

SUPPLEMENTARY MATERIALS

TABLE S1: ANTIBODIES AND TAGS

ANTIBODY	APPLICATION	SOURCE
Primary		
Acetylated-Histone H3, rabbit anti-human	ChIP	Upstate (06-599)
Acetylated-Histone H4, rabbit anti-human	ChIP	Upstate (06-598)
CD31, rat monoclonal	IHC	BD Biosciences (550389)
Digoxigenin, FITC conjugated	In situ TUNEL	Chemicon (S7110)
HDAC1, mouse monoclonal	ChIP, IP	Cell Signaling
Fas, rabbit polyclonal	IHC	Calbiochem (PC69)
FasL, rabbit polyclonal	IHC	Calbiochem (PC78)
I κ B α , mouse monoclonal	Western blot	Cell Signaling (9242)
NFATc2, rabbit polyclonal	ChIP	A Gift from JM Redondo
NF κ B p50, rabbit polyclonal	ChIP, IP, Western blot	Upstate (06-886)
p65, rabbit polyclonal	IHC, Western blot	Santa Cruz C-20 (sc-372)
p65, rabbit polyclonal	ChIP, IP	Santa Cruz F-6 (sc-8008)
p65, acetylated	WB	Cell signaling
p300, rabbit polyclonal	ChIP	Santa Cruz (sc-585x)
phospho-I κ B α (Ser32/36), mouse monoclonal	Western blot	Cell Signaling (9246)
RNA pol II, mouse monoclonal	ChIP	Upstate
TATA binding protein, mouse monoclonal	Western blot	Covance
Tubulin, rabbit polyclonal	Western blot	Neomarkers (MS-581-P0)
Secondary		
Donkey anti-rabbit, Cy3 conjugated	IHC	Jackson Immunoresearch
Donkey anti-Rat, Cy2 conjugated	IHC	Jackson Immunoresearch
Donkey anti-rabbit, HRP conjugated	Western blot	Jackson Immunoresearch
Rabbit anti-mouse, HRP conjugated	Western blot	Jackson Immunoresearch

TABLE S2: PCR PRIMERS

DESCRIPTION	SEQUENCE, 3' - 5'	Annealing T ^o
cFLIP promoter κ B site, conventional	F: TTGGTACATAAC TTGGCTGTGACT R: TAAAGCCTTTGA AATGGTCCTAAG'	60°C
cFLIP promoter κ B site, real-time	F: GGGGTGAAA GTGATGGAGAA R: AGGGCAAACAGCAAATGAAG	58°C
cFLIP promoter NFAT site, real-time	F: TCACGTTTGCTATGACTCCCAGAC R: TCCACGCGTTAGGAGTAAACACTG	60°C
FasL promoter, κ B site, conventional	F: TGTAATTCCAGCACTTTGGG R: AAACCAGTGGCCAAACACG	60°C
FasL promoter, κ B site, real-time	F: GCCTACTAACCTGTTTGGGTAGCA R: TGGGTAATTGAAGGGCTGCTGCAT	59°C
FasL cDNA, real-time	F: GGGCTGCCATGTGAAGAG R: GAATGAAATGAGTCCCCAAAACA	59°C
GAPDH cDNA, real-time	F: TTCGACAGTCAGCCGCATCTTCTT R: AGCCTTGACGGTGCCATGGAATTT	59°C
L19 ribosomal protein	F: CCATGAGTATGCTCAGGCTTCAGA R: TACAGGCTGTGATACATGTGGCGA	59°C

SUPPLEMENTARY MATERIALS

METHODS:

Cells and Reagents:

Human microvascular endothelial cells (HMVEC) (Lonza, Allendale NJ) were grown on gelatinized surface in MCDB131 medium (Sigma, St. Lois MO), 5% FBS, EGMTM-MV and SingleQuots supplements (Clonetics, North Brunswick NJ). EPC mouse bone marrow cells were isolated as described^{71,72}. Recombinant cytokines were from R&D Systems (Minneapolis MN). PEDF was isolated as described⁷³ or purchased (Bioproducts, West Palm Beach FL). PEDF 34-mer peptide³⁸ was from GenScript (Piscataway NJ). For TSP1, we used ABT-510 (Abbott, North Chicago IL) a peptide, which retains TSP1 anti-angiogenic function⁷⁴. BMS-345541 was from Calbiochem (San Diego CA), Vorinostat (SAHA) from Merck (Whitehouse Station NJ) and Valproic Acid (VA) from Sigma. For the antibodies see Table 1S.

Animals:

Nude mice were obtained from Harlan and maintained under conditions specified by the National Institute of Health guidelines. All the protocols were approved by Northwestern University Animal Care and Use Committee at the Center for Comparative Medicine. Four to five nude mice per treatment group were used in both the matrigel plug and DIVAA assays. C57Bl/6 mice from Harlan were used for the corneal micropocket assay, 3-6 mice per group. All *in vivo* experiments were repeated at least three times to ensure reproducibility. The animals were anesthetized with IP injections of Pentobarbital (60-80 mg/kg) and given Buprenex subcutaneously (SC), 2 mg/kg, prior to recovering and every 6 hours as needed, to control pain.

Isolation of endothelial precursor cells (EPCs) from bone marrow (1, 2).

Bone marrow from the tibia, fibia, and spine was isolated from 8-12 week old mice by dissociating the bones with mortar and pestle and collecting cellular fraction in DPBS. Mononuclear cells were purified by density gradient centrifugation in Histopaque-1083 followed by erythrolysis in ammonium chloride buffer (Stem Cell Technologies). Cells were resuspended in EBM-2 medium supplemented with EGM-2 MV SingleQuots (Clonetics). Six millions EPCs were plated in 10 cm tissue culture dishes coated with vitronectin (2.5 µg/mL) and cultured 4-7 days before treatment/analysis.

1. Asahara, T., T. Takahashi, H. Masuda, C. Kalka, D. Chen, H. Iwaguro, Y. Inai, M. Silver, and J.M. Isner. 1999. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *The EMBO journal* 18:3964-3972.
2. Li, M., H. Nishimura, A. Iwakura, A. Wecker, E. Eaton, T. Asahara, and D.W. Losordo. 2005. Endothelial progenitor cells are rapidly recruited to myocardium and mediate protective effect of ischemic preconditioning via "imported" nitric oxide synthase activity. *Circulation* 111:1114-1120.

SUPPLEMENTARY MATERIALS

SUPPLEMENTARY FIGURES:

Figure S1. Characterization of NF κ B activation by PEDF and TSP1

HMVECs were grown to near confluence, treated with the indicated combinations of VEGF, PEDF and TSP1, nuclear extracts collected and tested for NF κ B activation by Electrophoretic Mobility Shift Assay (EMSA). Unlabelled competitor oligonucleotide (Cmp) was used to control specificity. Two distinct oligonucleotides were used, with κ B consensus sequence specific for p65 (S1A) and p50 (S1B). Note increased NF κ B activity in VEGF-stimulated HMVECs in the presence of TSP1 and PEDF. Each experiment was performed three times with similar results.

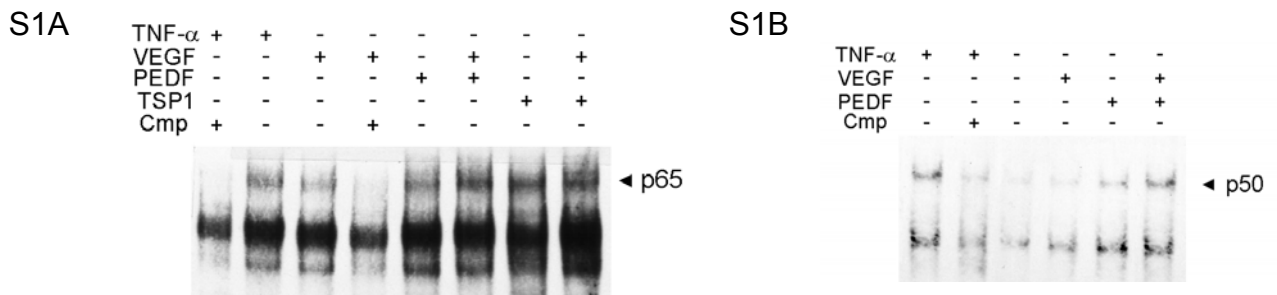


Figure S2. CD95/Fas expression was unchanged by PEDF or BMS-345541.

Nude mice received subcutaneous Matrigel plugs supplemented by bFGF (250 ng/ml), PEDF 34-mer (100nM) and BMS-345541, where indicated. On day 11 the plugs were extracted, snap-frozen and stained for CD31 (red) and CD95/Fas (green). Images were obtained by fluorescent microscopy and overlaid using MetaMorph software package. Ten fields per section (one for each Matrigel plug) were evaluated.

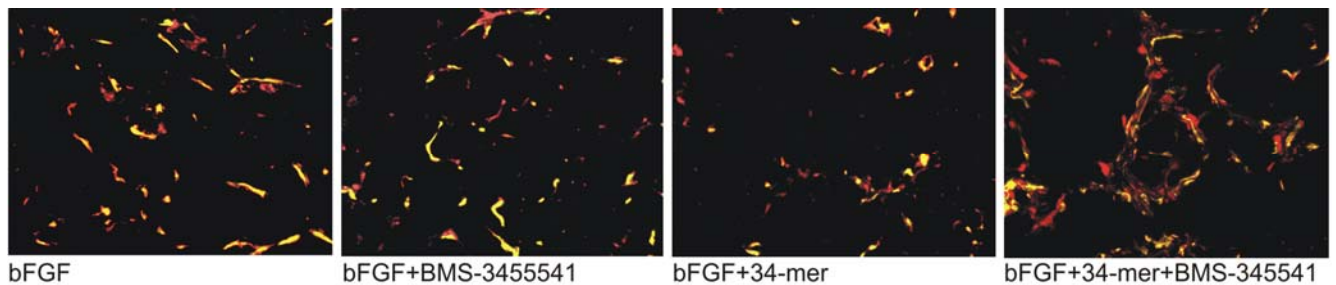


Figure S3. cFLIP activation was NF κ B dependent

HMVECs were grown to near confluence, stimulated with VEGF and treated overnight with TSP1 or PEDF. (S2A) cFLIP levels were assessed by Western blotting. (S2B) NF κ B was blocked with an IKK inhibitor, BMS-345541. As shown previously, TSP1 and PEDF decreased cFLIP protein levels in VEGF-stimulated endothelial cells (S3A and S3B). Although BMS-345541 by itself blocked FLIP

SUPPLEMENTARY MATERIALS

induction by VEGF, it reversed cFLIP decrease due to PEDF. Loading was assessed by re-probing with β -actin antibodies. Each experiment was performed three times, with similar results.

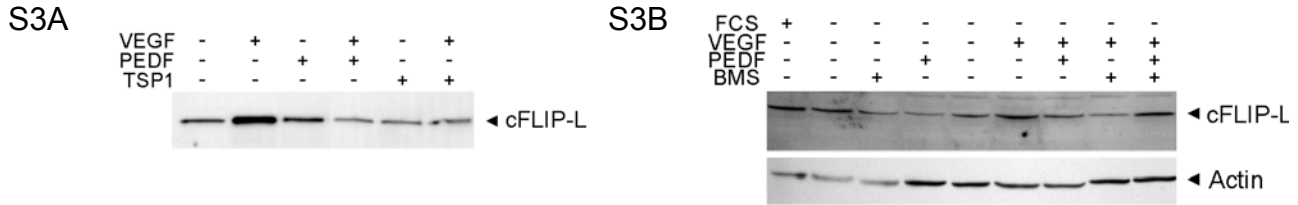


Figure S4. HDACi and PEDF have biphasic effect on cFLIP production

HMVECs were grown to near confluence and subjected to the indicated combinations of the fixed PEDF dose (10 nM) and/or increasing concentrations of SAHA (μ M). (S4A) cFLIP was measured by Q-PCR, with L19 as internal reference. Note the lack of effect by SAHA alone on cFLIP levels. The combination of low doses SAHA with PEDF further decreases cFLIP message; the highest dose of SAHA (100 μ M) reverses PEDF dependent decrease in FLIP. (S4B) FasL was measured by Q-PCR as in (S4A). Note dose dependent increase in FasL in response to SAHA alone, and the lack of improvement at high dose (100 μ M). Each data point was measured in duplicate and each experiment performed in triplicate, with similar results.

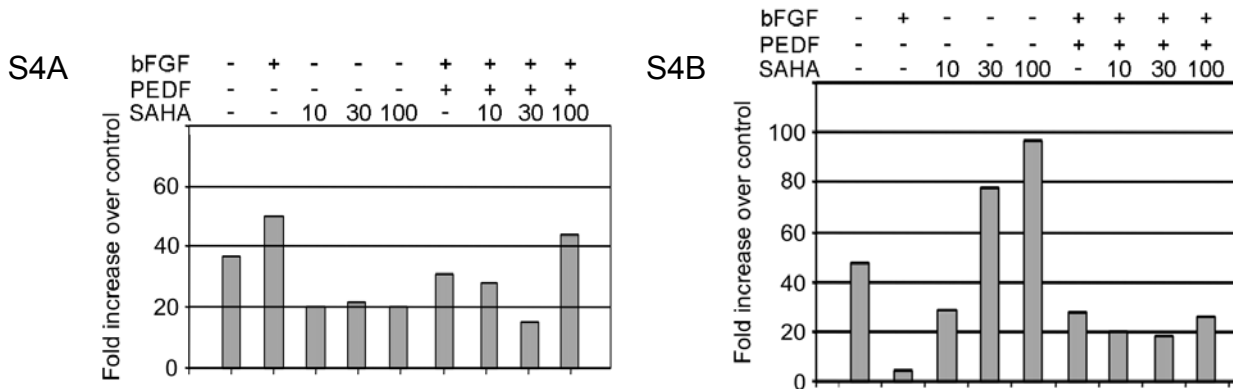


Figure S5. The effect of VA on anti-angiogenesis by PEDF.

(S5A) ECs were subjected to VA alone (0.1 and 1.0 mM) and/or PEDF (10 nM). Apoptosis was detected by in situ TUNEL and quantified by MetaMorph software (a minimum of 600 cells per condition). Note a dose-dependent increase in apoptosis by VA alone and the reversal of apoptosis by PEDF by 1 mM VA. (S5B) DIVAA. Mice were implanted with angioreactors containing VEGF/bFGF mix \pm PEDF (20 nM). The animals were treated with IP injections of VA (100 and 400 mg/kg) where indicated. Note a mild anti-angiogenic effect by VA alone, significant inhibition of angiogenesis by PEDF ($P=0.0004$) and a reversal of the inhibitory effect by high dose of VA (400 mg/kg). Statistical significance was evaluated by a one-way ANOVA.

SUPPLEMENTARY MATERIALS

