Contrasting roles of E2F2 and E2F3 in endothelial cell growth and ischemic angiogenesis

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Online Supplement

Supplemental Methods

siRNA transduction

Human and mouse E2F2 and E2F3 siRNAs were synthesized by Dharmacon, Inc. (Lafayette, CO) and transfected HUVECs and mouse lung ECs by following manufacturers' protocols [1]. Cells with a knockdown efficiency of over 85% at 16 h after transfection were chosen for functional analyses.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed in HUVECs 16 h after siRNA transduction with a ChIP assay kit (Upstate, NY) as previously described [1]. Briefly, cells were lysed and sheared by sonication; then, the cell extracts were pre-cleared with protein A agarose/salmon sperm DNA and immunoprecipitated overnight with an anti-E2F3 antibody (Santa Cruz, sc-878) or no antibody as negative control. The precipitates were washed with buffer for 5 minutes and extracted twice with 1% SDS in 0.1 M $NaHCO_3$; then, the elutes were pooled and heated at 65°C for 5 hours to reverse the formaldehyde cross-linking, and DNA fragments were purified via phenol/chloroform extraction. The purified DNA (2 μL) was added to a standard real-time PCR mixture and amplified. The 3' end UTR of human DHFR was amplified as negative control. Primer sequences are listed in the Online Supplemental Table S1.

Tube formation assay

After transduction with siRNAs for 16 h, 25×10^3 mouse lung ECs were seeded in each well of a 48-well plate coated with 120 μL of growth-factor-reduced Matrigel™ (BD Biosciences, San Jose, CA, USA). Tube formation was examined by phase-contrast microscopy 6 h later as previously described [2].

Migration assay

Cell migration was evaluated with a modified Boyden's chamber (Neuro Probe, Inc., Gaithersburg, MD, USA) as previously described [2]. Briefly, a polycarbonate filter (5-μm pore size; GE Infrastructure, Fairfield, CN, USA) was coated with a solution containing 2.5 μg/mL vitronectin (Sigma-Aldrich Co.) and 0.15% gelatin (Sigma-Aldrich Co.) and inserted between the chambers, then 5×10^4 cells per well were seeded in the upper chamber and the lower chamber was filled with EBM-2 medium that contained 20 ng/mL VEGF. Cells were incubated for 8 h at 37°C, then viewed under 20X objective,

and the number of cells that had migrated to the lower chamber were counted in 3 fields per well; migration was reported as the mean number of migrated cells per 20X objective field.

Supplemental Results

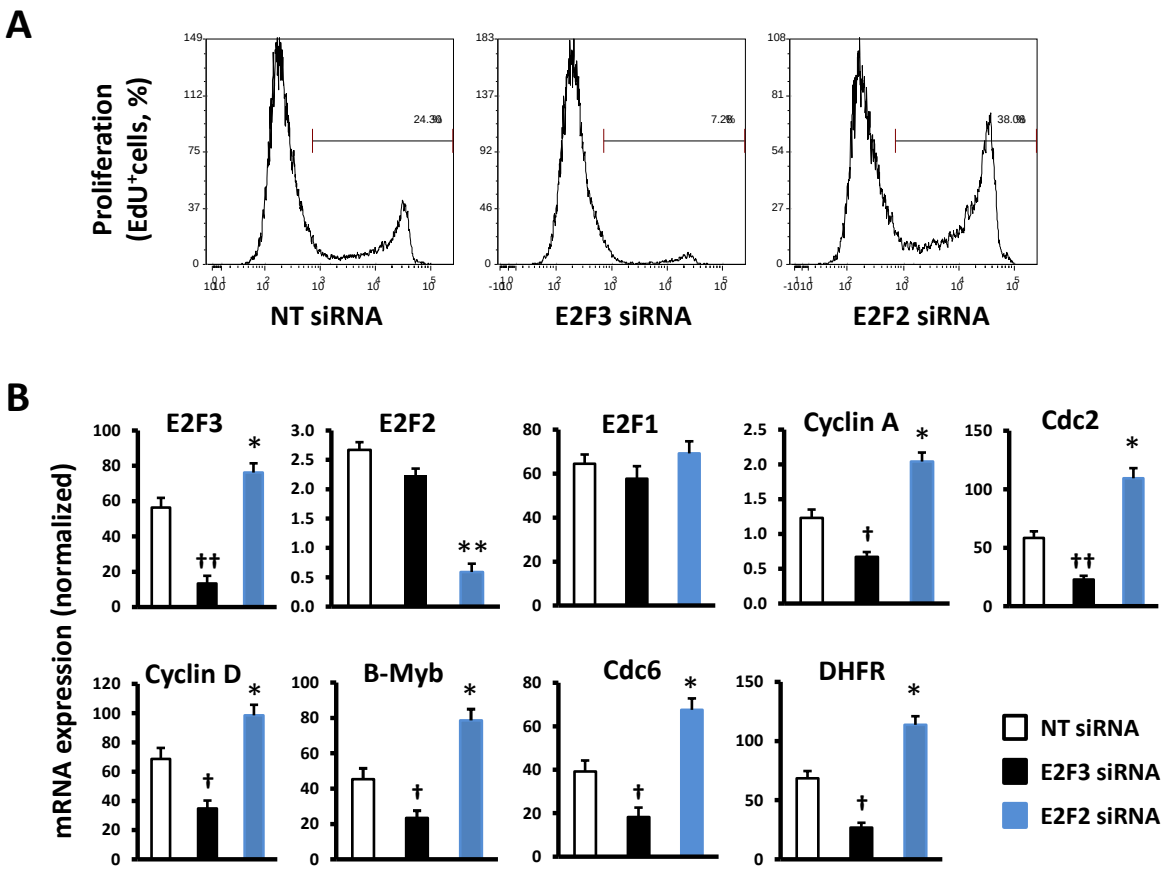

Figure S1: EC cell-cycle progression and G1/S-phase gene expression are increased by E2F2 knockdown and decreased by E2F3 knockdown. Murine lung ECs were isolated from WT mice, then treated with non-targeting (NT) siRNA and siRNAs for E2F2 and E2F3, and subjected to serum starvation for 24 h and stimulation for 16 h as described in Figure 2. (**A**) Cells were treated with EdU and cultured for additional 4 h; then, proliferation was evaluated via flow cytometry and quantified as the proportion of cells stained positively for EdU incorporation. (**B**) The expression of E2F2, E2F3, and the G1/S-phase genes Cyclin A, Cyclin D, Cdc2, Cdc6, B-Myb, and DHFR was determined via quantitative RT-PCR and normalized to18S rRNA levels (n=3). *P<0.05, **P<0.01, †P<0.05, ††P<0.01 vs. NT siRNA.

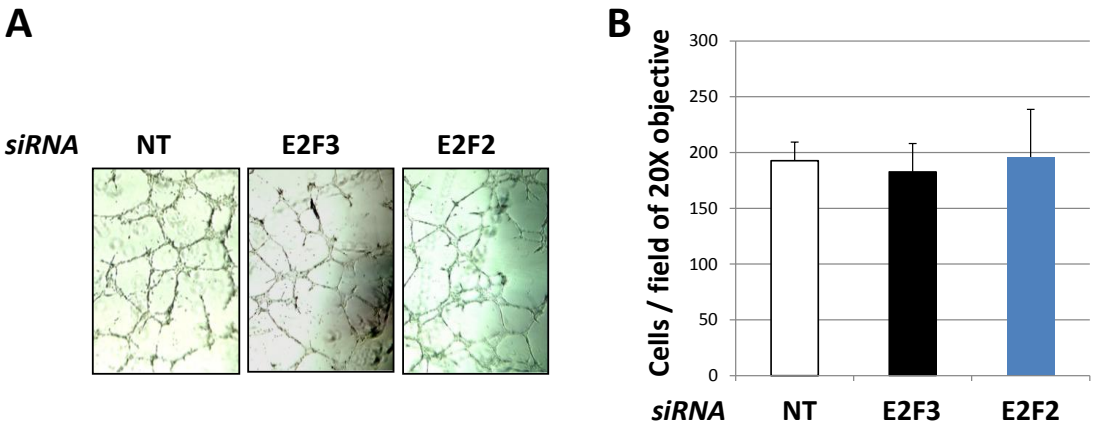

Figure S2: Knockdown of E2F2 and E2F3 expression does not affect EC tube formation and migration. Mouse ECs were transduced with non-targeting (NT) siRNA and siRNAs for E2F2 and E2F3 and 16 h later, subjected to assessments of tube formation (**A**) and migration (**B**). (**A**) $2.5x10^4$ cells were seeded on each well of the 48-well plate pre-coated with growth-factor–reduced Matrigel, and 6 h later, tube formation was assessed under a phase-contrast microscope. Shown are representatives of 3 independent experiments. (**B**) $5×10^4$ cells were seeded in the upper chamber of a modified Boyden chamber, and the lower chamber was filled with EBM-2 medium containing 20 ng/mL VEGF. Cell migration was quantified 8 h later by counting the number of cells per 20X objective field that had migrated through the membrane. N=4, no significant difference was found between the 3 treatment groups.

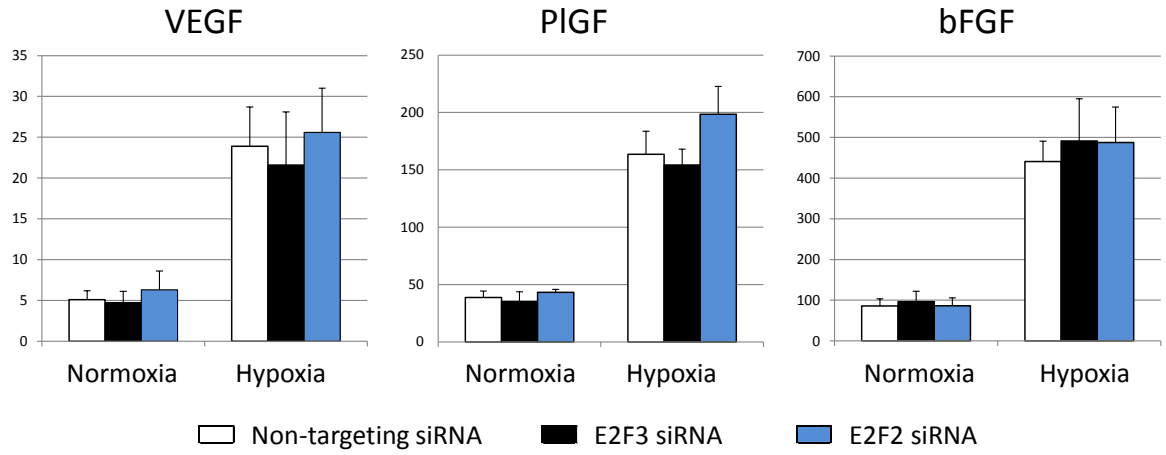

Figure S3. Knockdown of E2F2 and E2F3 expression does not affect the expression of angiogenic growth factors VEGF, PlGF, and bFGF. WT mouse lung ECs were transduced with non-targeting siRNA and siRNAs for E2F2 and E2F3 and 16 h later, exposed to hypoxia (0.5% oxygen) or cultured under normal condition for an additional 16 h. The expression of VEGF, PlGF, and bFGF was determined via quantitative RT-PCR and normalized to18S rRNA levels (n=4). No significant difference was detected between non-targeting, E2F2, and E2F3 siRNA treatment groups at both basal and hypoxia conditions.

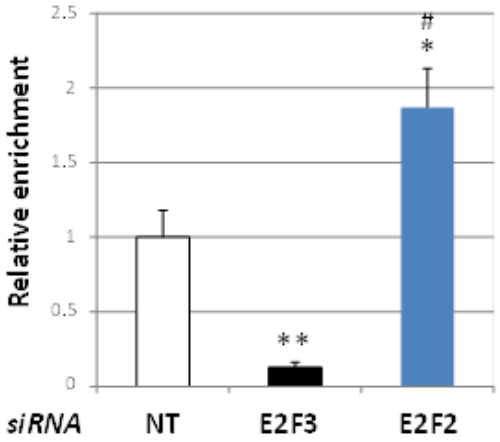

Figure S4: Knockdown of E2F2 in ECs leads to an enhanced occupancy of E2F3 at cyclin A promoter. ChIP analysis using an anti-E2F3 antibody on cross-linked extracts from HUVECs treated with non-targeting (NT) siRNA and siRNAs for E2F2 and E2F3. The E2F3 occupancy of the E2F site on the cyclin A promoter was analyzed by qPCR with primers listed in online supplemental table S1. Relative enrichments are calculated as fold change over the No Antibody control and normalized to ECs treated with NT siRNA. *p<0.05, **P<0.01 vs. NT siRNA; #P<0.001 vs. E2F2 siRNA; n=4.

Table S1: Primer sequences used for real-time RT-PCR and ChIP assays.

Gene	Forward primer	Reverse primer
Real-Time RT-PCR for gene expression assays		
murine E2F1	GAGGCTGGATCTGGAGACTG	GAGTCCTCCGAAAGCAGTTG
murine E2F2	GCCGAGGTGCTGGATGTG	TTTGGACTTCTTGCGGATGAG
murine E2F3	CGTCCAACCTTGAAGGACCTT	CCCAGGCTCAGCAGGTAGTC
murine Cyclin A	GGCTTTTAATGCAGCTGTCTCTTT	CAAAACTGCCATCCATTGGA
murine Cyclin D	GCTCTGTGCGCTACCGACTT	CACGCTTCCAGTTGCAATCA
murine Cdc2	CCGATCTCCAGAAGTGTTGCT	CAGTTCTGCAAATATGGTCCCTATAC
murine Cdc6	CAGCAGAAGATCCTGGTTTGC	CCAGGGTGACCTCTTTGATTTT
murine B-Myb	AACAGTGGACGCTGATAGCCA	TTCACAGCATTGTCCGTCCTC
murine VEGF	GCAGGCTGCTGTAACGATGA	GCATGATCTGCATGGTGATGTT
murine PlGF	TGGTGCCTTTCAACGAAGTG	TTCATCCAAGATGTACACCAGCTT
murine bFGF	GTCACGGAAATACTCCAGTTGGT	CCGTTTTGGATCCGAGTTTATACT
murine GAPDH	CGTGTTCCTACCCCCAATGT	TGTCATCATACTTGGCAGGTTTCT
murine 18S	CGGGTCGGGAGTGGGT	GAAACGGCTACCACATCCAAG
Chromatin immunoprecipitation (ChIP) assays		
human Cyclin A	CAGCCTTCGGACAGCCTCGC	CAAACTGGCTGGGGCGGGAG
human DHFR	TTCTGCTGTAACGAGCGGGCTCGGA	CTACAAGTTAGAGAAACAGCGTTACTCGAA
human 3' end DHFR	CTGATGTCCAGGAGGAGAAAGG	AGCCCGACAATGTCAAGGACTG

Supplemental References

[1] Zhou J, Zhu Y, Cheng M, Dinesh D, Thorne T, Poh KK, et al. Regulation of vascular contractility and blood pressure by the E2F2 transcription factor. Circulation. 2009;120:1213-21.

[2] Renault MA, Roncalli J, Tongers J, Thorne T, Klyachko E, Misener S, et al. Sonic hedgehog induces angiogenesis via Rho kinase-dependent signaling in endothelial cells. Journal of molecular and cellular cardiology. 2010;49:490-8.