JAGGED1 Signaling Regulates Hemangioma Stem Cell–to–Pericyte/Vascular Smooth Muscle Cell Differentiation

Elisa Boscolo, Camille L. Stewart, Shoshana Greenberger, June K. Wu, Jennifer T. Durham, Ira M. Herman, John B. Mulliken, Jan Kitajewski, Joyce Bischoff

Objective—The aim of our study is to determine the cellular and molecular origin for the pericytes in infantile hemangioma (IH) and their functional role in the formation of pathological blood vessels.

Methods and Results—Here we show that IH-derived stem cells (HemSCs) form pericyte-like cells. With in vitro studies, we demonstrate that HemSC-to-pericyte differentiation depends on direct contact with endothelial cells. JAGGED1 expressed ectopically in fibroblasts was sufficient to induce HemSCs to acquire a pericyte-like phenotype, indicating a critical role for JAGGED1. In vivo, we blocked pericyte differentiation with recombinant JAGGED1, and we observed reduced formation of blood vessels, with an evident lack of pericytes. Silencing JAGGED1 in the endothelial cells reduced blood vessel formation and resulted in a paucity of pericytes.

Conclusion—Our data show that endothelial JAGGED1 controls HemSC-to-pericyte differentiation in a murine model of IH and suggests that pericytes have a fundamental role in formation of blood vessels in IH. (Arterioscler Thromb Vasc Biol. 2011;31:2181-2192.)

Key Words: angiogenesis ■ Jagged1 ■ infantile hemangioma ■ pericytes ■ vasculogenesis
We report here the direct involvement of JAGGED1 in the HemSC-to-pericyte differentiation, suggesting an important role for pericytes in the formation of IH blood vessels. These results provide novel insight into mechanisms of human postnatal vasculogenesis.

Methods

Cell Isolation and Culture
Specimens of IH were obtained under a protocol approved by the Committee on Clinical Investigation, Children’s Hospital Boston. The clinical diagnosis was confirmed in the Department of Pathology, Children’s Hospital Boston. Informed consent was obtained, according to the Declaration of Helsinki. Single-cell suspensions were prepared from proliferating phase IH specimens, and HemSCs were selected and expanded as described. Briefly, HemSCs were selected using anti-CD133–coated magnetic beads (Miltenyi Biotech). CD133-selected cells were cultured on fibronectin-coated (1 μg/cm²) plates with EBM-2 (CC-3156, Cambrex). EBM-2 was supplemented with 20% fetal bovine serum, endothelial growth medium-2 SingleQuot (CC-4176; Cambrex). Hereafter, this supplemented medium is called EBM-2/20% fetal bovine serum. Hemangioma-derived endothelial cells (HemECs) and human umbilical cord blood endothelial progenitor cells (cbEPCs) were isolated as described. cbEPCs were shown to form the endothelial lining of blood vessels when injected in combination with smooth muscle cells or bone marrow–derived mesenchymal progenitor cells, in Matrigel, subcutaneously in mice. Human dermal fibroblasts (HDFs) were purchased from ATCC.

JAGGED1 expressing HDFs were produced by transfecting (Fugene HD, Roche) the cells with JAGGED1 vector (Origene); selection was done with G418 (Sigma-Aldrich). Short hairpin RNA (shRNA) lentiviral particles for JAGGED1 targeting (NM_00214) and nontargeting shRNA (control) were used to infect cbEPCs (Sigma-Mission). cbEPCs with stable expression of JAGGED1 shRNA were subjected to puromycin selection, according to the manufacturer’s instructions. The pool used for in vivo experiments was selected after testing 5 different shRNA sequences for silencing efficacy.

Coculture Differentiation Protocol
The in vitro pericytic differentiation was performed by seeding HemSCs together with cbEPCs at a ratio of 1:1 and a total density of 10⁴ cells/cm² on fibronectin-coated plates in EBM-2/20% fetal bovine serum. To inhibit the HemSC pericytic differentiation, we used recombinant human Fc-JAGGED1 and Fc-DLL4, and mouse anti-JAGGED1 (R&D Systems). Inhibitors were added 4 hours after seeding. cbEPCs were selected and expanded as described. To augment the HemSC-to-pericyte differentiation, we injected green fluorescent protein (GFP)–labeled HemSCs (GFP-HemSCs). The presence of α-SMA+/GFP+ pericytes confirmed their HemSC origin (Figure 1d). We previously reported that HemSCs can differentiate into endothelial cells. To verify that pericytes and endothelial cells are derived from the same progenitor cell, we prepared 3 single-cell–derived clonal populations of GFP-HemSCs derived from 2 different IH samples (Supplemental Figure Ib) and analyzed cellular fate(s) in vivo. All 3 GFP-HemSC clonal populations formed pericytes (Figure 1e and Supplemental Figure 1c) and endothelial cells (Supplemental Figure Id).

HemSCs Differentiate Into Pericytes When Combined With cbEPCs
In previous studies, we described a model to bioengineer human blood vessels using bone marrow–derived mesenchymal progenitor cells and cbEPCs. In this model, bone marrow–derived mesenchymal progenitor cells differentiate into α-SMA+ pericytes, whereas cbEPCs form the endothelium. To augment the HemSC-to-pericyte differentiation, we implanted HemSCs together with cbEPCs into mice. This resulted in a blood vessel density 4- to 5-fold higher than HemSCs implanted alone. GFP-HemSC+cbEPC produced vessels surrounded by GFP+/α-SMA+ cells (Figure 2a), providing direct evidence that GFP-HemSCs became pericytes. Furthermore, 3 of 3 GFP-HemSC clonal populations differentiated into α-SMA+ pericytes when injected with cbEPCs (Supplemental Figure Iia). Quantitative analysis showed that 82% (82±4) of blood vessels had α-SMA+ pericytes and 33% (33±2.1) had HemSC-derived pericytes (GFP+/α-SMA+). Of the total GFP+ blood vessels, 73% (33±4) coexpressed α-SMA (Figure 2a, bottom). We hypothesize that the remaining 27% of the GFP+ vessels are composed of GFP-HemSCs that underwent endothelial differentiation or remained undifferentiated. To confirm that cbEPCs contributed exclusively to the endothelial population, GFP-cbEPCs were coimplanted with unlabeled-HemSCs. No...
colocalization of eGFP with α-SMA was seen around blood vessels, confirming that cbEPCs do not differentiate into pericytes (Figure 2b).

To examine the contribution of murine cells to the pericytic population, we implanted unlabeled-HemSCs/cbEPCs into GFP-SCID mice27 (Figure 2c), as well HemSCs alone (Figure 2d). In both models, we observed a mosaic of vessels with murine- and HemSC-derived α-SMA+ pericytes. When HemSC+cbEPCs were injected into GFP-SCID mice, α-SMA+ pericytes were derived mostly from the HemSCs (Figure 2c, bottom). When HemSCs alone were injected into GFP-SCID mice, no statistical difference was seen between host (murine) and HemSC (human) contribution to the α-SMA+ pericytes (Figure 2d, bottom). This shows that, when in contact with cbEPCs, HemSCs prefer to take on the pericytic phenotype.
Pericytes in the murine model of IH also stained for calponin, most of erythrocyte-filled blood vessels were surrounded by calponin-stained cells, and calponin staining signal overlapped with -SMA (Supplemental Figure IIb).

HemSCs Cocultured With cbEPCs Differentiate Into Pericytes

Data in Figure 2 showed that HemSCs combined with cbEPCs resulted in HemSC-to-(α-SMA+) pericyte differentiation. This prompted us to analyze the role of cbEPC when cocultured with HemSCs. After 10 days, anti-α-SMA and -SMHC identified pericyte-like cells (Figure 3a, left). HemSCs and cbEPCs cultured alone did not express pericyte/smooth muscle cell markers (Supplemental Figure IIIa). To confirm that HemSCs differentiate into pericyte-like cells, we cocultured cbEPCs with GFP-HemSCs and showed that all of the α-SMA+ and -MHC+ cells were derived from the GFP-HemSCs (Figure 3a, right); and GFP-HemSCs cultured alone did not express α-SMA or smMHC (Supplemental Figure IIIb). Calponin-1 and sm22α are markers of pericyte-like cells.28,29 HemSC+cbEPC cocultures showed significant induction of calponin-1, sm22α, α-SMA protein (Figure 3b), and calponin-1 and smMHC mRNA (P<0.001) (Figure 3c) compared with HemSCs alone. The expression of calponin-1, smMHC, and α-SMA gradually increased from day 3 to day 10 (Figure 3c). In addition, hemangioma-derived endothelial cells (HemECs) also caused HemSC-to-pericyte differentiation (Supplemental Figure IIIc).

To further confirm that the HemSCs acquired a pericytic phenotype, we cocultured HemSC+cbEPC for 5 days and, after trypsinization, selected the cells based on the expression of CD31 (CD31+ indicates endothelial cells), or lack of CD31 (CD31− indicates HemSCs and HemSC-derived pericytes). Real-time analysis showed that CD31− cells from coculture significantly upregulated PDGFRβ (P=0.004) and

Figure 2. Green fluorescent protein (GFP)-IH-derived stem cells (HemSCs) form α-smooth muscle actin (α-SMA)+ pericytes when coinjected with cord blood endothelial progenitor cells (cbEPCs). Shown are Matrigel explants stained for α-SMA (red) and GFP (green) (asterisks mark blood vessel lumens); nuclei counterstained in blue (4',6-diamidino-2-phenylindole). a, GFP-HemSCs coinjected with unlabeled cbEPCs. Perivascular GFP-HemSCs (green) expressing α-SMA (red) showed yellow fluorescence (arrows). b, HemSCs coinjected with GFP-cbEPCs. GFP staining showed no overlap with α-SMA (arrows). c, Unlabeled HemSC+cbEPCs coinjected into GFP-SCID mouse. d, Unlabeled HemSCs alone injected into GFP-SCID mouse. In a and b, bottom: Quantification of α-SMA+ (red columns), GFP+ (green columns), and GFP+/α-SMA+ (yellow columns) vessels in percentage relative to total number of vessels. c and d, bottom: Quantification of GFP+ (green columns) and α-SMA+ (red columns) vessels, quantification of GFP+/α-SMA+ (yellow columns) (pericytes of murine origin), and no-overlap vessels (gray columns) (perivascular cells of human origin) in GFP-SCID mice. *P=0.031; n.s. [not significant], P=0.677. n=10 fields/section, n=5 animals/group. Scale bar=30 μm. Data expressed as mean±SEM.
neural glial antigen-2 (P=0.004), and CD31 expression was not detected (Figure 3d).

We next wanted to test the HemSC-derived pericytes for contractility to assess the specific acquisition of pericyte/smooth muscle cell function. GFP-HemSCs were cultured with cbEPCs to obtain differentiation, and after 10 days the cbEPCs were removed with CD31 magnetic beads. The CD31− cells were assessed for contractility on a silicon-based substrate. CD31− cells obtained from the coculture showed almost a 3-fold increase in the capacity to form wrinkles in the silicon, compared with CD31− cells from GFP-HemSCs cultured alone (Figure 3e). This indicates that the HemSC-derived pericytes acquired expression of pericyte/smooth muscle markers that functionally correlate with contractile force.

Next, to assess that each single HemSC has the ability to differentiate into a pericyte, we used single-cell–derived HemSC clones. Six of 6 HemSC clones tested, cocultured with cbEPCs, showed induction of α-SMA and smMHC (Supplemental Figure IIIc) and calponin-1 (Supplemental Figure IIIe). These results exclude the possibility that mixed populations of unpotentential cells were responsible for the pericyte-like phenotype.

Direct and Continuous HemSC-cbEPC Contact Is Required for Pericycle Differentiation

The endothelial component in the coculture was crucial in the HemSC-to-pericycle differentiation. We next tested whether HemSC-derived pericytes could maintain their phenotype if endothelial cells were removed. We used GFP-HemSCs because of their puromycin resistance induced by lentiviral insertion of a vector containing GFP and the puromycin-resistance genes. GFP-HemSCs + cbEPCs were cocultured for 10 days to obtain α-SMA/smMHC expressing cells; after replating, the cells were treated with puromycin (Figure 4a). As expected, cbEPCs died after 2 days, confirmed by the absence of von Willebrand factor staining. The surviving cells (puromycin-resistant GFP-HemSCs) were cultured for up to 20 days. smMHC expression became undetectable after 15 days. α-SMA expression was retained until day 20, but cellular morphology, beginning at day 15, reverted to a spindle shape (Figure 4b). Results were confirmed by gradual decrease of calponin-1 (Figure 4c) and calponin-1/tubulin (Figure 4d) from day 5 to day 20. α-SMA expression in control GFP-HemSC+cbEPC (normal and puromycin-resistant) cocultures with and without addition of puromycin is shown in Supplemental Figure IVa and IVb. GFP-HemSCs cultured alone did not show α-SMA expression, with or without puromycin treatment (Supplemental Figure IVc).

This experiment indicates that continuous physical contact with endothelial cells is needed for the GFP-HemSCs to maintain the pericyte phenotype.

To test whether HemSC differentiation could occur without direct cellular contact with cbEPCs, we cultured HemSCs alone on the bottom well and cbEPCs or HemSCs+cbEPCs on the top well of a 0.4-μm-pore Transwell. HemSCs did not express smMHC under these conditions (Supplemental Figure IVd). We tested specific factors reported to induce smooth muscle differentiation (transforming growth factor-β1, platelet-derived growth factor-BB, type IV collagen-coated wells). After 10 days, smMHC was not detected, and only rare α-SMA+ cells were seen (Supplemental Figure IVe). Without direct endothelial cell contact, the HemSCs were unable to differentiate toward the pericyte/smooth muscle phenotype under the culture conditions tested.

JAGGED1 Is Required for HemSC-to-Pericyte Differentiation and Blood Vessel Formation

We and others recently reported high JAGGED1 expression in IH endothelium. We further confirmed these findings here, as shown by the high JAGGED1 mRNA and protein expression in HemECs (Supplemental Figure Va) and JAGGED1 in endothelium of a proliferating IH specimen (Supplemental Figure Vb). We used different approaches to explore the role of the endothelial JAGGED1 in the HemSC-to-pericycle differentiation. The first was to inhibit membrane-bound JAGGED1 binding to its ligand/s by adding recombinant human (rh) Fc-JAGGED1 (Fc-JAGGED1) to the HemSC+cbEPC cocultures. Fc-JAGGED1 (0.1 μg/mL) limited HemSC-to-pericycle differentiation as indicated by reduced calponin-1 and smMHC expression, compared with human recombinant Fc-domain (Figure 5a).

On NOTCH receptor signaling, the intracellular domain of NOTCH is cleaved by γ-secretases and is translocated to the nucleus where it associates with the RBP-Jk proteins to induce transcription of the basic helix-loop-helix genes HES and HEY, which are the most prominent NOTCH effector molecules. We assessed NOTCH ligand-induced signaling by measuring HES and HEY. Treatment with Fc-JAGGED1 significantly decreased (P<0.05) HES1 and HEYL mRNA expression (Figure 5a). Fc-JAGGED1 also prevented calponin-1 and sm22α protein upregulation in HemSCs purified from the cocultures (Figure 5b). Additionally, the number of α-SMA+ cells in cocultures treated with Fc-JAGGED1 showed an 80% reduction compared with the

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**Figure 3 (Continued).** IH-derived stem cells (HemSCs) differentiate into pericytes when cocultured with cord blood endothelial progenitor cells (cbEPCs). a, HemSC+cbEPC cocultures at day 10 stained for von Willebrand factor (vWF) (red) and α-smooth muscle actin (α-SMA) or smooth muscle myosin heavy chain (smMHC) (green) (left). Green fluorescent protein (GFP)-HemSC+cbEPC cocultures at day 10 stained for GFP (green) and α-SMA or smMHC (red) (right). Scale bar=100 μm. Nuclei counterstained in blue (4',6-diamidino-2-phenylindole), b, Immunoblots for calponin-1, α-SMA, and sm22α in HemSCs alone or in HemSCs+cbEPCs (tubulin was used as the loading control). c, Quantitative real-time polymerase chain reaction (PCR) analysis of calponin-1 (left) and smMHC (right) in GFP-HemSCs+cbEPC (gray columns) compared with GFP-HemSCs alone (black columns). *P<0.001. Results are representative of 3 independent experiments repeated with HemSCs from 3 different infantile hemangiomia (IH) samples. d, HemSC+cbEPC cocultured for 5 days and then subjected to immunoseparation based on CD31 expression (schematic). The CD31− fraction was analyzed by real-time PCR for relative expression of neural glial antigen-2 (NG2), platelet-derived growth factor receptor (PDGFR)-β, and CD31, and results were compared with HemSCs cultured alone. e, GFP-HemSCs alone (top panels) and CD31− cells immunoseparated from GFP-HemSC+cbEPC coculture (bottom panels) cultured in silicon based substrata. Shown are phase, fluorescence, and merge. Right panel, quantification of wrinkle-forming cells. Data expressed as mean±SD.
Figure 4. IH-derived stem cell (HemSC)–derived pericytes dedifferentiate after cord blood endothelial progenitor cell (cbEPC) removal. a, Schematic of experimental procedure. b, Puromycin-resistant green fluorescent protein (GFP)-HemSCs cocultured with cbEPCs and treated with puromycin (2 µg/mL) were stained for von Willebrand factor (vWF) (red) and smooth muscle myosin heavy chain (smMHC) (top) or α-smooth muscle actin (α-SMA) (bottom) (green). c, Immunoblots for calponin-1 and tubulin. d, Densitometric quantification of calponin-1/tubulin band intensity. A representative is shown of n=3 independent experiments. Scale bar=100 µm.
Figure 5. JAGGED1 activity is required for IH-derived stem cell (HemSC)-to-pericyte differentiation. a, Real-time polymerase chain reaction (PCR) for calponin-1, smooth muscle myosin heavy chain (smMHC), HES1, and HEYL in Fc-JAGGED1 (0.1 mg/mL)-treated cultures (gray columns) compared with matching concentration of Fc-domain treated cultures (black columns). b, HemSC/cord blood endothelial progenitor cell (cbEPC) cocultures treated with 2 μg/mL Fc-JAGGED1 for 5 days. HemSCs were separated from cbEPCs using anti-human CD31-magnetic beads and analyzed by immunoblot for calponin-1 and sm22α after treatment with Fc-JAGGED1 or control Fc-domain. c, Immunofluorescence for von Willebrand factor (vWF) (red) and α-smooth muscle actin (α-SMA) (green) in cocultures treated with Fc-domain or Fc-JAGGED1 with quantification of α-SMA+ cells shown in the bar graph. Data are expressed as mean, normalized to control, of 3 independent experiments with n=3; *P<0.006. d, Hematoxylin/eosin-stained sections of Matrigel explants containing HemSC/cbEPCs treated with 1 mg/mL Fc-domain (top) and Fc-JAGGED1 (bottom) and quantification of MVD and human CD31+ vessels (right). Scale bar=100 μm. e, Immunoblot for JAGGED1 in human dermal fibroblasts (HDFs) after transfection with control (empty) and JAGGED1 vector. f, Real-time PCR showing calponin-1, smMHC, NOTCH3, HES1, HEYL, neural glial antigen-2 (NG2), platelet-derived growth factor receptor (PDGFR)-β, α-SMA, and sm22α in HemSCs cocultured with JAGGED1-expressing HDFs (gray columns) compared with HDFs expressing empty vector (black columns). n=3 independent experiments. Data are expressed as mean±SD (a, c, and e) or as mean±SEM (d).
Fc-domain control (Figure 5c). In vivo, Fc-JAGGED1 affected vessel integrity, suggesting a disruption in the perivascular coverage (Figure 5d). Fc-JAGGED1 significantly ($P<0.05$) reduced the total microvessel density (MVD) and the human CD31 $+$ MVD (Figure 5d).

The NOTCH ligand DLL4 was reported to have a role in the pericyte/smooth muscle differentiation of bone marrow cells and in the vessel maturation. Therefore, we investigated the effect of DLL4 blockade in the HemSC-to-pericyte differentiation. HemSC $+$ cbEPC and HemSC $+$ HemEC cocultures were treated with rhJAGGED1, rhDLL4, or a combination of both. Results showed that DLL4 blockade did not inhibit calponin, $\alpha$-SMA, or sm22a expression, and we did not detect any additional reduction in calponin, $\alpha$-SMA, or sm22a when rhDLL4 was added with rhJAGGED1 (Supplemental Figure Vc). These results are in line with our previous report on modest levels of DLL4 in HemEC compared with human dermal microvascular EC, and they suggest that, as DLL4 expression is low in HemEC, DLL4 blockade may not affect HemEC-induced HemSC-to-pericyte differentiation.

To confirm that blockade of JAGGED1 signaling inhibits HemSC-to-pericyte differentiation, we treated cbEPC $+$ HemSC cocultures with anti-JAGGED1 antibody. Results showed significant decreases ($P<0.05$) in calponin-$1$, smMHC, and HES1 and HEYL mRNA compared with cocultures treated with control mouse IgG (Supplemental Figure Vd). Fc-JAGGED1 blocked rather than induced pericytic differentiation, as reported in the literature. Therefore, we tested whether cell-bound JAGGED1 was sufficient to stimulate differentiation. We transfected HDFs with an expression plasmid for JAGGED1 (Figure 5e), and cocultured them with HemSCs. After 5 days, HemSC $+$ JAGGED1-HDF cocultures exhibited increased ($P<0.05$) mRNA expression of calponin-$1$, smMHC, NOTCH3, HES1, HEYL, neural glial antigen-$2$, PDGFR-$\beta$, $\alpha$-SMA, and sm22a compared with HemSC $+$ empty vector-HDF cocultures (Figure 5f). Therefore, JAGGED1 on a nonendothelial membrane was sufficient to stimulate HemSCs to differentiate toward a pericyte-like phenotype in vitro.

To test whether JAGGED1 is necessary in the HemSC-to-pericyte differentiation, we downregulated JAGGED1 in cbEPCs using shRNA-mediated silencing. Two cbEPCs pools with downregulated JAGGED1 (shJAGGED1-pool1 and shJAGGED1-pool2) (Figure 6a), were combined with HemSCs and coinjected into mice. The shJAGGED1 cbEPCs showed decreased MVD and human-derived CD31 $+$ MVD compared with the control nontargeting shRNA (Figure 6b and Supplemental Figure VIa) that correlated with the level of decreased JAGGED1. To assess pericytic coverage of the vessels, sections were immunostained for human CD31 in combination with anti-$\alpha$-SMA antibody. Specificity of anti-human CD31 is shown in Supplemental Figure VIIa. Quantification of the human CD31 $+$ vessels with $\alpha$-SMA $+$ pericytes versus murine vessels (human CD31 $+$) with $\alpha$-SMA $+$ pericytes revealed that JAGGED1 silencing affected only the pericyte coverage of the human-CD31 $+$ vessels (Figure 6c). These results confirmed that JAGGED1 in cbEPCs is responsible for the HemSC-derived vasculogenesis. In addition, we tested Matrigel explants for presence of GFP-HemSC-derived $\alpha$-SMA $+$ pericytes. Explants of GFP-HemSC $+$ shJAGGED1 cbEPCs exhibited a significant reduction ($P\leq0.004$) of GFP/$\alpha$-SMA $+$ cells (compared with control, nontransfected cells, and nontargeting shRNA-treated cells). (Representative pictures are shown in Figure 6d and quantification in Figure 6e).

**Discussion**

In this study, we showed that HemSCs can differentiate into pericytes in vitro and in vivo, suggesting that pericytes in IH originate from HemSCs. Furthermore, we present the first evidence of a functional role for JAGGED1 in the pericytic differentiation of HemSCs, suggesting that JAGGED1 may be involved in the pathogenesis of IH. Because we showed previously that HemSCs can differentiate into endothelial cells, the HemSC-to-pericyte differentiative capability provides, for the first time to our knowledge, evidence for a human postnatal vascular progenitor cell.

For many years, IH has been thought to originate from aberrant endothelium and excessive angiogenesis. However, attempts to create an IH model by injecting HemECs into immune-deficient mice have failed. Our HemSC-IH model represents an important advance to previously published models because the patient-derived HemSCs form hemangioma-like GLUT1 $+$ vessels within 7 days and adipoctyes by 8 weeks, thereby recapitulating important events in the IH life-cycle. HemSCs have also been shown to be the cellular target of corticosteroids, supporting the critical role of HemSCs during the growth of IH. We propose that IH arises by HemSC-driven vasculogenesis, defined as the de novo formation of blood vessels from stem/progenitor cells.

IH vessels are surrounded by closely associated perivascular cells that express markers of pericytes (neural glial antigen-$2$, PDGFR-$\beta$, and $\alpha$-SMA) and smooth muscle cells ($\alpha$-SMA, calponin and smMHC) (Figure 1a and 1b). In contrast, tumor vessels have perivascular cells that are often...
loosely associated or appear detached.\textsuperscript{32–45} Tumor blood vessels expand through an angiogenic program in which VEGF-A negatively regulates perivascular coverage preventing tumor endothelial cell maturation.\textsuperscript{46} In contrast, we show here that pericytes in IH are critical because inhibition of HemSC-to-pericyte differentiation severely compromised blood vessel assembly in our IH murine model (Figures 5d and 6).

Embryonic vascular progenitor cells have been shown to differentiate into endothelial and pericytic phenotypes in response to VEGF-A and transforming growth factor-\(\beta\) or platelet-derived growth factor-BB.\textsuperscript{30–32} In contrast, cellular contact with an endothelial cell, cbEPC or HemEC, was needed for HemSC-to-pericyte differentiation (Figures 3 and 4). In addition, JAGGED1-expressing fibroblasts were sufficient to induce the HemSCs to acquire a pericyte-like phenotype (Figure 5e and 5f). Furthermore, soluble recombiant Fc-JAGGED1 thwarted differentiation of the HemSCs (Figure 5a to 5c) and inhibited blood vessel formation in the IH murine model (Figure 5d). This was perhaps due to disruption of juxtacrine interactions between endothelial JAGGED1 and a NOTCH receptor on HemSCs.\textsuperscript{16}

Silencing of JAGGED1 in the cbEPCs resulted in a dose-dependent decrease in the vascular density and pericytes in the IH murine model (Figure 6). Endothelial JAGGED1 is crucial for vascular smooth muscle development.\textsuperscript{23,47} Furthermore, angiogenic sprouting in the retina is inhibited in the endothelial \textit{Jag1} loss of function model.\textsuperscript{18} In this model, the authors reported a lack of \(\alpha\)-SMA+ smooth muscle cells on developing arteries, but the pericyte coating seemed unaffected. Our results may differ because IH blood vessels form through a vasculogenic process, with angiogenic sprouting as secondary event.

We also tested inhibitors of receptor-ligand pathways that have been implicated in vascular development, including \(\gamma\)-secretase inhibitors to prevent NOTCH juxtacrine signaling. Recombinant Tie2, an ERK inhibitor (U0126), and a PDGFR\(\beta\) inhibitor (AG1295) did not prevent HemSC-to-pericyte differentiation in the coculture assay (Supplemental Figure VII), but 2 different \(\gamma\)-secretase blockers (\(N\)-[\(3,5\)-difluorophenacetyl]-L-alanyl]-S-phenylglycine \(t\)-butyl ester and Compound E) were inhibitory (Supplemental Figure VIII). DAPT was tested in the IH murine model and resulted in reduced MVD and reduced \(\alpha\)-SMA+ cells in proximity to blood vessels. Importantly, DAPT had little effect on proliferation or viability of HemSCs and cbEPCs in vitro. This supplemental data supports the idea that JAGGED1 is exerting an effect on NOTCH signaling.

In summary, our study demonstrates that HemSCs differentiate into pericytes as well as endothelial cells and are thus akin to a vascular progenitor cell. We speculate that the first event of IH vasculogenesis is the differentiation of the HemSCs into HemECs. HemECs in turn induce neighboring HemSCs to become pericytes, thereby initiating a feedforward mechanism for vascular assembly and further growth. Recruitment of circulating endothelial cells, through VEGF-A and matrix metalloproteinase-9, into the hemangioma could further contribute to the vasculogenesis\textsuperscript{48,49} and would diminish the need for HemSC-to-endothelial differentiation, enabling them to differentiate into pericytes.

VEGF-A is highly expressed in HemSCs and is required for the HemSC-derived blood vessel formation in the IH murine model.\textsuperscript{3} VEGF-A plays a positive role in angiogenesis in concert with NOTCH signaling\textsuperscript{50,51}; furthermore, authors have reported VEGF-A as a negative regulator of pericyte and vascular smooth muscle cells recruitment.\textsuperscript{46} In contrast, pericytes are prevalent in the proliferating phase of IH\textsuperscript{8} despite high levels of VEGF-A (Figure 1a and 1b), Furthermore, preliminary data showed that when HemSCs and cbEPCs were cocultured, VEGF-A was upregulated and that DAPT inhibited this upregulation. We thereby speculate that in our model, VEGF-A expression can also be regulated downstream of NOTCH signaling.

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**Disclosures**

None.

**References**


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SUPPLEMENTARY MATERIAL:

Expanded Methods:

Cell Isolation and Culture

GFP-HemSC clonal populations: One clone (clone B) was grown from seeding 1 GFP-HemSC per well (Supplementary Fig.1 b) (patient 125) and two were obtained by GFP-labeling expanded clonal populations (same clonal populations used in 1) (clone 9 and clone A, patient 106).

To obtain TurboGFP 2-expressing cells, 1.6x10^4 HemSCs were plated into wells of a 96-well plate. After 24 h, fresh medium containing 8 µg/mL hexadimethrine bromide (Sigma-Aldrich) was added and cells were infected with 10 µl lentiviral-based particles containing TurboGFP™ encoding vector (Sigma-Aldrich, Mission®). To obtain stable GFP expression, cells were maintained in 2 µg/mL puromycin (Sigma-Aldrich) medium. eGFP-cbEPCs were obtained as described 3.

For some experiments, HemSC were cocultured with cbEPC for 5 days and then were re-separated with the use of immunomagnetic beads conjugated with anti-CD31 (Invitrogen), to obtain two populations CD31+ (endothelial cells), and CD31- (HemSC and HemSC-derived pericytes).

qRT-PCR

RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA synthesis was performed with iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Primers used are listed in Supplementary Table I. All reactions were performed for 35 cycles with
the following temperature profiles: 95 °C for 2 min (initiation; 30 s/cycle thereafter), annealing step for 25 s, and extension step at 72 °C for 30 s.

**In Vivo Model of Infantile Hemangioma**

Experiments were carried out with 2 x 10⁶ cells per animal as described ¹. Briefly, cells were grown in EBM2/20%FBS until subconfluent, trypsined, washed and suspended in 200 µl of Matrigel™ (BD Bioscience) on ice. The cell/Matrigel solution was injected subcutaneously on the back of 6-7 weeks old male athymic nu/nu mice (Massachusetts General Hospital, Boston, MA) or GFP-SCID mice (kindly provided by Taturo Udagawa) (n = 5-10/group). For treatments, DAPT (10 or 50 µM) and Fc-JAGGED1 (1mg/ml) were added to the cell/Matrigel suspension before injection.

**Immunohistochemistry**

Paraffin sections of Matrigel explants or frozen sections of proliferating IH were used. The sections were stained with the following antibodies: human-specific CD31 monoclonal antibody (1:50, DakoCytomation and 1:50, Santa Cruz) and VE-cadherin (1:200, Santa Cruz) for detection of microvessels. Perivascular cells were stained with anti-αSMA (1:1000, Sigma), Calponin1 (1:100, Abcam), PDGFRβ (1:100, Santa Cruz), NG2 (1:200, R&D Systems) and smMHC (1:100, Sigma). T-GFP expression was detected with anti-turboGFP antibody (1:2000, Evrogen, AB511), eGFP-cbEPCs and mouse tissue eGFP expression was detected with anti-eGFP antibody (1:200, Abcam). Incubation with primary antibody was followed by FITC or Texas Red-labeled secondary antibody (1:200, Vector Laboratories). The sections were mounted with DAPI (Vector Laboratories).
**Immunocytochemistry**

Cells were fixed in methanol (GFP fluorescence is lost with methanol treatment) or paraformaldehyde (to preserve GFP activity). cbEPCs and HemSCs, alone or in coculture, were next double-stained with anti-vWF (1:300, Dako), TurboGFP (1:1000, Evrogen), and αSMA or smMHC (1:2000 and 1:100, Sigma). Cells were then incubated with FITC and Texas Red-labeled secondary antibody (1:200, Vector Laboratories) and nuclei counterstained with DAPI (Vector Laboratories).

**Microscope Image acquisition**

Images were taken with Axiophot II microscope (Zeiss, Germany) equipped with AxioCam MRc5 (Zeiss) and with the use of AxioVision Rel.4.6.3 Software.

Fluorescence images were taken with Leica TCS SP2 Acousto-Optical Beam Splitter confocal system equipped with DMIRE2 inverted microscope (Diode 405 nm, Argon 488 nm, HeNe 594 nm; Leica Microsystems, Germany), Leica Confocal Software Version 2.61, Build 1537. Images were taken at room temperature (about 20°C) and files always exported as 8 bit format.

**Western Blot**

Cells were lysed with RIPA buffer (Boston Bioproducts), containing a protease inhibitor cocktail Complete Mini tablet (Roche, Indianapolis, IN). Lysates were subjected to SDS-PAGE and transferred to Immobilon-P membrane. Membranes were incubated with anti-human αSMA (1:2000; Sigma-Aldrich), anti-human Calponin1 (1:500, Sigma-Aldrich), anti-
sm22α (1:500, Abcam), anti-Jagged-1, anti-NOTCH3 (1:500, Santa Cruz), anti-NG2 (Chemicon) and anti-human Tubulin (1:10000, Sigma-Aldrich). Membranes were then incubated with secondary antibodies (1:5000, peroxidase-conjugated anti-specie isotype; Vector Laboratories). Antigen-antibody complexes were visualized using Lumiglo and chemiluminescent sensitive film.

**Microvessel Density (MVD)**

For the assessment of MVD, ten fields from mid-Matrigel H&E sections of each of the animals in the group were quantified by counting luminal structures containing red blood cells. MVD is expressed as vessels/millimeter².

**Wrinkle Assay**

Deformable silicone substrata for analysis of cell contractility were prepared as described previously. Briefly, eight microliters of silicone (Sigma) was pipetted onto glass coverslips and then thermally crosslinked. A glow plasma discharge apparatus was used to generate a plasma discharge onto silicone-coated coverslips. The silicone surface was coated with 100 µl of 0.1 mg/ml Type I collagen (BD Biosciences) in PBS. Approximately 4x10³ cells were seeded onto silicone-coated coverslips and cultured for 12 h prior analysis. Quantification of contractile force production was performed by counting the number of cells that induced deformation of silicone substratum (wrinkles).
**Assays for In Vitro Cellular Proliferation (Supplementary Data)**

Proliferation was assessed after seeding the cells at $10^4$ cell/cm$^2$ on fibronectin-coated 48-well plates and culturing in EBM-2/20% FBS. Following attachment (24 h), plating efficiency was determined, and DAPT, Compound E and DMSO were added to the media. Cell number was determined using a Coulter Counter® (Beckman).

**Statistical Analysis**

The data were expressed as means ± s.d. or means ± s.e.m. and analyzed by ANOVA followed by Student's t-test where appropriate. Differences were considered significant at $p$ values < 0.05.
## SUPPLEMENTARY FIGURES AND FIGURE LEGENDS

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**Supplementary Table I**
Supplementary Figure I

Boscolo E. et al., ATVB
JAGGED1 signaling regulates hemangioma stem
cell-to-pericyte/vascular smooth muscle cell differentiation

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Supplementary Figure I
**Supplementary Figure I.**

**a-** Real Time PCR analysis results for the relative (to CD31) gene expression for IH samples (8 proliferating 2.5-13 months, and 6 involuting 1.5-5 years). Transcripts analyzed are NG2, PDGFRβ, Calponin1, αSMA and smMHC.

**b-** GFP-HemSC plated as single cell (left), and after clonal expansion. (right).

**c-** Clonal populations of GFP-labeled HemSCs, designated clone 9 and clone B, implanted in vivo in Matrigel, were stained for αSMA (red) and GFP (green). Phase contrast images show blood vessel location (arrows). Scale bar = 30 µM.

**d-** 3/3 GFP (green)-labeled HemSC clones, designated clone 9, clone A and clone B, formed CD31+ (red) endothelial cells lining perfused blood vessels when injected alone into nude mice. * mark vessel lumens.
Supplementary Figure II
Supplementary Figure II. a- 3/3 GFP (green)-labeled HemSC clones formed αSMA+ (red) pericytes, when injected together with cbEPCs into nude mice. Red blood cell-filled vessels are seen in the corresponding phase images (arrows). Scale bar= 50 µM. b- Matrigel explants stained for Calponin (green) and αSMA (red) nuclei counterstained in blue (DAPI). Corresponding phase images are shown on the bottom panels.
Supplementary Figure III
Supplementary Figure III.  

a- HemSCs do not express pericyte/smooth muscle or endothelial markers when cultured alone, cbEPCs and human SMCs serve as positive and negative controls (left panel). Real Time PCR analysis for expression of pericyte/smooth muscle genes in HemSCs (HemSC97, HemSC125) and HemECs (HemEC26, HemEC133) compared to normal cells bone marrow mesenchymal progenitor cells (bmMPC), smooth muscle cells (SMC), cbEPC and human umbilical vein endothelial cells (HUVEC) (right panel). Protein expression of Calponin, αSMA, sm22α, and PDGFRβ and loading control Tubulin was also analyzed by immunoblot (left, lower panel).  

b- GFP-HemSC were cultured alone for 10 days and analyzed for expression of αSMA (upper panel), smMHC (lower panel) and GFP.  

c- HemSCs in contact with HemECs express pericyte/smooth muscle markers, HemECs alone do not express α-SMA or smMHC.  

d- αSMA or smMHC (green) and vWF (red) staining at day 10 of HemSC clones co-cultured with cbEPCs (two upper rows) and HemSC clones cultured alone (two lower rows). Nuclei counterstained in blue (DAPI). Scale bar= 100 µM.  

e- Immunoblot for Calponin-1 and Tubulin (loading control) in HemSC clones alone or co-cultured with cbEPCs.
Supplementary Figure IV
**Supplementary Figure IV.**  

- **a-** GFP-HemSC (puromycin resistant) + normal cbEPC (non puromycin resistant) or  
  - **b-** GFP-HemSC+cbEPC shNon target (puromycin resistant), were  
  cocultured for 10 days, then re-plated in medium with or without puromycin.  
  Immunostaining shows expression of vWF (red) and αSMA (green) at days 5, 10, 15 and  
  20.  
- **c-** GFP-HemSC alone were cultured for 10 days, replated with or without puromycin,  
  and analyzed for αSMA expression.  
- **d-** HemSCs do not express smooth muscle myosin heavy chain (smMHC) marker when cultured without direct contact with cbEPCs.  
- **e-** HemSCs treated with TGFβ1 (top) and plated on collagen IV with PDGF-BB (bottom) do  
  not express smMHC; low levels of α-SMA were detected. Scale bar=100 µM.
Supplementary Figure V

JAGGED1 signaling regulates hemangioma stem cell-to-pericyte/vascular smooth muscle cell differentiation
Supplementary Figure V.  a- HemECs express higher levels of Jagged1 compared to HUVECs, cbEPCs and HemSCs. Western blot on top and real time PCR on bottom. HemECs isolated from three different IH (26, 130, and 133) were analyzed. b- JAGGED1 immunostaining of proliferating IH. c- Immunoblot analysis for Calponin I, αSMA, sm22α and loading control tubulin at day 8 of coculturing HemSC+HemEC or HemSC+cbEPC in medium containing 2 µg/ml Fc-domain protein (control), recombinant human (rh)JAGGED1, rhDLL4, and rhJAGGED1+rhDLL4 d- Real time PCR of HemSC+cbEPC co-cultures treated with 10µg/ml blocking antibody anti-JAGGED1 (grey bars) compared to mouse IgG control (black bars).
Supplementary Figure VI. a- shRNA-expressing-cbEPCs were co-injected with HemSCs into nude mice; at day 7 implants were stained for hematoxylin and eosin (top), and human-specific CD31 (bottom). Scale bar= 50 µM. b- Staining for human CD31 using two different commercially available antibodies (Santa Cruz, top; Dako, bottom). Right panels show murine tissues to verify specificity of the human specific anti-CD31. Scale bar= 100 µM.
Supplementary Figure VII.  a- Recombinant Tie-2, MAPK and PDGFR inhibitors do not prevent HemSC-to-pericyte differentiation (vWF in red, smMHC in green). Scale bar 100 µM.
Supplementary Figure VIII. γ-secretase inhibitors prevent pericytic differentiation and blood vessel formation.

a- HemSC+cbEPC cocultures treated for 10 days with DMSO, 10 µM DAPT or Compound E (Calbiochem) stained for vWF (red) and smMHC (green, top) or αSMA (green, bottom), and b- immunoblots for Calponin-1 (top), NG2, JAGGED1 (bottom) and relative Tubulin. c- Real time PCR shows Calponin-1 and smMHC mRNA expression at day 10 of DAPT or Compound E (10 µM) treatment. d- Matrigel explants at day 7 after injection of GFP-HemSCs+cbEPCs, with control DMSO treatment (top), HemSCs pretreated with DAPT 10 µM for 24 hours and addition of 10 (middle) or 50 µM (bottom) DAPT prior injection; and e- quantification of microvascular density (MVD). f- GFP (green) and αSMA (red) (top) staining of Matrigel explants from experiment in d. Phase contrast images show blood vessel location (arrows, bottom). (Asterisks mark blood vessel lumens.) Nuclei counterstained in blue (DAPI). Scale bar=100 µM. g- Quantification of vessels containing GFP-HemSC derived αSMA-positive pericytes. (n=10 fields/section, 5 animals/group). h- DAPT does not affect proliferation of HemSCs and cbEPC.

Data expressed as means ± s.e.m.
Inhibiting NOTCH signaling prevents HemSC-to-pericyte differentiation (text to further explain experiments in Supplemental Fig VII and VIII)

To elucidate pathways involved in the HemSC-pericytic differentiation, we tested a panel of inhibitors of receptor-ligand pathways that have been implicated in vascular development. We focused on the NOTCH pathway because the results obtained with in vitro studies (Fig.4 and Supplementary Fig.IV) revealed that direct cellular contact with endothelial cells was required for HemSCs to differentiate into pericytes. NOTCH family members and the cognate ligands are membrane bound proteins that interact in a juxtacrine manner. Therefore, γ-secretase inhibitors 5, which inhibit NOTCH signal activation, were tested. HemSC+cbEPC co-cultures were treated with two γ-secretase inhibitors, DAPT or Compound E. At day 10, smMHC was completely absent in co-cultures treated with either γ-secretase inhibitor (Supplementary Fig.VIII a, top row), and only a few spindle-shaped HemSCs acquired αSMA expression (Supplementary Fig.VIII a, bottom row). Calponin1 was strongly detected in the untreated or DMSO-treated co-cultures whereas no expression was seen in cells treated with γ-secretase inhibitors (Supplementary Fig.VIII b, top). The pericyte marker NG2, whose expression was induced in HemSC+cbEPC cultures, was down-regulated by DAPT; additionally, DAPT downregulated JAGGED1 in the coculture (Supplementary Fig. VIII b, bottom). Calponin-1 and smMHC mRNAs were also significantly downregulated (p<0.05) in response to DAPT and Compound E compared to untreated and DMSO treated cocultures (Supplementary Fig.VIII c). In contrast, human recombinant Tie2, an ERK inhibitor (U0126), and PDGFRβ inhibitor (AG1295), did not dramatically inhibit smMHC expression in the tested conditions (Supplementary Fig.VII a).
Next we tested the effect of γ-secretase inhibitors in vivo. DAPT was used to test the effect of NOTCH signaling blockade on blood vessel formation in the murine model of IH. GFP-HemSCs were treated with 10 µM DAPT (or DMSO as a control) for 24 hours. At the time of implantation, 10 or 50 µM DAPT was added to the cell/Matrigel suspension. Matrigel explants with control DMSO-treated cells appeared robustly vascularized at the macroscopic level (Supplementary Fig.VIII d, top row). In contrast, explants containing DAPT treated cells exhibited much less vascularization (Supplementary Fig.VIII d, middle and bottom rows). Quantitative analysis showed a significant decrease in the microvascular density (MVD) for both 10 and 50 µM DAPT treated groups, compared to the DMSO treated cells (control) (Supplementary Fig.VIII e). To evaluate DAPT effects on HemSC-to-pericyte differentiation, we tested Matrigel explants for presence of pericytes. DAPT-treated explants (10 and 50 µM) did not exhibit αSMA+ cells in a perivascular location (Supplementary Fig.VIII f, top row). Interestingly, in response to 10 µM DAPT, many GFP-HemSCs within the Matrigel were aligned along the abluminal surface of blood vessels, but did not acquire αSMA expression. In some areas there was also a marked decrease in perfused blood vessels, as seen in the phase images (Supplementary Fig.VIII f, bottom row). Quantitative analysis showed that DAPT treatment, at both concentrations, significantly impaired the number of vessels with HemSC-derived αSMA+ pericytes (Supplementary Fig.VIII g). Importantly, DAPT had no or very mild effect on the proliferation or viability of HemSCs and cbEPCs in vitro (Supplementary Fig.VIII h).
REFERENCES


