Regulation of Endothelial Hemoglobin Alpha Expression by Kruppel-Like Factors.

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Supplemental Methods, Tables, and Figures

Supplemental Methods

Adenoviral infection and plasmid DNA transfection

For overexpression in human ECs, cells at passage 3–9 were infected with control or adenovirus (10 multiplicity of infection or less) carrying mouse *Klf2* or human *KLF4* genes. For transient plasmid DNA expression, BAECs (passage 3–9) were transfected with 50 ng of control vector, KLF2 or KLF4 plasmid along with 200 ng of a luciferase construct driven by control, wild type or mutant HBA promoter by using FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instruction with minor modifications. Experiments were performed 48 hours after infection or transfection.

EC isolation

ECs were isolated from the heart of mice using a standard technique as previously described with a minor modification.[1] Briefly, the hearts were washed in cold PBS, minced with blades, digested in PBS containing 1%BSA, collagenase type I, 1mM $CaCl_2$, and 1 mM $MgCl_2$ at 37^{o}C for 45 minutes. ECs were purified using Dynabeads-conjugated with anti-CD31antibody (catalog 553370, BD Biosciences, San Jose, CA, USA).

RNA isolation and Real time-PCR

Total RNA from cells was isolated by using High Pure RNA Isolation Kit and reverse transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Quantitative real-time PCR (qPCR) was carried out using TaqMan Master Mix (Applied

Biosystems Inc., Foster City, CA, USA) and the reactions were performed using the StepOnePlus Real-Time PCR System (Applied Biosystems Inc.). Endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference (normalizer) for the gene expression. Primers are listed in Table S1. Relative mRNA expression levels (fold changes) between groups were calculated using the delta-delta Ct method.

Western blot

EC, MEJ, and SMC were harvested and lysed with ice-cold RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing protease and phosphatase inhibitor cocktail. Protein concentration was measured by using BCA Protein Assay Reagent Kit (Thermo Fisher Scientific). Protein lysates were subjected to protein electrophoresis using 4-12% Bis-Tris gels (Life Technologies, Carlsbad, CA) and transferred to nitrocellulose membrane. The membrane was incubated overnight at 4^{o}C with primary antibodies: goat anti-hemoglobin α (V-13) (catalog sc-31109, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-GAPDH (catalog G9545, Sigma-Aldrich, St. Louis, MO), mouse anti- eNOS/NOS Type III (catalog 610297, BD Biosciences, Franklin Lakes, NJ), rabbit anti-KLF2 (catalog 09-820, EMD Millipore, Billerica, MA), and goat anti-KLF4 (catalog AF3640, R&D Systems, Inc., Minneapolis, MN, USA). Secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) are donkey anti-goat IgG, IRDye 800CW conjugated (catalog 926-32214), donkey anti-rabbit IgG, IRDye 680RD conjugated (catalog P/N 925-68073), goat anti-mouse IgG, IRDye 680RD conjugated (catalog 926-68070), and goat anti-rabbit IgG, IRDye 800CW conjugated (catalog 926-

32211). Protein was detected using Odyssey imaging systems (LI-COR Biosciences). Protein quantification was performed using Bio-Rad Quantity One software.

Protein Digestion

Gel pieces cut from SDS-polyacrylamide gels were first destained with 50% acetonitrile in 100 mM ammonium bicarbonate, and 100% acetonitrile. Then, the protein was reduced by 20 mM DTT at room temperature for 60 min followed by the alkylation using 50 mM idoacetamide for 30 min in the dark. The reaction reagents were removed and the gel pieces were washed with 100 mM ammonium bicarbonate and dehydrated in acetonitrile. Sequencing grade modified trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate was added to the dried gel pieces and incubated at 37°C for overnight. Proteolytic peptides extracted from the gel with 50% acetonitrile in 5% formic acid were then dried and re-suspended in 0.1% formic acid.

Mass Spectrometry

Liquid chromatography-tandem mass spectrometry analysis of the resulting peptides was performed on Orbitrap Elite Hybrid Mass Spectrometer (ThermoFisher Scientific, Waltham, MA) coupled with a Waters nanoAcquity UPLC system (Waters, Taunton, MA). The spectra were acquired in the positive ionization mode by data-dependent methods consisting of a full MS scan in high-mass accuracy FT-MS mode at 120 000 resolution, and MS/MS on the twenty most abundant precursor ions in CID mode with the normalized collision energy of 35%. Mascot Daemon (version 2.4.0; Matrix Science, London, UK) was used to identify the peptides, and the data were searched against

SwissProt human database. Mass tolerance was set at 10 ppm for precursor ions and 0.8 Da for product ions. Carbamidomethylation of cysteines was set as a fixed modification, and oxidation of methionine as a variable modification. The significance threshold p value was set to < 0.05. Proteins hits with at least two unique peptides at Mascot score > 20 were considered to be identified.

Luciferase assay

Expression plasmid containing the luciferase reporter driven by a 953 bp (counting from the transcription start site) fragment of the human hemoglobin alpha gene (HBA) promoter or a control empty vector (SwitchGear Genomics, Carlsbad, CA, USA) was co-transfected with KLF2, KLF4 or both expression plasmids into BAECs. The cells were grown for 48 hours to allow sufficient expression of luciferase protein, and luciferase activity (bioluminescence) was measured on a luminometer according to the manufacturer's instruction with a minor modification. Background signal was determined from wells without cells. The luciferase activity was normalized with protein concentration and values were presented in % Relative Light Units (RLU).

Site-directed mutagenesis

KLF binding sites in the HBA promoter were generated by QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) using the luciferase reporter driven by a 953 bp fragment of the HBA promoter plasmid as a template. Forward and reverse site-directed mutagenesis primers containing the desired mutation

flanked by unmodified nucleotide sequences, were designed to anneal on opposite strands of DNA (Table S2).

Chromatin immunoprecipitation

DNA-protein complexes were isolated from HUVECs overexpressing KLF4 after fixation with formaldehyde. Antibody against KLF4 (catalog AF3640, R&D Systems Inc., Minneapolis, MN, USA) was used to capture KLF4-DNA protein complexes. These immune-complexes were precipitated by protein A/G magnetic beads. The immunoprecipitated DNA was reverse crosslinked and analyzed by qPCR for enrichment of KLF4 on the promoter region of HBA. Target gene enrichment was determined by qPCR (SYBR Green). The qPCR primer sequences for the promoter region of HBA are 5'- GGGTGGAGGGTGGAGACG -3' (Forward) and 5'-CGCCAGGGTTTATGCTTGGG -3' (Reverse). Five percent of DNA input was used as the normalization. Relative levels (fold changes) between groups were calculated using the delta-delta Ct method.

Reference

1. Lim YC, Garcia-Cardena G, Allport JR, et al. Heterogeneity of endothelial cells from different organ sites in T-cell subset recruitment. *Am J Pathol*. 2003; 162: 1591-601.

Supplemental Tables

Table S1. Primer sequences for qPCR (TaqMan) analysis.

Gene	Forward (5' to 3')	Reverse (5' to 3')	Probe number
KLF2	CATCTGAAGGCGCATCTG	CGTGTGCTTTCGGTAGTGG	11
KLF4	GGGAGAAGACACTGCGTCA	GGAAGCACTGGGGGAAGT	52
NOS3	GCATCCCTACTCCCACCAG	TTCTTCACACGAGGGAACTTG	23
HBA	CCGACAAGACCAACGTCAA	GGGGAAGGACAGGAACATC	81
GAPDH	TGTTCGTCATGGGTGTGAAC	GGTGCTAAGCAGTTGGTGGT	90
Klf2	CTAAAGGCGCATCTGCGTA	TAGTGGCGGGTAAGCTCGT	48
Klf4	CGGGAAGGGAGAAGACACT	GAGTTCCTCACGCCAACG	62
Gapdh	TGTCCGTCGTGGATCTGAC	CCTGCTTCACCACCTTCTTG	80
Hba	CTCTGGGGAAGACAAAAGCA	GTGGGGAAGCTAGCAAACAT	27

KLF2: Human kruppel-like factor 2; *KLF4*: Human kruppel-like factor 4; *NOS3*: Human nitric oxide synthase 3; *HBA*: Human hemoglobin subunit alpha; *GAPDH*: Human Glyceraldehyde 3-phosphate dehydrogenase; *Klf2*: Mouse kruppel-like factor 2; *Klf4*: Mouse kruppel-like factor 4; *Gadph*: Mouse glyceraldehyde 3-phosphate dehydrogenase; *Hba*: Mouse hemoglobin subunit alpha; qPCR: Quantitative real-time PCR.

Table S2. The HBA promoter mutagenic oligonucleotide primers (5'-3')

HBA-KLF site 1	
Sense	CCGGGCGTGCCCCCGCGCCAAAAGCATAAACCCTGGCGC
Antisense	GCGCCAGGGTTTATGCTTTTGGCGCGGGGGCACGCCCGG
HBA-KLF site 2	
Sense	CGGTCCAGGCCGCGCGACGGGCTCCGCGCCAG
Antisense	CTGGCGCGGAGCCCGTCGCGCGGCCTGGACCG
HBA-KLF site 3	
Sense	CCGCGCAGGCCCCGGACGGGACTCCCCTGCG
Antisense	CGCAGGGGAGTCCCGTCCGGGGCCTGCGCGG
HBA-KLF site 4	
Sense	CGTCCTGGCCCCCGGACCGCGTGCACCCCCAG
Antisense	CTGGGGGTGCACGCGGTCCGGGGGCCAGGACG

HBA: Hemoglobin subunit alpha; KLF: Kruppel-like factor.

Table S3. Identified Peptides of HBA Protein in the MEJ of KLF4 overexpressing HCAECs using LC-MS/MS

Number	Peptide	Mr (expt)	Mr (calc)	ppm	Score	
					Monomer[a]	Dimer[a]
2-12	VLSPADKTNVK	1170.6596	1170.6608	-1	45	
13-32	AAWGKVGAHAGEYGAEALER	2041.9962	2041.9969	0	36	
18-32	VGAHAGEYGAEALER	1528.7267	1528.7270	0	59	50
18-41	VGAHAGEYGAEALERMFLSFPTTK	2597.2592	2597.2584	0	67	
33-41	MFLSFPTTK	1086.5415	1086.5420	0	40	40
42-57	TYFPHFDLSHGSAQVK	1832.8844	1832.8846	0	68	
63-91	VADALTNAVAHVDDMPNALSALSDLHAHK	3011.4750	3011.4771	-1	27	
92-100	LRVDPVNFK	1086.6184	1086.6186	0	46	34
94-100	VDPVNFK	817.4324	817.4334	-1	27	24

[a]The monomer (16 kDa) and the dimer (32 kDa)-like bands on a gel.

HBA: Hemoglobin subunit alpha; MEJ: Myoendothelial junction; KLF4: Kruppel-like factor 4; HCAECs: Human coronary artery endothelial cells; Mr (expt): Experimental molecular weight of each peptide; Mr (calc): Calculated molecular weight of each peptide; ppm: parts per million.

Supplemental Figures

Figure S1

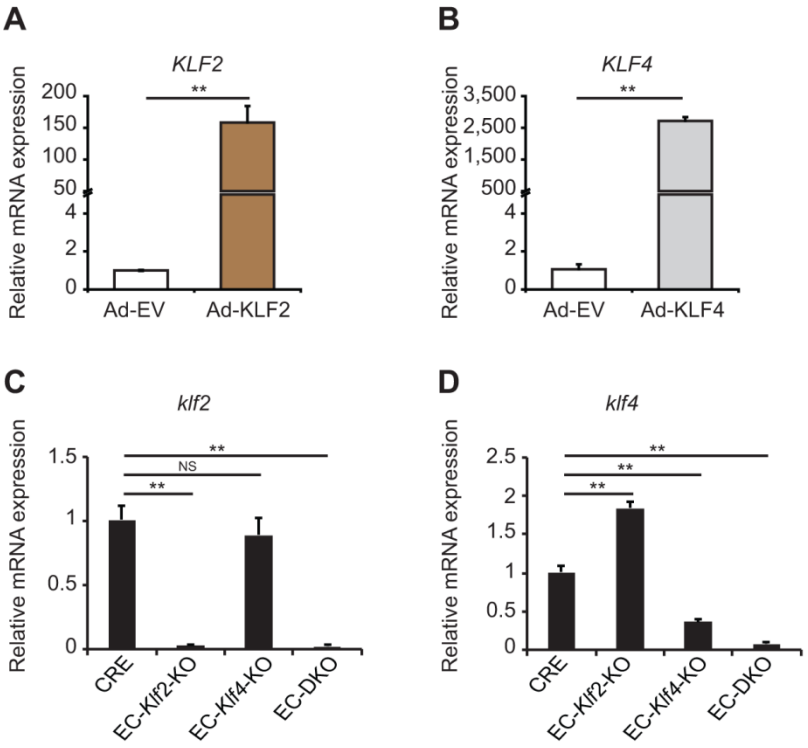

Figure S1. **Expression of KLF2 or KLF4 mRNA in ECs. (A-B)** Human coronary artery endothelial cells were infected with adenovirus carrying empty vector (Ad-EV), mouse KLF2 (Ad-KLF2), or human KLF4 (Ad-KLF4) and cultured on the petri dishes for 48 hours. Expression of mRNA was measured by TagMan real-time PCR. A representative data of three independent experiments is shown. **(C-D)** Cardiac microvascular ECs were isolated from EC-specific deletion of *Klf2* (EC-*Klf2*-KO), *Klf4* (EC-*Klf4*-KO), both *Klf2* and *Klf4* (EC-DKO), and CRE control (*n*=8-9 per genotype, each *n* was pooled form 2 mice). CRE: Cdh5(PAC)-CreERT2; EC: Endothelial cell; KLF: Kruppel-like factor. Data are presented as mean ± SEM values. * *: $P < 0.01$. NS: Not significant.

Figure S2

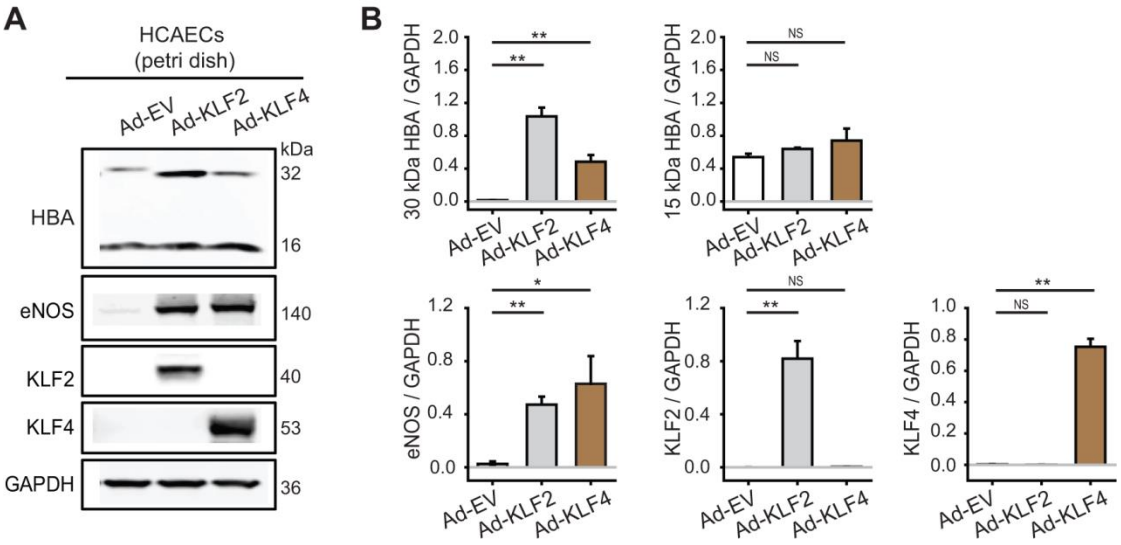

Figure S2. KLF2/KLF4-mediated hemoglobin alpha protein expression in the EC cultured alone on a petri dish.

(**A**) Representative western blot analysis of HBA, eNOS, KLF2, KLF4, and GAPDH when human coronary artery endothelial cells were cultured alone (*n*=3 independent experiments). The first band in HBA panel was likely non-specific due to its higher molecular weight. (B) Quantification of western blot analysis (normalized to GAPDH). Ad-EV: Control (empty) adenovirus; Ad-KLF2: Adenovirus carrying Kruppel-like factor 2; Ad-KLF4: Adenovirus carrying Kruppel-like factor 4; HBA: Hemoglobin subunit alpha; eNOS: Endothelial nitric oxide synthase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase. Data are presented as mean ± SEM values. *: $P < 0.05$, * *: $P < 0.01$. NS: Not significant.

Figure S3

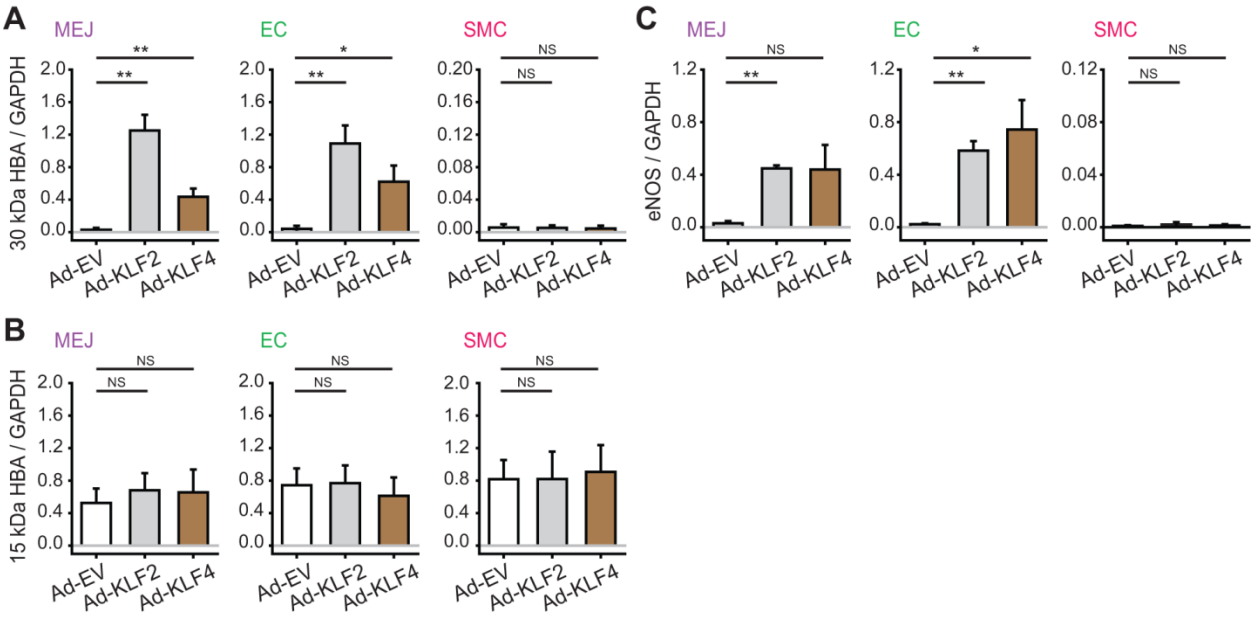

Figure S3. KLF2/KLF4-mediated hemoglobin alpha protein expression in the EC and the MEJ obtained from the co-cultured model.

(**A-C**) Quantification of western blot analysis (normalized to GAPDH) from HCAECs/HCASMCs co-culture where ECs were overexpressed with either KLF2 or KLF4 (*n*=3-4 independent experiments). Ad-EV: Control (empty) adenovirus; Ad-KLF2: Adenovirus carrying Kruppel-like factor 2; Ad-KLF4: Adenovirus carrying Kruppel-like factor 4; HBA: Hemoglobin subunit alpha; eNOS: Endothelial nitric oxide synthase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; HCAECs: Human coronary artery endothelial cell; HCASMCs: Human coronary artery smooth muscle cells; EC: Endothelial cells; MEJ: Myoendothelial junction; SMC: Smooth muscle cell. Data are presented as mean ± SEM values. *: $P < 0.05$, * *: $P < 0.01$. NS: Not significant.