Supplementary Figure Legends:

S 1: **miR-424 expression**:

(**A**) Hypoxia mediated changes in miR-424 expression in human dermal microvascular endothelial cells (MVEC) under normoxia and hypoxia. Expression of miR-424 was determined by q- PCR. Values represent mean ± standard deviation (SD), n=3, ** denotes ($p \leq 0.02$).

S 2: Effect of miR-424 on CUL2 transcript:

(A) Changes in CUL2 transcript levels by miR-424 was determined by q-PCR . HUVEC were transfected with miR-424 and total RNA was isolated at 0, 24 and 48 hr time points and q -PCR was used to determine transcript levels.

S 3: Reduction in CUL2 level destabilizes VCBCR complex

(A) Western blot analysis showing RBX1 levels in the whole cell lysates of HUVEC expressing either a miR-control (lane 1), or miR-210 (lanes 2, 3), or miR-424 (lanes 4, 5). β-actin was used as a loading control.

(B) Western blot analysis indicating that knocking down CUL2 destabilizes the VCBCR complex. HUVEC cells were transfected either with scrambled control RNA (scRNA) or with siRNA specific to CUL2 under normoxia. Whole cell lysates were prepared at indicated time points and immunoblotting was performed using antibodies specific to CUL-2, RBX1 and VHL1. β-actin was used as a loading control.

S 4: Effect of miR-424 on endothelial cell proliferation and Angiogenesis:

(A) The HUVEC were transfected with either no morpholino (left panels), or control morpholino (middle panels) or 424-morpholino (right panels) and then kept under

hypoxia in the presence of VEGF (50 ng/ml). After 48 hours cells were fixed and stained for PCNA (green) and nucleus (DAPI, blue). Representative images are shown. The lower panel shows the respective processed images to determine colocalization of PCNA and DAPI. Scale bar represents 20 μm.

(B) The gastrocnemius muscle from sham and femoral artery ligated mice were perfused and fixed in neutral formalin. Paraffin embedded sections (10 μm) were processed and stained for blood vessels using tomato-lectin conjugated to Texas red. Vessel length per field was calculated as previously described.

Values represent mean ± standard deviation (SD), ** denotes ($p \leq 0.02$).

(C) Western blots analysis of Hif-1α expression in the tissues of sham (S) operated and ligated (L) mice.

(D) Quantification of HIF-1α levels from Western blots. The HIF-1α levels were determined by pixel density after normalizing to β-actin. * denotes ($p \leq 0.05$).**S 5: In situ hybridization:**

Representative confocal images of in situ hybridization using a specific 3´DIG-labelled LNA probe miR-322 (red) on the formalin fixed sham (upper panel) and ligated (lower panel) muscle tissues are shown. Sections were counterstained for lectin (green), and DAPI (blue). Scale bar represents 10μm.

S 6: PU.1 levels in RUNX-1 knockdown HUVEC:

Western blots showing the expression of PU.1 in HUVEC after knocking down RUNX-1 using two RUNX-1 specific shRNA. Cells were kept under normoxia for 24 hr (lane 1), or hypoxia for 24 and 48 hr (lane 2, 3). Cells were transfected with RUNX-1 shRNA 1 and kept under hypoxia for the indicated time (lanes 4-7). β-actin was used as a loading control.

S 7: Effect of proteasome inhibitor on HIF-1α:

HUVEC were transfected with either miR-control or miR-424 and treated with or without 10 uM of MG132 for 8 hours. Cultures were exposed to either normoxia or hypoxia as indicated. Cell lysates were analyzed for HIF-1α levels by western blot. β-actin was used as a loading control.

Supplementary Procedures :

Reporter constructs: Human genomic DNA was used to isolate the 3'UTR of CUL2 by PCR using the forward primer, GGCTAGCTGTGAGAAGATCATTGCCATCA and reverse primer, GGCTCGAGTATTTGACAAGCAGCAGTATCATG. PCR product was digested and cloned into Nhe I and Xho I sites of pDM128/RRE vector(1). Target site of miR-424 in the CUL2 3' UTR was mutated by deleting CTGC seed sequence using site-directed mutagenesis. All cloned products were verified by sequencing.

Luciferase reporter assay (Promoter): Following constructs were used; 1) miR-424-PU.1 construct wherein, a 500bp sequence of human miR-424 genomic DNA containing PU.1 bindinig sites was cloned into Xho I-Bgl II sites of PGL4.10 (Luc 2) vector (PROMEGA). 2) PU.1-URE-construct wherein, a 800bp sequence of human PU.1 genomic DNA containing RUNX-1 binding site was PCR amplified and cloned into Xho 1-Bgl II sites of PGL4.10 (Luc 2) vector (PROMEGA). 3) PU.1-URE-Promoter construct wherein, a 300bp sequence of human PU.1- genomic DNA from the promoter which includes an C/EBPα binding site was PCR amplified and fused to the PU.1-URE construct.

Transient expression studies: Primary miR-424 was PCR amplified from human genomic DNA and cloned into EcoRI-BamHI sites of pGSU6 vector (Gene therapy systems Inc).

Expression of miR-424: PCR-amplified primary miR-424 from genomic DNA was cloned into the MDH1-PGK-GFP 2.0 retroviral vector. The production of amphotropic viruses and infection of target cells was carried out as previously described (2). HUVEC cultures infected with the recombinant virus resulted in >70 % transduction efficiency as determined by the GFP positive cells. RNA was isolated from the infected cells and miR-424 expression was determined by RT-PCR analysis.

Immunofluorescence: Cells were fixed with Phemo [(PIPES (0.068 M), HEPES (0.025 M) EGTA (0.015 M), MgCl2 (0.003M), DMSO (10%), pH 6.8)] buffer containing 3.7 % formaldehyde, 0.05 % glutaraldehyde and 0.5 % Triton X-100) for 10 minutes at room temperature. After 3 washes, cells were blocked with 5% BSA. PU.1, HIF-1α and PCNA were visualized by indirect immunofluorescence using Alexa Fluor 488–labeled secondary antibodies. Cell nuclei were stained with DAPI (InVitrogen). Fluorescence images were captured using a Nikon C1 Digital Eclipse (TE 2000-U) confocal microscope system and camera (Nikon Instruments). Images were acquired under the same conditions for each experiment, and no digital manipulation of images was performed other than cropping and merging.

Immunoblotting: Cells were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.5, 1% NP-40, 1% deoxycholate, 0.1% SDS, protease inhibitor cocktail (Roche). Proteins from total cell lysates were resolved by the SDS-PAGE 10% or 12% gel, transferred to PVDF membranes, blocked with 5% non-fat milk in PBS/Tween-20, and blotted with the antibodies to PU.1 (Santa Cruz Biotechnology, Inc), CUL2 (Santa Cruz Biotechnology, Inc) , RUNX-1 (Sigma), HIF-1α (BD Transduction Laboratories), VHL (Cell Signalling), Rbx-1 (ROC1, Thermo Scientific), C/EBPα (Santa Cruz Biotechnology, Inc), HIF-2α (Novus Biologicals) and β-actin (Millipore and Santa Cruz), Histone 1A (Santa Cruz Biotechnology, Inc).

Transfections: All transfections were carried out using either Amaxa HUVEC Nucleofector Kit (Lonza) or Superfect Transfection Reagent (Qiagen) according to manufacturer's instructions. Morpholino specific for miR-424, TAGAACACTTCAAAACATGAATTGCTGCTGT and control morpholino were obtained from Gene Tools, LLC. HUVEC were cultured at 80% confluence and the spent medium was replaced with fresh medium, then 1-2 μM of morpholino and 6 μM of endoporter (Gene Tools, LLC) was added per ml of media.

Chromatin immunoprecipitation (ChIP) assay: ChIP assay was carried out as previously described (3). Briefly, HUVEC cells were kept under normoxic or hypoxic conditions and ChIP was performed using ChIP assay kit (Upstate), according to the manufacturer's instructions. DNA/protein cross-linking was obtained by incubating the cells for 20 min at 37°C in 1% formaldehyde. After sonication, chromatin was immunoprecipitated overnight with 10μl of anti-PU.1 antibody (Santa Cruz Biotechnology, Inc). q-PCRs of genomic region containing the putative PU.1 binding site were performed using oligo primers as described (3) previously.

In vitro Scratch wound Assay (Migration), proliferation and Tube formation assays: Migration and tube formation assays were carried out as described previously (4). For all the assays, HUVEC were transfected with either pGSU6-miR424 or pGSU6-miR-control vector (Gene Therapy Systems). Proliferation was determined by BrdU incorporation into DNA (Roche).

Luciferase Assays (3'UTR and promoter activity): HUVEC cells of ~60% confluence were transfected with Firefly luciferase reporter gene construct and pRL-SV40 *Renilla* luciferase construct (10:1 ratio) using Superfect reagent (Qiagen). Cell extracts were prepared 24hrs after transfection, and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

Immunohistochemistry and Vascular density: Formalin fixed sections were processed and stained for blood vessels with tomato lectin (InVitrogen) linked to Texas Red. HIF-1α was detected by a mouse monoclonal antibody (NeoMarkers), and visualized with Alexa Fluor 488–labeled goat anti-mouse IgG. Images were acquired under the same conditions for each experiment, and no digital manipulation of images was performed other than cropping and merging. Vessel length was calculated as previously described (5).

In situ hybridization: In situ hybridization was done to detect miR-322 expression, in muscle tissues from sham and ligated animals as previously described by Obernosterer et al. (6). Breifly, paraffin embedded muscle tissues were deparaffinized first the sections were washed 3 times for 5 min and incubated with protein kinase K (Sigma) for 5 min. Probes (2 µl 3`-DIG labeled LNA probes, Exiqon) were mixed with 200 µl denaturation buffer, heated to 80 °C for 5 min, chilled on ice and added to the sections followed by incubation over night at 51 °C. The LNA probes used from Exiqon were miR-322 (39520-00), U6 (99004-00) as positive control probe and scrambled (99002-00) as negative control probe. All the probes used were unlabeled and 3′-DIG nucleotides were added afterwards by a terminal transferase reaction (Roche DIG High-Prime kit). The tissue sections were incubated with anti-DIG alkaline phosphatase and with antibodies to CUL-2 (Santa Cruz biotechnology). The anti-mouse or rabbit secondary antibody linked to Alexa Fluor 488 was used. The sections were subsequently stained with Fast Red substrate (Sigma) containing levamisol and finally mounted in DAPI containing medium (InVitrogen).

References

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6. Obernosterer, G., Martinez, J., and Alenius, M. 2007. Locked nucleic acid-based in situ detection of microRNAs in mouse tissue sections. *Nat Protoc* 2:1508-1514.

Figure S1

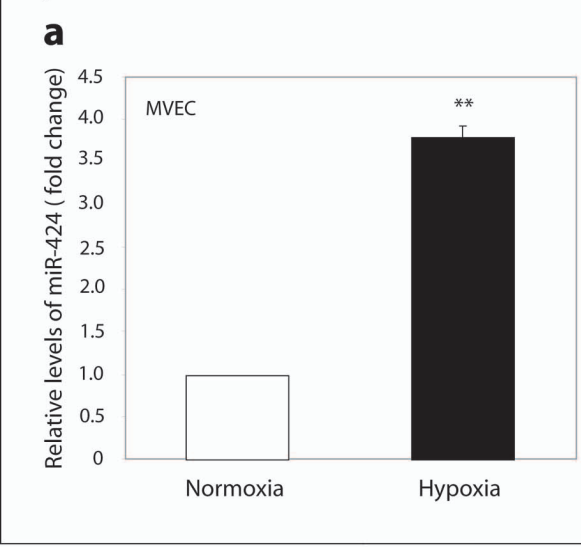

a
MVEC
**
Relative levels of miR-424 (fold change)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Normoxia
Hypoxia

Figure S2

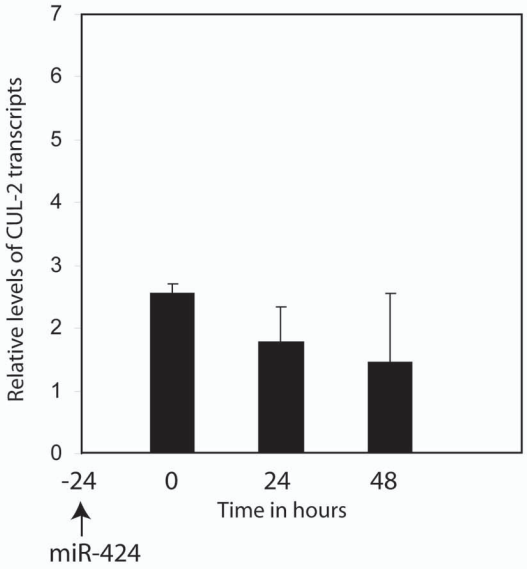
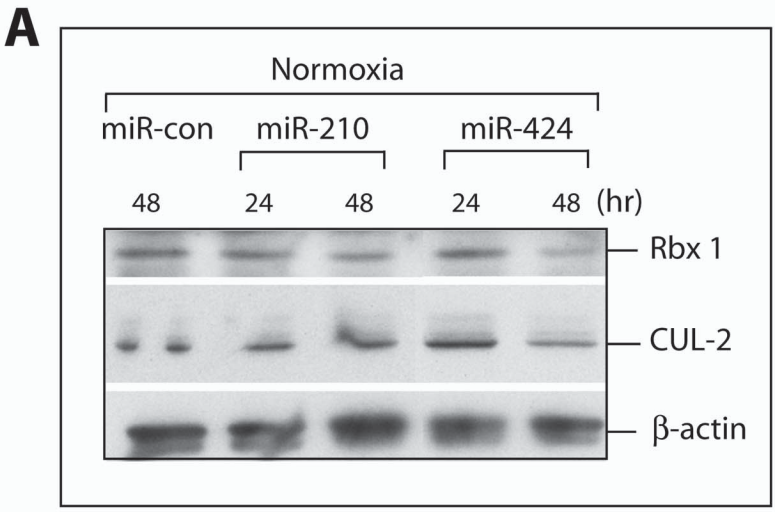

7
6
5
4
3
2
1
0
Relative levels of CUL-2 transcripts
-24
0
24
48
Time in hours
miR-424

Figure S3


A
Normoxia
miR-con
miR-210
miR-424
48
24
48
24
48
(hr)
Rbx 1
CUL-2
β-actin

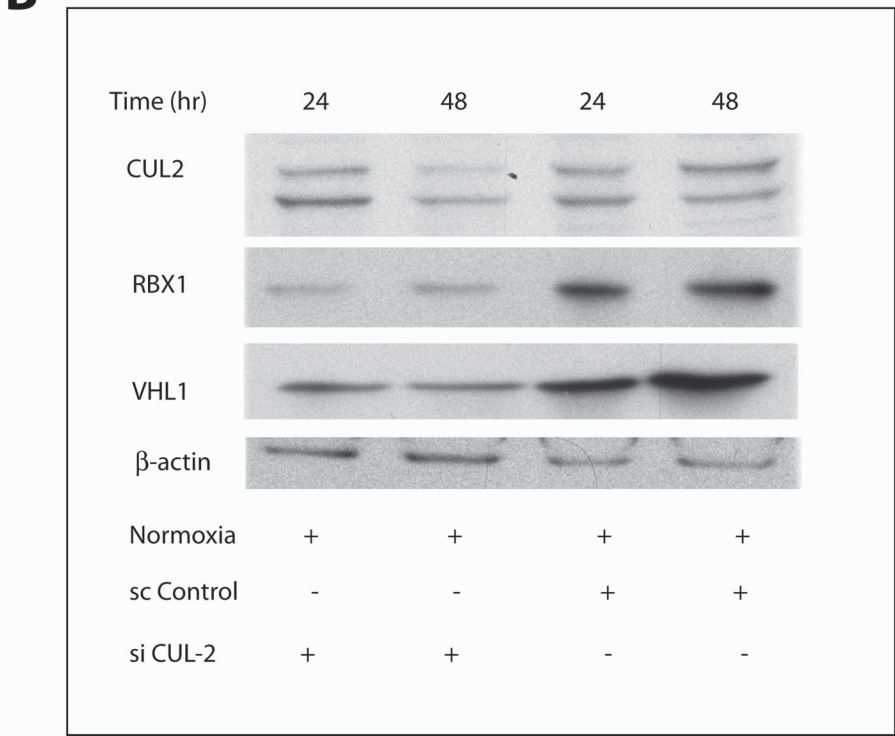

B
Time (hr)
24
48
24
48
CUL2
RBX1
VHL1
β-actin
Normoxia + + + +
sc Control - - + +
si CUL-2 + + - -

Figure S4

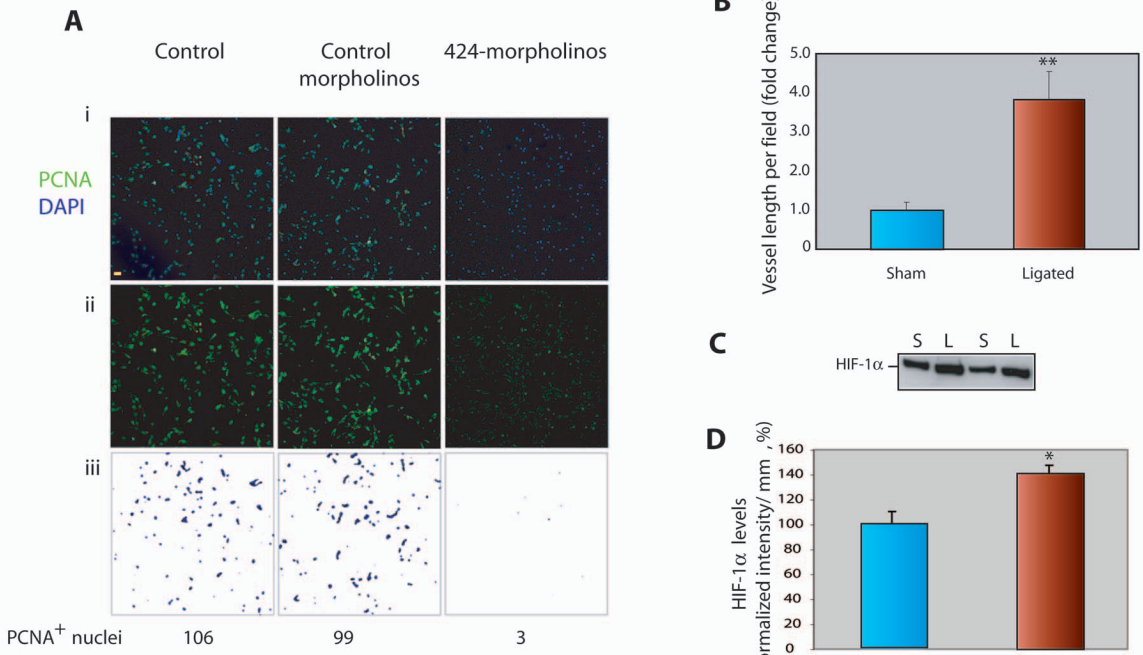

A
Control
Control morpholinos
424-morpholinos
i
PCNA
DAPI
ii
iii
PCNA+ nuclei
106
99
3
B
Vessel length per field (fold change)
5.0
4.0
3.0
2.0
1.0
0
**
Sham
Ligated
C
S L S L
HIF-1α
D
HIF-1α levels (Normalized intensity/ mm , %)
160
140
120
100
80
60
40
20
0
*
Sham
Ligated

Figure S5

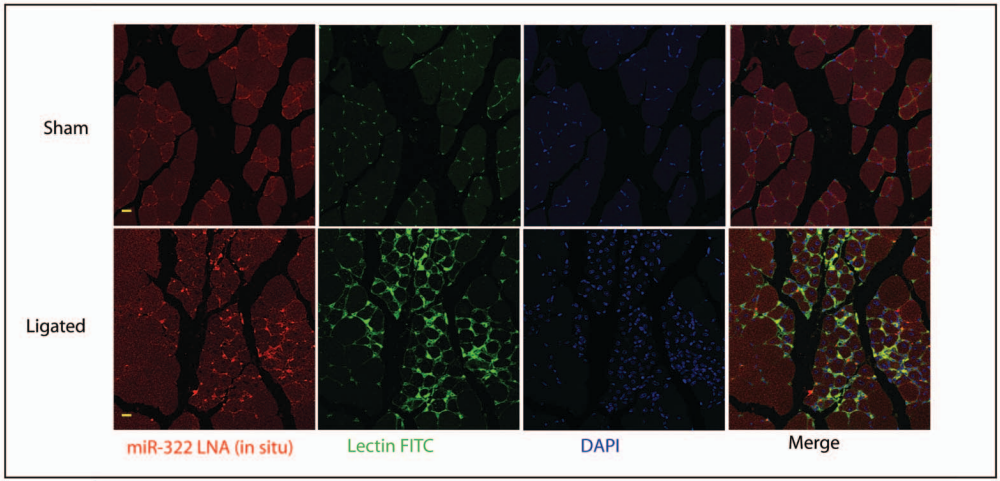


Figure S6

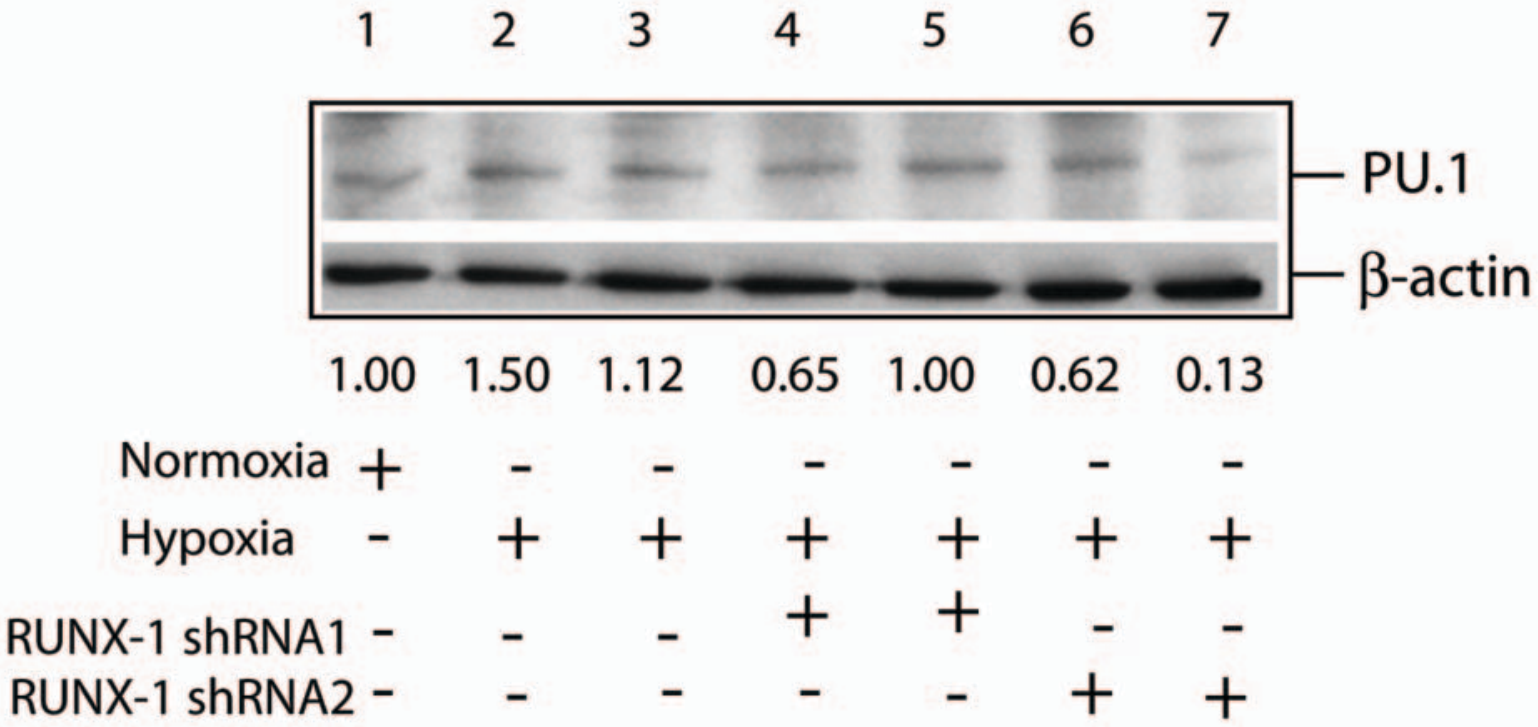
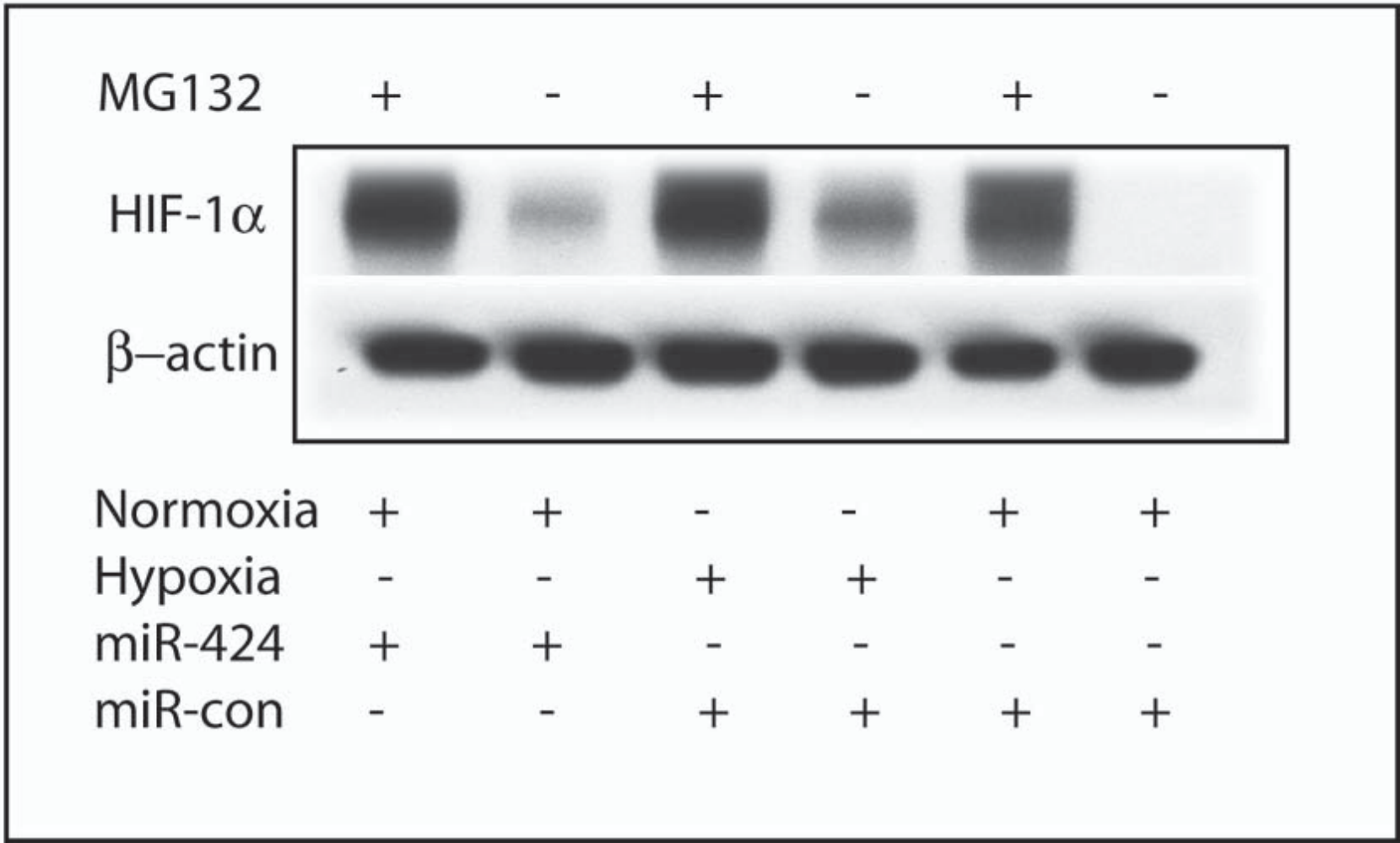

1 2 3 4 5 6 7
PU.1
β-actin
1.00 1.50 1.12 0.65 1.00 0.62 0.13
Normoxia + - - - - - -
Hypoxia - + + + + + +
RUNX-1 shRNA1 - - - + + - -
RUNX-1 shRNA2 - - - - - + +

Figure S7



S Table 1: Relative expression of miRNAs under hypoxia

miRNA	HUVEC (normoxia)	HUVEC (hypoxia)
hsa-let-7a	13.03	13.17
hsa-let-7b	3.337	4.5
hsa-let-7c	4.738	5.314
hsa-let-7d	7.13	8.424
hsa-let-7e	5.096	5.513
hsa-let-7f	8.8	8.423
hsa-let-7g	4.561	4.602
hsa-let-7i	1.702	1.687
hsa-miR-100	7.811	7.237
hsa-miR-101	2.266	4.109
hsa-miR-103	4.12	4.964
hsa-miR-106a	5.689	7.164
hsa-miR-106b	1.626	2.925
hsa-miR-107	3.51	4.14
hsa-miR-10a	6.045	6.695
hsa-miR-10b	3.066	2.847
hsa-miR-125a	3.592	3.118
hsa-miR-125b	8.252	8.452
hsa-miR-126*	2.464	3.549
hsa-miR-126	34.5	36.69
hsa-miR-130a	3.422	5.336
hsa-miR-130b	5.032	5.63
hsa-miR-136	0.915	0.335
hsa-miR-138	0.624	0.947
hsa-miR-151	0.351	0.574
hsa-miR-152	0.383	0.309
hsa-miR-154	0.812	1.323
hsa-miR-155	0.808	1.207
hsa-miR-15a	0.627	1.008
hsa-miR-15b	0.763	1.238
hsa-miR-16	5.112	5.006
hsa-miR-17-3p	1.044	1.271
hsa-miR-17-5p	2.985	3.517
hsa-miR-181a*	0.31	0.447
hsa-miR-181a	1.427	1.569
hsa-miR-181b	1.606	1.882
hsa-miR-181c	0.309	0.999
hsa-miR-181d	1.472	1.399
hsa-miR-182	0.38	0.694
hsa-miR-184	2.824	3.089
hsa-miR-185	1.497	1.831
hsa-miR-187	0.343	0.292
hsa-miR-188	0.459	0.661
hsa-miR-18a	1.341	2.13
hsa-miR-18b	0.583	0.804

hsa-miR-191	0.39	0.814
hsa-miR-195	3.585	5.028
hsa-miR-196b	0.792	0.25
hsa-miR-198	1.271	1.171
hsa-miR-199a*	1.673	1.689
hsa-miR-19a	5.96	5.19
hsa-miR-19b	4.839	6.211
hsa-miR-202	20.83	15.89
hsa-miR-206	1.153	1.313
hsa-miR-20a	5.23	5.313
hsa-miR-20b	4.462	4.871
hsa-miR-21	69.69	81.45
hsa-miR-210	**0.538**	**1.172**
hsa-miR-211	0.353	0.437
hsa-miR-214	7.345	8.159
hsa-miR-216	1.524	1.867
hsa-miR-219	0.272	0.236
hsa-miR-22	4.363	5.102
hsa-miR-221	5.918	5.581
hsa-miR-222	5.455	4.493
hsa-miR-23a	16.46	11.17
hsa-miR-23b	19.91	16.8
hsa-miR-24	3.644	4.462
hsa-miR-25	0.971	1.249
hsa-miR-26a	3.377	3.003
hsa-miR-26b	2.865	3.518
hsa-miR-27a	2.181	2.661
hsa-miR-27b	3.756	3.795
hsa-miR-28	2.582	1.774
hsa-miR-29a	8.301	8.784
hsa-miR-29b	10.36	11.61
hsa-miR-29c	7.029	5.553
hsa-miR-301	1.004	0.644
hsa-miR-302c*	0.699	0.866
hsa-miR-302d	0.485	0.217
hsa-miR-30a-5p	1.928	1.936
hsa-miR-30b	2.998	2.597
hsa-miR-30c	2.308	2.707
hsa-miR-30d	1.897	2.983
hsa-miR-31	0.397	1.184
hsa-miR-320	13.74	13.34
hsa-miR-331	1.242	1.677
hsa-miR-335	0.454	0.697
hsa-miR-339	0.271	0.27
hsa-miR-33b	0.299	0.206
hsa-miR-342	0.567	0.806
hsa-miR-34a	0.663	0.714
hsa-miR-34b	0.333	0.365
hsa-miR-361	0.745	0.899
hsa-miR-363*	4.15	4.981

hsa-miR-365	1.729	1.626
hsa-miR-368	2.028	2.468
hsa-miR-369-3p	0.428	0.476
hsa-miR-371	0.324	0.235
hsa-miR-373*	5.095	6.38
hsa-miR-374	1.803	1.907
hsa-miR-375	0.309	0.37
hsa-miR-376a	3.13	3.451
hsa-miR-376b	1.541	0.222
hsa-miR-377	1.55	2.479
hsa-miR-381	0.349	0.399
hsa-miR-383	0.425	0.103
hsa-miR-422a	0.739	0.0811
hsa-miR-422b	0.499	0.398
hsa-miR-423	0.325	0.515
hsa-miR-424	**6.596**	**10.87**
hsa-miR-452	0.596	0.568
hsa-miR-453	0.349	0.599
hsa-miR-484	0.614	0.712
hsa-miR-487b	0.887	0.932
hsa-miR-491	0.479	0.496
hsa-miR-492	0.426	0.762
hsa-miR-494	34.85	40.77
hsa-miR-495	0.38	0.565
hsa-miR-498	1.179	0.924
hsa-miR-500	0.455	0.643
hsa-miR-503	0.552	0.812
hsa-miR-504	0.321	0.335
hsa-miR-513	0.69	0.488
hsa-miR-516-5p	0.596	0.842
hsa-miR-518c	0.526	1.028
hsa-miR-518c*	0.575	0.628
hsa-miR-518f*	0.459	0.666
hsa-miR-521	0.282	0.26
hsa-miR-524*	0.309	0.418
hsa-miR-525	0.277	0.499
hsa-miR-526a	0.459	0.484
hsa-miR-526b	0.458	0.633
hsa-miR-526c	1.21	1.244
hsa-miR-532	0.296	0.362
hsa-miR-542-5p	0.441	0.437
hsa-miR-548a	0.44	0.581
hsa-miR-550	0.309	0.662
hsa-miR-557	1.102	1.06
hsa-miR-565	0.975	1.036
hsa-miR-574	1.203	1.252
hsa-miR-575	0.606	1.157
hsa-miR-583	0.391	0.775
hsa-miR-584	1.375	1.472
hsa-miR-589	0.363	0.407

hsa-miR-590	0.921	0.976
hsa-miR-601	0.902	0.889
hsa-miR-602	0.831	0.964
hsa-miR-604	0.519	0.921
hsa-miR-615	0.57	0.478
hsa-miR-634	1.49	1.688
hsa-miR-635	0.371	0.454
hsa-miR-638	5.279	6.883
hsa-miR-650	0.499	0.515
hsa-miR-658	3.019	2.159
hsa-miR-660	0.325	0.482
hsa-miR-661	0.265	0.272
hsa-miR-663	7.81	8.674
hsa-miR-671	0.544	0.747
hsa-miR-765	21.78	21.22
hsa-miR-766	0.823	0.874
hsa-miR-768-3p	1.067	2.221
hsa-miR-768-5p	5.668	4.92
hsa-miR-92	0.498	0.593
hsa-miR-93	1.849	1.673
hsa-miR-98	3.73	4.635
hsa-miR-99a	2.267	3.438
hsa-miR-99b	1.957	2.671

Data from a representative experiment are shown.