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Supplemental Information

Epigenetic Modification of MicroRNA-200b Contributes to Diabetic Vasculopathy

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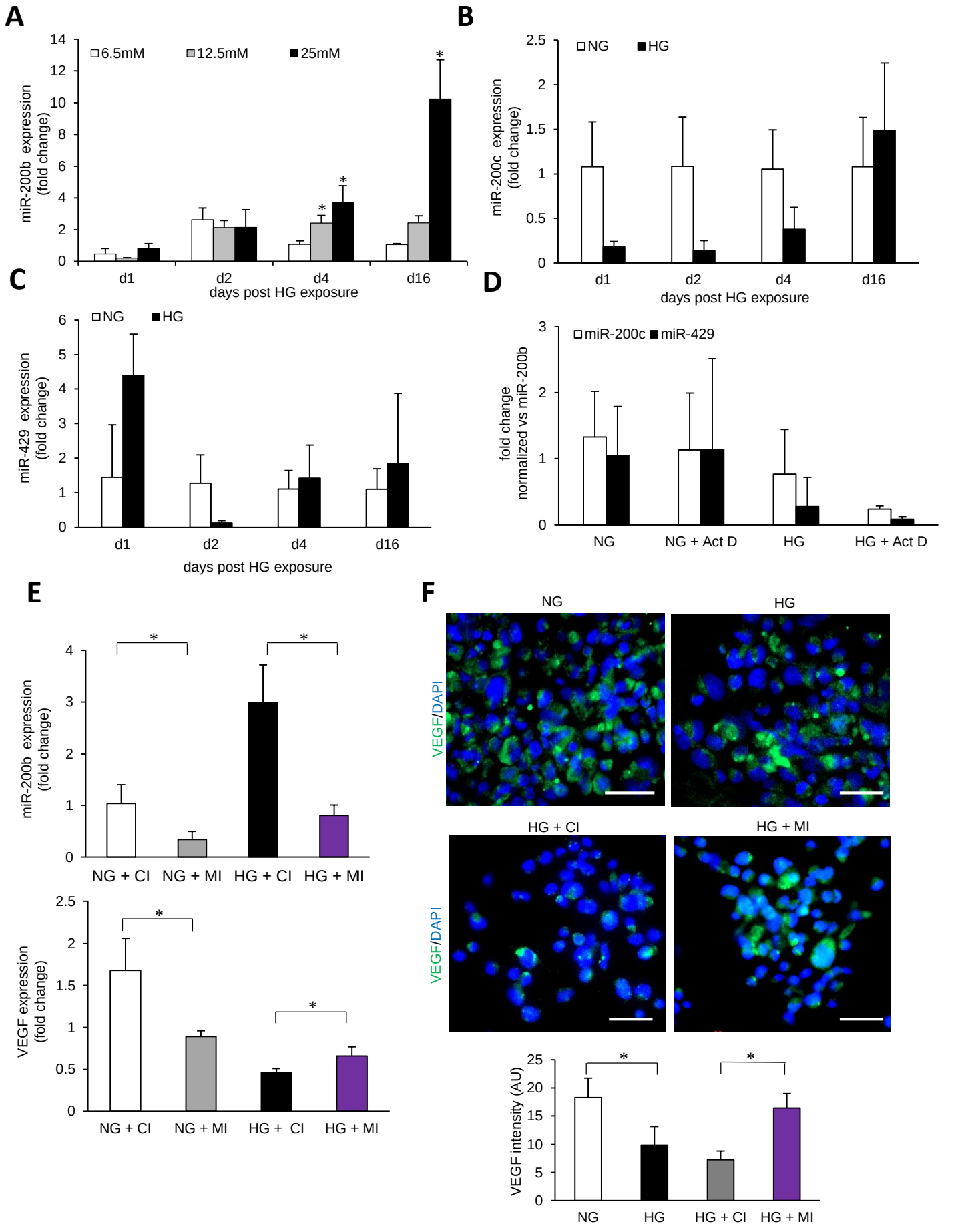
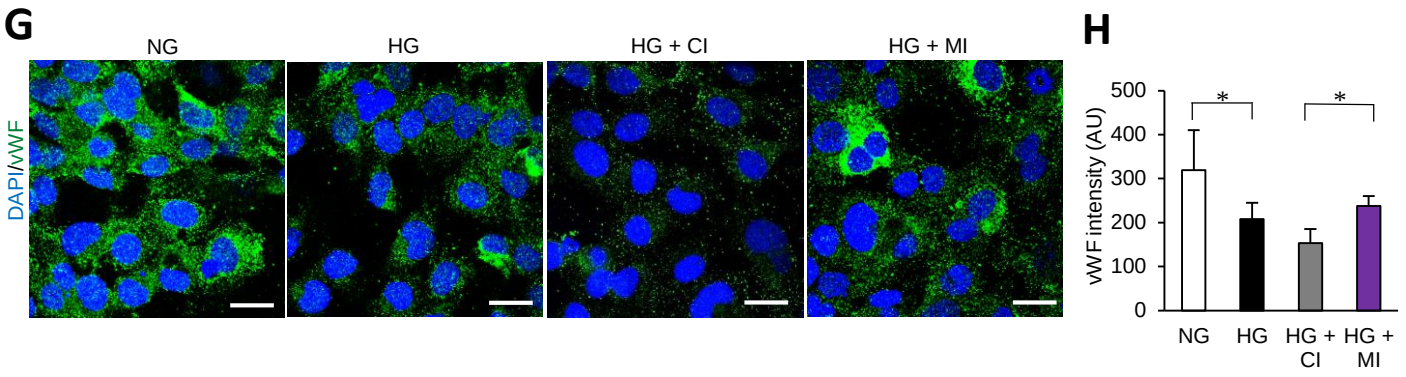
Supplementary Figure 1

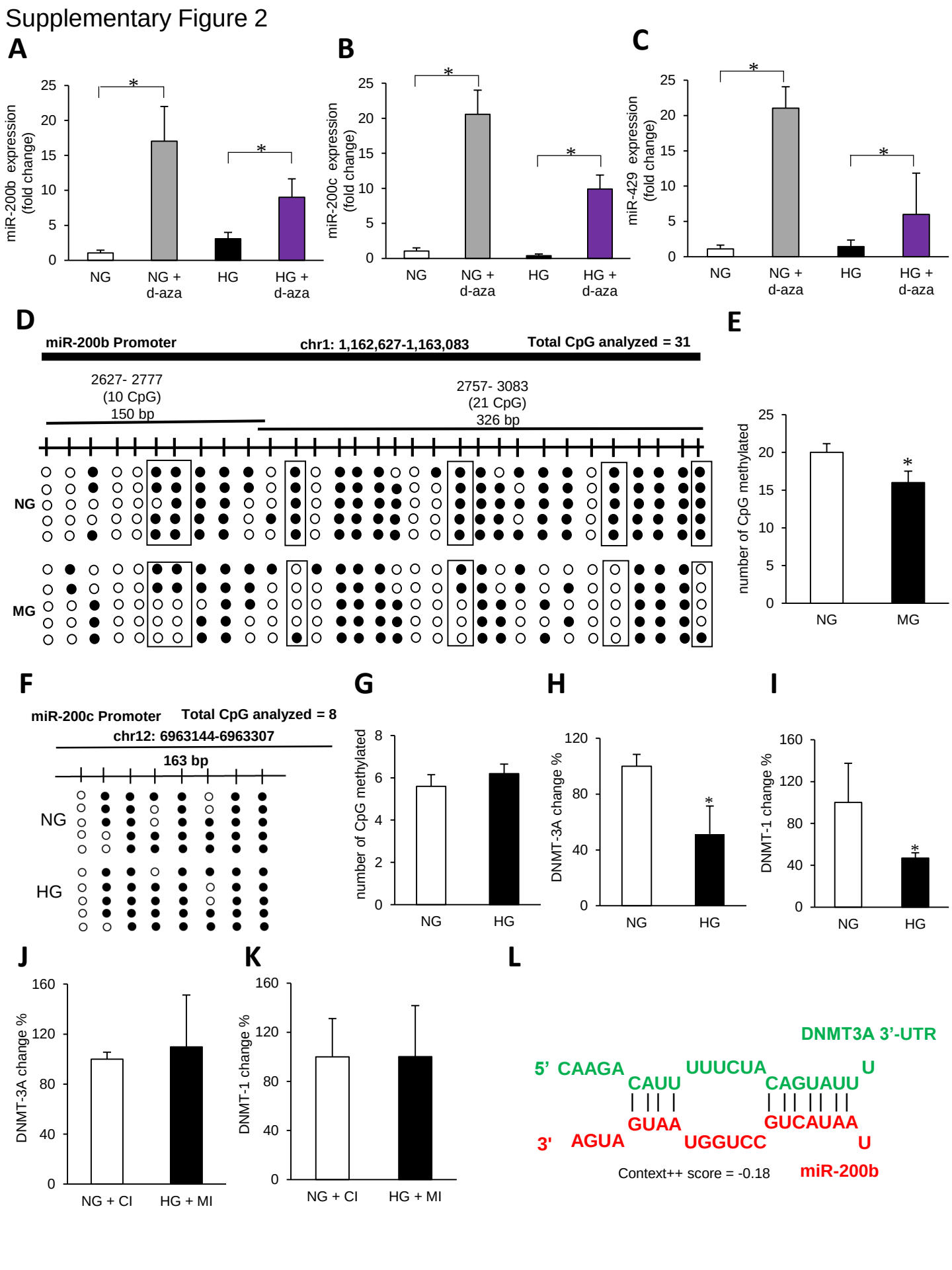


Supplementary Figure 1

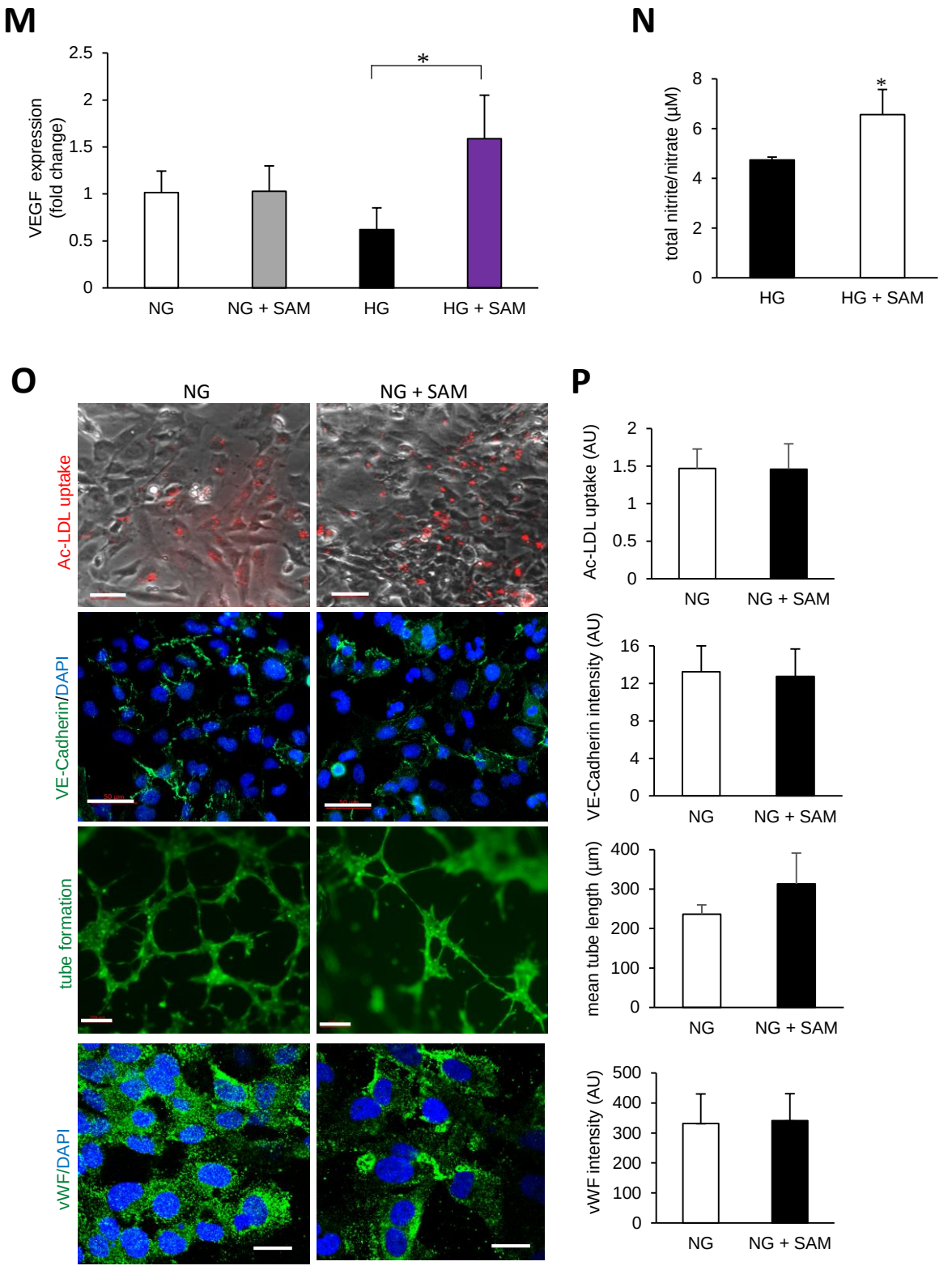


Supplementary Figure 1: Elevated miR-200b in response to hyperglycemia caused endothelial cell dysfunction. (A) qRT-PCR analysis of miR-200b expression in HMEC as a function of time (d1-d16) and varying concentrations (6.25, 12.5 or 25 mM) after exposure to high glucose (HG) or normal glucose (NG) conditions for 16 days. N = 3, ***p*< 0.05 (Student's t test). **(B)** qRT-PCR analysis of miR-200c expression in HMEC after exposure to HG/NG conditions for 16 days. N = 3 **(C)** qRT-PCR analysis of miR-429 expression in HMEC after exposure to HG/NG conditions for 16 days. N = 3 **(D)** qRT-PCR analysis of miR-200c and miR-429 expression in HMEC after exposure to HG/NG conditions for 4 days followed by actinomycin D treatment (2.5µg/ml, 4h). N = 5 **(E)** qRT-PCR analysis of miR-200b and VEGF expressions in HMEC after exposure to HG/NG conditions after cotretament with control (CI) or miR-200b inhibitor (MI) for 4 days. N = 4, ***p*< 0.001, F = 31.82 (miR-200b); 49.51 (VEGF) (one way ANOVA) **(F)** Immunocytochemical analysis of VEGF expression in HMEC under NG/HG and cotreatment with control (CI) or miR-200b inhibitor (MI). N = 4, ***p*< 0.001, F = 14.56 (one way ANOVA). Scale bar, 100 µm. **(G, H)** Immunocytochemical analysis of vWF expression in HMEC under NG/HG and cotreatment with control (CI) or miR-200b inhibitor (MI). Scale bar, 20 µm. N = 3, ***p*< 0.05, F = 6.08 (one way ANOVA). Data represented as the mean ± S.D.

Supplementary Figure 2

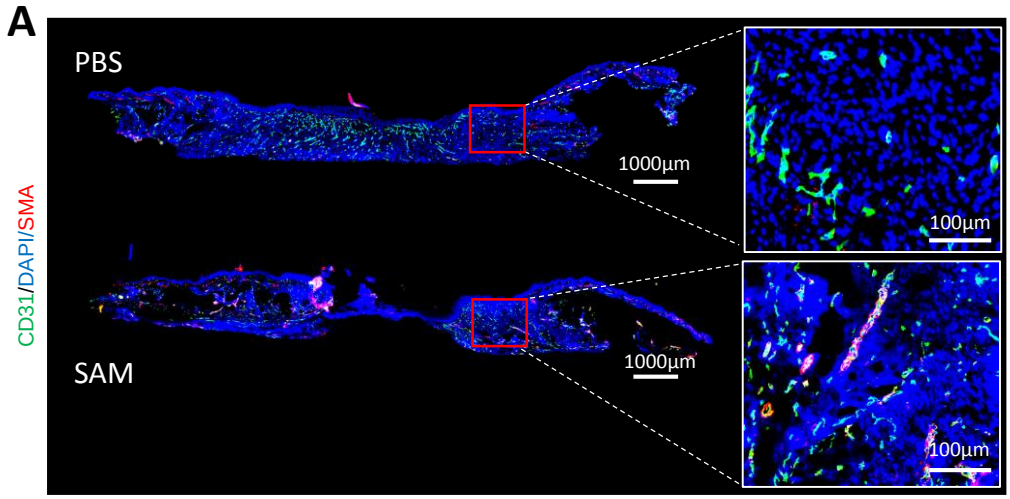


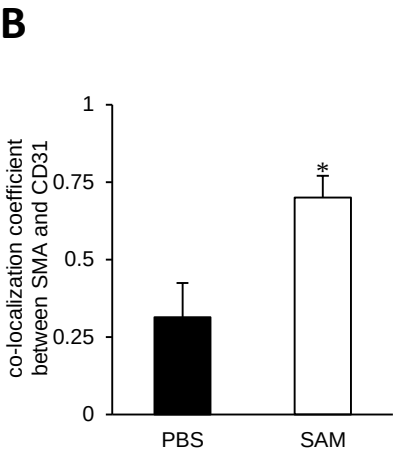
Supplementary Figure 2

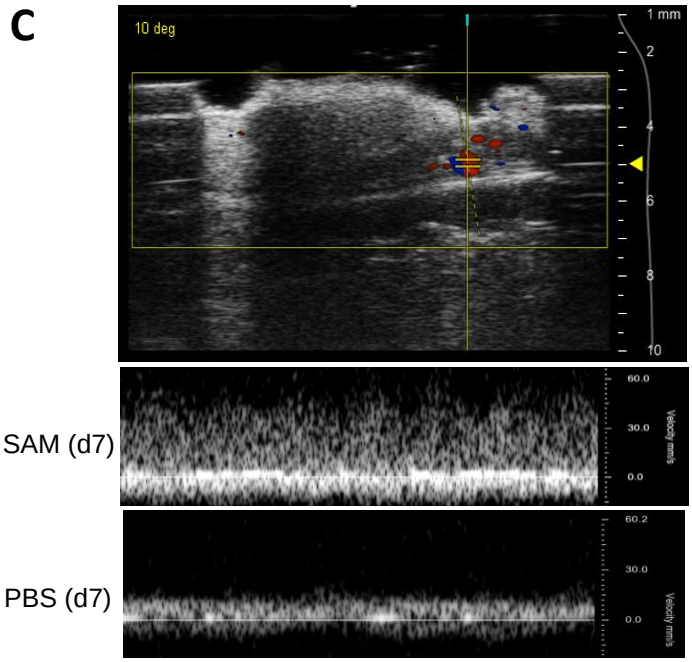


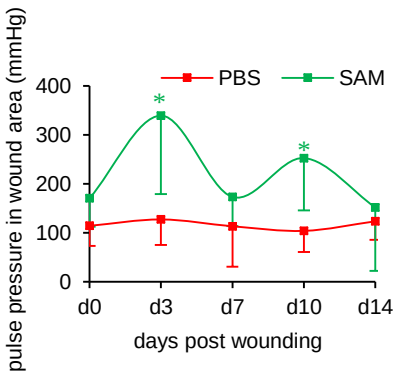
Supplementary Figure 2: Methylation regulates the expression of miR-200 family genes. **(A - C)** qRT-PCR analysis of miR-200b, miR-200c and miR-429 expression after 48 hr exposure to 5-Aza-2′-deoxycytidine (d-aza) under high glucose (HG) or cultured in normal glucose (NG) conditions. N = 3, ***p*< 0.05 (one way ANOVA). **(D)** Schematic diagram showing the regions (1, 2) of miR-200b promoter analyzed through bisulfite genomic sequencing of DNA. Methylation profile of the miR-200b promoter in HMEC cultured in normal glucose (NG) or exposed to methylglyoxal (500 µM, 48 h). (methylated CpG = black, unmethylated CpG = white). Number of clones = 5. **(E)** Total number of methylated CpG sites obtained from bisulfite sequencing analysis. N = 5, ***p*< 0.05 (Student's t test). **(F)** Schematic diagram showing the region of miR-200c promoter analyzed by bisulfite genomic sequencing of DNA. **(G)** Methylation profile of the miR-200c gene promoter is shown under HG/NG conditions (methylated CpG = black, unmethylated CpG = white). **(H)** Nuclear DNMT3A protein and **(I)** DNMT1 protein expression analysis in NG/HG exposed HMEC. N = 3, ***p*< 0.05 (Student's t test). **(J)** Nuclear DNMT1 and **(K)** DNMT3A protein expression analysis in NG/HG condition after treatment with control (CI) or miR-200b inhibitor (MI). N = 3 **(L)** Schematic representation of the alignment of the DNMT3A mRNA depicting the miR-200b binding site in its 3′-UTR (position in UTR: 709-715; Context score = - 0.18). Top strand, DNMT3A mRNA; bottom strand, miR-200b. **(M)** qRT-PCR analysis of VEGF expression in HMEC administered with S-Adenosyl methionine (SAM) administration in NG/HG condition after treatment with control (CI) or miR-200b inhibitor (MI). N = 5, *p< 0.05, F = 7.39 (one way ANOVA). **(N)** Total nitrate/nitrite production in HMEC after SAM treatment in HG condition. N = 5, ***p*< 0.05 (Student's t test). Data represented as the mean ± S.D. **(O, P)** Endothelial function analysis: ac-LDL uptake (upper panel, Scale bar, 50 µm), VE-cadherin expression (middle panel, Scale bar, 50 µm), matrigel tube length (middle panel, Scale bar, 200 µm), and vWF expression (lower panel, Scale bar, 30 µm) after SAM treatment in HG condition. N = 3, p>0.05 (Student's t test).

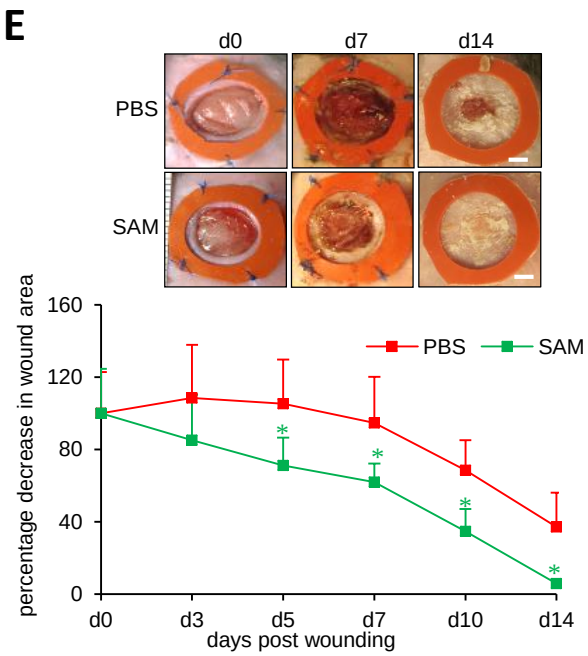
Supplementary Figure 3:

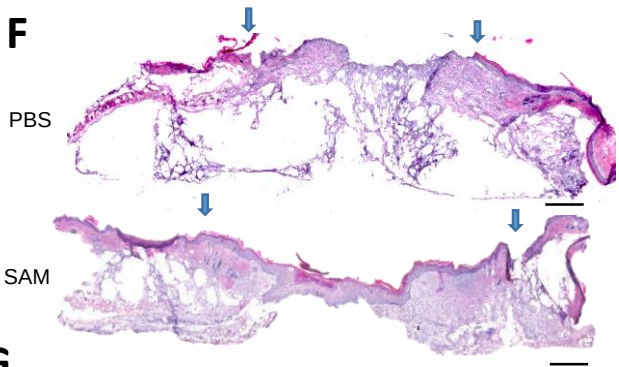


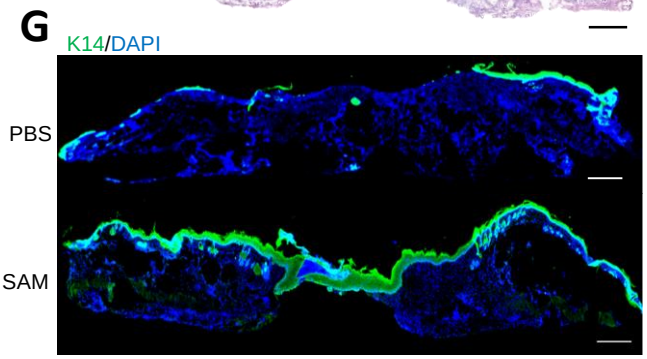




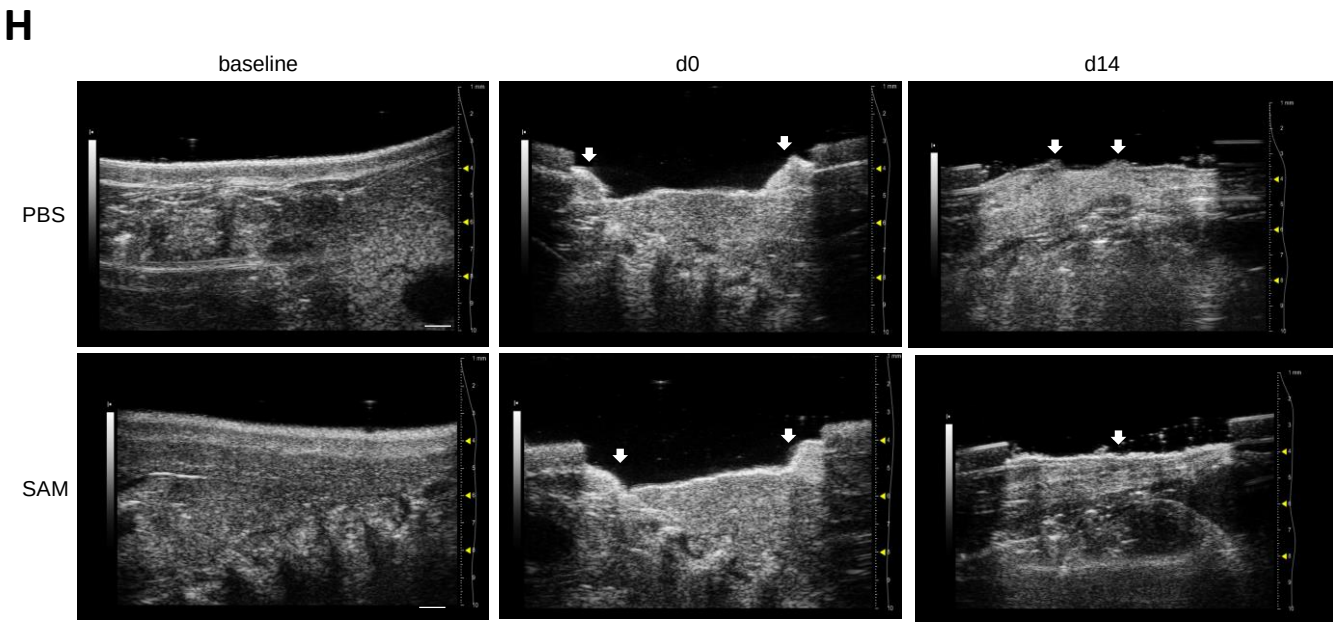






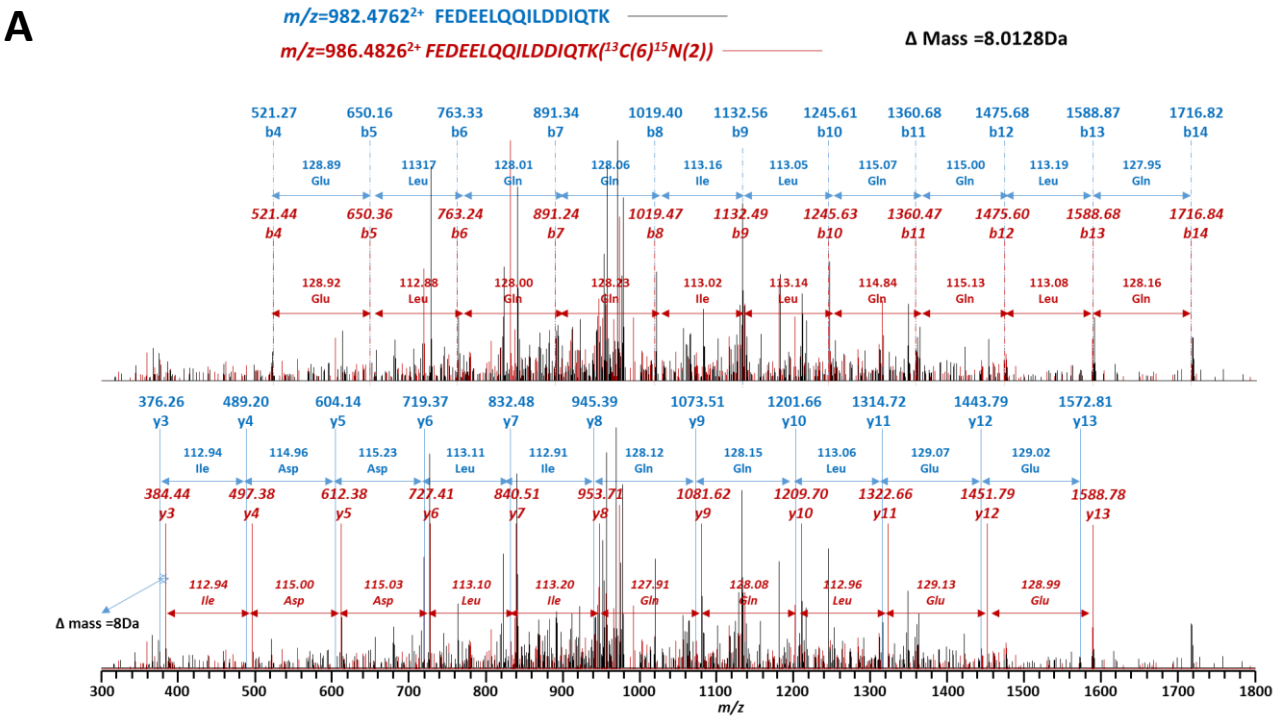


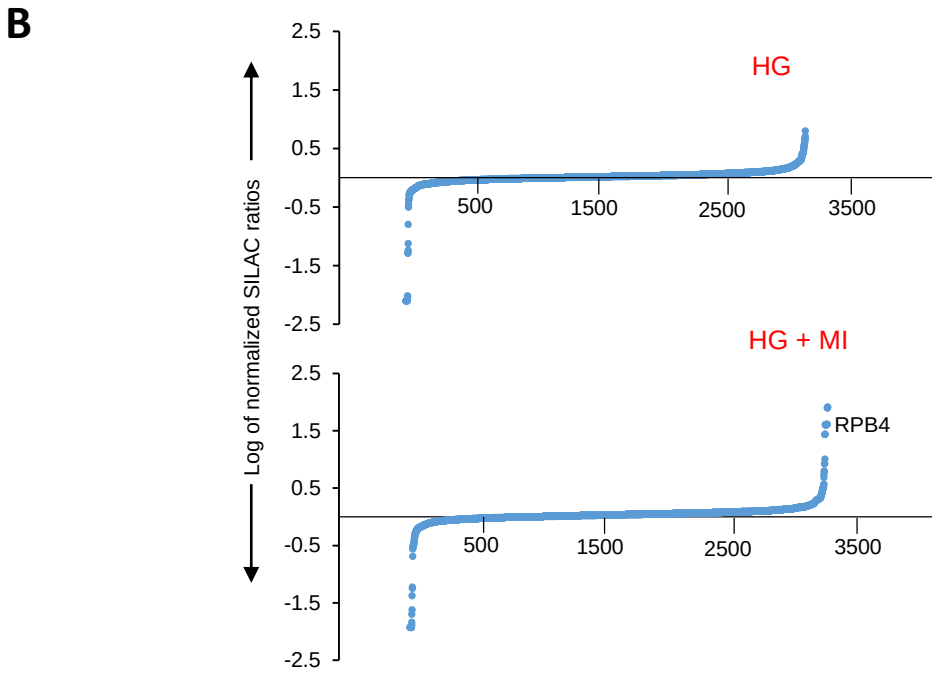
Supplementary Figure 3:



Supplementary Figure 3: SAM restored vascular function in diabetic wounds. **(A - B)** Immunohistochemical analysis showing $SMA^+/CD31^+$ co-expression in SAM administered diabetic wounds**.** N = 3, *p< 0.05 (Student's t test). **(C – D)** Wound edge blood flow and pulse pressure was measured using color Doppler feature of Vevo-2100. N = 3, *p< 0.05 on days 3 and 10 (Student's t test). **(E)** Wound closure was monitored on days 3, 5, 7, 10 and 14 days post-wounding after treatment with SAM or PBS by digital planimetry and was presented as percentage of wound closure. N = 3, *p< 0.05 on days 5, 7, 10 and 14 (Student's t test).**(F)** Representative images of formalin-fixed paraffin-embedded biopsy tissue sections (10 µm) of diabetic wounds (day 14) stained using hematoxylin (blue) and eosin (red), and **(G)** immunostained with anti-keratin-14 (green) and DAPI (blue). Scale bar, 200 µm. **(H)** *In vivo* wound imaging using Vevo-2100 for monitoring wound contraction and re-epithelialization in SAM treated (lower panels) or placebo (upper panels) diabetic wounds. Scale bar, 1 mm. Data represented as the mean ± S.D.

Supplementary Figure 4





Supplementary Figure 4

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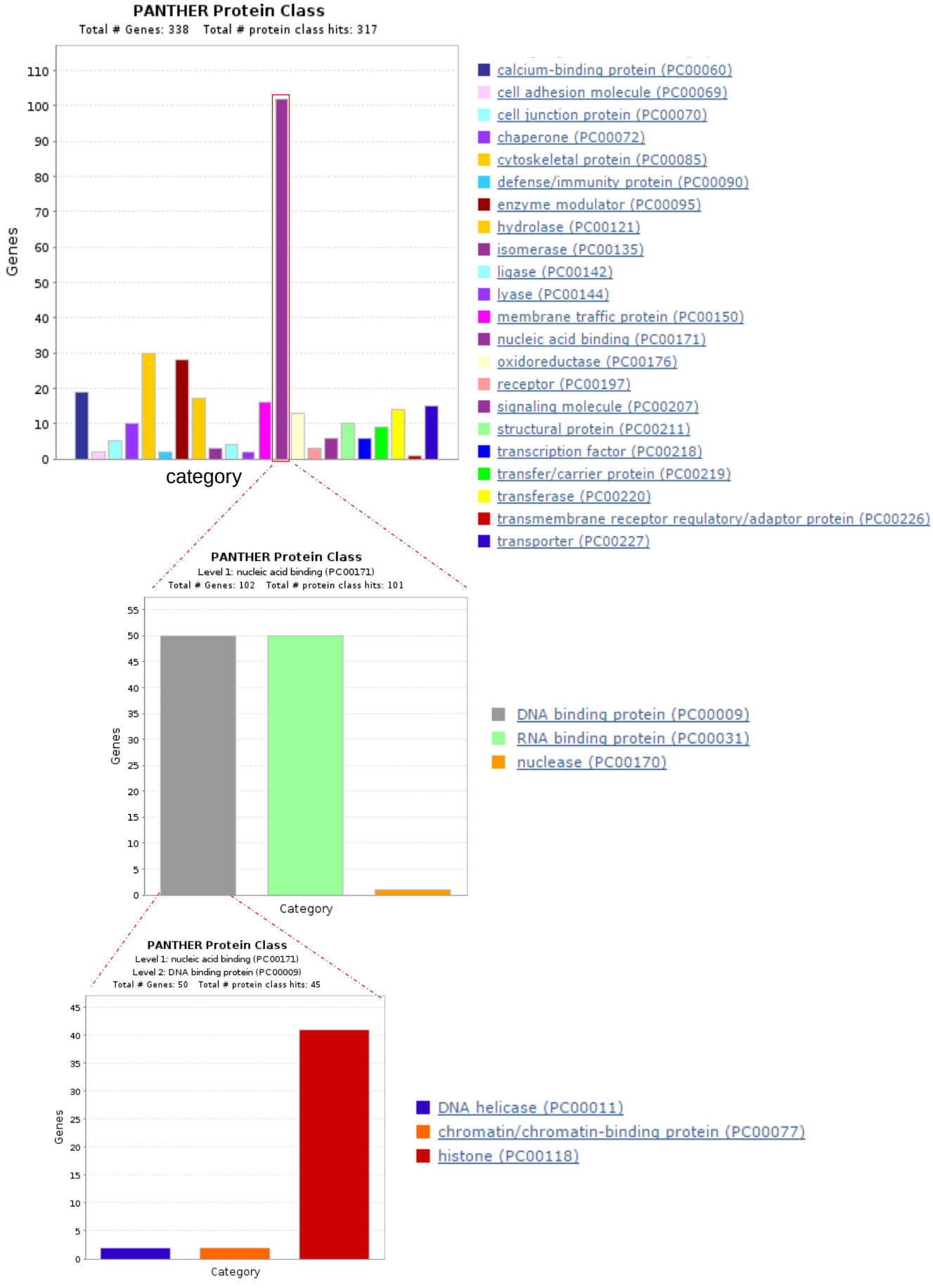
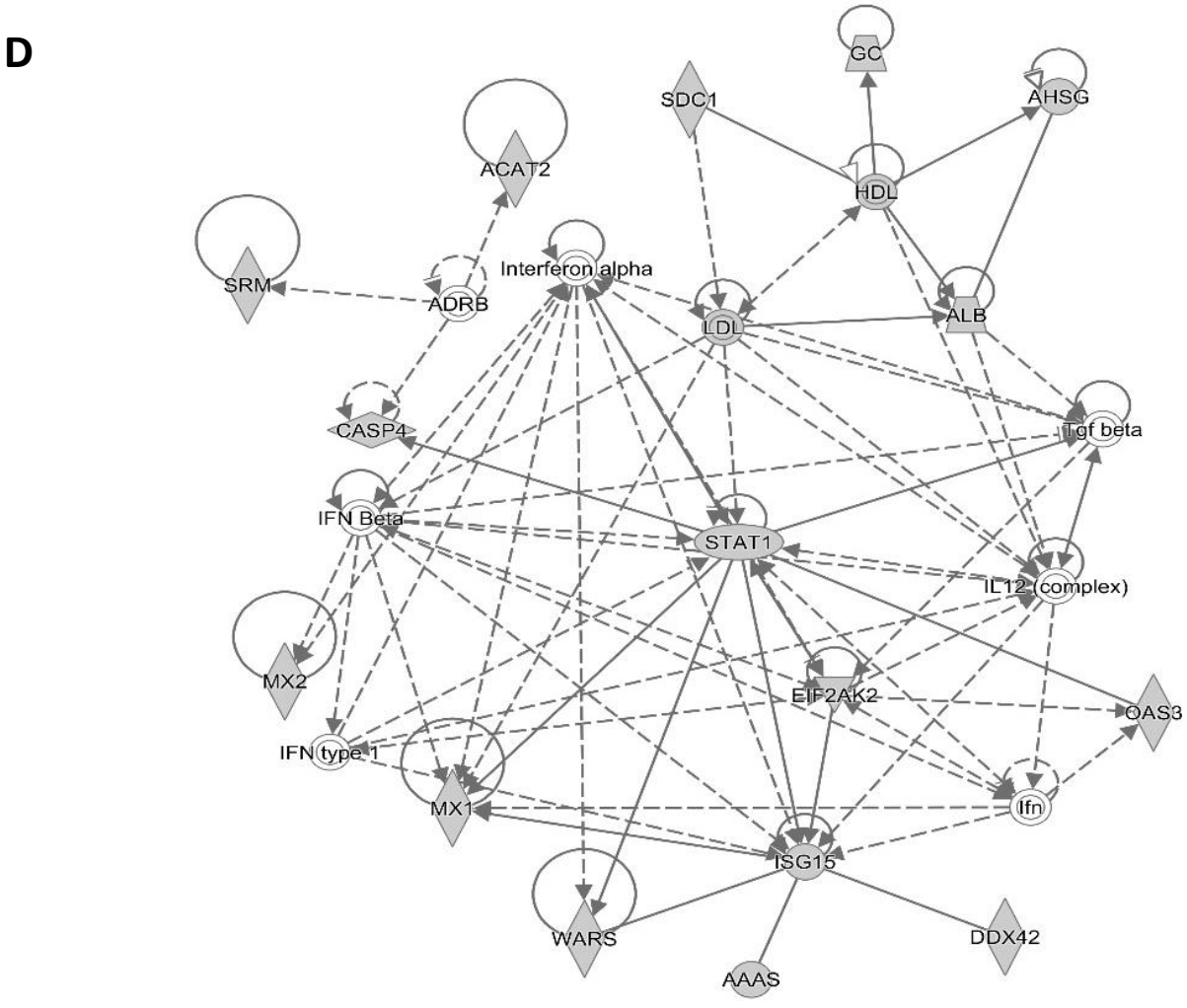
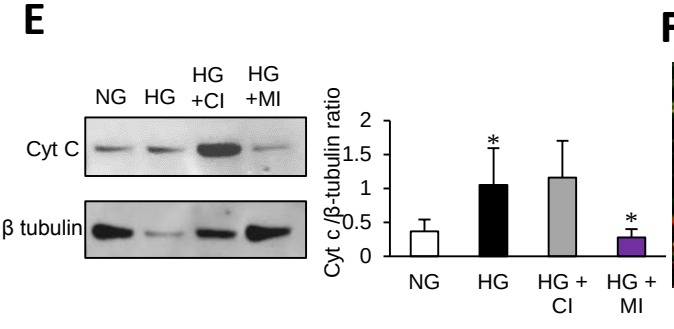
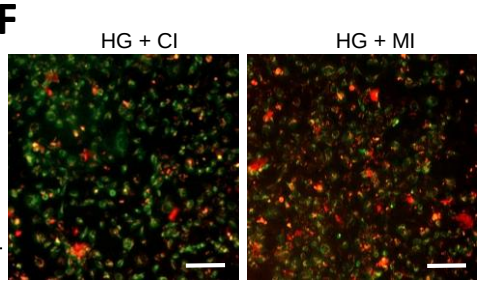
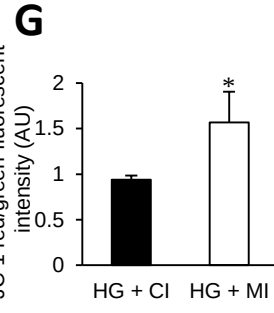


Supplementary Figure 4









Supplementary Figure 4

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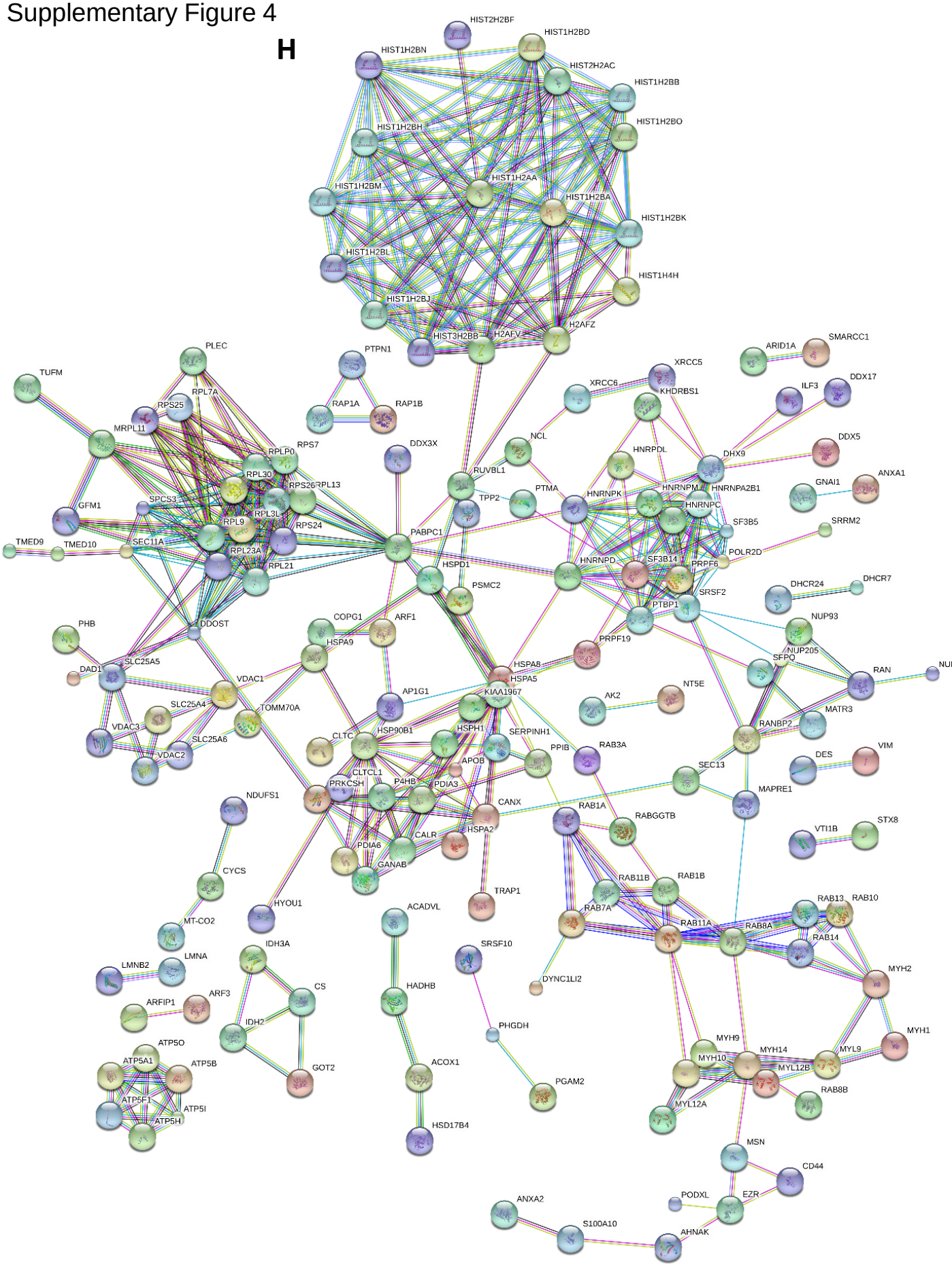

Supplementary Figure 4: Proteomic identification of candidates and pathways responsive to miR-200b inhibition during hyperglycemia. **(A)** MSMS mass Spectra of peptides FEDEELQQILDDIQTK (black) and FEDEELQQILDDIQTK (13C(6)15N(2))(red), a mass difference of 8 Da was observed between the *y* ions generated from heavy and light isotopic labeled peptides. **(B)** Normalized log ratio distribution of all quantitated proteins. **(C)** Functional classification of genes significantly upregulated due to inhibition of miR-200b in HG microenvironment. The bar graphs shown below represents the proportion of each functional group and the total number of genes in each group. **(D)** Ingenuity pathway analysis (IPA) showing that pathway of downregulated proteins due to inhibition of miR-200b in HG microenvironment. **(E)** Western blot analysis of cellular cytochrome C release from HMEC under NG/HG and co-treatment with control (CI) or miR-200b inhibitor (MI). N = 3 $*p < 0.05$ (Student's t test). **(F, G)** Mitochondrial potential using JC-1 dye in HMEC under HG conditions and co-treatment with control (CI) or miR-200b inhibitor (MI). Scale bar, 100 µm. N = 3 $*p < 0.05$ (Student's t test). Data represented as the mean ± S.D. **(H)** STRING version 10.0 was used to construct protein-protein interaction network of significantly upregulated proteins in HMEC under HG conditions and cotreatment with miR-200b inhibitor (MI). Lines or strings are indicative of protein interactions. Proteins with no interacting partners were omitted.

Supplementary Table:

1. Total proteins identified after SILAC labeling in hyperglycemia (HG) and HG + miR-200b inhibitor and their abundance ratios.

2. Upregulated proteins after miR-200b inhibition in HMEC (p value < 0.05; % change > 10%)

3. Down-regulated proteins after miR-200b inhibition in HMEC (p value < 0.05; % change > 10%)

4. Table showing functional classification of upregulated proteins after miR-200b inhibition during hyperglycemia using ingenuity pathway analysis

5. PFAM domain based network analysis of upregulated proteins after miR-200b inhibition during hyperglycemia using Search Tool for the Retrieval of INteracting Genes/ proteins (STRING).

6. Functional network analysis of upregulated proteins after miR-200b inhibition during hyperglycemia using Ingenuity Pathways Analysis (IPA).

7. Functional network analysis of down-regulated proteins after miR-200b inhibition during hyperglycemia using Ingenuity Pathways Analysis (IPA).

8. Table showing proteins upregulated through SILAC quantitative proteomics after miR-200b inhibition during hyperglycemia exposure which are also supported by established predictive computational algorithms miRnalyze and TargetScan.

9. Table showing clinical and demographic details of the human subjects included in the study.

Supplementary Table 4:

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Ingenuity Toxicity Lists	-log(p-value)	Ratio	Molecules
Mitochondrial Dysfunction	4.78	0.0568	ATP5H,ATP5B,ATP5A1,ATP5O,CYCS,VDAC3,VDAC1,ATP5F1,ATP5I,VDAC2
Fatty Acid Metabolism	2.85	0.0513	ACSL3,ACADVL,IWS1,ACOX1,HADHB,HSD17B4
Cardiac Hypertrophy	2.67	0.0276	SLC25A4,RAB1A,RAB4A,RAB2A,NT5E,MYH14,SERPINE1,H2AFZ,DES,ATP2A2,EEF1D,S100A10
Cholesterol Biosynthesis	1.93	0.125	FDFT1,DHCR7
Increases Transmembrane Potential of Mitochondria and Mitochondrial Membrane	1.82	0.06	ARID1A,PHB,CYCS
Decreases Depolarization of Mitochondria and Mitochondrial Membrane	1.5	0.0741	VDAC1,ATP2A2
Liver Necrosis/Cell Death	1.49	0.0242	SPTBN1,SLC25A4,SERPINH1,PTPN1,HSPD1,SERPINE1,SLC25A5
Increases Renal Proliferation	1.24	0.0288	UBE2M,LARP1,HSPD1,VDAC1
Mechanism of Gene Regulation by Peroxisome Proliferators via PPARα	1.11	0.0316	ACOX1,MRPL11,HSD17B4
Increases Permeability Transition of Mitochondria and Mitochondrial Membrane	1.09	0.125	FAM162A
Primary Glomerulonephritis Biomarker Panel (Human)	0.963	0.0909	SERPINE1
Increases Liver Hepatitis	0.937	0.0357	SERPINE1,CYR61
PPARα/RXRα Activation	0.911	0.022	HSP90B1,PDIA3,ACOX1,GOT2
Increases Liver Damage	0.909	0.0256	PTPN1,CD44,SERPINE1
Decreases Transmembrane Potential of Mitochondria and Mitochondrial Membrane	0.878	0.0248	SLC25A6,IMMT,VDAC1
Recovery from Ischemic Acute Renal Failure (Rat)	0.865	0.0714	COL4A1
Increases Bradycardia	0.837	0.0667	CALR
Swelling of Mitochondria	0.837	0.0667	DES
Cardiac Necrosis/Cell Death	0.804	0.0183	CALR,PHB,MANF,HSPD1,CYR61
Renal Glomerulus Panel (Human)	0.787	0.0588	PODXL
Hypoxia-Inducible Factor Signaling	0.782	0.0286	P4HB,UBE2M
Increases Depolarization of Mitochondria and Mitochondrial Membrane	0.765	0.0556	CYR61
Biogenesis of Mitochondria	0.723	0.05	RAB3A
Glutathione Depletion - Phase II Reactions	0.723	0.05	GSTK1
Genes associated with Chronic Allograft Nephropathy (Human)	0.704	0.0476	COL4A1
Increases Liver Steatosis	0.611	0.022	ATP5A1,CD44
NRF2-mediated Oxidative Stress Response	0.598	0.0163	HSP90B1,DNAJC9,PPIB,GSTK1
Positive Acute Phase Response Proteins	0.569	0.0333	SERPINE1
Renal Necrosis/Cell Death	0.526	0.0135	FDFT1,HSP90B1,GLS,CD44,CYCS,DDX17,VDAC1
Cardiac Fibrosis	0.487	0.0155	SERPINE1,DES,ATP2A2
Increases Cardiac Proliferation	0.41	0.0213	RAB1A
Acute Renal Failure Panel (Rat)	0.321	0.0161	CD44
LPS/IL-1 Mediated Inhibition of RXR Function	0.308	0.0119	ACSL3,ACOX1,GSTK1
Aryl Hydrocarbon Receptor Signaling	0.305	0.0126	HSP90B1,GSTK1
Increases Renal Damage	0.242	0.0123	CD44
RAR Activation	0.228	0.0105	ARID1A,RDH11
TGF-β Signaling	0.214	0.0111	SERPINE1

Table showing functional classification of upregulated proteins after miR-200b inhibition during hyperglycemia using ingenuity pathway analysis

Supplementary Table 5:

PFAM Protein Domains			
pathway ID	**pathway description**	**count in gene set**	**false discovery rate**
PF00071	Ras family	22	1.06E-15
PF00125	Core histone H2A/H2B/H3/H4	17	6.57E-15
PF00076	RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain)	13	1.67E-05
PF00038	Intermediate filament protein	5	6.29E-05
PF00012	Hsp70 protein	4	0.000419
PF00769	Ezrin/radixin/moesin family	3	0.00549
PF04732	Intermediate filament head (DNA binding) region	3	0.00549
PF00191	Annexin	3	0.0119
PF00270	DEAD/DEAH box helicase	5	0.0245
PF09379	FERM N-terminal domain	3	0.0296
PF09380	FERM C-terminal PH-like domain	3	0.0296
PF00271	Helicase conserved C-terminal domain	5	0.047
PF01576	Myosin tail	2	0.047
PF02736	Myosin N-terminal SH3-like domain	2	0.047

PFAM domain based network analysis of upregulated proteins after miR-200b inhibition during hyperglycemia using Search Tool for the Retrieval of INteracting Genes/ proteins (STRING).

Supplementary Table 6:

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Symbol	Entrez Gene Name	GenPept/UniProt/ Swiss-Prot Accession	Location	Family	Entrez Gene ID for Human
ATP5A1	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	P25705	Cytoplasm	transporter	498
DDX39B	DEAD-box helicase 39B	Q13838	Nucleus	enzyme	7919
DNAJC9	DnaJ heat shock protein family (Hsp40) member C9	Q8WXX5	Nucleus	other	23234
GOT			Other	group	
GOT2	glutamic-oxaloacetic transaminase 2	P00505	Cytoplasm	enzyme	2806
H2AFZ	H2A histone family member Z	P0C0S5	Nucleus	other	3015
HIST1H2BA	histone cluster 1, H2ba	Q96A08	Nucleus	other	255626
HIST1H2BD	histone cluster 1, H2bd	P58876	Nucleus	other	3017
HIST1H2BJ	histone cluster 1, H2bj	P06899	Nucleus	other	8970
HIST1H2BK	histone cluster 1, H2bk	O60814	Nucleus	other	85236
HIST1H2BL	histone cluster 1, H2bl	Q99880	Nucleus	other	8340
HIST2H2AC	histone cluster 2, H2ac	Q16777	Nucleus	other	8338
HSPA1L	heat shock protein family A (Hsp70) member 1 like	P34931	Cytoplasm	other	3305
HSPA2	heat shock protein family A (Hsp70) member 2	P54652	Cytoplasm	other	3306
HSPA5	heat shock protein family A (Hsp70) member 5	P11021	Cytoplasm	enzyme	3309
HSPA8	heat shock protein family A (Hsp70) member 8	P11142	Cytoplasm	enzyme	3312
HSPA9	heat shock protein family A (Hsp70) member 9	P38646	Cytoplasm	other	3313
HSPD1	heat shock protein family D (Hsp60) member 1	P10809	Cytoplasm	enzyme	3329
MACF1	microtubule-actin crosslinking factor 1	Q9UPN3	Cytoplasm	enzyme	23499
MANF	mesencephalic astrocyte derived neurotrophic factor	P55145	Extracellular Space	other	7873
MDH2	malate dehydrogenase 2	P40926	Cytoplasm	enzyme	4191
NCEH1	neutral cholesterol ester hydrolase 1	Q6PIU2	Plasma Membrane	enzyme	57552
PCK2	phosphoenolpyruvate carboxykinase 2, mitochondrial	Q16822	Cytoplasm	kinase	5106
PSMC2	proteasome 26S subunit, ATPase 2	P35998	Nucleus	peptidase	5701
RPL23A	ribosomal protein L23a	P62750	Cytoplasm	other	6147
RUVBL1	RuvB like AAA ATPase 1	Q9Y265	Nucleus	transcriptio n regulator	8607
SLC25A5	solute carrier family 25 member 5	P05141	Cytoplasm	transporter	292
TUFM	Tu translation elongation factor, mitochondrial	P49411	Cytoplasm	translation regulator	7284

Functional network analysis of upregulated proteins after miR-200b inhibition during hyperglycemia using Ingenuity Pathways Analysis (IPA).

Supplementary Table 7:

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Symbol	Entrez Gene Name	GenPept/UniProt/Swiss-Prot Accession	Location	Family	Entrez Gene ID for Human
AAAS	aladin WD repeat nucleoporin	Q9NRG9	Nucleus	other	8086
ACAT2	acetyl-CoA acetyltransferase 2	Q9BWD1	Cytoplasm	enzyme	39
AHSG	alpha 2-HS glycoprotein	P02765	Extracellular Space	other	197
ALB	albumin	P02768	Extracellular Space	transporter	213
CASP4	caspase 4	P49662	Cytoplasm	peptidase	837
DDX42	DEAD-box helicase 42	Q86XP3	Cytoplasm	enzyme	11325
EIF2AK2	eukaryotic translation initiation factor 2 alpha kinase 2	P19525	Cytoplasm	kinase	5610
GC	GC, vitamin D binding protein	P02774	Extracellular Space	transporter	2638
HDL			Plasma Membrane	complex	
Ifn			Extracellular Space	group	
IFN Beta			Extracellular Space	group	
IFN type 1			Other	group	
IL12 (complex)			Extracellular Space	complex	
Interferon alpha			Extracellular Space	group	
ISG15	ISG15 ubiquitin-like modifier	P05161	Extracellular Space	other	9636
LDL			Plasma Membrane	complex	
MX1	MX dynamin like GTPase 1	P20591	Cytoplasm	enzyme	4599
MX2	MX dynamin like GTPase 2	P20592	Nucleus	enzyme	4600
OAS3	2'-5'-oligoadenylate synthetase 3	Q9Y6K5	Cytoplasm	enzyme	4940
SDC1	syndecan 1	P18827	Plasma Membrane	enzyme	6382
SRM	spermidine synthase	P19623	Cytoplasm	enzyme	6723
STAT1	signal transducer and activator of transcription 1	P42224	Nucleus	transcription regulator	6772
Tgf beta			Extracellular Space	group	
WARS	tryptophanyl-tRNA synthetase	P23381	Cytoplasm	enzyme	7453

Functional network analysis of down-regulated proteins after miR-200b inhibition during hyperglycemia using Ingenuity Pathways Analysis (IPA)

Supplementary Table 8:

Protein name
Ras-related protein Rab-7a
heat shock protein family A (Hsp70) member 9 (HSPA9)
DnaJ homolog subfamily (HSP40)
ATP synthase
collagen type IV
ATP-dependent RNA helicase
Calumenin
heterogeneous nuclear ribonucleoprotein
talin 2
cyclin-dependent kinase

Table showing proteins upregulated through SILAC quantitative proteomics after miR-200b inhibition during hyperglycemia exposure which are also supported by established predictive computational algorithms miRnalyze and TargetScan.

Supplementary Table 9:

Parameter	Control	Diabetic
Age (years)	53.66 ± 10.04	49 ± 12.66
Gender	M = 1; F = 2	M = 2; F = 1
Weight (lbs)	221 ± 40.11	199.33 ± 33.57
HbA1c (%)	n/a	6.46 ± 0.29
Ethnicity	Not Hispanic or Latino = 3	Not Hispanic or Latino = 3
Race	Caucasian = 3, African American = 0	Caucasian = 2, African American = 1
wound etiology	pressure = 1; surgical = 2	pressure = 1; surgical = 2
infection status	non-infected =2, infected = 1	non-infected =3, infected = 0

Table showing clinical and demographic details of the human subjects included in the study.