Supplemental data

Inhibition of GPR39 Restores Defects in Endothelial Cell-Mediated Angiogenesis under the Duress of Chronic Hyperglycemia: Evidence for Novel Regulatory Roles of the Sonic Hedgehog Signaling Axis

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Running title: GPR39 regulates angiogenesis in hyperglycemia

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Expanded materials and methods

Animals

GPR39 knockout mice ($GPR39^{KO}$, on the C57BL/6 background) and their wildtype littermates ($GPR39^{WT}$) were kindly provided by Dr. Diederik Moechars of Janssen Pharmaceutical and transferred from Dr. Michal Hershfinkel's lab at the Ben-Gurion University of Negev, Israel. To create an animal model of chronic hyperglycemia, we injected the animals with streptozotocin (STZ) at 50 mg/kg for five consecutive days (STZ group). Citrate buffer was injected into the control group. After confirming that the animals were hyperglycemic (≥250 mg/dL), they were maintained for three months. To create an animal model of type 2 diabetes (T2D), mice were maintained on a high-fat diet (Research Diets, D12492) for 10 weeks. They then received additional low-dose STZ *via* intraperitoneal injections for 7 days (day 1 at 50 mg/kg, days 2-7 at 25 mg/kg). After confirming that these animals were hyperglycemic (≥250 mg/dL), they were maintained for two months before use in experiments. In another set of experiments, diet-induced obese (DIO) C57BL/6 mice maintained on a high-fat diet for 10 weeks were purchased from Jackson Laboratories (Bar Harbor, ME), and were continued on a high-fat diet until their weight reached 45-50 gm at which point they received STZ injections to create the T2D model. All animal experiments were performed according to Wayne State University Institutional Animal Care and Use Committee (IACUC) guidelines.

Cell culture

Human aortic endothelial cells (ECs) from healthy and T2D donors were purchased from Lonza (healthy ECs, CC-2535, n=5, age 53.67 ± 1.12 years, male/female = 3:2, BMI =22.7±1.34 ; T2D ECs, CC-2920, n=5, age 60.6 ± 4.31 years, male/female = 3:2, BMI =24.1±3.24) and were cultured in endothelial growth media-2 (EGM-2, Lonza, CC-3162) at 37 ºC, 5% CO2. Mouse ECs were

isolated and expanded *in vitro* in a DMEM culture medium supplied with endothelial cell growth supplement (ECGS, 75 mg/L, Sigma) and 10% fetal bovine serum (FBS) using the previously published protocol (1) **.** The human and mouse ECs were used between passages 5 and 7. In some experiments, the cells were treated with the GPR39 agonist, TC-G 1008 (C18H19ClN6O2S; Tocris,UK, #5355) at 5 μM for 24 h 35. For ligand-dependent SHH pathway activation, in human ECs, recombinant human SHH (C24II) N-terminus peptides (R&D Systems,# 1845-SH-100/CF) were used at 0.1 ng/ml for 90 min; in mouse ECs, recombinant mouse SHH (C25II) N-terminus peptides (R&D Systems, #464-SH-200/CF) were used at 0.1 ng/ml for 90 min. The control group was treated with DMSO. These cells were used to evaluate endothelial cell functions (tube formation, migration, and proliferation).

Small interfering RNA transfection

One day before transfection, ECs (2×10^6 cells/well) were seeded into 6-well plates to grow to reach 70 to 80% confluency. They were then transfected using DharmaFECT™ transfection reagents with human GPR39siRNA or scrambled siRNA (Ambion Inc). After siRNA treatment, the cells were incubated with the siRNA for 24 – 48h at 37°C in a 5% CO2 incubator. Knockdown of the targeted gene was confirmed by real-time PCR analysis after 48 h of transfection. Cells with the siRNA treatment were then used to evaluate EC function (tube formation, migration, proliferation).

Adenovirus transfection

The cells were seeded in 6-well plates. After 24 h, cells were transduced with adenovirus carrying human GPR39 (Ad-h-GPR39, Vector Biolabs) at a multiplicity of infection (MOI) of 30 with Ad-GFP as the control for 48 h. GPR39 mRNA levels was measured *via* real-time PCR. These cells

were then further used to evaluate EC functions (tube formation, migration, and proliferation) as above.

Hypoxia exposure

The cells were maintained in a humidified modular incubator chamber infused with gas mixture containing 95% N_2 and 5% CO_2 at 37 °C for 24 h. Cells cultured at 37 °C in 5% CO_2 incubator at 21% O_2 were used as control.

Cell functional assays

Proliferation assay (BrdU): EC proliferation was evaluated using the BrdU cell proliferation assay kit (Cell Signaling, #6813) according to the manufacturer's instructions. Briefly, ECs were plated on a 96 well plate at 5 x 10^4 / well density before receiving different treatments, followed by 16-hour incubation with BrdU agent. Optical density at 450 nm was recorded using a BioTek Epoch Spectrophotometer

Migration assay (modified Boyden chamber): Migration assays were performed in Boyden chambers (2) (Transwell Costar, 6.5 mm diameter, 8 μm pore size). Briefly, the inserts were coated with 0.1% gelatin (Sigma) for 1 h. The ECs (3×10^4 cells) transfected with adenovirus carrying human GPR39 plasmid, GPR39 siRNA and/or siRNA against SerpinE1, EGF, VEGF, IL-8, Cog factor III, and angiopoietin-1 with respective controls were suspended in a serum-free medium and were plated into the upper chamber. 10% FBS was added to the lower chamber to promote migration. After 7 h of incubation at 37°C, 5% CO_2, the inserts were removed, and unmigrated cells were wiped off with a cotton swab. Cells that migrated to the other side of the insert membrane were fixed using 4% paraformaldehyde and stained with 0.5% crystal violet. The number of migrated cells was counted using an EVOS fluorescence microscope under 20X magnification at four random high power fields (HPF).

3D-tube formation assay: EC angiogenesis was determined using a 3D-tube formation assay (3, 4). 3.8 x 10^4 cells of cell suspension (ECs transfected with adenovirus carrying human GPR39 plasmid and GPR39 siRNA with respective controls) were mixed with collagen and placed into the center of a 48-well plate. After 1h incubation at 37°C, 5% CO2, EGM-2 medium with 30 ng/mL VEGF-A165 (Peprotech, #100-20) was added to the wells. On days 6-8, the medium was removed, and the cell pellet was washed with PBS and fixed with 4% paraformaldehyde, and the cells then were permeabilized using 0.2% Triton- X100 in PBS. The cells were stained with 10 μg/mL FITC-conjugated lectin (Sigma, L9006) in PBS for 1 h in the dark at 4°C. Pictures of the tubes were taken at five random fields per well under 200X magnification using an EVOS fluorescence microscope.

Transcriptional activities of GLI by GLI-luciferase reporter assay

The activation of the SHH pathway was measured by detecting the expression of a luciferase reporter gene under the control of a GLI-responsive promoter (GLI-luc). The reporting lentivirus has a luminescent firefly luciferase 3 reporter under a minimal CMV promoter (mCMV) embedded with optimized tandem repeats of GLI responsive element sequence (5′-GACCACCCAC). It also expresses GFP as a fluorescent selection/normalization marker under the constitutive RSV promoter. When GLI proteins bind to the Gli responsive element sequence, the downstream reporter is expressed as the result of activation for the minimal promoter. According to the manufacturer's protocol, the cells were transfected with the GLI-luc lentivirus for 3 days. The SHH signaling was measured by detecting the luminescence before and 90 mins after adding SHH ligand at 0.1 ng/ml. The luminescent signaling was recorded using a GloMax® Illuminometer (Promega).

Western blot analysis

Cell lysates were collected by adding 80 µl of cell lysis buffer supplemented with protease and phosphatase cocktail inhibitors (Thermo Fisher Scientific) to the cells. Protein concentration in cell lysates was evaluated using the Bradford method. Equal amounts of protein for each sample were subjected to SDS-PAGE. After electrophoresis, proteins were transferred onto nitrocellulose membranes, and the membranes were subsequently blocked with intercept blocking buffer (Li-Cor) for 1 h at room temperature. Membranes were then incubated overnight at 4°C with primary antibodies against the proteins of interest. The primary antibodies were Sonic hedgehog (SHH), (#2207,1:1000), SUFU (#2522,1:1000), β-actin (#12262,1:10000), GLI1 (#2553 ,1:1000) from cell signaling, Patched-1 (PTCH-1) (#PA-46222,1:500) from Invitrogen, Smoothened (Anti-SMO), (#235183,1:1000) from Abcam. After 3 washes with TBST, membranes were incubated with horseradish peroxidase (HRP) - conjugated secondary antibody or IRDye® 800CW or 680CW secondary antibody at room temperature for 1 h. The signals were visualized using Odyssey CLx Imaging System (Li-Cor). Densitometry analysis was conducted using Image Studio 5.2. (Li-Cor).

Co-Immunoprecipitation assay

Healthy ECs were transfected with the adenovirus carrying human GPR39 with GFP tag (Ad-GFP-GPR39) or the adenovirus with only GFP tag (Ad-GFP) as control. A total of 400 µg of protein was used for each pull-down reaction. We used the GFPTrap ® Agarose Kit (Chromotek, #gtak-20) to immunoprecipitate the GFP-tagged GPR39 from cell lysates. The GFP-Trap is a ready-to-use reagent that consists of a GFP Nanobody conjugated to beads. The Co-IP was performed according to the manufacturer's protocol. After elution with the SDS-sample buffer, the samples were analyzed by Western blot using antibodies against PTCH-1) (Invitrogen #PA- 46222,1:500), SMO (Abcam #235183,1:1000), SUFU (Cell signaling, #2522,1:1000), SHH (Cell signaling

#2207,1:1000). After 3 washes, membranes were incubated with horseradish peroxidase (HRP) - conjugated secondary antibody or IRDye® 800CW or 680RD secondary antibody at room temperature for 1 h. The signals were visualized under the Odyssey CLx Imaging System (Li-Cor). The densitometry analysis was made using Image Studio 5.2. (Li Cor).

Human pro-angiogenic protein array

The Proteome Profiler Human Angiogenesis Array (R&D Systems, #ARY007) allows simultaneous detection of 55 human angiogenesis-related proteins. Studies followed the manufacturer's instructions. Briefly, ECs at 80% confluence were transfected with Ad-h-GPR39 or Ad-GFP as control. The membrane, spotted with 55 angiogenesis-related antibodies, was blocked for 1 h at room temperature. Cell lysates (200 μg) were mixed with a cocktail of biotinylated detection antibodies (15 μL), added to the membrane, and incubated overnight at 4°C. Membranes were exposed to streptavidin-HRP for 30 min, followed by chemiluminescence detection reagents, and exposed for 10 min. The intensity of protein expression signals was quantified with an Odyssey CLx Imaging System (Li-Cor).

Hindlimb ischemic surgery

The femoral artery was isolated from the nerves and vein. The proximal portion of the femoral artery and the distal portion of the saphenous vein was ligated while the animal was under anesthesia. The arterial branches between the ligation were destroyed. The overlying skin was closed with sutures. Blood flow was measured before, immediately after, and at 1, 7, 11, 14, 21, and 28 days after femoral arterial ligation using Laser Doppler imaging and a PeriScan PIM 3 System (Perimed). Tissue necrosis was assessed and scored on daily basis. Gastrocnemius muscle tissues were harvested for immunofluorescence studies at the end of the experiments. Supplemental Table 2 delineates the severity of ischemia injury. The animals were promptly

euthanized when they displayed the following complications: 1) necrosis Score ≥ 5; 2) ulceration depth to muscle; 3) bone exposure; 4) sign of infection, such as purulent discharge from the wound

Immunofluorescent staining

Muscle samples from the upper thigh and calf were collected and embedded in OCT. 8 μm sections were cut using a cryostat and immediately transferred to poly-L-lysine coated slides (FisherScientific,12-550-15). Sections were fixed for 15 min at room temperature (RT) with 4% paraformaldehyde (Thermo Fisher Scientific J19943-K2) and washed with TBST; 0.2% Triton X-100 in TBS was used to permeabilize tissue. After 15 mins, the slides were washed and blocked with 5% BSA for 1 h. The sections were then incubated with a goat anti-mouse CD31 conjugated Alexa 488 antibody (R&D Systems, #FAB 3628G, 1:150 dilution), or incubated with a rabbit anti-mouse α-smooth muscle actin (SMA) conjugated Alexa 594 antibody (Cell Signaling Technology, #36110, 1: 60 dilution) overnight at 4°C. The slides were washed, mounted with DAPI fluorescent medium (Southern Biotech, 0100-20), and air-dried. Fluorescent images were recorded by fluorescence microscopy (EVOS, Thermo Fisher Scientific). For arterial morphologies, Only arterial structures identified by the presence of SMA and CD31 positive stainings present between muscle bundles and fibers and with a mathematically derived area of 500 μm x 500 μm were counted. The number of arteries present in each thigh was expressed as the ratio of the number of arteries to the surface area. The diameter of the artery was acquired by tracing the inner circumference of the artery and calculated using the formula Diameter = Circumference / π .

Supplemental Table S1. Real-time PCR Primer information.

Gene	Primer sequence
Human.GAPDH, forward	5’-CAACTTTGGCATTGTGGAAGG-3’
Human.GAPDH, reverse	5’-ACACATTGGGGGTAGGAACAC-3'
Mouse.Gapdh, forward	5’-AATGGTGAAGGTCGGTGTG-3’
Mouse. Gapdh, reverse	5’-GTGGAGTCATACTGGAACATGTAG-3’
Human. GPR39, forward	5’-CATCTTCCTGAGGCTGA-3'
Human. GPR39, reverse(5)	5’-ATGATCCTCCGTCTGGTTG-3'
Mouse.Gpr39, forward	5’-GACAGCAAGAAGACAGACCAT-3’
Mouse.Gpr39, reverse	5’-ATCATGTATGCCCTGAAGTACG-3’
Human.VEGF, forward	5’-GTACAAGATCCGCAGACGTG-3’
Human.VEGF, reverse	5’-TTCTGTATCAGTCTTTCCTGGTG-3’
Human.PIGF, forward	5’-TGATGGTAGGAGGCAGTAGT-3’
Human.PIGF, reverse	5’-CGTTATCTTTCATGGTGTTTTCTTG-3’
Human.IL-8 , forward	5’-GAGACAGCAGAGCACACAAG-3’
Human.IL-8 , reverse	5’-CTTCACACAGAGCTGCAGAA-3’
Human.Coagulation Factor-III, forward	5’-ACCCGTCAATCAAGTCTACAC-3’
Human.Coagulation Factor-III, reverse	5’-GTCTGCTTCACATCCTTCACA-3'
Human.EGF, forward	5’-ACAGAATCTCAACACATGCTAGT-3’
Human.EGF, reverse	5’-CATCCTCTCCCTCTGAAATACAC-3’
Human.Endothelin-1 , forward	5’-GTGTCTACTTCTGCCACCTG-3’
Human.Endothelin-1 , reverse	5’-AAGTAAATTCTCCAAGGCTCTCT-3’

Human.MCP-1,forward	5’-AGCAGCCACCTTCATTCC-3’
Human.MCP-1,reverse	5’-GCCTCTGCACTGAGATCTTC-3’
Human.Pentraxin,forward	5’-GTTGGAGCGGATGTATTGGAT-3’
Human.Pentraxin,reverse	5’-CTTGTAGCCTACTCTGAATCAGC-3’
Human.PDGF-AB, forward	5’-CCACACTCCTTGCCCTTT-3’
Human.PDGF-AB, reverse	5’-CACAGACTCAATCACCTTCCA-3’
Human.IGFBP-1,forward	5’-GCTCTCCATGTCACCAACA-3’
Human.IGFBP-1,reverse	5’-CTTCTCCTGATGTCTCCTGTG-3’
Human.Artimin,forward	5’-GGAAAGGTGCCTAGAAGAACA-3’
Human.Artimin,reverse	5’-GTCCAAGTTCCATCTCTATCAACA-3’
Human.Angiopoietin-1,forward	5’-AACCAGCCTCCTCTCTCAG-3’
Human.Angiopoietin-1,reverse	5’-GCAGCTGTATCTCAAGTCGA-3’
Human.Angiopoietin-2,forward	5’-CAAAATAAGCAGCATCAGCCA-3’
Human.Angiopoietin-2,reverse	5’-AGTACATTCCGTTCAAGTTGGA-3’
Human.Activin A,forward	5’-ACAGTGCCAATACCATGAAGA-3’
Human.Activin A,reverse	5’-TTTTCCTTCTCCTCTTCAGCA-3’
Human.Serpin-E1,forward	5’-TGACAACAGGAGGAGAAACC-3’
Human.Serpin-E1,reverse	5’-GAGCTCCTTGTACAGATGCC-3’
Human.DPPIV,forward	5’-GACATGGGCAACACAAGAAAG-3’
Human.DPPIV,reverse	5’-CCACTAAGAAGTTCCATCTTCC-3’
Human.SHH, forward	5’-GAGTCCAAGGCACATATCCAC-3'
Human.SHH, reverse	5’-TCAGGTCCTTCACCAGCTT-3’
Human.GLI-1, forward	5’-CATCAGGGAGGAAAGCAGAC-3’

Human.GLI-1, reverse	5’-CATTGCCAGTCATTTCCACAC-3’
Human.PTCH-1, forward	5’-GAACCTCGAGACCAACGTG-3’
Human.PTCH-1, reverse	5’-ATAGCCTCTTCTCCAATCTTCTG-3’
Human.SMO, forward	5’-AGATCAACGAGACCATGCTG-3'
Human.SMO, reverse	5’-CATTGGCCTGACATAGCACA-3'
Human.SUFU, forward	5’-GCCCTTCTGCTAACATCCC-3'
Human.SUFU, reverse	5’-GTCAACTCAAAGCCAAAACCAC-3’
Human.FGF2, forward	5’-AGCGGCTCTACTGCAAGAAC-3’
Human.FGF2, reverse	5’-AGCAGACATTGGAAGAAACAGT-3’
Human.MMP1, forward	5’-ATAACTACGATTCGGGGAGAAG-3’
Human.MMP1, reverse	5’-TCTATGGGAAACCTCATAAGCA-3’
Human.GM-CSF, forward	5’-CACTGCTGCTGAGATGAATGAAA-3’-3’
Human.GM-CSF, reverse	5’-GTCTGTAGGCAGGTCGGCTC-3’
Human.TGFB1, forward	5’-TCGCCAGAGTGGTTATCTT-3’
Human.TGFB1, reverse	5’-TAGTGAACCCGTTGATGTCC-3’
Human.TGFB2, forward	5’-ACACTCAGCACAGCAGGGTCCT-3’
Human.TGFB2, reverse	5’-TTGGGACACGCAGCAAGGAGAAG-3’
Human.TGFB3, forward	5’-TGAGTGGCTGTTGAGAAGAGA-3’
Human.TGFB3, reverse	5’-ATTGTCCACGCCTTTGAATTTGAT-3’
Human.ICAM1, forward	5’-GTGGTAGCAGCCGCAGTC-3’
Human.ICAM1, reverse	5’-GGCTTGTGTGTTCGGTTTCA-3’
Human.VCAM1, forward	5’-AATGGGAATCTACAGCACCTTT-3’
Human.VCAM1, reverse	5’-ATATCCGTATCCTCCAAAAACT-3’

Mouse. Shh, forward	5’-GAATCCAAAGATCACATCCAC-3’
Mouse. Shh, reverse	5’-CGTAAGTCCTTCACCAGATTG-3’
Mouse. Gli-1, forward	5’-CCTTTCTTGAGGTTGGGATGA-3’
Mouse. Gli-1, reverse	5’-TTGGATTGAACATGGCATCT-3’
Mouse. Ptch-1, forward	5’-ACAAGCCCATCGACATTAGTC-3’
Mouse. Ptch-1, reverse	5’-CAAGCGGTCAGGTAGATGTAG-3’
Mouse. Smo, forward	5’-GCTGCCACTTCTATGACTTCT-3’
Mouse. Smo, reverse	5’-GCCGATTCTTGATCTCACAGT-3’
Mouse. Sufu, forward	5’-CACTGGCACTACATCAGCTT-3’
Mouse. Sufu, reverse	5’-CGTCAACTCAAAGCCAAATCC-3’
Mouse. Serpine1, forward	5’-CGTGTCAGCTCGTCTACAG-3’
Mouse. Serpine1, reverse	5’-CTATGGTGAAACAGGTGGACT-3’
Mouse. Il8, forward	5’-GCTTCATTGCCGGTGGAA-3’
Mouse. Il8, reverse	5’-ACAGAAAGGAAGTGATAGCAGT-3’
Mouse. Cog factor III, forward	5’-GCCTTCTTCTGCTCATCCTT-3’
Mouse. Cog factor III, reverse	5’-ACATTCAGACGGACATCACAG-3’
Mouse. Vegfa, forward	5’-GCTACTGCCGTCCGATTGAGAC-3’
Mouse. Vegfa, reverse	5’-GTGCTGGCTTTGGTGAGGTTTG-3’
Mouse. Egf, forward	5’-GAGTTGCCCTGACTCTACCG-3’
Mouse. Egf, reverse	5’-CCACCATTGAGGCAGTATCC-3’
Mouse. Angiopoietin-1, forward	5’-CCATGCTTGAGATAGGAACCAG-3’
Mouse. Angiopoietin-1, reverse	5’-TTCAAGTCGGGATGTTTGATTT-3’

Supplemental Table S2. The assessment of the severity of tissue necrosis

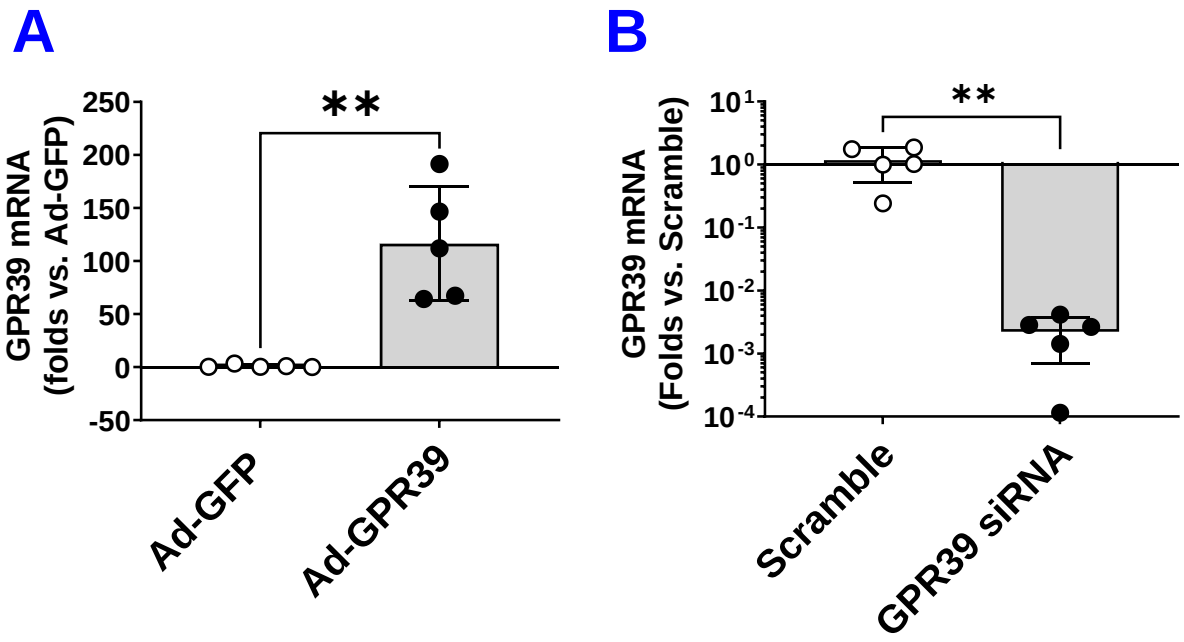
Necrosis Score
7 Auto-amputation of leg
6 Leg necrosis
5 Foot necrosis
4 Discoloration of > two toes
3 Discoloration of one toe
2 Discoloration of > two nails
1 Discoloration of one nail
0 No necrosis

The animals were promptly euthanized when they displayed the following complications: 1) Necrosis Score ≥ 5; 2) Ulceration depth to muscle; 3) Bone exposure; 4) Sign of infection, such as purulent discharge from the wound.

Supplemental Table S3. Antibody used in western blot analysis

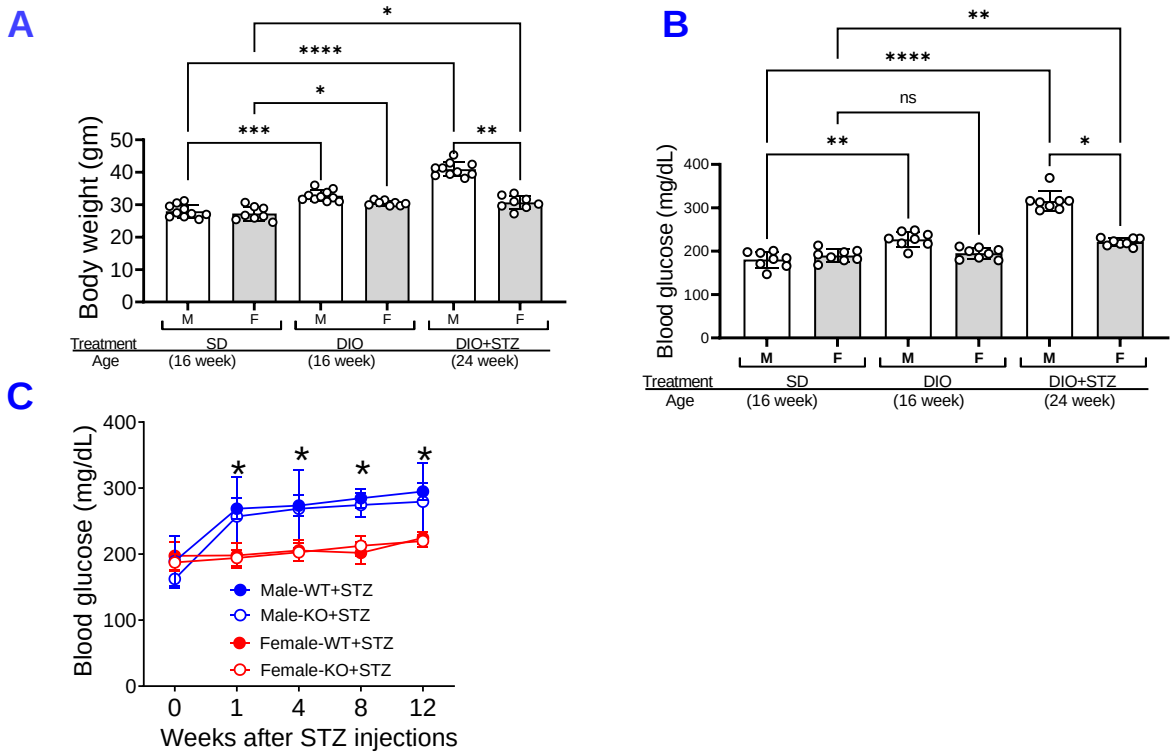
Target antigen	Vendor or Source	Catalog #	Working concentration
Sonic hedgehog (SHH)	Cell Signaling	2207	1:1000
Suppressor of fused (SUFU)	Cell Signaling	2522	1:1000
Glioma associated gene (GLI-1)	Cell Signaling	2553	1:1000
β-actin	Cell Signaling	12262	1:10000
Patched-1(PTCH-1)	In vitrogen	PA-46222	1:500
Smoothened (Anti-SMO)	Abcam	235283	1:1000
HRP horse anti-mouse IgG	Cell Signaling	7076S	1:3000
HRP goat anti-rabbit IgG	Cell Signaling	7074S	1:3000
IRDye 680RD goat anti-mouse	Li-Cor	926-68170	1:15000
IRDye 800CW goat anti-rabbit	Li-Cor	926-32211	1:15000

Supplemental Figure S1. The GPR39 mRNA levels after adenovirus-mediated GPR39 overexpression or siRNA-mediated GPR39 gene knockdown.



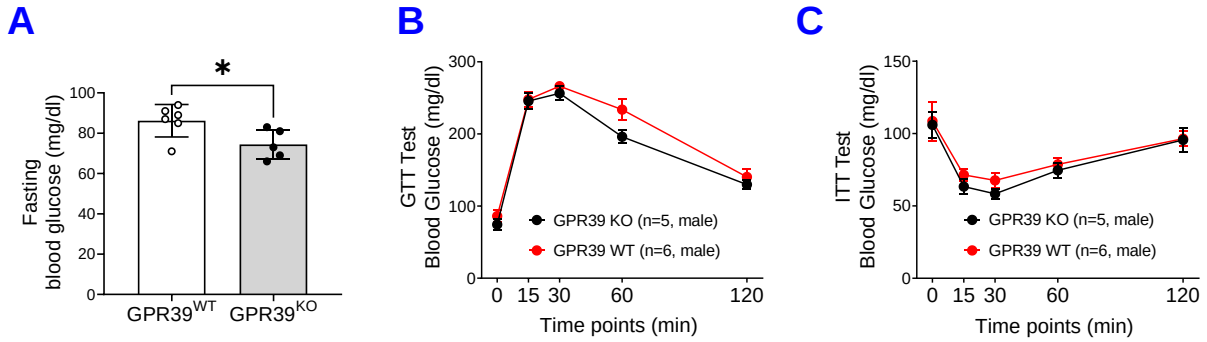
(A) Overexpression of GPR39 mRNA levels in healthy ECs by the transfection of adenovirus carrying human GPR39 (Ad-GPR39) using adenovirus carrying GFP (Ad-GFP) as control, measured by real-time PCR. n=5,**p < 0.01. **(B)** Decreased GPR39 mRNA levels in human ECs by the transfection of siRNA against GPR39 (GPR39 siRNA), using scramble oligo as control, measured by real-time PCR. n=5 per group. **p < 0.01. Data are presented as mean±SD. *P* values were determined by the Mann-Whitney U test.

Supplemental Figure S2. The body weight and blood glucose in male and female mice with Diet induced obesity and add-on Stroptozotocin-induced hyperglycemia.



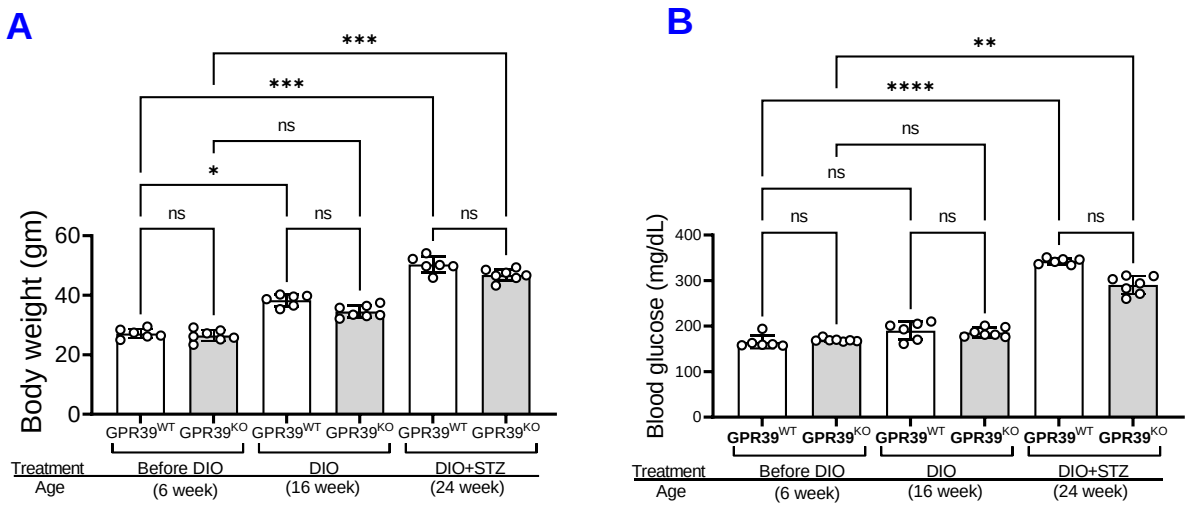
(A) Body weights of male and female GPR39^{WT} mice in SD, DIO, and DIO+STZ groups. n=10 in the male group; n=8 in the female group. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. **(B)** Blood glucose levels of male and female GPR39^{WT} mice in SD, DIO, and DIO+STZ groups. n=8 per group. *p < 0.05; **p < 0.01; ****p < 0.0001. **(C)** Blood glucose levels of GPR39^{WT} and GPR39^{KO} mice of both sexes at various time points after STZ injections. n = 5 per group. *p < 0.05 vs. females receiving the same procedure at the same time point. Data are represented as mean±SD. When a comparison was made between two groups, the *p* values were determined by the Mean-Whitney U test. When comparison was made between more than two groups of treatments, the p values were first determined by the Kruskal-Wallis test across all the groups and, if significant, the pairs of primary interest, based on scientific rationale, were assessed using the Mann-Whitney U test with Benjamini Krieger, and Yekutieli's adjustment for multiple comparisons. The significant differences that came from *posthoc* comparisons of groups were noted.

Supplemental Figure S3. Glucose metabolism profiles of adult male GPR39^{KO} and GPR39^{WT} mice.



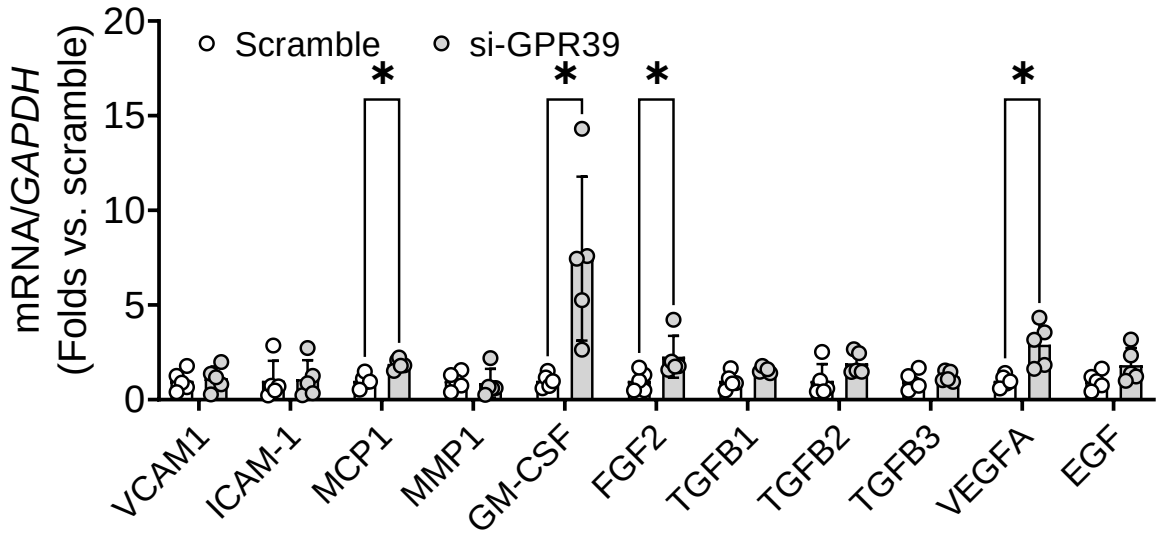
(A) The fasting blood glucose of male GPR39^{WT} and GPR39^{KO} mice. n=6 in GPR39^{WT} group, n=5 in GPR39^{KO} group. *$p < 0.05$. **(B)** Blood glucose recordings of male GPR39^{WT} and GPR39^{KO} mice in glucose tolerance test (GTT). n=6 in GPR39^{WT}; n = 5 in GPR39^{KO}. Each animal was given an intraperitoneal injection of glucose at 2.5 g/kg bodyweight. **(C)** Blood glucose recordings of male GPR39^{WT} and GPR39^{KO} mice in insulin tolerance test (ITT). n=6 in GPR39^{WT}; n = 5 in GPR39^{KO}. Each animal was given an intraperitoneal insulin injection at 0.75 U/kg body weight, followed by blood glucose monitoring. Data are presented as mean±SD. The p values were assessed using the Mann-Whitney U test. The comparisons at each time point were adjusted with Benjamini Krieger and Yekutieli's method.

Supplemental Figure S4. Bodyweight gain and blood glucose levels in GPR39^{KO} and GPR39^{WT} mice receiving high-fat diet-induced obesity (DIO) and low-dose streptozotocin (STZ)-induced hyperglycemia.



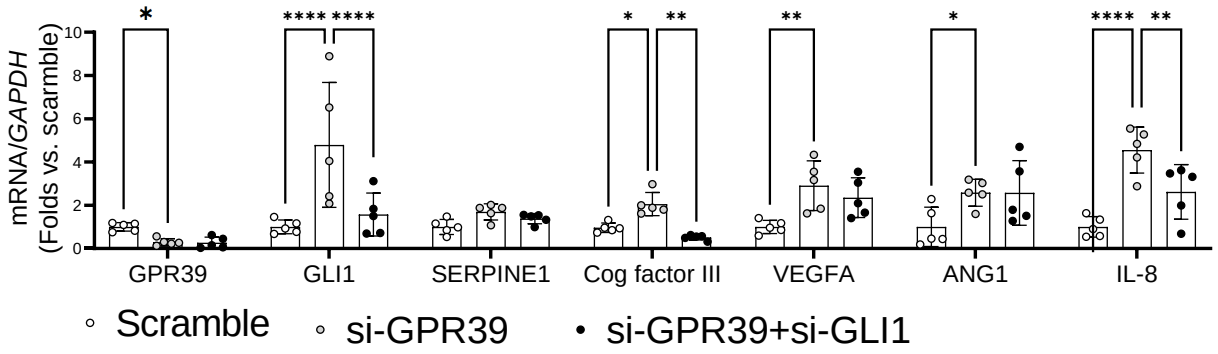
(A) The body weights of male GPR39^{WT} and GPR39^{KO} mice before and after DIO and DIO+STZ. n=6 in GPR39^{WT} group, n=7 in GPR39^{KO} group. *p < 0.05; ***p < 0.001; ns, not significant. **(B)** Blood glucose recordings of male GPR39^{WT} and GPR39^{KO} mice before and after DIO and DIO+STZ. n=6 in GPR39^{WT}; n = 7 in GPR39^{KO}. **p < 0.01; ****p < 0.0001; ns, not significant. Data are presented as mean±SD. The p values were first determined by the Kruskal-Wallis test across all the groups, and, if significant, the pairs of primary interest, based on scientific rationale, were assessed using the Mann-Whitney U test with Benjamini Krieger, and Yekutieli's adjustment for multiple comparisons. The significant differences that came from *posthoc* comparisons of groups were noted.

Supplemental Figure S5. EC-derived arteriogenic factors upon GPR39 siRNA transfection.



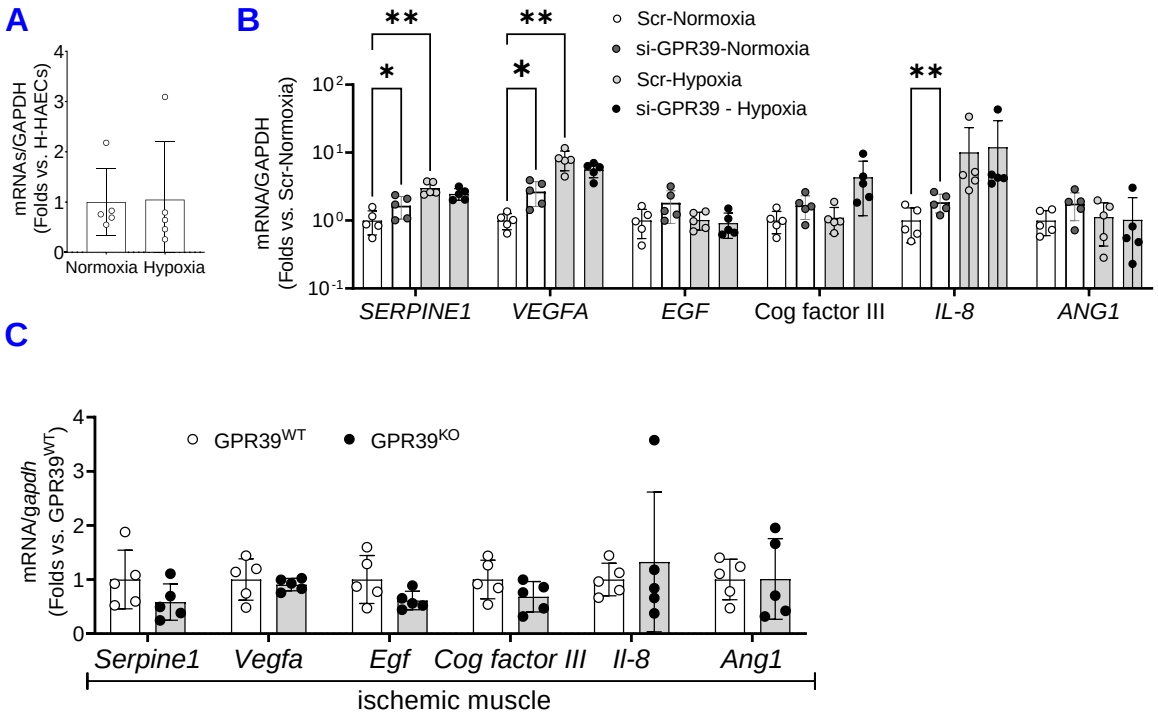
The mRNA levels of EC-derived arteriogenic factors in human healthy ECs transfected with GPR39 siRNA or scramble control, measured by real-time PCR. n=5 per group. *$p < 0.05$. Data are presented as mean±SD. *p* values were determined by the Mann-Whitney U test.

Supplemental Figure S6. GPR39-regulated Cog factor III and IL-8 production in ECs were mediate by GLI1.



To test whether these cytokines/growth factors were due to SHH/GLI1 mediated transcriptional activation, we knocked down GLI1 in human aortic ECs and tested tested the transcriptions of these molecules. The mRNA levels were measured by real-time PCR. n=5 per group. *p < 0.05; **p < 0.01; **** p < 0.0001. Data are presented as mean±SD. The *p* values were first determined by the Kruskal-Wallis test across all the groups and, if significant, the pairs of primary interest, based on scientific rationale, were assessed using the Mann-Whitney U test with Benjamini Krieger, and Yekutieli's adjustment for multiple comparisons. The significant differences that came from *posthoc* comparisons of groups were noted.

Supplemental Figure S7. The potential impact of hypoxia on angiogenic factors in ECs and in ischemic skeletal muscle.



(A) GPR39 mRNA levels in human ECs exposed to hypoxia or normoxia for 24 h. n=5. **(B)** mRNA levels of pro-angiogenic factors in human ECs with siRNA transfection following hypoxia or normoxia exposure. n=5, *p < 0.05, **p < 0.01. **(C)** mRNA levels of angiogenic factors in ischemic gastrocnemius muscle from $GPR39^{KO}$ and $GPR39^{WT}$ mice on day 7 after hindlimb ischemia surgery. n=5. Data are presented as mean±SD. The *p* values were first determined by the Kruskal-Wallis test across all the groups and, if significant, the pairs of primary interest, based on scientific rationale, were assessed using the Mann-Whitney U test with Benjamini Krieger, and Yekutieli's adjustment for multiple comparisons. The significant differences that came from *posthoc* comparisons of groups were noted.

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