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Pathogenesis of infantile haemangioma

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Hemangioma is a vascular tumor of infancy that is well-known for its rapid growth during the first weeks to months of a child's life followed by a spontaneous but slow involution. During the proliferative phase, the vessels are disorganized and composed of immature endothelial cells but are not leaky [1], perhaps due to the abundance of α-smooth muscle actin (α-SMA)-positive perivascular that circumscribe the vessels (Figure 1). When the tumor involutes, the vessels mature and enlarge but are reduced in number. Fat, fibroblasts and connective tissue replace the vascular tissue, with few large feeding and draining vessels evident.

Both angiogenesis [2] and vasculogenesis [3] have been proposed as mechanisms contributing to the neovascularization in hemangioma tumors. Angiogenesis is defined as the growth of new vessels from pre-existing vessels, requiring degradation of the basement membrane, migration of endothelial cells and tubulogenesis, followed by recruitment of perivascular cells. Vasculogenesis is the *de novo* formation of blood vessels from stem or progenitor cells. In recent years, several of the "building blocks", the cells comprising the hemangioma, have been isolated. Among them are hemangioma progenitor/stem cells (HemSC), endothelial cells (HemEC) and pericytes (HemPericytes). This review will focus on these cell types, as well as molecular pathways within these cells that have been implicated in driving the pathogenesis of IH.

Cellular Components of IH

Stem Cells

The concept that IH arises from a dysregulated differentiation of embryonic cells has been considered for several decades[4–6]. Further, Smoller and Apfelberg speculated that primitive cells within interstitial regions of the tumor are capable of differentiating into endothelial cells and pericytes[7]. In 2008, our group isolated a primitive mesenchymal cell with these properties from proliferating phase IH [8] using anti-CD133-coated magnetic beads (CD133 is a cell surface membrane glycoprotein expressed on many types of human stem and progenitor cells.) The CD133+ cells are rare, comprising between 0.1–1% of the cells in proliferating phase IH. We call the CD133-selected cells hemangioma stem cells (HemSC) based on their ability to self-renew and undergo multi-lineage differentiation, two essential properties of stem cells. When implanted sub-cutaneously into immune-deficient (nude) mice, the HemSC form GLUT1+ vessels (a specific marker of IH) within 7–14 days. Using Green Fluorescent Protein (GFP)-labeled HemSC we showed that the cells differentiate into endothelium, adipocytes [8] and into pericytes[9] in mice and in culture dishes. Two other groups have isolated HemSC using similar techniques and have reported similar vasculogenic properties[1011]. Others have found evidence for primitive mesodermal cells in

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IH [12–14]. That HemSC form vessels de novo strongly implicates vasculogenesis as an important mechanism underlying hemangioma-genesis[15].

Endothelial Cells

In the proliferative phase of hemangioma, the vessels are small with lumens that are sometimes difficult to see by histology. The endothelium is plump and appears metabolically active, suggesting an immature phenotype [16]. North and colleagues showed that glucose transporter-1 (GLUT1) is consistently expressed on hemangioma endothelium, and indeed provides a definitive immunohistochemical test for distinguishing IH from other types of vascular tumors and vascular malformations[17] Beginning in 1982 [18] Mulliken and colleagues showed that hemangioma-derived endothelial cells (HemEC) proliferate readily in the culture dish and form capillary-like tubes – a phenomenon called “in vitro angiogenesis” [19]. Subsequent in vitro studies revealed that HemEC resemble fetal endothelial cells more than neonatal endothelial cells[20]. The HemEC and fetal endothelial cells displayed a spindle-shaped morphology and the endothelial adhesion molecule CD31/PECAM-1 was detected on intracellular membranes, suggesting it had not reach the cell surface, where it resides in mature endothelium. Boye and colleagues provided evidence that HemEC are clonal, suggesting they arise from a common precursor[21]. The contribution of HemEC to the total non-hematapoietic (CD45-) cell population in proliferating phase hemangioma ranges from 24% when VEGFR-2/KDR is used to quantify endothelial cells [22] and up to 30% when a cocktail of antibodies against endothelial markers (CD31, VE-cadherin, CD34 and VEGFR-2) are used to quantify the endothelial compartment (Lan Huang, unpublished data). A small percentage (0.1–2%) of these endothelial cells co-express the human stem cell marker CD133, suggesting that the endothelial cells may be transitioning from an immature to mature phenotype[22].

Pericytes

The perivascular cells surrounding the nascent vessels in the proliferative phase express the pericyte markers α-SMA[23], neural glial antigen-2 (NG2), platelet-derived growth factor receptor-β (PDGFRβ), calponin, and smooth muscle myosin heavy chain[9]. NOTCH3, typically associated with smooth muscle cells, is also detected in the perivascular layer[24], with colocalized expression of the NOTCH target gene HEYL[15]. Thus - the phenotype of the perivascular cells in hemangioma is typical of both pericytes and smooth muscle cells. They have also been shown to have characteristics of mesenchymal stem cells [25]. For simplicity, we will refer to the perivascular cells in infantile hemangioma as pericytes.

The pericytes are abundant in the proliferating phase and appear to undergo a maturation process concurrently with the endothelial cells (Figure 1). Recently, pericytes were isolated from proliferating phase (n=4) and involuting phase (n=4) IH specimens from different patients[26]. In vitro, the hemangioma-derived pericytes were found to express all of the markers detected in cells surrounding hemangioma vessels in histological sections(e.g. NG-2, PDGFRβ, calponin, αSMA, NOTCH3), consistently over several in vitro passages. When hemangioma pericytes were combined with endothelial cells and implanted in mice, the cells assembled into vessels that connected with murine vessels within 7 days. When compared to normal human pericytes isolated from retina or placenta, hemangioma pericytes proliferated more rapidly, expressed more VEGF-A, but expressed reduced levels of angiopoietin-1 (ANGPT1). In co-culture, hemangioma pericytes showed a reduced ability to suppress the proliferation and migration of normal human endothelial cells. Taken together, the increased VEGF-A, decreased ANGPT1, increased proliferation, increased vessel formation in vivo, and decreased ability to suppress proliferation and migration of endothelial cells indicates that hemangioma pericytes are pro-angiogenic [26].

Figure 2 depicts the key cellular features of HemSC, HemEC, and HemPericytes.

Mast cells

Mast cells are present in IH[27,28]. Their number predominates in the early to middle involuting phase, whereas lower numbers are seen in the proliferative and the involuted phases[1]. This difference has led to the hypothesis that mast cells play a role in the regression of IH. However, little is known about these cells. Moreover, it has recently been demonstrated that mast cells are present in comparable numbers in various skin tumors, including basal cell carcinoma, squamous cell carcinoma, melanomas and nevi[29]. Accordingly, the unique role of mast cells in IH requires further study.

Molecular Basis of IH

Genetics

In contrast to other vascular anomalies, in which a germline or somatic mutations have been identified as a cause [30], the etiology of hemangioma of infancy still remains obscure. It is likely that the origin of IH is multi-factorial, with genetic factors being part of the contributing triggers. In most cases, IH are sporadic. However, autosomal dominant inheritance pattern and a linkage to 5q have been reported in few families [3111]. Recently, in a study of large population database, a 2-fold increased relative risk for hemangiomas among siblings of an affected proband has been shown, further supporting the hypothesis of a genetic cause as a contributing factor[32]. Somatic mutations leading to uncontrolled proliferation of hemangioma cells have been also proposed. In support of this theory, a clonality of ECs from hemangioma lesions have been shown on a small set of IH, using an X-chromosome inactivation assay[21]. Evidence for clonality was also found in cells from hemangioma tissue sections, supporting the somatic mutation hypothesis[33]. Mutations in the integrin-like receptor Tumor Endothelial Marker 8 (TEM8) and in VEGFR2 were identified in a small subset of HemEC and corresponding blood sample from IH patients, using a targeted candidate gene approach [34], identifying these as germline, potentially “risk-factor” mutations, that may contribute to IH.

The existence of IH as part of syndromes such as PHACE (posterior fossa abnormalities, infantile hemangioma, arterial abnormalities, cardiac anomalies, and eye colobomas) [35] or SACRAL (spinal dysraphism, anogenital, cutaneous, renal and urologic anomalies, associated with an angioma of lumbosacral localization) [36] also suggests a germline or somatic mutation. No specific mutation was identified for these syndromes, however genomic copy number variation (CNV) has been found in PHACE syndrome[37].

VEGF and VEGF-receptors

Several signaling pathways have been linked with IH pathogenesis, with the VEGF (Vascular endothelial growth factor A) pathway being the key one. VEGF-A is a master regulator of angiogenesis and vasculogenesis[38]. It is present at higher levels in proliferating phase compared to the involuting phase of IH [139404142] and its level in the serum of IH patients is decreased following systemic steroid therapy [43]. The high expression of VEGF might be related to hypoxia, as increased hypoxia inducible factor-1α (HIF-1α) stabilization was reported in patients with proliferating IH [40]. We recently found that the corticosteroids dramatically down-regulate VEGF-A secretion by hemangioma stem cells (HemSC). Furthermore, silencing the expression of VEGF-A or VEGF receptor 1 (VEGFR-1) in HemSC by short hairpin RNA (shRNA) was sufficient to block blood vessel formation in vivo [4144]. In contrast, HemEC express and secrete very little VEGF and these low levels are not affected by corticosteroid treatment.

VEGF-A binds to three tyrosine kinase receptors, VEGFR-1, VEGFR-2 and VEGFR-3. VEGFR-2 appears to mediate almost all of the known angiogenic responses to VEGF-A. In contrast, the function and signaling of VEGFR-1, which is present on endothelial and non-endothelial cells, is less understood. VEGFR-1 is considered to be a VEGF-A trap, by virtue of its high binding affinity for VEGF and its relatively low kinase activity[45]. HemEC[46], as well as hemangioma specimens[47], express relatively low levels of VEGFR-1. This low expression (i.e., fewer "VEGF traps") results in increased VEGF-dependent activation of VEGFR-2 and downstream signaling pathways. In summary, several lines of research point to a critical role for VEGF and its receptors in the pathological vasculogenesis and angiogenesis in IH.

Angiopoietins and TIE2

Angiopoietin-1 (ANGPT1) and angiopoietin-2 (ANGPT2) signal through the endothelial membrane receptor TIE2 to regulate distinct steps in vascular remodeling, vessel maturation and vascular inflammation [48,49] TIE2 mutations have been identified in inherited form of venous malformation (VM) [50] and 50% of sporadic VM [51]. Similar mutations were not found in IH. However Tie2 mRNA and protein were shown to up-regulated in IH tissues and in HemEC. In accordance, increase in cellular responsiveness to Ang1 was observed[52]. A related study in a murine endothelioma tumor model has shown that blocking Ang2 function with soluble Tie2 receptor or down-regulating Ang2 pharmacologically inhibited growth of a tumor[53]. Calicchio and colleagues found ANGP2 mRNA significantly increased in hemangioma endothelium isolated by laser capture micro-dissection [54]. This, coupled with the diminished expression of ANGPT1 by hemangioma pericytes, would be permissive for angiogenesis and hinder vessel maturation. Still, confirmatory studies in animal models of IH as well as mechanistic studies are needed to clearly define the role of angiopoietins and TIE2 in the growth and involution of IH.

The Notch Pathway

The Notch pathway has also been implicated in hemangiogenesis. This evolutionarily conserved signaling system regulates cell-fate determination during development and in stem cells. In the vascular system, interaction of Notch receptors (Notch1 to Notch4) with their ligands (Delta-like 1, Delta-like 3, Delta-like 4, Jagged-1and Jagged-2) regulates the specification of endothelial cells into arterial and venous phenotypes during development [55]. JAGGED1 has been reported to be highly expressed in IH endothelium [2454] whereas its receptor Notch3 is expressed by HemSC and becomes prominently expressed in the perivascular cells in the involuting phase[24]. Also, we have recently shown that JAGGED1 directs the differentiation of the HemSC into the "endothelium-coating" cells, the pericytes [56]. Based on these observations, it is tempting to speculate that juxtacrine signaling between hemangioma endothelial cells and pericytes is mediated by the Notch pathway.

The mTOR Pathway

Mammalian target of rapamycin complex (mTOR) is a major intersection that translates signals from the extracellular milieu, such as glucose, amino acids and growth factors to corresponding changes in basic intracellular processes including proliferation, protein synthesis and autophagy [57]. Rapamycin, an mTOR inhibitor, is known to have an anti-angiogenic effect on ECs in pathological settings [5859] and has shown efficacy in the treatment of complicated vascular malformations[60]. We found that rapamycin inhibits the proliferation and the self-renewal activity of the HemSC. This inhibition, in turn, prevented HemSCs, either alone or combined with endothelial cells, from forming blood vessels in vivo [61]. Beside its anti-vasculogenic effect on HemSC, rapamycin has anti-angiogenic effect on the HemEC, suppressing their proliferation and leading to regression of pre-existing IH

vessels. Rapamycin also decreases expression of HIF-1α, reducing already low levels of VEGF-A in HemEC even further [62].

Other pathways

Various other factors have been found to be differentially expressed in the proliferating phases of IH but their role is less defined. These include MCP-1 [6342], IL-6 and uPAR [42] and Insulin-like growth factor 2 (IGF-2) [64,65]. The SKI oncogene, a transcriptional repressor that inhibits expression of TGFβ family members, was found to be highly expressed in the endothelium of proliferating phase IH, but was not detected in several vascular malformation specimens [66], an intriguing suggestion that TGFβ signaling may be suppressed in IH. The cell adhesion molecule E-selectin, normally only expressed in inflamed endothelium, is strongly expressed on vessels in proliferating phase IH [67]. E-selectin is also constitutively expressed by proliferating phase HemEC and appears to mediate interactions with HemSC [68].

The Renin-Angiotensin system has recently been suggested to play a role in IH pathogenesis as well as in the response to propranolol [69]. Specifically, it was suggested that angiotensin II could drive proliferation of endothelial progenitor cells into mitotically active cells that characterize IH. The hypothesis was based on the clinical observation of a higher incidence of IH in premature babies, female infants and infants of mixed European descent that have been shown to have higher circulating renin activity compared to their age and sex-matched controls [70]. Indeed, angiotensin-converting enzyme and angiotensin II receptor-2 were shown to be expressed in IH [69]. However, support to this theory from in-vivo studies is lacking. Also against this theory is the low renin activity found in IH patients [71] and the poor effect of the ACE inhibitor captopril on IH [72].

Drug treatment of IH

Corticosteroids have been the first-line treatment for complicated IH for many years [7374]. In recent years, β-adrenergic receptor blockers have emerged as an effective and safe pharmacological treatment of proliferating IH. We have recently published a detailed review of these drugs' mechanism of action [75]. Here, we will focus on new findings regarding the effect of these drugs on the constituent cells and the molecular pathways involved in IH.

Beta blockers

Propranolol, the most commonly used beta-blocker against IH, is an orthosteric antagonist of both β_1- and β_2-adrenergic receptors. Despite its widespread use, since 2008, propranolol's mechanism of action remains uncertain. Expression of all three β-adrenergic receptors have been shown in IH tumors [767778]. By immunohistochemical analysis, the expression of β_2 receptor was located mainly to the endothelial cells [78]. However, in-vitro studies show expression of β_1 adrenergic receptors in HemEC and β_2 adrenergic receptors in HemEC, Hem Pericytes and at low levels in HemSC (Lee, Greenberger and Bischoff, unpublished data). As a rapid change in both the color and the consistency of the tumor is sometimes noticed following the initiation of propranolol, it is reasonable that propranolol exerts its effect via vasoconstriction of the high-flow blood vessels feeding the IH tumor. In the skin, adrenaline-induced vasoconstriction has been shown to be increased by oral propranolol [79]. The target cells for this effect might be the tumor's pericyte.

Additional mechanisms might be responsible for the slower, long-term effect of propranolol. Among them is VEGF suppression. Noradrenaline has been shown to enhance VEGF-A production by several cell types, both normal and cancerous [808182]. These effects of the catecholamines are mediated by the β_1 and β_2 adrenergic receptors and are blocked by propranolol[808183 82]. In IH, Ji and his colleagues have recently shown that the Noradrenaline

agonist isoprenaline increased HemECs proliferation via regulation of thee cell-cycle proteins cyclin D1 and its associated kinases, CDK-4 and CDK-6. These effects were reversed by β_2-adrenergic receptor antagonists. Furthermore, isoprenaline increased the expression of VEGF-A and the phosphorylation of VEGFR-2 in HemECs in a β-adrenergic receptor- and extracellular-signal-regulated kinase (ERK) -dependent manner.

Additional pro-angiogenic effects of noradrenaline blocked by propranolol are up-regulation of HIF-1α protein [82], matrix metalloproteinases (MMPs) 2 and 7 and 9 [81,84,85] and IL-6 [86]. However, these effects have not been studied in IH-derived cells or in IH animal models. Several groups have demonstrated a direct pro-apoptotic or anti-migratory activity of propranolol on HemEC. However, these effects were achieved in high concentrations of propranolol that are not likely to be present in the tumor's microenvironment[87].

Corticosteroids

Corticosteroids affect the growth of IH by modifying the "pro-angiogenic" environment of the tumor, mostly likely by suppressing the high levels of VEGF-A secreted by the HemSC. By using IH animal model, we found that steroids lead to dose-dependent reduction in the tumor's vascularity. Corticosteroids, including dexamethasone, prednisone, prednisolone and methylprednisolone dramatically down-regulate VEGF-A secretion by HemSC and this suppression was sufficient to block blood vessel formation in vivo[41]. Several other pro-angiogenic genes were modified as well by steroids. These include Monocyte chemotactic protein-1 (MCP-1), Interleukin-6, matrix metalloproteinase-1 (MMP-1) and urokinase-type plasminogen activator receptor (uPAR)[41]. These target genes have high expression level in proliferating versus involuting IH tissue. The down-regulation of these pro-angiogenic cytokines, including VEGF-A might be mediated by corticosteroids interference with Nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) activity [42].

Concluding Remarks

Substantial progress has been made on understanding the cells and molecular pathways that are prominent in IH. There remain, however, several unsolved mysteries. What is the nature and origin of the HemSC – is this normal post-natal vasculogenic stem cell perturbed by an external stress or has it acquired a mutation that expands its proliferative and differentiative capacity? Do HemSC arise in situ or do they come from a distant location such as the placenta or bone marrow? Do the hemangioma stem cells, ECs or pericytes exert dominant effects on surrounding vessels, subverting and/or increasing their angiogenic and vasculogenic capability? And finally, what triggers involution? The answers to these questions may help to devise therapies that can stop hemangioma growth in its tracks, providing enormous relief and reassurance to patients and families. Such therapies might consist of combinations of drugs given concurrently or perhaps sequentially to target cells that are most active in the proliferating phase. Another goal would be to identify biomarkers that would predict early in the proliferative phase which tumors should be treated and which could be left untreated to follow the natural cycle of growth and involution.

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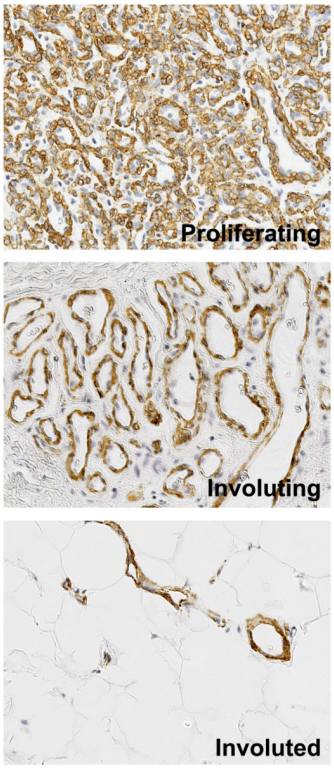

Figure 1.
α-Smooth muscle actin (α-SMA) highlights vessel maturation and regression during the life cycle of IH. Paraffin-embedded sections from proliferating, involuting and involuted phase IH were immunostained with anti-α-SMA, images taken at 40X.

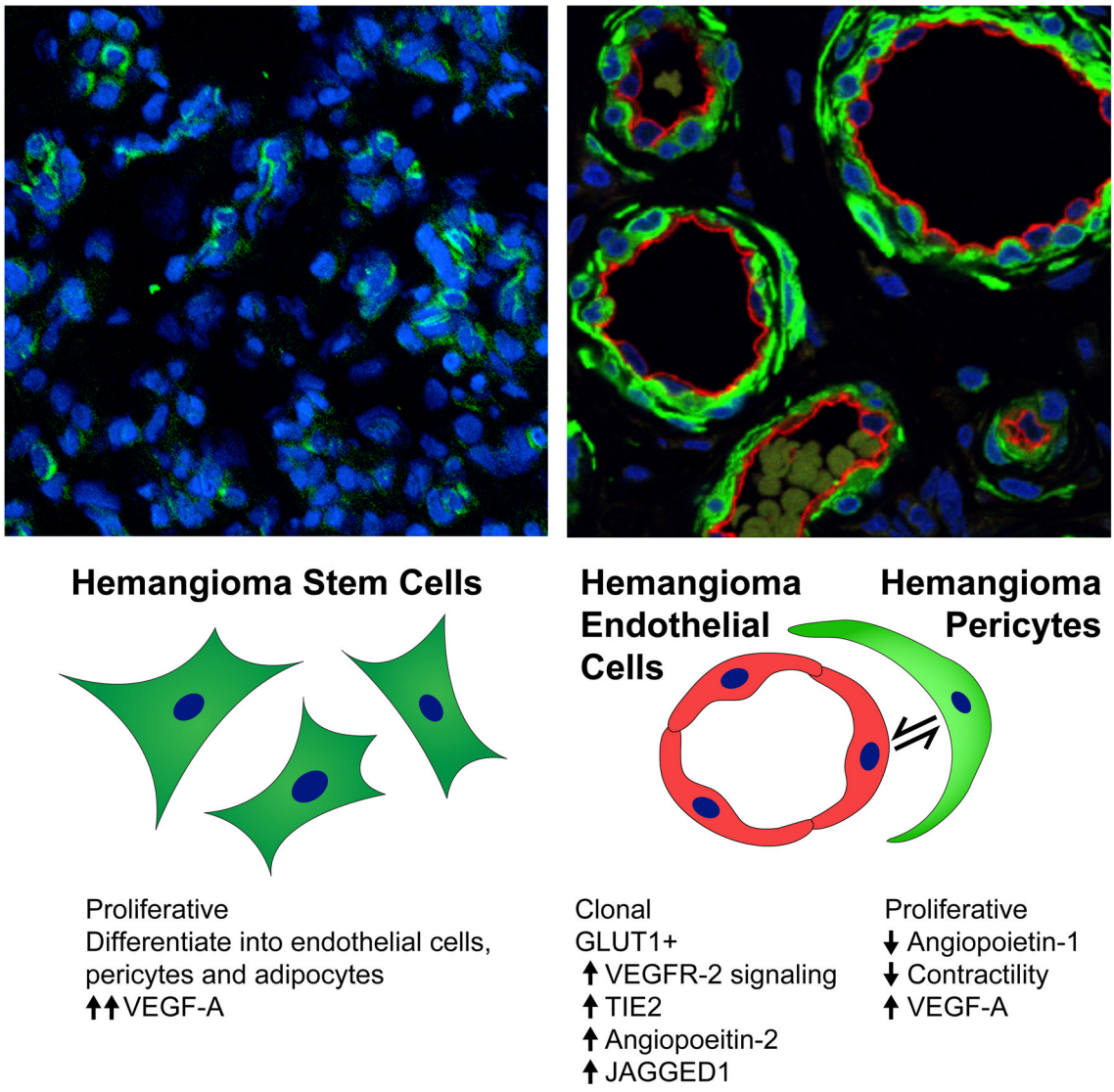

Figure 2.

Cellular Components of IH. Left panel shows a proliferative phase IH tumor section stained for the human stem/progenitor cell marker CD133 (CD133+ cells, green; nuclei, stained blue). Staining and image provided by Dr. Arnaud Picard. CD133+ cells are sparse and not associated with well-formed luminal structures. Right panel shows IH tumor section double-stained for the endothelial marker CD31 (red) and the smooth muscle marker calponin (green). Nuclei are stained blue with DAPI. Lumens are lined with plump endothelial cells surrounded by perivascular cells. Staining and image provided by Dr. Elisa Boscolo. The schematic below depicts 1) the mesenchymal morphology of HemSC and 2) the interaction between HemEC and HemPericytes and 3) cellular and molecular features of each cell type.