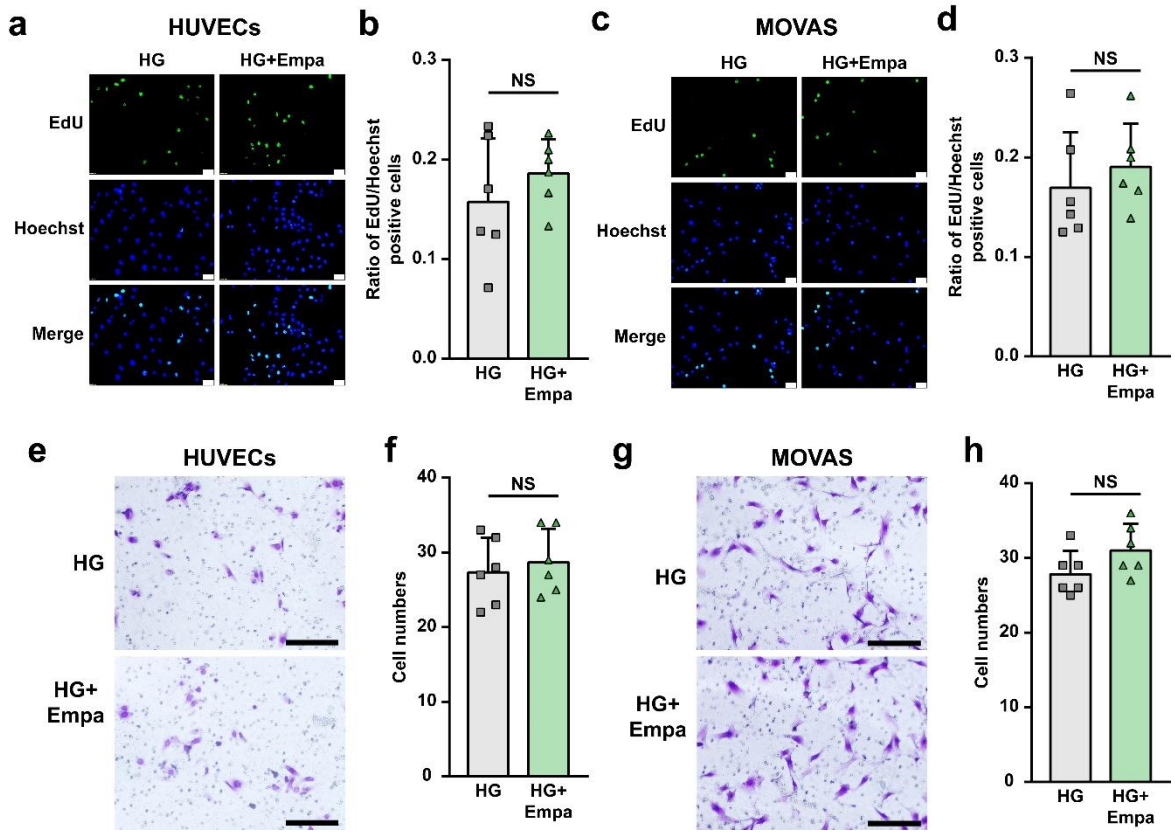
Supplementary Fig. S5. Empagliflozin promotes GPX4 expression under normoglycemia. (a–b) GPX4 protein expression in C2C12 cells treated with 10 μM empagliflozin under normoglycemia, as examined using western blotting. Representative images (a) and quantification results (b) were shown. β-Actin was used as western blotting loading control. Data were presented as mean ± SD ($n = 3$). NG: normoglycemia; *$P < 0.05$.

Supplementary Figure S6



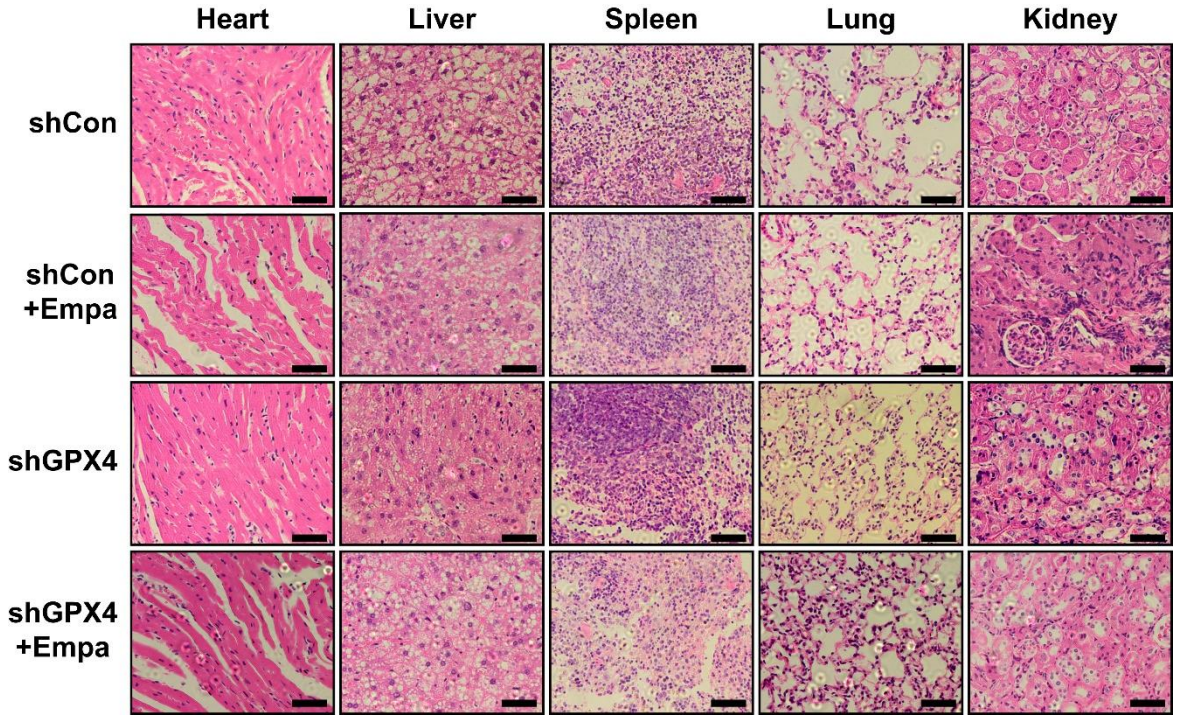
Supplementary Fig. S6. Hyperglycemia reduces angiogenic potential. mRNA expression levels of ANG1, FGF2, HGF, VEGF-A, and PDGF-B in C2C12 cells cultured under hyperglycemia, as examined using qRT-PCR. β-Actin was used for qRT-PCR normalization. Data were presented as mean ± SD (n = 3). NG: normoglycemia; HG: hyperglycemia; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

Supplementary Figure S7



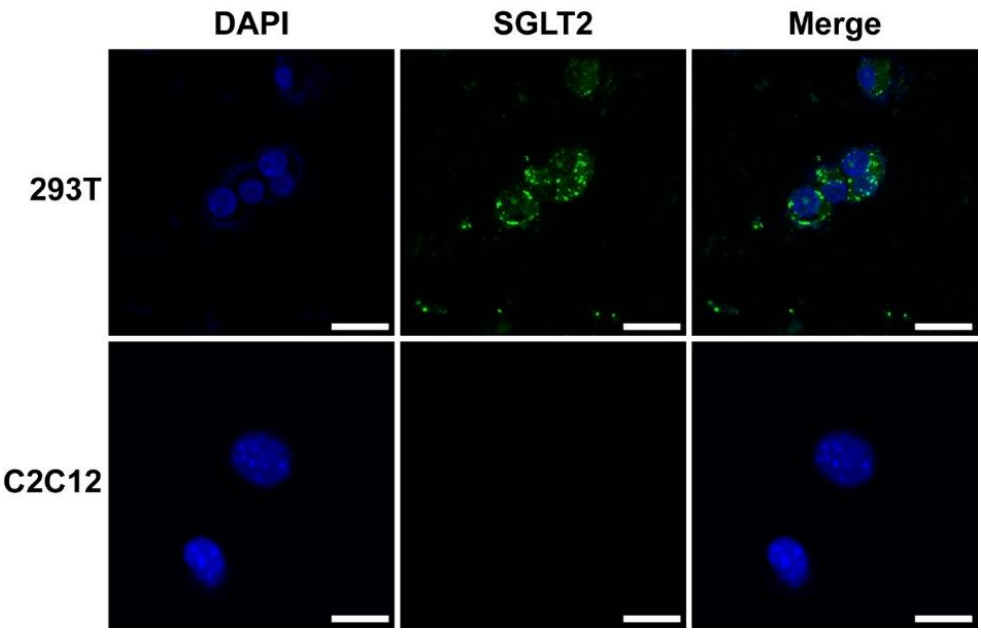
Supplementary Fig. S7. The effect of direct empagliflozin treatment on blood vessel-forming cells. (a–b) Proliferation potential of empagliflozin-treated HUVECs cells, as examined using EdU incorporation assay. Representative images (a; scale bars: 100 μm) and quantification results (b) were shown. **(c–d)** Proliferation potential of empagliflozin-treated MOVAS cells, as examined using EdU incorporation assay. Representative images (c; scale bars: 100 μm) and quantification results (d) were shown. **(e–f)** Migration potential of empagliflozin-treated HUVECs cells, as analyzed using transwell migration assay. Representative images (e; scale bars: 200 μm) and quantification results (f) were shown. **(g–h)** Migration potential of empagliflozin-treated MOVAS cells, as analyzed using transwell migration assay. Representative images (g; scale bars: 200 μm) and quantification results (h) were shown. Data were presented as mean ± SD ($n = 6$). All experiments were performed under hyperglycemia. HG: hyperglycemia; NS: not significant.

Supplementary Figure S8



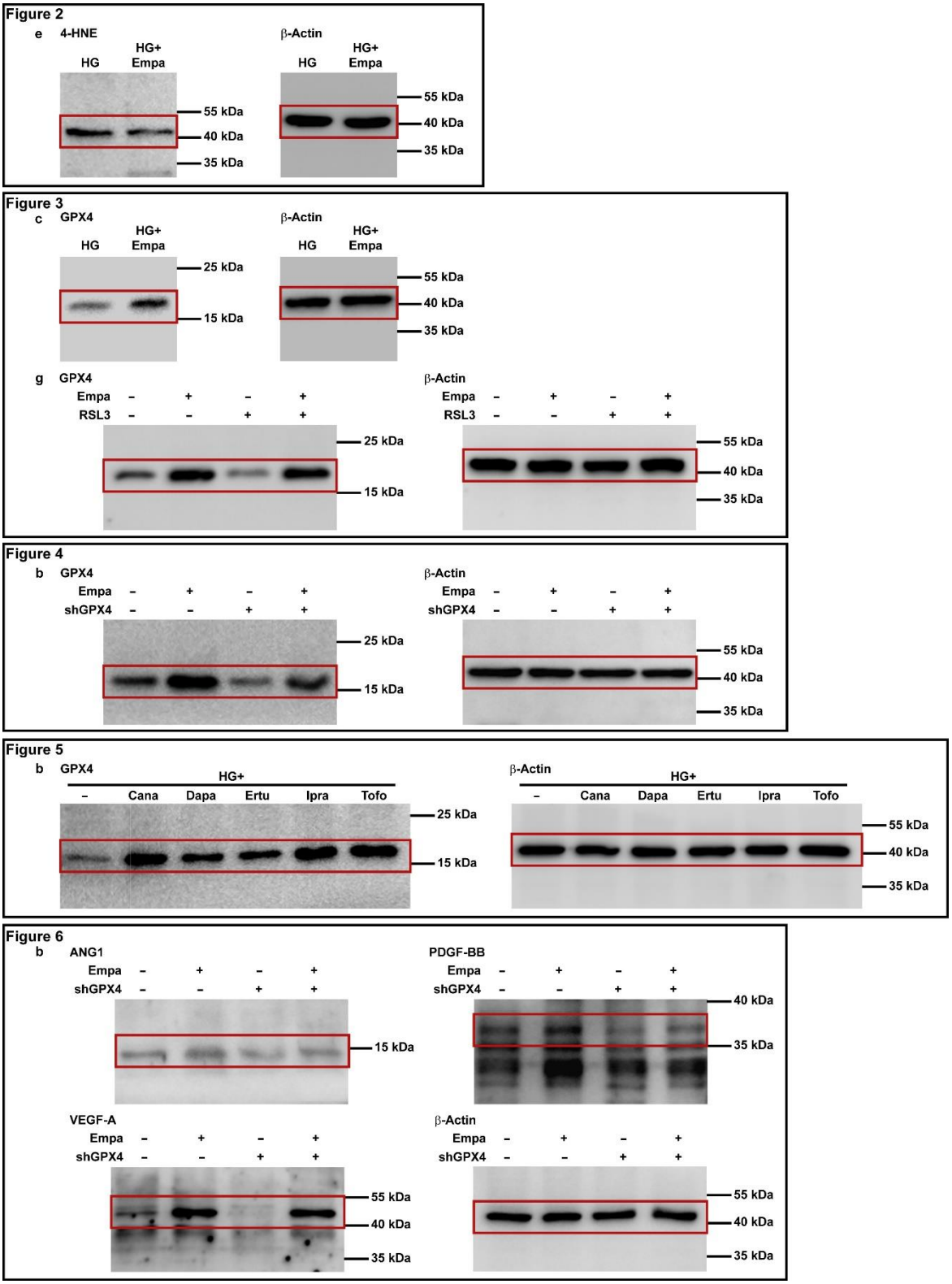
Supplementary Fig. S8. H&E staining of different organs in diabetic HLI mice treated intramuscularly with empagliflozin. Effect of empagliflozin treatment (10 mg/kg body weight) and shCon or shGPX4 vectors on different organs in diabetic HLI mice, as examined using H&E staining. Representative images were shown (scale bars: 50 μm).

Supplementary Figure S9



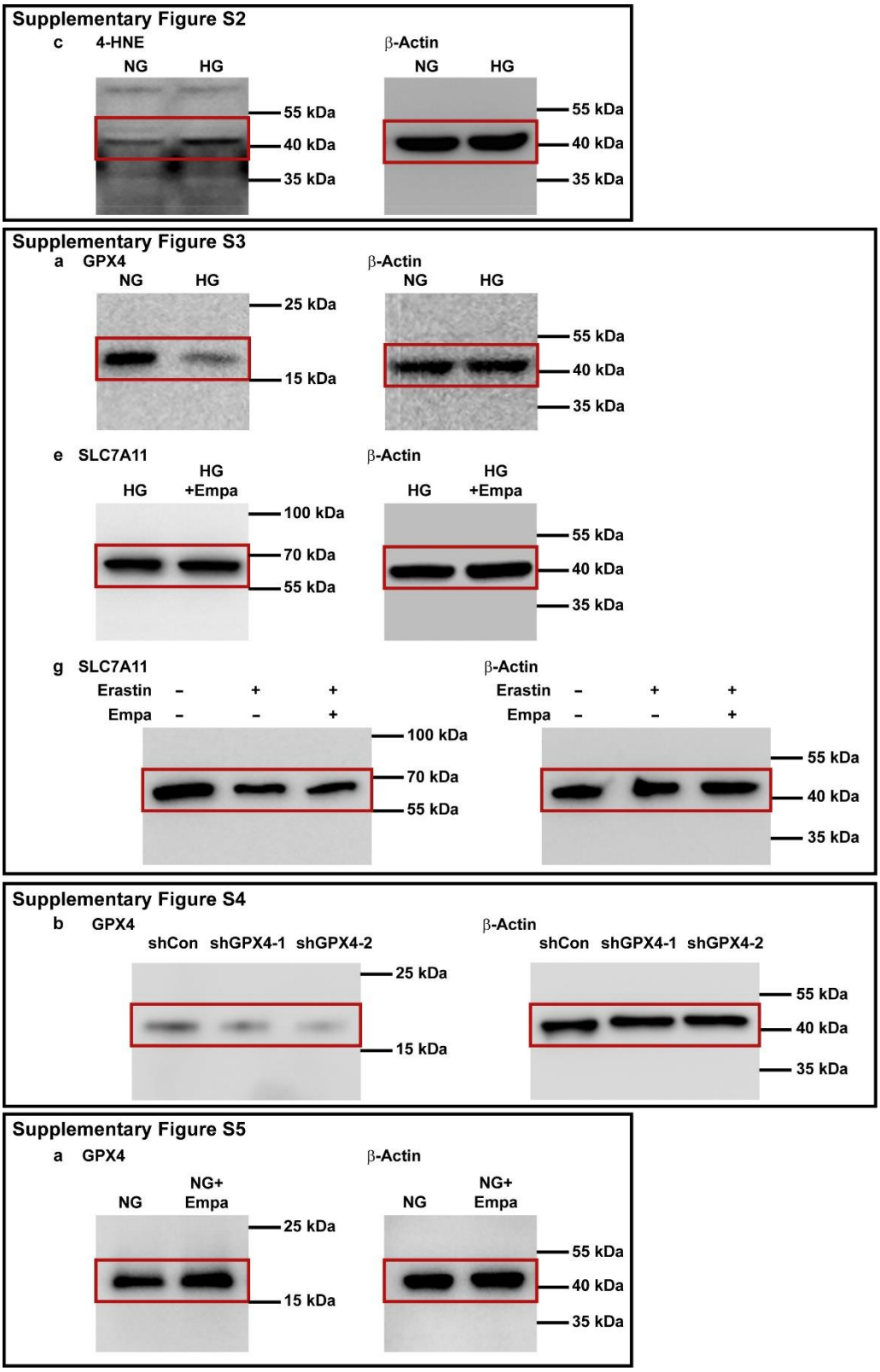
Supplementary Fig. S9. SGLT2 expression level in kidney and skeletal muscle cells. Representative images of SGLT2 expression in 293T cells and in C2C12 cells, as examined using immunofluorescence (scale bars: 12.5 μm).

Supplementary Figure S10



Supplementary Fig. S10. Uncropped western blot membranes for Figures 1-7.

Supplementary Figure S11



Supplementary Fig. S11. Uncropped western blot membranes for Supplementary Figures S1-9.

Supplementary Table S1. Primer pairs used in qRT-PCR

Genes	Refseq No.	Forward primer (5' – 3')	Reverse primer (5' – 3')
MyoD1	NM_010866.2	AGCACTACAGTGGCGACTCA	GGCCGCTGTAATCCATCAT
MyoG	NM_031189.2	CCTTGCTCAGCTCCCTCA	TGGGAGTTGCATTCACTGG
GPX4	NM_001037741.4	GTTTCGTGTGCATCGTCACC	CCCTTGGGCTGGACTTTCAT
GCLM	NM_008129.4	TTGAAGCCCAGGATTGGGTG	TAAGAGCCCCTCCTTTTGGC
GSS	NM_008180.2	GCTGGCTGGGACTAAGAAGG	AAGTGGCTAGGAGCAGCAAG
SLC3A2	NM_008577.4	CTCTCTGTTGCACGGTGACT	TTATGCCAGCAGGGAGGTTG
GCLC	NM_010295.2	TCCAGGTGACATTCCAAGCC	GAAATCACTCCCCAGCGACA
SLC7A11	NM_011990.2	TCCCTGGTTTTCTGGTCTGC	GAGCAGTTCCACCCAGACTC
FTL1	NM_010240.2	GGCAACCATCTGACCAACCT	GAGTGAGGCGCTCAAAGAGA
FTH1	NM_010239.2	CCAGAACTACCACCAGGACG	CAGAGCCACATCATCTCGGT
SLC40A1	NM_016917.2	GGCACTTTGCAGTGTCTGTG	GTCACCAATGATGGCTCCCA
ANG1	NM_009640	TTGTGATTCTGGTGATTGTGG	CTTGTTTCGCTTTATTTTTGT
FGF2	NM_008006	GTCACGGAAATACTCCAGTTGGT	CCCGTTTTGGATCCGAGTT
HGF	NM_010427	TGAATGAGTCTGAGTTATGTGC	GAACAATGACACCAAGAACC
VEGF-A	NM_001025250.3	GCAGAAGTCCCATGAAGTGAT	GTCTCAATCGGACGGCAGTAG
PDGF-B	NM_011057.4	AGCAGAGCCTGCTGTAATCG	GGCTTCTTTCGCACAATCTC
β-Actin	NM_007393.5	AGATGTGGATCAGCAAGCAG	GCGCAAGTTAGGTTTTGTCA

Supplementary Table S2. Antibodies used for western blotting, immunohistochemistry and immunofluorescence.

Antibody	Company	Cat #	Experiment	Dilution
Anti-4-HNE	Bioss	bs-6313R	Western blotting Immunohistochemistry	1/2,000 1/500
Anti-GPX4	ZEN BIO	ZEN381958	Western blotting Immunohistochemistry	1/2,000 1/200
Anti-SLC7A11	Proteintech	26864-1-AP	Western blotting	1/1,500
Anti-ANG1	Santa Cruz Biotechnology	SC-74528	Western blotting	1/300
Anti-PDGF-BB	Wanlei Biotechnology	WL01625	Western blotting	1/1,000
Anti-VEGF-A	Wanlei Biotechnology	WL03335	Western blotting	1/1,500
Anti-β-Actin	Proteintech	60008-1-Ig	Western blotting	1/50,000
Goat Anti-Mouse IgG	ZSGB-BIO	ZB2305	Western blotting	1/10,000
Goat Anti-Rabbit IgG	ZSGB-BIO	ZB2301	Western blotting	1/10,000
Anti-PECAM-1	BD Pharmingen	550274	Immunofluorescence	1/100
Monoclonal anti-murine α-SMA Cy3 conjugate	Sigma-Aldrich	C6198	Immunofluorescence	1/100
Anti-SGLT2	Proteintech	24654-1-AP	Immunofluorescence	1/200
Alexa Fluor 488 Donkey Anti-rabbit IgG	Invitrogen	A21206	Immunofluorescence	1/500

Supplementary Table S3. Body weight of diabetic HLI mice

shCon (g)						shCon+Empa (g)					
No	**Day-0**	**Day-3**	**Day-7**	**Day-14**	**Day-21**	**No**	**Day-0**	**Day-3**	**Day-7**	**Day-14**	**Day-21**
1	18.68	19.33	21.03	20.81	22.21	1	20.85	21.00	21.34	22.00	24.23
2	18.80	19.12	20.70	21.29	21.93	2	21.07	21.88	23.34	24.48	23.34
3	20.50	21.07	24.35	23.66	24.15	3	17.14	18.67	22.46	23.5	22.86
4	19.80	20.18	21.18	23.38	22.56	4	18.28	19.03	19.69	20.96	22.71
5	19.52	19.94	19.64	24.37	23.72	5	21.04	21.42	22.20	22.80	24.45
6	19.90	20.08	21.06	21.10	24.62	6	18.31	19.41	21.88	22.77	24.56
7	18.70	19.65	22.24	21.68	22.00	7	21.92	20.52	19.95	23.97	24.60
Mean	**19.41**	**19.91**	**21.46**	**22.33**	**23.03**	**Mean**	**19.80**	**20.28**	**21.55**	**22.93**	**23.82**
Stdev	**0.71**	**0.64**	**1.49**	**1.44**	**1.11**	**Stdev**	**1.84**	**1.25**	**1.33**	**1.20**	**0.83**
***P* value**	——	——	——	——	——	***P value**	**0.613**	**0.504**	**0.903**	**0.413**	**0.155**
shGPX4 (g)						**shGPX4+Empa (g)**					
No	**Day-0**	**Day-3**	**Day-7**	**Day-14**	**Day-21**	**No**	**Day-0**	**Day-3**	**Day-7**	**Day-14**	**Day-21**
1	21.00	19.96	19.23	20.16	20.36	1	23.55	23.40	23.56	24.00	22.96
2	19.31	19.31	19.55	19.66	19.64	2	21.87	21.66	21.23	21.67	24.71
3	20.00	20.20	21.89	22.32	21.93	3	19.78	20.39	19.61	19.54	22.37
4	18.75	19.13	20.82	21.64	22.23	4	17.70	18.75	22.62	22.46	21.92
5	15.52	17.59	20.75	21.45	22.51	5	19.21	19.26	19.36	19.16	25.06
6	18.73	20.68	22.40	23.36	24.00	6	17.22	18.13	21.33	22.63	21.96
7	20.03	20.34	20.83	21.54	21.76	7	19.05	19.97	21.73	22.47	21.84
Mean	**19.05**	**19.60**	**20.78**	**21.45**	**21.78**	**Mean**	**19.77**	**20.22**	**21.35**	**21.70**	**22.97**
Stdev	**1.75**	**1.04**	**1.14**	**1.25**	**1.43**	**Stdev**	**2.25**	**1.81**	**1.51**	**1.75**	**1.36**
***P value**	**0.618**	**0.518**	**0.359**	**0.244**	**0.093**	**#P value**	**0.516**	**0.447**	**0.443**	**0.757**	**0.135**

shCon: diabetic HLI mice administered with shCon vectors and treated with 10% DMSO; shCon+Empa: diabetic HLI mice administered with shCon vectors and treated with empagliflozin (10 mg/kg body weight); shGPX4: diabetic HLI mice administered with shGPX4 vectors and treated with 10% DMSO; shGPX4+Empa: diabetic HLI mice administered with shGPX4 vectors and treated with empagliflozin (10 mg/kg body weight).

**P* value was calculated versus mice from shCon group in corresponding days using one-way ANOVA.

#*P* value was calculated versus mice from shGPX4 group in corresponding days using one-way ANOVA.

Supplementary Table S4. Blood glucose level of diabetic HLI mice

shCon (mM)				shCon+Empa (mM)			
No	**3w HFD**	**1d pre-surgery**	**3w post-surgery**	**No**	**3w HFD**	**1d pre-surgery**	**3w post-surgery**
1	8.4	29.7	29.8	1	7.8	24.8	26.5
2	7.6	27.7	30.1	2	8.1	22.1	23.1
3	8.8	26.9	27.1	3	6.9	26.7	26.9
4	7.9	28.5	28.4	4	7.4	32.4	30.9
5	9.1	24.6	27.3	5	8.7	24.0	28.6
6	7.2	24.4	26.7	6	8.1	29.5	27.6
7	8.2	23.7	26.3	7	6.2	22.0	22.8
Mean	**8.2**	**26.5**	**28.0**	**Mean**	**7.6**	**25.9**	**26.6**
Stdev	**0.67**	**2.30**	**1.51**	**Stdev**	**0.84**	**3.88**	**2.89**
***P* value**	——	**0.00000000012***	**0.19#**	***P* value**	——	**0.000000040***	**0.71#**
shGPX4 (mM)				**shGPX4+Empa (mM)**			
No	**3w HFD**	**1d pre-surgery**	**3w post-surgery**	**No**	**3w HFD**	**1d pre-surgery**	**3w post-surgery**
1	7.2	28.6	26.7	1	9.2	17.0	19.7
2	7.3	26.9	27.5	2	6.9	27.8	28.3
3	7.7	28.4	30.3	3	7.7	28.7	25.5
4	8.9	27.3	30.5	4	9.7	27.9	32.5
5	9.8	28.9	29.0	5	6.8	21.6	25.2
6	6.5	23.5	23.7	6	7.1	27.7	32.3
7	9.3	27.1	29.4	7	7.9	18.4	19.1
Mean	**8.1**	**27.2**	**28.2**	**Mean**	**7.9**	**24.2**	**26.1**
Stdev	**1.23**	**1.83**	**2.41**	**Stdev**	**1.14**	**5.02**	**5.41**
***P* value**	——	**0.00000000003***	**0.44#**	***P* value**	——	**0.0000024***	**0.50#**

HFD: high fat died; shCon: diabetic HLI mice administered with shCon vectors and treated with 10% DMSO; shCon+Empa: diabetic HLI mice administered with shCon vectors and treated with empagliflozin (10 mg/kg body weight); shGPX4: diabetic HLI mice administered with shGPX4 vectors and treated with 10% DMSO; shGPX4+Empa: diabetic HLI mice administered with shGPX4 vectors and treated with empagliflozin (10 mg/kg body weight).

*P value was calculated versus 3w HFD in the same group using one-way ANOVA.

#P value was calculated versus 1d pre-surgery in the same group using one-way ANOVA.