Supplementary Appendix

Methods

Hemangioma-derived stem cell (HemSC), hemangioma-derived endothelial progenitor cell (HemEPC) and cord blood endothelial progenitor cell (cbEPC) isolation procedures.

HemSC, HemEPC isolation was done as before[1] [2] [3]. Briefly, freshly obtained proliferating hemangioma specimens were digested with 0.2% collagenase A (Roche Diagnostics, Indianapolis, IN) in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 2% FBS, $Ca^{++}Mg^{++}$ and PSF (Invitrogen; 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin) for 30 minutes at 37°C. The tissue homogenate was filtered through a 100-μm cell strainer (Fisher Scientific, Hampton, NH). Red blood cells were lysed by incubating the sample in NH_4Cl (0.8% NH_4Cl/0.1 mM EDTA; StemCell Technologies, Vancouver, BC, Canada) for 10 minutes on ice. Cells were filtered through a 40-μm strainer to obtain a single-cell suspension. HemSC $CD133^+$ cells, constituting approximately 0.2% of the hemangioma cells[5] were isolated using anti-CD133–coated magnetic beads (Miltenyi Biotec, Auburn, CA) and cultured on fibronectin (FN; 1 $\mu g/cm^2$) coated plates with Endothelial Basal Medium (EBM-2; CC-3156; Cambrex Bio Science, Walkersville, MD) supplemented with 20% FBS, SingleQuot (CC-4176; Cambrex Bio Science) and PSF. HemSCs display a morphology that is similar to bone-marrow mesenchymal stem and express CD90, a mesenchymal cell marker[1].

To purify the HemEPC, subconfluent $CD133^+$ containing colonies of cobble-stone appearing cells were trypsizined and incubated with CD31 coated beads (Dynabeads®

CD31 Endothelial Cell, invitrogen). HemEPCs were cultured and expanded in the EBM-2/20% FBS on FN-coated plates as described for $CD133^+$ cells. HemEPC express endothelial cell markers CD31, CD146, CD34, and VEGFR-2.

Cord-blood EPCs (cbEPCs) were isolated, as described previously [4] [5]. In brief, mononuclear cells were seeded on 1% gelatin–coated tissue culture plates for 48 hours. Unbound cells were removed following the 48 hours of culture and the bound fraction was cultured for another 2 weeks in EBM-2/20% FBS. Colonies of endothelial-like cells were trypsinized and then selected using either Ulex- or CD31-coated magnetic beads (Dynal Biotech, Brown Deer, WI).

Cell culture

All cells in the study were cultured on fibronectin-coated (1 μg/cm^2, Millipore) dishes with EBM-2 (Lonza). EBM-2 was supplemented with 20% fetal bovine serum (Hyclone), endothelial growth media-2 SingleQuots (Lonza), and 1× GPS (Invitrogen). For all the *in-vitro* experiments cells were plated on non-FN coated dishes. Twenty-four hours later, medium replaced with EBM-2 supplemented with 20% FBS without SingleQuots, with the indicated concentration of dexamethasone (D2915, Sigma) for three days. For VEGF-A quantitative RT-PCR and ELISA, media containing dexamethasone was removed and cells were serum-starved over-night before assay.

Angiogenesis antibody array

Conditioned media from HemSCs, treated or untreated with dexamethasone, were analyzed for protein expression using RayBio® Human Angiogenesis Antibody Array C Series 1000 (RayBiotech; http://www.raybiotech.com/map/human_angio_1000_map.pdf)

according to the manufacturer's instructions. Blots were analyzed by with ImageJ software (NIH)

Quantitative PCR for uPAR, MCP-1, IL-6 and MMP-1

All primers were designed by PerlPrimer[6]. Total RNA was extracted using RNeasy (Qiagen) according to the manufacturer's instructions. A quantitative RT-PCR was done with the following primers MCP-1 5' CAC CTG CTG TTA TAA CTT CAC 3' and 3' AAT GGT CTT GAA GAT CAC AG 5', IL-6 5' GAG AAG ATT CCA AAG ATG TAG CC 3' and 3' GCA AGT CTC CTC ATT GAA TCC 5', MMP-1 5' GAC TTA GTC CAG AAA TAC CTG and 3' CTT TCA GCC CAA AGA ATT CC 5' Reactions were carried out for 35 cycles at an annealing temperature of 58°C.

TUNEL Assay

HemSCs were plated on chamber slides and incubated three days with increasing concentrations of dexamethasone. Direct TUNEL labeling assay was done by In Situ Cell Death Detection Kit, TMR red (Roche) according to the manufacturer's instructions.

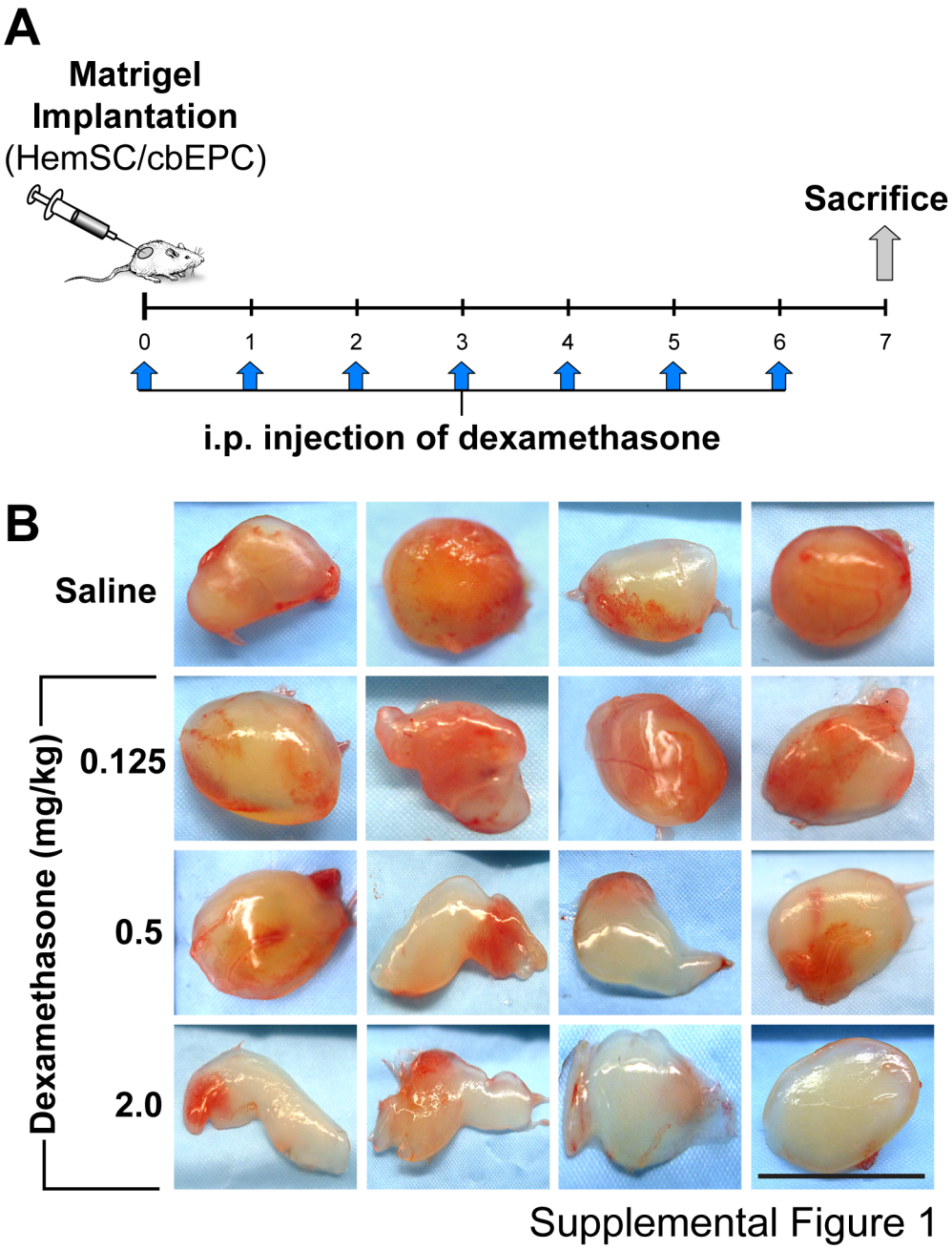
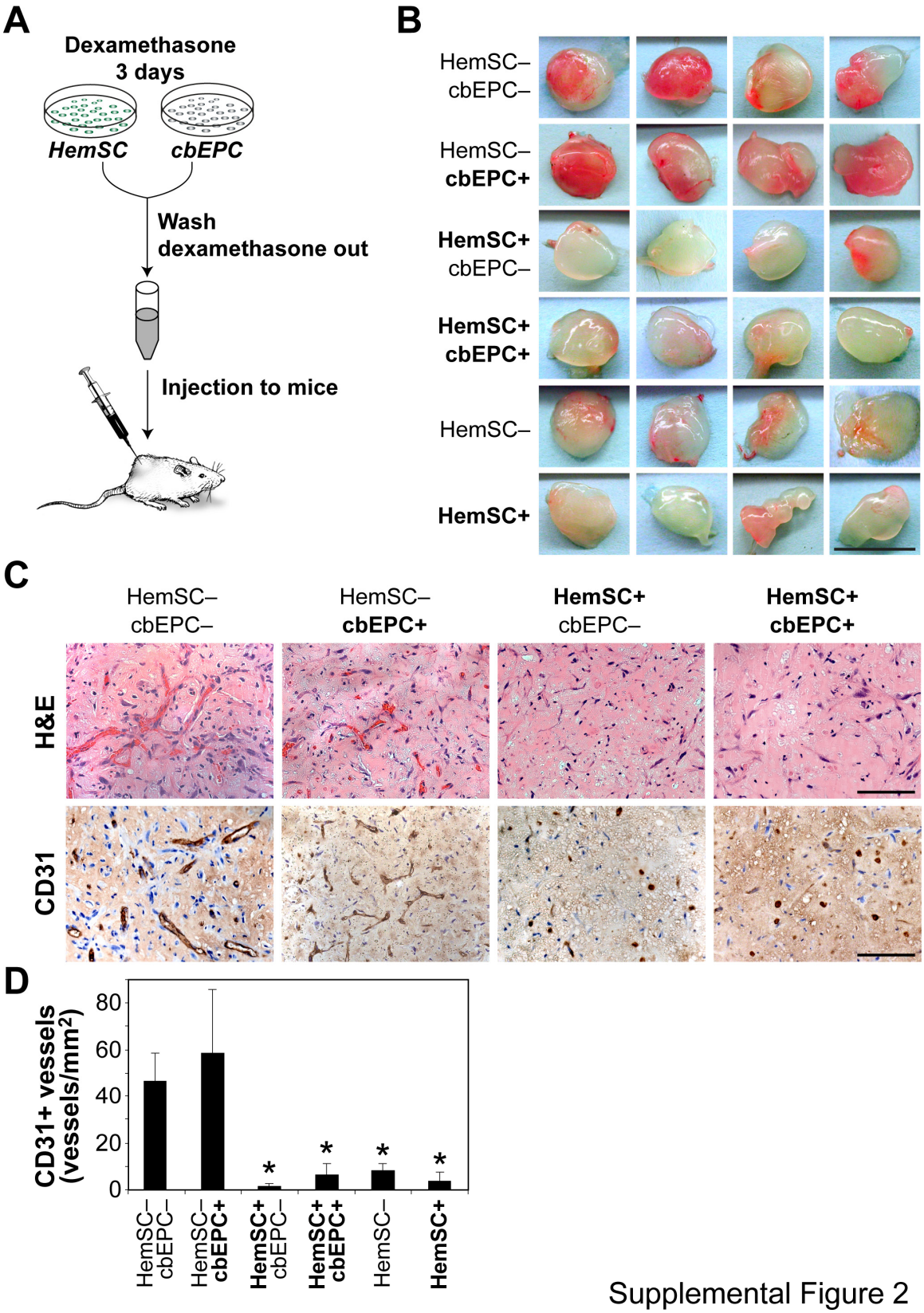
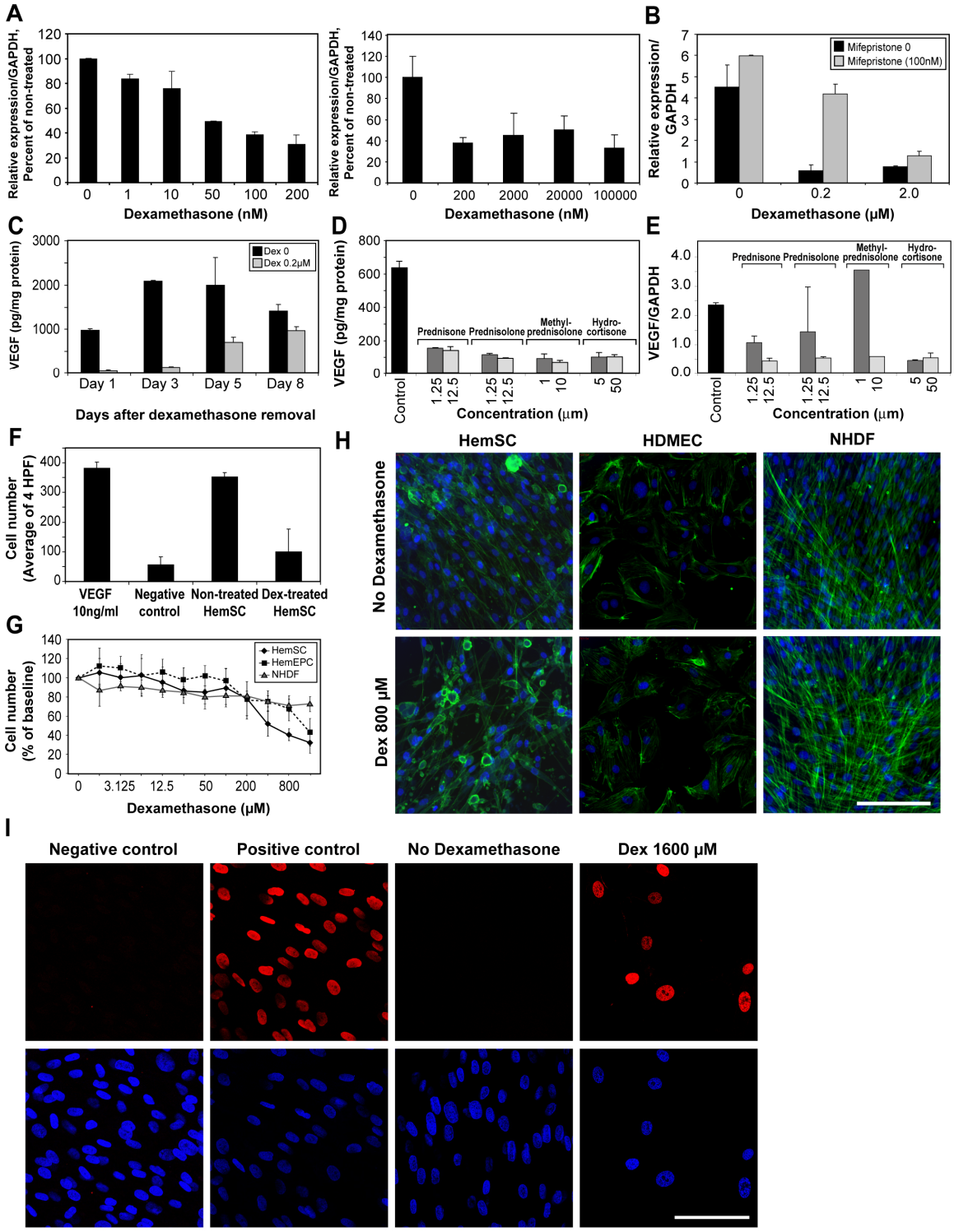


Figure S1 Panel A, Schematic of the experimental design for dexamethasone dose response experiment. Panel B, cell/Matrigel explants from each animal. Scare bar: 1 cm. The macroscopic images shown for saline (far right) and 2.0 mg/kg dexamethasone (far right) are also shown in Figure 1B.



Supplemental Figure 2

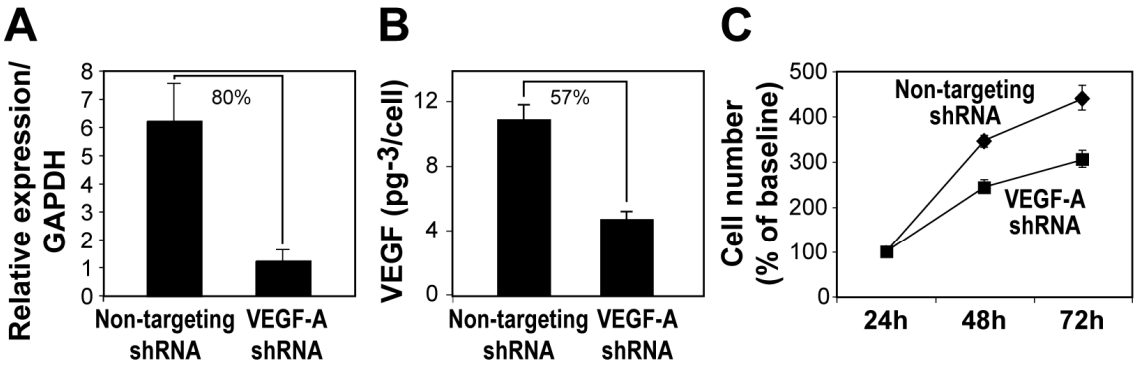
Figure S2 - The experimental design is illustrated in Panel A. HemSCs or cbEPCs or both cell types were treated by 200nM dexamethasone for 3 days. Dexamethasone was washed out and cells implanted in nude mice. Panel B shows the explants from animals in each treatment group on day 7. Bold font and the + symbol indicate cells were treated with dexamethasone as shown in Panel A. Scale bar: 1 cm. Panel C, top row shows H&E of Matrigel explants from Panel B. Erythrocyte-filled vessels were seen only when untreated HemSCs were implanted (first two H&E panels). Panel C, bottom row shows staining with anti-human CD31 antibody. Immunostaining with anti-human CD31 was done in order to isolate vasculogenesis (*de novo* creation of human blood vessels) from angiogenesis (attraction of mouse vessels to grow into the Matrigel). All images were taken at the same magnification, Scale bars: 100 μm. Panel D shows quantification of CD31-positive vessels from Panel C. N=4-6/group, $*P < .05$ compared with the saline-treated cells. This experiment was repeated twice with similar results.



Supplemental Figure 3

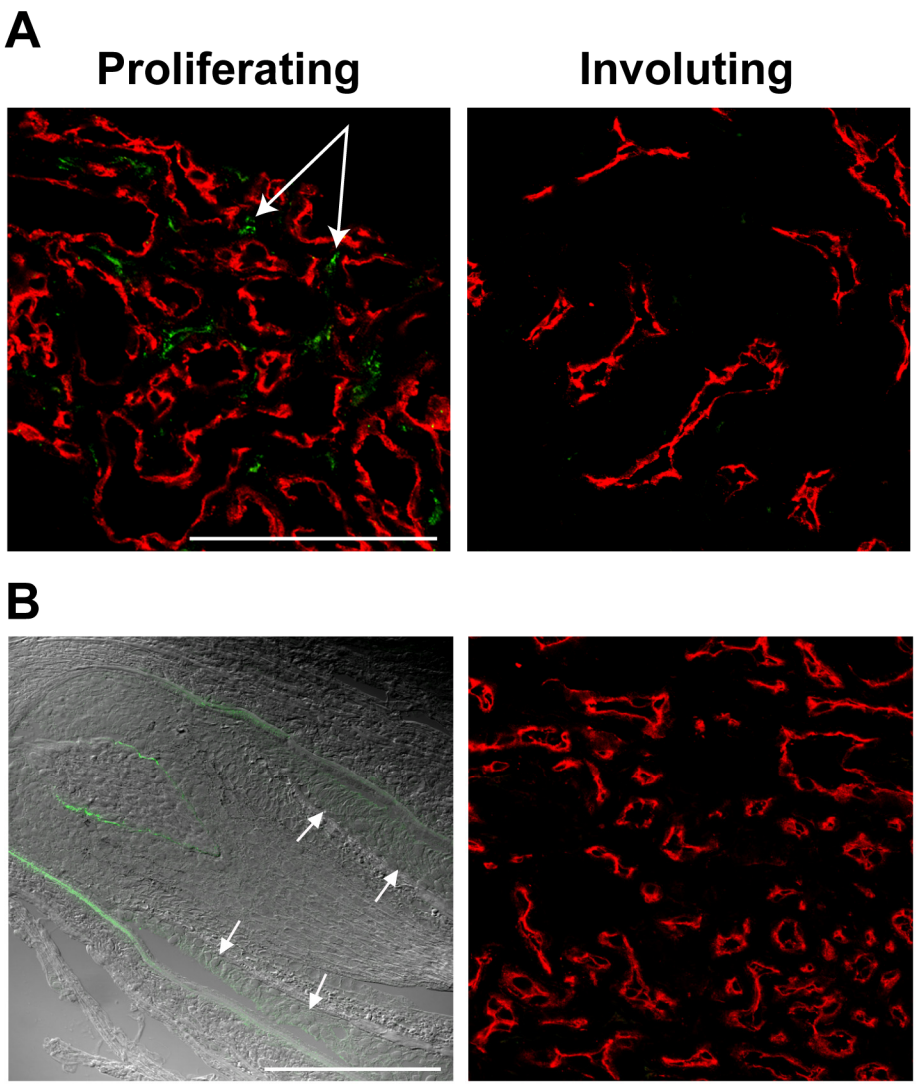
Figure S3 – Effects of corticosteroid treatment on HemSCs. In Panel A, quantitative RT-PCR was used to measure VEGF-A mRNA levels in HemSCs treated with dexamethasone for three days. Dose-response suppression was noted with maximum inhibition achieved at 200 nM. Panel B shows that mifepristone, a glucocorticoid receptor antagonist, reversed the dexamethasone-induced suppression of VEGF-A. Panel C shows determination of VEGF-A protein levels secreted from the HemSCs 1, 3, 5 and 8 days after removal of dexamethasone. Panel D shows VEGF-A protein levels in the conditioned media from HemSCs treated with different corticosteroids. HemSC were treated for three days with prednisone, prednisolone, methylprednisolone or hydrocortisone at equivalent concentrations to dexamethasone 0.2 and 2μM according to their relative potencies. VEGF-A was measured by an ELISA. Panel E shows VEGF-A mRNA levels in cells treated as in D. Relative mRNA levels were determined by quantitative RT-PCR. In Panel F, the endothelial chemotactic activity present in conditioned media from HemSCs treated with or without dexamethasone was measured in a migration assay using cbEPCs. Panel G shows cell number in response to increasing doses of dexamethasone. HemSCs and HemEPC were isolated from the same IH; NHDF are normal human dermal fibroblasts. The IC50 in HemSCs was achieved at ~400μM, 2000-fold higher than the concentration that caused anti-vasculogenic effects. For Panels A-G, data are presented as the mean value ± standard deviation. Panel H shows morphology of HemSCs incubated with dexamethasone for three days. Green fluorescence corresponds to phalloidin staining (cytoskeletal actin microfilaments) while blue fluorescence corresponds to Hoechst staining (cellular nuclei). In concentrations higher than 400 μM, disarrangement of actin filaments and cell death was noted.

Fibroblast and endothelial cells were resistant to dexamethasone compared to HemSCs. Scale bar: 50 μm. In Panel I, TUNEL assay was done in order to verify that high dose, dexamethasone-induced changes in HemSCs cell morphology were caused by apoptosis. At 1600μM, cells were positive (red) for apoptosis. The positive control shows cells incubated with DNase to induce DNA strand breaks. For the negative control, the TdT enzyme was omitted from the reaction mix Scale bar: 50 μm. Experiments in Panels A-I were performed on HemSCs isolated from at least three different IH specimens.



Supplemental Figure 4

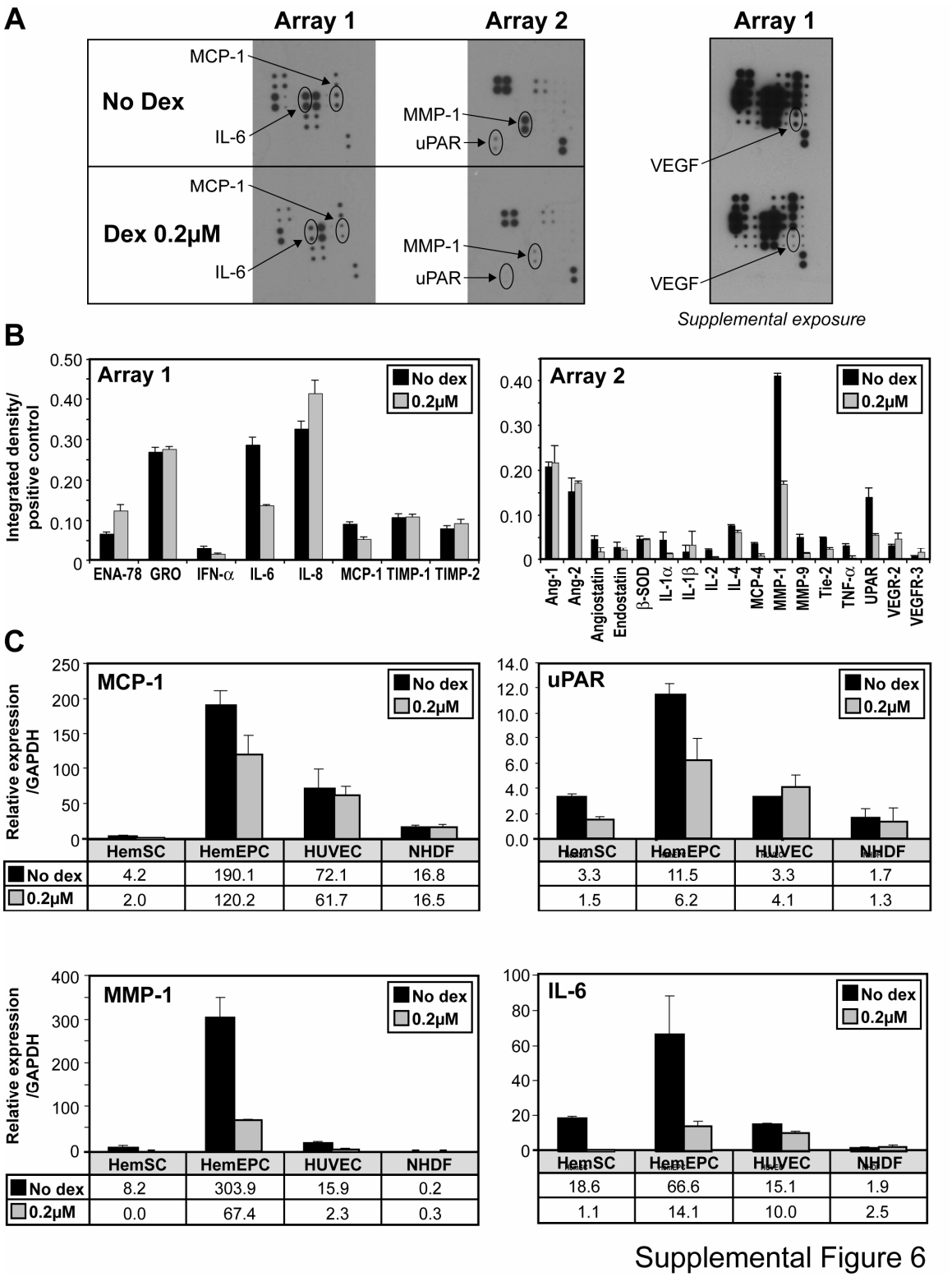
Figure S4 – HemSC were stably infected with a VEGF-A shRNA or with control, non-targeting shRNA. In Panel A, quantitative RT-PCR shows 80% suppression of VEGF-A mRNA transcript levels compared to the non-targeting shRNA infected cells. Panel B shows corresponding effect on VEGF-A protein, measured by ELISA. In panel C, the proliferation curve shows that VEGF-A silencing leads to slower proliferation of HemSCs compared to the control cells. The experiment was repeated twice with similar results.



Supplemental Figure 5

Figure S5 – VEGF-A in proliferating but not involuting IH. Panel A, representative images of proliferating and involuting infantile hemangioma tissue sections immunostained simultaneously with anti-human VEGF-A (green) and anti-human CD31 (red) are shown. Arrows point to VEGF-A (green) located in regions outside of the CD31-positive (red) blood vessels in the proliferating hemangioma. VEGF-A (green) was not detected in involuting hemangioma (right panel). Scale bar, 100 μM. Panel B shows positive and negative controls for the immunofluorescent staining of VEGF. Left

panel – hair follicle stained with anti-VEGF-A shows staining of follicular keratinocytes [7]. Scale bar: 100 μm. Right panel - isotype-matched negative control for anti-VEGF-A staining (green) and anti-CD31 (red) in a proliferating IH tissue section.



Supplemental Figure 6

Figure S6 – uPAR, MCP-1, IL-6 and MMP-1 are down-regulated in HemSCs treated with dexamethasone - Angiogenesis antibody array and verification of the positive hits.

To investigate other pro-angiogenic factors in HemSC that might be regulated by corticosteroid, we used a human angiogenesis antibody array. This enabled detection of 43 different pro-angiogenic proteins in dexamethasone-treated (0.2 μM) and non-treated HemSCs. Panel A shows blots for Array 1 and 2 and a second, longer exposure of Array 1 to show the VEGF-A signal. In addition to VEGF-A, we found a marked suppression of urokinase plasminogen activator receptor (uPAR), monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6) and matrix metalloproteinase-1 (MMP-1). In Panel B, image analysis software was used to quantify levels of the positive hits on the angiogenesis antibody array: dexamethasone-treated HemSCs (grey bars) and non-treated HemSCs (black bars). In Panel C, the expression of these factors in HemSCs as well as the suppressive effect of dexamethasone on these genes in hemangioma-derived endothelial progenitor cells (HemEPC), human umbilical vein endothelial cells (HUVEC) and NHDF was verified by quantitative RT-PCR. Interestingly, HemEPCs exhibited relatively elevated expression of these four factors and the expression levels of each were suppressed by dexamethasone. In contrast, levels of these four mRNA transcripts in normal human endothelial cells and fibroblasts were not affected by dexamethasone.

	Patient number	Gender	Age	Location	Steroid treatment (systemic or local)
Proliferating	68	F	3.5 months	Scalp	No
	88	F	7 months	Back	No
	129	F	3 months	Upper chest	No
	137	F	6 months	Behind ear	No
	138	F	3 months	Chest	No
	140	F	11.5 months	Forehead	No
	142	F	5.5 months	Jaw	No
Involuting	I-30	F	23 months	Lip	No
	I-41	M	2.5 years	Scalp	No
	I-53	F	5 years	Right chick	No
	I-59	F	3.5 years	Lower lip	Yes
	I-68	F	3 years	Forehead	No

Table S1 – Clinical data for proliferating and involuting IH tissue samples used to analyze VEGF expression.

Supplementary references

1. Khan ZA, Boscolo E, Picard A, et al. Multipotential stem cells recapitulate human infantile hemangioma in immunodeficient mice. J Clin Invest 2008;118(7):2592-9.

2. Khan ZA, Melero-Martin JM, Wu X, et al. Endothelial progenitor cells from infantile hemangioma and umbilical cord blood display unique cellular responses to endostatin. Blood 2006;108(3):915-21.

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4. Wu X, Rabkin-Aikawa E, Guleserian KJ, et al. Tissue-engineered microvessels on three-dimensional biodegradable scaffolds using human endothelial progenitor cells. Am J Physiol Heart Circ Physiol 2004;287(2):H480-7.

5. Hasan Q, Ruger BM, Tan ST, Gush J, Davis PF. Clusterin/apoJ expression during the development of hemangioma. Hum Pathol 2000;31(6):691-7.

6. Marshall OJ. PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. Bioinformatics 2004;20(15):2471-2.

7. Yano K, Brown LF, Detmar M. Control of hair growth and follicle size by VEGF-mediated angiogenesis. J Clin Invest 2001;107(4):409-17.