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Characterization of Hemangioma-initiating Stem Cells

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Abstract

Infantile hemangioma (IH) is the most common vascular tumour of infancy. IH undergoes a unique life cycle consisting of robust endothelial cell proliferation and vessel formation in the proliferating phase, followed by spontaneous regression in the involuting phase. Our laboratory has shown that IH arises from multipotential stem cells termed hemangioma stem cells (HemSCs). However, the phenotype of HemSCs has not been fully elucidated. Here, I examined HemSCs and compared these lesion-derived cells to a panel of normal counterparts. My results show that HemSCs share similar gene expression profiles with human fetal liver-derived stem cells (FLSCs) and postnatal bone marrow mesenchymal/mesodermal progenitor cells (BM-MPCs). Specifically, all three precursor cell types expressed endothelial, mesenchymal, stem/progenitor, and hematopoietic lineage genes to varying degrees. Furthermore, for the first time, I show that proliferating IH lesions are immunoreactive to markers associated with hematopoiesis; namely, RUNX1, GATA2, GPR56, CD45 and CD150. However, HemSCs failed to produce hematopoietic colonies when assessed using *in vitro* hematopoietic activity assays. Taken together, my studies suggest that HemSCs express hematopoiesis-specific markers but their ability to undergo hematopoiesis is suppressed. Although my findings have provided greater characterization of HemSCs, more studies are needed to fully understand the mechanisms that regulate HemSC differentiation paths, and ultimately IH pathogenesis.

Keywords: infantile hemangioma, hemangioma stem cells, fetal liver stem cells, bone marrow progenitor cells, hematopoiesis, endothelial-to-hematopoietic transition

Co-Authorship Statement

All work shown in this document was performed by Natalie Montwill. Dr. Zia A. Khan contributed to the experimental design and data interpretation.

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α-SMA	Alpha-smooth muscle actin
AGM	Aorta-gonad-mesonephros
ALCAM	Activated leukocyte adhesion molecule
APC	Activated protein C
BFU	Blast forming unit
BM	Bone marrow
BMP	Bone morphogenetic factor
C/EBP	CCAAT/enhancer-binding protein
CD	Cluster of differentiation
CD133	Cluster of differentiation 133, Prominin-1
c-KIT	Tyrosine-protein kinase Kit
CVS	Chorionic villus sampling
CXCL	C-X-C motif chemokine ligand
D3	Type III iodothyronine deiodinase
DAPI	4',6-diamidino-2-phenylindole
DPPA4	Developmental pluripotency associated 4
E	Embryonic day
EBM-2	Endothelial basal media-2
EC	Endothelial cell
ECFC	Endothelial colony forming cell
EDTA	Ethylenediaminetetraacetic acid
EHT	Endothelial-to-hematopoietic transition
EndMT	Endothelial-to-mesenchymal transition
ENG	Endoglin
EPC	Endothelial progenitor/precursor cell
ERG	ERG, ETS transcription factor
ES	Embryonic stem cell
ES-BCs	Embryonic stem cell-derived blast cells

ETV6	ETS variant 6
FABP4	Fatty acid binding protein 4
FGF	Fibroblast growth factor
FL	Fetal liver
FLI1	Fli-1 proto-oncogene
FLK1	Fetal liver kinase-1 (Vascular endothelial growth factor receptor 2)
FLSC	Fetal liver stem cell
FLT1	Fms related tyrosine kinase 1 (Vascular endothelial growth factor receptor 1)
GATA	GATA binding protein (Globin transcription factor)
GDF3	Growth differentiation factor 3
GLUT1	Glucose transporter 1
GPR56	G protein-coupled receptor 56
GSC	Glioblastoma stem-like cells
HE	Hemogenic endothelium
HemEC	Infantile hemangioma-derived endothelial cell
HemPER	Infantile hemangioma-derived pericyte
HemSC	Infantile hemangioma-derived stem cell
HIF-1α	Hypoxia inducible factor-1α
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
IAC	Intra-aortic clusters
IDO	Indoleamine 2,3-deoxygenase
IFN	Interferon
IH	Infantile hemangioma
KDR	Kinase insert domain receptor (Vascular endothelial growth factor receptor 2)
LDL	Low-density lipoprotein
LEFTY1	Left-right determination factor 1

LEP	Leptin
LMO2	LIM domain only 2
LT-HSC	Long-term hematopoietic stem cells
LYL1	Lymphoblastic leukemia associated hematopoiesis regulator 1
MMP	Matrix metalloproteinase
MNC	Mononuclear cell
MPC	Mesenchymal progenitor/precursor cell
MSC	Mesenchymal stem cell/Mesenhymal stromal cell
NANOG	Nanog homeobox
NFAT	Nuclear factor of activated T cells
NG2	Nerve/glial antigen 2
NGFR	Nerve growth factor receptor
NT5E	5`-nucleotidase
OCT4/POUF5	Octomer-binding transcription factor 4
PBS	Phosphate-buffered saline
PDGFR	Platelet-derived growth factor receptor
PHACES	Posterior fossa-hemangiomas–arterial lesions/anomalies–cardiac defects–eye abnormalities–sternal cleft and supraumbilical raphe syndrome
PODXL	Podocalyxin like
PPARɣ	Peroxisome proliferator-activated receptor gamma
PSF	Penicillin-streptomycin-funizone
PTPRC	Protein tyrosine phosphatase receptor C
RT-PCR	Reverse transcription-polymerase chain reaction
ROS	Reactive oxygen species
RUNX	Runt related transcription factor
SC	Stem cell
SCA1	Spinocerebellar ataxia 1
SCF	Stem cell factor
SCL/TAL1	Stem cell protein/T-cell acute lymphocytic leukemia protein 1
SDF-1α	Stromal cell-derived factor-1α

SOX2	Sex determining region Y-box 2
TBX2	T-box transcription factor 2
TEM8	Tumor endothelial marker 8
TF	Transcription factor
TGF	Transforming growth factor
THY	Thy-1 cell surface antigen
TNF-α	Tumor necrosis factor-α
Ulex	Ulex europaeus 1
VCAM-1	Vascular cell adhesion molecule-1
VE-Cadherin	Vascular endothelial-Cadherin (also known as Cadherin 5)
VEGF	Vascular endothelial growth factor
VEGFA	Vascular endothelial growth factor A
VEGFR	Vascular endothelial growth factor receptor
vWF	von Willebrand Factor
YS	Yolk sac
ZFP42	Zinc-finger protein 42

Chapter 1

1 INTRODUCTION

1.1 Infantile hemangioma

Infantile hemangioma (IH) is the most common vascular tumour of infancy, affecting approximately 5-10% of newborns each year.[1-3] Although the mechanisms underlying the pathophysiology of IH are currently unknown, there is a higher incidence rate associated with infants that are female, Caucasian, born prematurely and with a low birth weight.[4-8] The majority of IH lesions are found in the head and neck regions, followed by the trunk and extremities.[9]

IHs are assessed clinically based on how deep they reside in the dermis or subcutaneous tissue (as in, superficial, deep or combined), as well as their anatomical distribution.[10,11] The majority of IHs are localized, arising from a single focal point. Segmental IHs are distributed throughout a larger area. Lesions that are neither completely localized nor segmental are called indeterminate, and those that appear in multiple anatomic regions are known as multifocal.[12,13] Based on their anatomical location, multifocal IHs can be indicative of complications associated with other parts of the body. For example, large, segmental, facial IHs can be an indication of PHACES syndrome (Posterior fossa-hemangiomas–arterial lesions/anomalies–cardiac defects–eye abnormalities–sternal cleft and supraumbilical raphe syndrome).[14] Additionally, the presence of multiple cutaneous lesions can be indicative of hepatic IH,[15] which is the most common site of visceral IHs.[16] Despite the benign nature of IH, lesions can arise in areas that can cause functional impairment, such as in the lip or nose, or life-threatening complications, such as in the liver and trachea.[13,15]

Usually, no signs of IH are present at birth. Rather, lesions appear weeks to months postnatally within the first year of life[17] and then progress through a unique life cycle consisting of three phases: proliferation, involution, and then the involuted phase (Figure 1.1). In the first phase, endothelial cells (ECs) rapidly proliferate to form an immature

vascular network.[13,18] Histologically, ECs appear enlarged and are surrounded by plump pericytes, and cellular proliferation markers are prominent.[13] IHs will typically remain in this phase for up to 1 year, after which ECs will show a flattened morphology and vessels will attain a more mature appearance, thus entering the involuting phase. During this time, apoptosis offsets cellular proliferation and contributes to IH regression.[19] This period will persist for 3 to 5 years before entering the involuted phase, in which blood vessels are replaced by fibroadipose tissue.[20] Most tumours almost completely regress by 8 years of age, leaving a fibrofatty residuum or scar depending on the size and behaviour of the IH.[15]

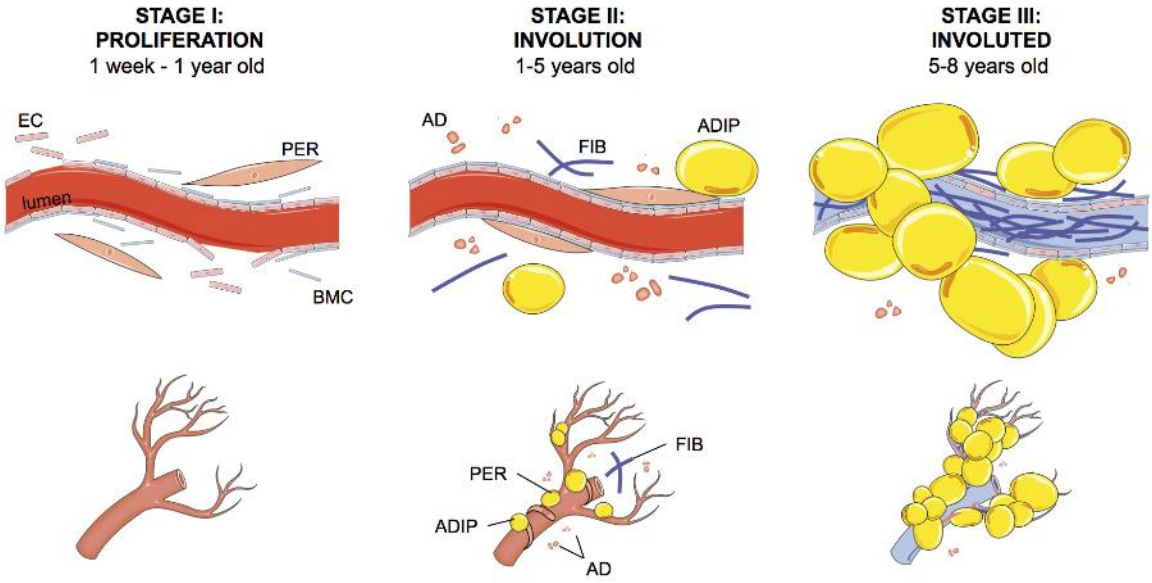


Figure 1.1 The life cycle of IH.

Schematic diagram depicting the three stages of IH. The proliferating phase begins within the first few weeks after birth and is characterized by rapidly-proliferating ECs and recruitment of pericytes. Newly-formed blood vessels are disorganized and immature, until pericytes and basal membrane components stabilize the vasculature. After about one year, the IH lesion begins to involute. Cellularity diminishes through apoptosis, and remaining cells commence differentiation into adipocytes. By 8 years of age, most, if not

all, of the vasculature is replaced by fibrofatty tissue. EC = endothelial cell; PER = pericyte; BMC = basement membrane component; AD = apoptotic debris; FIB = fibrous tissue; ADIP = adipocyte

1.1.1 Diagnosis and treatment of IHs

Vascular 'birthmarks' are commonly encountered in children and are classified as IHs or vascular malformations. In contrast to IHs, vascular malformations are not neoplasms but rather, permanent developmental abnormalities of capillaries, veins, arteries or lymphatic vessels. The most commonly used diagnostic/confirmation tool for IH is endothelial glucose transporter-1 (GLUT1) immunohistochemical staining. GLUT1 is an erythrocyte-type transporter that is expressed by hemangioma endothelium in all three phases of IH development. Normally, endothelial GLUT1 expression is restricted to microvessels in placenta as well as those with blood-tissue barrier function. For this reason, GLUT1 staining has become the gold standard for IH diagnosis.[21]

Given the unique ability of IH lesions to regress on their own, practitioners will often leave lesions untreated, though the size and nature of the lesion does not predict its outcome. Up to 20% of IHs result in complications such as ulceration or functional impairment (both of which are potentially life-threatening), or permanent disfigurement.[22] Furthermore, 40-80% of cases will leave a permanent residual mark.[23,24] Faint traces of vasculature may be present if vessels did not regress completely, and ulcerated lesions tend to leave discoloration or scars.[25,26] For these reasons, early therapeutic intervention is recommended to reduce pain, prevent complications from developing and to avoid potential psychosocial effects.

Currently, the first-line therapy for treating IH lesions is propranolol, a ß-adrenergic receptor blocker. Discovered serendipitously for its antiproliferative effects on IH,[27] it has been shown to be clinically superior compared to other therapeutic options in terms of resolution rate and reports of adverse effects.[28,29] Although the definite mechanism of action of propranolol in IH regression is not known, it has been suggested to involve

vasoconstriction, inhibition of angiogenesis, as well as induction of apoptosis.[30] When treated with propranolol, patients experience a visible colour change in their IHs within 1-3 days. This is due to inhibition of the ß1- and ß2-adrenergic receptors found on ECs, which results in vessel constriction and subsequently reduced blood flow to IH lesions.[27] Propranolol also suppresses production of proangiogenic factors implicated in proliferating IHs, such as vascular endothelial growth factor-A (VEGF-A) and matrix metalloproteinases (MMPs) 2 and 9.[31] These effects and the fact that it acts on many cell types that comprise IH (specifically ECs, stem cells, and pericytes) has resulted in a decrease in the use of other treatments and dampened efforts to discover new options. However, in rare cases where propranolol does not reduce the IH lesion and/or serious adverse effects are observed, alternative therapies may be considered. Currently, these alternative therapies include corticosteroids, topical ß-blockers and pulsed dye laser therapy.[32-34] Occasionally, surgery will be used if the patient does not respond to pharmacotherapy. Other indications for surgical excision include obstruction caused by the lesion, deformation, bleeding or ulceration, lack of response to medical or laser therapy and redundant residual tissue left after IH involution.[22,25] Since these treatments present a much higher likelihood of producing adverse effects in comparison to propranolol however, they are no longer recommended as a standard for managing IH.[22]

1.1.2 Cellular components of IHs

IHs are predominantly composed of ECs surrounded by perivascular cells known as pericytes. As the IH progresses through its three characteristic phases, organization of the IH endothelium changes dramatically. During this time, various endothelial cell surface markers such as CD31, CD34, CD146, vascular endothelial growth factor receptor 2 (VEGFR2), vascular endothelial-cadherin (VE-CADH), and von Willebrand factor (vWF) are highly expressed.[20,21] In addition, studies have shown that ECs derived from proliferating IHs exhibit increased proliferation and migration *in vitro.*[23,25,26] This altered behavior has led to the speculation that IH arises from clonal expansion of a single endothelial precursor cell, referred to as an endothelial progenitor cell (EPC, now called endothelial colony forming cell (ECFC)), harbouring a somatic mutation.[35] Indeed, a

study by Yu and colleagues in 2004 found that 0.1 to 2% of ECs derived from IH samples co-expressed endothelial cell markers (CD34 and VEGFR2) and the stem cell antigen CD133, supporting the existence of EPCs in IH.[36]

Pericytes are elongated cells that surround ECs in microvessels including IH blood vessels.[37] Though well-known for their role in vessel stabilization and hemodynamic processes,[38] their involvement in IH remains obscure. Pericytes derived from proliferating IH lesions (termed HemPER from hereon) can be identified based on their expression of alpha smooth muscle actin (α-SMA),[39] the pericyte marker nerve/glial antigen-2 (NG2),[40-42] platelet-derived growth factor receptor-β (PDGFRβ),[43] calponin,[20,42] and NOTCH3.[44] In normal tissue, pericytes modulate EC proliferation, vessel maturation and vasoconstriction.[38,45] However, a study that isolated pericytes from proliferating and involuting phase IH found that, when co-cultured with normal cord blood-derived ECFCs, HemPERs induced ECFC proliferation and migration.[41] Even though this study utilized cord blood-derived ECFCs and not mature vessel-derived ECs, inclusion of retinal pericytes as control confirmed that HemPERs possess a pro-angiogenic phenotype. Furthermore, contractile ability by HemPERs was suppressed, which may explain the high-flow characteristics of IH. This suggests that pericytes may have a proangiogenic role in hemangiogenesis, due to their inability to regulate vessel maturation.

A significant number of mast cells have also been reported in IHs, but observations have been inconsistent in terms of when in IH development they are most abundant. Glowacki and Mulliken (1982) found the greatest mast cell numbers in proliferating lesions,[46] whereas others have reported highest counts during involution coinciding with EC apoptosis and capillary dropout.[47-49] The current literature has not yet elucidated an exact role for mast cells in hemangiogenesis; however, a growing body of evidence suggests that they are involved in IH regression. Mast cells release various interferons and transforming growth factors such as interferon (IFN)-α, IFN-β, IFN-γ, and transforming growth factor-β (TGF-β), that are known to suppress IH proliferation.[50,51] Notably, contradicting studies also exist arguing for a proangiogenic role of mast cells, owing to their ability to secrete VEGF-A and fibroblast growth factor (FGF)-2.[52,53] These

discordances in findings may perhaps imply that the roles of mast cells in IH changes depending on the developmental stage of the lesion. Hence further investigation is required to fully understand how mast cells contribute to the IH life cycle.

Recently, multipotential stem cells have been isolated from proliferating IH lesions that were able to form blood vessels and IH-like lesions *in vivo*.[54] These cells express the stem cell marker CD133 and undergo clonal expansion, and are thus referred to as HemSCs. Making up 0.2% of proliferating IHs, HemSCs have robust vessel-forming capabilities that have been demonstrated both *in vitro* and in immunodeficient mouse models. Vessels formed by HemSCs express characteristic IH markers GLUT1 and merosin, and naturally transition into adipocytes – mimicking the involuting and involuted phases of IH. The stem cell phenotype and plasticity of these HemSCs has been confirmed by *in vivo* studies.[54] Clonally expanded (single cell) HemSCs were injected into immunodeficient mice. Human CD31(endothelial cell marker)-expressing cells were then isolated and injected into secondary immunodeficient mouse recipients. After 14 days, new blood vessels had formed, confirming the robust vasculogenic potential of HemSCs. Since then, similar results demonstrating the vessel-forming properties of HemSCs have been observed in other studies.[55,56] Interestingly, HemSCs are also capable of differentiating into pericytes both *in vitro* and *in vivo*.[57] In the study by Boscolo et al (2011), CD133+ cells were isolated from proliferating IHs, expanded, and injected into nude mice. After 7 days, pericytes had developed around the newly-formed blood vessels, as was confirmed by the pericyte marker α-SMA. To confirm that these pericytes were of HemSC origin, GFP-labelled HemSCs were also injected. The co-expression of α-SMA and GFP confirmed that HemSCs give rise to pericytes. Moreover, another study showed that HemPER interaction with ECs was vital for proper vascular assembly in IH.[41] This unique ability to give rise to both HemECs and HemPERs therefore emphasizes the principal role of HemSCs in IH pathogenesis.

1.1.3 Signaling pathways in IHs

Due to the excessive development and disorganization of blood vessels characteristic of early phase IHs, many studies have focused on the role of VEGF-A, a universal proangiogenic factor, in IH. Numerous studies have confirmed that VEGF-A is the major growth factor responsible for HemEC proliferation.[58-60] As higher VEGF-A serum concentrations are observed in children with proliferating IH versus involuting, this conclusion is not surprising.[60-62] Furthermore, VEGF-A levels have been reported to drop following steroid treatment.[60,62]

VEGF-A binds primarily to two tyrosine kinase receptors: VEGFR1/Flt-1 and VEGFR2/KDR. Both are found on the surface of ECs; however, they are believed to exert opposing effects.[63] VEGFR1 is indirectly antiangiogenic, binding to VEGF-A with higher affinity but without transmitting a signal. In doing so, it acts as a "trap" that prevents VEGF-A from binding to VEGFR2, which activates downstream signals resulting in EC proliferation.[64] However, it is the downregulation of VEGFR1 expression in IHs that is believed to contribute to increased VEGF-A levels and thus VEGF-A activity. A study found that low VEGFR1 expression resulted in increased VEGFR2 activity due to higher VEGF-A/VEGFR2 binding.[65] This reduction in VEGFR1 is due to downregulation of nuclear factor of activated T cells (NFAT), a transcription factor regulating VEGFR1 expression. More specifically, missense mutations encoding VEGFR2 and tumor endothelial marker-8 (TEM8) have been found in HemECs and are implicated in the suppression of NFAT by altering the interactions between VEGFR2, TEM8 and ß1-integrin, which regulate NFAT activity. This imbalance in VEGFR1 and VEGFR2 results in constitutive VEGFR2 signaling in HemECs, inducing EC proliferation and tumour development.[65,66] Abnormal VEGF-A/VEGFR2 interaction may also be important in HemEC survival as VEGFR2 prevents ECs from undergoing apoptosis, whereas VEGFR1 mediates the proapoptotic effects of VEGF-A.[67,68] Hence the imbalance of VEFR1 and VEGFR2 activity may mediate IH formation by affecting HemEC survival.

Another signaling pathway involved in IH development is the NOTCH pathway, which orchestrates cell fate differentiation as well as angiogenesis.[44,69] The NOTCH system

depends on juxtacrine interactions between the NOTCH receptors (NOTCH-1 to -4) and ligands (Delta-like (DLL)1, DLL3, DLL4, JAGGED1 and JAGGED2). A study by Boscolo et al (2011) showed that JAGGED1 is highly upregulated in HemECs taken from proliferating IHs and that this expression is required for HemSC-to-HemPER differentiation.[57] Furthermore, silencing *JAGGED1* gene expression in HemECs abolished HemSC-to-HemPER differentiation and reduced blood vessel formation *in vivo*. The authors proposed that IH vasculogenesis begins with differentiation of HemSCs into HemECs, which then promote HemSC-to-HemPER differentiation and ultimately vascular development. In addition, VEGF-A is known to interact with NOTCH receptors and ligands that promote EC survival and angiogenesis.[70,71] Since high levels of VEGF-A are found in IH,[60] this may lead to NOTCH pathway activation and induce a proangiogenic signaling cascade, propagating tumour development.

Low oxygen tension (hypoxia) has been associated with tumour angiogenesis and neovascularization in many cancers.[72-74] Though its involvement in IH is poorly defined, a number of studies have associated hypoxia with IH development.[75,76] Clinically, a blanched area of skin that precedes the IH is presented at birth. Such lesions lack normal blood flow and may be an area of local ischemia, the causes of which are still unknown.[77] During hypoxia, the expression of transcription factor hypoxia inducible factor-1α (HIF-1α) is upregulated in tumour cells and induces production of other proangiogenic factors, such as VEGF-A and stromal cell-derived factor-1α (SDF-1α).[78] These factors are known to recruit ECFCs to ischemic areas and induce vasculogenesis.[79] MMP-9 and estrogen also play a role in ECFC mobilization.[80-82] These mediators, as well as HIF-1α, SDF-1α and VEGF-A, were measured in blood and tissue samples of children with proliferating IH and were found to be elevated.[66,83] Moreover, low oxygen conditions have been shown to upregulate GLUT1 expression in various cell types.[84,85] Thus hypoxia may induce ECFC localization and homing to the IH lesion, where the microenvironment further supports their maturation and development.[86]

1.1.4 Current theories on the origin of IH

Various hypotheses have arisen in the past two decades regarding the origin of IH as investigations have been conducted. To date, it has been suggested that IH may arise from the placenta, from intrinsic defects or somatic mutations, or be induced by extrinsic factors. A unified hypothesis also exists combining both intrinsic and extrinsic theories. In the placenta theory, IH is believed to be caused by the embolization of placental precursors to the developing fetus from chorionic villus sampling (CVS) or placental trauma.[13,87,88] Similarities in molecular marker expression as well as developmental timelines reinforce this notion. For example, microvessels in both placental tissue and IH are immunoreactive for GLUT1, Lewis Y antigen, merosin, Fc-γ receptor-IIb, indoleamine 2,3-deoxygenase (IDO), and type III iodothyronine deiodinase (D3).[21,89] The placenta, like IH, undergoes a period of robust blood vessel proliferation and then stabilizes itself.[90] Mihm Jr. and Nelson (2010) have also added a 'metastatic niche' component to this theory, in which the placenta secretes substances preparing a site (or 'niche') for the homing and growth of IH precursor cells (in this case, placental precursors).[91] However, further investigation is needed to show a conclusive role of the placenta in the pathogenesis of IH.

The intrinsic theory speculates that genetic alterations in a progenitor cell are a contributing factor to hemangiogenesis.[69] Mutations in the 5q chromosome, which contains genes for EC proliferation and differentiation as well as tumour suppression, have been associated with IH development.[92] Missense mutations in VEGFR2, VEGFR3 and TEM8 were detected in IH cells but not in cells from normal adjacent tissue.[65,93] The progenitor origin of IH is further reinforced by nonrandom X-chromosome inactivation patterns exhibited in proliferating IH tissue, suggesting a monoclonal origin.[94] Additionally, elevated circulating ECFC levels have been documented in infants with IH and these ECFCs express GLUT1, merosin and CD32.[95] In 2008, Khan et al confirmed the progenitor origin of IH when CD133+ multipotential stem cells isolated from proliferating IH tissue were able to imitate all three stages of IH when implanted into mice.[54] The newly-formed blood vessels also expressed GLUT1 and merosin, adding further support to the intrinsic hypothesis of IH emergence.

Conversely, the extrinsic theory states that the microenvironment plays an essential role in inducing and regulating IH growth. Evidence shows that the epidermis overlying the tumour is altered in comparison to healthy, adjacent tissue.[96] Levels of IFN-β, an antiangiogenic regulator, were significantly lower in epidermis overlying proliferative phase IHs compared to normal skin. Furthermore, IFN-β levels increased as the lesions underwent involution, with levels reaching normalcy by 5 to 11 years of age. Although the source responsible for this change in IFN-β levels (ie. the epidermis or the IH tumour) was not determined, the authors suggest that the hyperplastic epidermis could very well play a role in disrupting angiogenic regulators in favour of IH development. Additionally, stromal cells in the tumour microenvironment may also influence IH growth by secreting VEGF[41,97] and by activating alternate angiogenic pathways.[98] Taken collectively, this evidence supports an extrinsic component in the development of IH.

Most probably, IH development is a multi-faceted process that involves both intrinsic and extrinsic components. We know that IH arises from HemSCs, which are multipotential stem cells that are able to form GLUT1-positive blood vessels *in vivo* and that later undergo adipogenesis to produce fibrofatty residuum.[54] Moreover, there is a higher incidence of IH in babies of mothers who underwent CVS.[99] It is supposed then, that HemSCs may become dislodged from the placenta during CVS and home to an area in the developing fetus permissive to IH development, where cell-secreted growth factors and low oxygen levels induce HemSC differentiation and growth.

1.2 Development of the circulatory system

Insights into the origin and pathogenesis of IHs can be gained by examining the development of the circulatory system. In mammals, the formation of blood vessels and blood cells occurs in parallel through processes called vasculogenesis and hematopoiesis, respectively.[100] Both events begin in the embryonic yolk sac (YS), where mesodermal cells form aggregates called blood islands. Here, the first primitive endothelial and hematopoietic cells emerge.[101] Due to their spatiotemporal similarity, there is a long-held

belief that both of these cell types emerge from a common bipotential precursor cell known as the hemangioblast.[102,103] In the YS, primitive ECs form the first vascular network, termed the capillary plexus,[100] of which the VEGF signaling pathway is the primary regulator.[104,105] Once the capillary plexus has formed, it begins to remodel itself through angiogenesis.[106] This process is carefully orchestrated by many molecular signaling pathways such as the Tie family of receptor kinases and the TGF-β superfamily.[107] Smooth muscle cells and pericytes are also recruited in order stabilize the rapidly-changing endothelium,[108] resulting in a highly organized network of arteries and veins that are structurally and functionally distinct. Through this remodeling process, each vascular component is able to serve a specialized purpose within the developing embryo.[107]

Concurrent with vasculogenesis is the process of hematopoiesis, in which hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs; distinct from HSCs with limited self-renewing capacity) give rise to all blood cells in the circulatory system. The emergence of these blood cell progenitors occurs in distinct waves and sites.[109] The first wave, termed 'primitive' hematopoiesis, transpires in the YS blood islands and produces nucleated erythroid progenitors.[109] Synchronous with the first embryonic heartbeat, it then quickly transitions into the second, 'definitive' wave. At this time, HSCs are produced *de novo* in the aorta-gonad-mesonephros region (AGM),[110] umbilical arteries,[111] and placenta;[112,113] however, the AGM and placenta contribute the most to the HSC pool.[114] Following definitive hematopoiesis, HSCs migrate to the fetal liver (FL), which becomes the principal hematopoietic organ for a considerable period during prenatal development. Shortly before birth, HSCs then home to the thymus, spleen, and finally the bone marrow (BM), which becomes the main source of HSCs after birth.[115]

1.2.1 Origins of hematopoietic progenitors

Similarities in marker expression and their synchronicity in appearance led to the postulation that endothelial and hematopoietic cells share a common precursor.[116-119] The earliest studies supporting the existence of the hemangioblast arise from work in chick

embryos, in which mesodermal cells selected for VEGFR2 could only give rise to endothelial or hematopoietic colonies, but not both.[120] Fetal liver kinase (FLK1, another term for VEGFR2), though not an exclusive marker, has been used as a means of potential hemangioblast isolation, as all hematopoietic and blood cells are derived from FLK1+ mesoderm.[121,122] Disruption of the *FLK1* gene in mice ablated the ability to form blood vessels and severely reduced hematopoietic stem and progenitor cell (HSPC) counts, supporting the existence of a hemangioblast-like cell.[123] Whilst the hemangioblast concept has been proven in various animal model systems such as the mouse and zebrafish,[102,124,125] the advent of human embryonic stem cells (hES) provided real insight as to whether the hemangioblast exists in humans. Work by Kennedy et al (2007) showed that early-stage hES cultures were able to form blast colonies with hematopoietic and endothelial potential.[126] In addition, these hES-derived blast cells (hES-BCs) were able to restore vascularization in ischemic areas when injected into mice.[127] Though not yet confirmed in hES, studies using murine-derived ES have elucidated the essential roles of runt-related transcription factor 1 (RUNX1), GATA binding protein 2 (GATA2), stem cell leukemia (SCL, typically referred to as SCL/TAL1), VEGFR2 and bone morphogenetic protein-4 (BMP4) transcription factors in generating hemangioblast-like colony forming cells.[122,128-131] Depending on intrinsic and extrinsic factors, the hemangioblast either gives rise to an endothelial-fated progenitor cell (ECFC or angioblast), or a HSC.[132,133] Originally, they were believed to exist only in the early stages of embryogenesis; however, recent evidence has emerged confirming the presence of hemangioblasts in the adult.[134,135] This exciting finding has major implications in elucidating not only how the adult body repairs itself during vascular injury, but also how it may contribute to vascular pathologies.

Alternatively, studies have shown an endothelial origin of HSCs. Namely, a subset of specialized ECs collectively known as the hemogenic endothelium (HE) gives rise to hematopoietic progenitors by forming intra-aortic clusters (IAC) or by budding.[136] Lineage tracing done by Zovein and colleagues (2008) revealed that, based on the expression of endothelial marker VE-cadherin, long-term, multilineage HSCs emerge from the ventral floor of the dorsal aorta of the AGM in a process known as endothelial-to-hematopoietic transition (EHT).[137] Once emerged, these HSCs migrate and seed

successive hematopoietic organs, where they differentiate. Indeed, multiple studies thereafter confirmed the AGM as a major source of HE in definitive hematopoiesis.[138-140] Currently, identification of HE has been based upon co-expression of endothelial and hematopoietic cell markers. Specifically, collective VE-cadherin, CD31, CD34 and CD45 positivity has been associated with hemogenic potential and HSC emergence.[109,138,141,142] Once HSCs have disengaged from the HE or HE-associated IACs, they progressively lose expression of EC marker VE-cadherin and upregulate the pan-hematopoietic marker CD45.[143,144]

Though the existence of HE has been consistently supported, the existence of a bipotential precursor giving rise to hematopoietic cells leaves researchers confused as to the true origin of HSCs. Originally, hemangioblasts were believed to exist only in the early stages of development, following which the HE became the main source of hematopoietic progenitors. Furthermore, questions remain unanswered regarding the origin of HE itself. Are ECs predetermined to have hemogenic potential, or do influences from the microenvironment instigate EHT in differentiated ECs? A middle-ground theory has also been proposed, in which HSCs emerge from the hemangioblast through an intermediate endothelial stage, the HE.[144,145] However, additional evidence is required to support this supposition.

1.2.2 Regulators of hematopoiesis

The process of hematopoiesis and EHT is complex and involves numerous pathways and factors. These include the VEGF, Wnt, NOTCH and BMP pathways;[146] fibroblast and angiopoietin growth factors;[147] transcription factors (TFs) RUNX1, GATA2, and SCL/TAL1; and the protein G protein-coupled receptor 56 (GPR56).[148-150] For my studies, I will focus on RUNX1, GATA2, SCL/TAL1, and GPR56.

During embryonic development, RUNX1 has been documented to be expressed in ECs of definitive hematopoietic sites – that is, the AGM, YS, the placenta, and in the umbilical and vitelline arteries – but not in ECs elsewhere.[151,152] Mice containing homozygous

mutations for RUNX1 show embryonic lethality at embryonic day 12.5 (E12.5) due to an inability of the embryo to undergo definitive FL hematopoiesis.[153] Moreover, recent studies have shown that RUNX1 is expressed in HE located in the AGM, umbilical and vitelline arteries, and placenta during definitive hematopoiesis,[101,154] as well as in IACs budding off of the HE.[138] However, once HSCs have emerged from HE, RUNX1 is no longer required for HSC maintenance.[155] Therefore RUNX1 is indispensable for HSC emergence, but not thereafter.

GATA2 is another pivotal regulator of hematopoiesis. This is highlighted by the fact that all HSCs and most HPCs express GATA2,[156] and that GATA2 knockout mice die at E10-11 due to severe anemia.[157] During hematopoiesis, GATA2 plays two functionally distinct roles: the production and expansion of HSCs in the AGM, and HSC proliferation in adult BM.[158] Both loss- and gain-of-function experiments have shown that GATA2 maintains HSC quiescence[159,160] and regulates their proclivity to apoptosis[161] – both of which are important in maintaining the HSC pool in embryonic and adult hematopoietic sites. It also conserves the immature state of HSPCs, as its expression decreases with differentiation.[162] The mechanism by which GATA2 accomplishes these roles is not yet fully defined; however, studies suggest that it forms a multiprotein complex with TFs SCL/TAL1, Fli-1 proto-oncogene (FLI1), LIM domain only 2 (LMO2), RUNX1, lymphoblastic leukemia associated hematopoiesis regulator 1 (LYL1) and ETS transcription factor ERG, collectively known as the HSC "heptad" (Figure 1.2).[163,164] This heptad binds to specific coding genes and microRNA promoters, activating a regulatory circuit that participates in lineage differentiation during hematopoiesis and EHT.[163-165] One of the downstream targets of the HSC heptad is GPR56. Whole transcriptome analysis done on mouse HSCs, hemogenic ECs, and ECs revealed that GPR56 is one of the most highly upregulated (38-fold) of the 530 genes investigated, along with the heptad TFs.[166] Furthermore, in human HSC-enriched cells, all seven heptad TFs were found bound to the GPR56 enhancer during EHT, thus regulating its expression. Though the exact function of GPR56 in hematopoiesis is currently unknown, studies suggest that it is required for HSC generation from HE,[166] for maintaining the HSC pool in BM,[150] as well as for HSC repopulation potential in HSC engraftment.[150,167] Despite these speculations however, contradicting evidence by Rao et al (2015) showed

that, despite high levels of expression in murine-derived HSCs, GPR56 knockout did not impair HSC proliferation or survival in mice.[168] Furthermore, GPR56-deficient HSCs were able to regenerate the hematopoietic system in BM of irradiated recipient mice to the same degree as wild-type HSCs. Hence the role of GPR56 in HSC development requires validation.

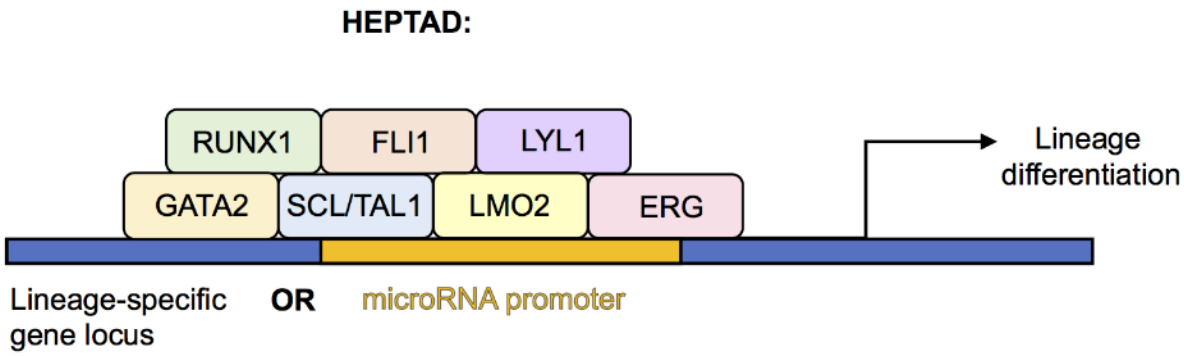


Figure 1.2 The heptad transcription factors control fate-determination of HSCs.

Schematic diagram illustrating the mechanism by which several TFs (RUNX1, FLI1, LYL1, GATA2. SCL/TAL1, LMO2, ERG) interact to form a heptad, which controls lineage differentiation in HSCs.

No combination of markers currently exist that explicitly define HSCs. Nevertheless, researchers have identified surface antigens that are consistently expressed on HSCs and that are thus used for their potential isolation. As described previously, HSCs derived from hematopoietic clusters in EHT share similar cell marker expression with the hemogenic ECs from which they are derived. The endothelial markers VE-cadherin,[169,170] CD31,[171,172] and CD34,[171,173] as well as the hematopoietic markers CD45,[174] GATA2,[116,175] SCL/TAL1,[116] and RUNX1[151] have been observed in both cell types. Other markers such as c-Kit[109,176] and CD150[177] have also been associated with HSC identity. However, these markers are not specific for HSCs and thus cell populations

isolated based on their expression are inevitably heterogeneous. Furthermore, the cell-surface phenotype changes as HSCs develop.[177] For these reasons, future studies are necessary in order to determine the true molecular signature of HSCs.

1.3 Organs involved in vascular development

1.3.1 The fetal liver

The fetal liver (FL) is a well-known site of definitive hematopoiesis. At approximately E11-12 in the mouse,[178] and day 23 and then again at day 30 in the human,[179] it becomes the primary source of HSCs in the embryo. These hematopoietic cells are believed to be derived from the YS and AGM; in other words, there is little evidence that the FL produces HSCs *de novo* or participates in EHT. Instead, the FL provides a niche, which is a local microenvironment that maintains the HSC pool through cellular interaction and factor secretion.[109,180] Chemoattractants such as C-X-C motif chemokine ligand (CXCL)12 in FL endothelium help regulate HSC homing,[181,182] while cell adhesion markers E-selectin and vascular cell adhesion molecule 1 (VCAM-1) control their retention from the circulation.[183] Once in the FL, ECs from the portal vessels encourage HSC survival and proliferation via activated protein C (APC).[184] Perivascular cells expressing Nestin and NG2 also contribute to HSC maintenance.[185]

During embryogenesis, the FL develops a complex vascular network that arises from *de novo* vessel generation and angiogenesis.[186] In the early stages of development, ECs arise from endodermal progenitor cells.[187] However, studies have also identified a cohort of FL-derived progenitor cells that express ECFC markers and are able to form endothelial colonies *in vitro*.[188,189] A study by Cherqui et al (2006) confirmed their existence when CD31+Sca1+ cells isolated from the FL possessed high angiogenic potential when transplanted into mice.[190] In this study, CD31 served as a marker of ECs and Sca1 as the stem cell antigen. Interestingly, ECs derived from murine FL at E12 (during the definitive hematopoiesis time frame) have demonstrated similar robust neovascularization abilities *in vivo*.[191] During liver organogenesis, the development of the

hepatic vascular network is regulated by VEGF, integrin interactions and extracellular matrix proteins.[192] Hence it is evident that the FL is not only a major site of hematopoiesis, but also of vascular development.

1.3.2 The bone marrow

As the skeletal system develops in the embryo, blood vessels invade and provide circulation throughout the developing bone. In mice this occurs at E12.5[193] and in human at about 10.5 weeks.[179] This vascular intrusion allows for homing of HSCs from the FL, and from hereon the BM becomes the primary source of HSCs postnatally. However, unlike FL-HSCs which are highly proliferative, those in the BM are largely quiescent.[194] This is to maintain an appropriate number of differentiated blood cells in the adult and renew HSCs on an as-needed basis.

In the BM, HSCs localize to sinusoids, where they interact with various cell types that regulate their quiescence and differentiation state.[185,195] These include osteoblasts,[196,197] perivascular cells,[198] ECs,[199] mesenchymal stem cells (MSCs),[200] adipocytes,[201] and neurons of the sympathetic nervous system.[202] Many of these cells express stem cell factor (SCF) and/or CXCL12, which are critical for HSC maintenance.[198,203,204] In addition to ligand-receptor interactions, the BM houses HSCs in the endosteum, which is a relatively hypoxic environment.[205-207] Though the mechanism by which low oxygen tension maintains HSC quiescence is not yet fully understood, studies suggest it could be by regulating factors important for hematopoiesis as well as for cell cycle progression. For example, hypoxia appears to upregulate expression of VEGF,[208] SCF,[209] and NOTCH1,[210] all of which are involved in HSC maintenance, by oxygen-sensitive HIF-1α. HIF-1α is also important in maintaining HSCs in G_0, as it upregulates cell-cycle inhibitors $p21^{Cip1}$[211] and $p57^{kip2}$[212]. Furthermore, the low-oxygen environment appears to protect HSCs from reactive oxygen species (ROS).[213,214] Therefore both the molecular and physical components of the BM make it an ideal microenvironment for adult HSCs.

Emerging evidence suggests that the BM is not only a source of HSCs, but also of ECFCs. These ECFCs can be mobilized from the BM, enter the circulation and travel to sites of ischemia and/or injury where they undergo neovascularization.[215,216] Shi et al (2008) isolated cells based on CD45 expression from human BM and cultured them in media supplemented with basic FGF (bFGF), insulin-like growth factor-1 (IGF-1), and VEGF.[189] They found that, after 15 to 20 days in culture, the CD45+ cells formed adherent EC-like colonies. Furthermore, these cells expressed vWF and incorporated acetylated low-density lipoprotein (LDL). The presence of ECFCs in the BM has also been repeatedly investigated *in vivo*. In these studies, cells isolated for certain endothelial and hematopoietic markers were implanted into animals with organ-specific ischemic injuries. In all cases, implanted ECFC-like cells restored the vasculature at the sites of injury,[217-219] reaffirming a vasculogenic role of precursor cells in the BM.

1.3 Rationale

Previous work done in our laboratory has shown that IH arises from multipotential stem cells, termed hemangioma stem cells (HemSCs).[54] However, the origin of HemSCs has not been elucidated. During embryogenesis, blood vessels and blood cells are believed to develop in parallel at various sites from a common bipotential precursor cell called a hemangioblast.[220] Based on the developmental time and the microenvironment, these hemangioblasts commit to becoming hematopoietic or endothelial precursors.[221,222] It was originally believed that these hemangioblasts are present only in mesoderm and in the early developing embryo (that is, the yolk sac blood islands),[223] and that angioblasts and hemogenic endothelium give rise to endothelial and hematopoietic cells, respectively, for the remainder of embryonic development and in adult life.[103,224] However, recent evidence suggests that adult hemangioblasts do in fact exist, and are able to form ECs both *in vitro* and *in vivo.*[103,135,225-227] Given that IHs arise from HemSCs with vasculogenic but not hematopoietic potential, I wanted to investigate whether HemSCs show similarity to the hemangioblast, and if so, what mechanisms are involved in its endothelial versus hematopoietic commitment. By doing so, I aimed to provide novel insight into the origin of HemSCs and how they develop into IH.

1.4 Hypothesis

I hypothesize that *IH stem cells will express markers of hematopoiesis but will be incapable of undergoing hematopoiesis.* If true, my studies will support the idea that IH represents abnormal homing of a bilineage mesodermal precursor, specifically along the migration from fetal liver to bone-marrow (Figure 1.3).

1.4.1 Specific Aims

In order to test my hypothesis, I established the following two aims:

1. To compare the expression profile of HemSCs and normal stem/progenitor cells, including bone marrow-mesodermal progenitor cells (BM-MPCs) and fetal liver stem cells (FLSCs).

2. To compare the differentiation potential of HemSCs, BM-MPCs, and FLSCs.

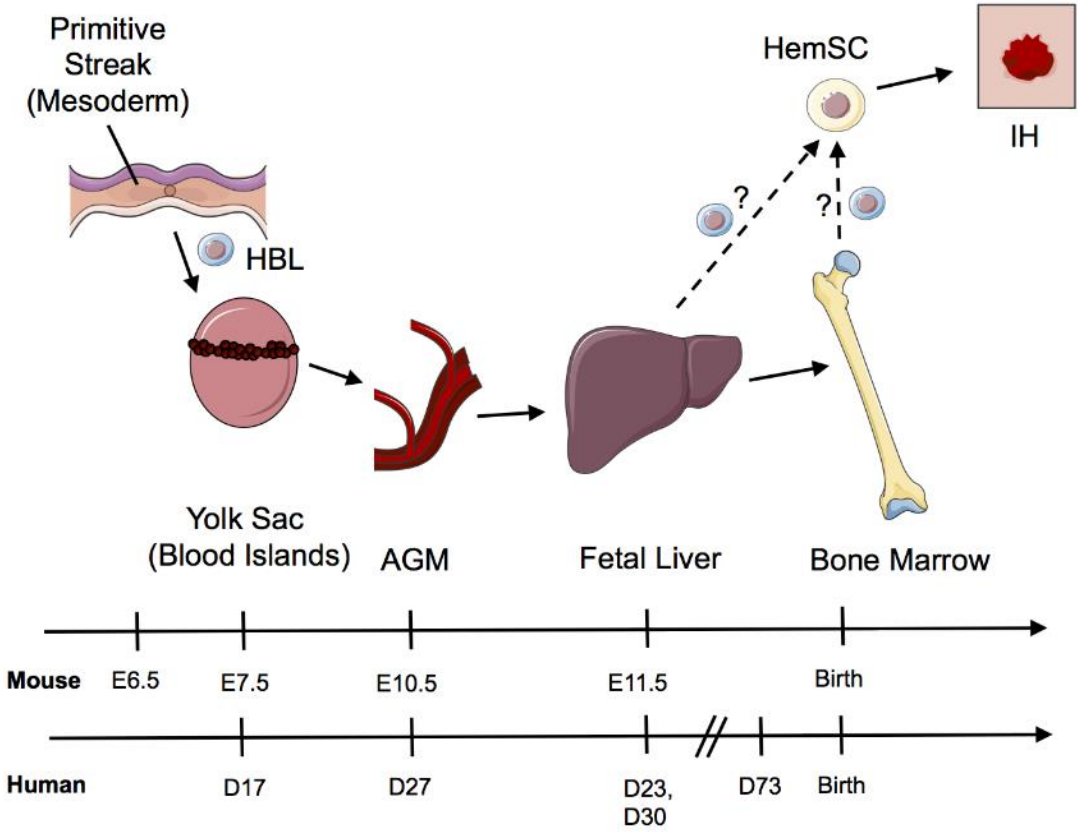


Figure 1.3 Stepwise migration of the hemangioblast during embryogenesis, potentially giving rise to IH.

The hemangioblast may divert from its path either from the fetal liver before birth, or from the bone marrow postnatally. E = embryonic day; D = day; HBL = hemangioblast; AGM = aorta-gonad-mesonephros region; HemSC = IH-derived stem cell; IH = infantile hemangioma

Chapter 2

2 Materials and Methods

2.1 IH cell culture

CD133-selected cells from proliferating IH specimens (HemSCs) were kindly provided by Dr. Joyce Bischoff (Children's Hospital Boston, Boston, MA). We have previously characterized these cells through RT-PCR, immunostaining, and cellular activity assays.[54] Bone marrow mesenchymal progenitor cells (BM-MPCs) were isolated from bone marrow mononuclear cell (BM-MNC) preparations (2M-125B, Lonza Inc., Walkersville, MD). For my hematopoiesis assay, fresh BM-MNCs were graciously given by Dr. David Hess (Western University, ON). Both BM-MPCs and BM-MNCs were used as normal stem/progenitor cell controls. CD133-selected human fetal liver cells (FLSCs) were obtained from Applied Biological Materials (Richmond, BC, Canada) and were also used as normal stem cell controls. All cells were cultured in complete EBM-2 media (Lonza, Walkersville, MD) supplemented with 20% fetal bovine serum (Lonza), EGM-2 SingleQuots (CC-4176, Lonza Inc.) and 1X antibiotic antimycotic media (PSF; Life Technologies). EGM-2 SingleQuots contain epidermal growth factor, VEGF-A, IGF-1, bFGF, hydrocortisone, ascorbic acid, and gentamycin/amphotericin B. Hereinafter this media is called EBM-2/20% FBS. Media was changed every other day. Cells were cultured under identical conditions and kept in an incubator with 5% CO_2 at 37^{o}C. Three biological replicates were used for my IH samples taken from three separate patients, whereas BM-MPCs and FLSCs had one biological replicate.

2.2 RNA isolation and RT-PCR

RNA was isolated using the RNeasy Micro Plus Kit (Qiagen, Mississauga, ON) and measured using Qubit RNA Broad Range Assay in a Qubit Fluorometer (Life Technologies). cDNA was then synthesized using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) in T100 Thermal Cycler (Bio-Rad). Genes associated with stem/pluripotent, endothelial, mesenchymal, hematopoietic and adipogenic cell lineages were analyzed, as were markers of EHT, using individual primers outlined below by RT-PCR (Table 2.1). Each RT-PCR reaction (individual gene) consisted of 10 µL RT^2 SYBR Green qPCR Mastermix (Qiagen), 2 µL primer mix (Qiagen), 1 µL cDNA, and 7 µL of H_2O for a total reaction volume of 20 µL. All reactions were performed for 40 cycles following the RT^2 protocol: 95°C for 10 minutes (initial denaturation and polymerase activation); and 60°C for 1 minute (annealing and extension). Gene expression was analyzed by CFX Manager Software (Bio-Rad Laboratories, Inc.) using the normalized ($\Delta\Delta$CT) method with β-actin as the housekeeping gene. Analysis via RT-PCR included three experimental replicates and one technical replicate per RNA sample. Melting curve analysis was performed to ensure specific amplification. For pluripotency genes, RNA from human embryonic stem cells H9 (ScienCell, Catalogue # 5825) was amplified and melting temperatures of amplicons were compared with HemSCs, BM-MPCs, and FLSCs. For mesenchymal genes, RNA from human umbilical artery smooth muscle cells (Lonza Inc., Catalogue # CC-2579) was amplified and melting temperatures compared. Lastly, for endothelial cell genes, RNA from human neonatal dermal microvascular endothelial cells (Lonza Inc., Catalogue # CC-2516) was used as control.

Table 2.1 List of primers used for RT-PCR

GENE	Description	Source (Catalogue #)
Stem Cell and Pluripotency Phenotype		
CD133	Cluster of differentiation 133, Prominin-1	Qiagen (QT00075586)
c-KIT	Tyrosine-protein kinase Kit	Qiagen (QT00080409)
DPPA4	Developmental pluripotency associated 4	Qiagen (QT00046515)
GDF3	Growth differentiation factor 3	Qiagen (QT00014952)
LEFTY1	Left-right determination factor 1	Qiagen (QT00037373)
NANOG	Nanog homeobox	Qiagen (QT01025850)
OCT4/POUF5	Octomer-binding transcription factor-4	Qiagen (QT00210840)
PODXL	Podocalyxin like	Qiagen (QT00005138)
SOX2	Sex determining region Y-box 2	Qiagen (QT00237601)
ZFP42	Zinc-finger protein-42	Qiagen (QT00051009)
Endothelial Phenotype		
CD34	Hematopoietic/endothelial cell surface glycoprotein	Qiagen (QT00056497)
VEGFR2/KDR	Vascular endothelial growth factor receptor 2	Qiagen (QT00069818)
CD31	Platelet endothelial cell adhesion	Qiagen (QT00081172)
VE-Cadherin	Vascular endothelial-cadherin	Qiagen (QT00013244)
vWF	von Willebrand factor	Qiagen (QT00051975)
Mesenchymal Phenotype		
NT5E/CD73	5'-nucleotidase	Qiagen (QT00027279)
ENG	Endoglin	Qiagen (QT00013335)
NGFR	Nerve growth factor receptor	Qiagen (QT00056756)
THY1	Thy-1 cell surface antigen	Qiagen (QT00023569)
Hematopoiesis & EHT Phenotype		
ALCAM	Activated leukocyte adhesion molecule	Qiagen (QT00026824)
PTPRC/CD45	Protein tyrosine phosphatase receptor C	Qiagen (QT00028791)
ETV6	ETS variant 6	Qiagen (QT00074648)
FEV	FEV, ETS transcription factor	Qiagen (QT00215887)

GATA2	GATA binding protein 2	Qiagen (QT00045381)
GATA3	GATA binding protein 3	Qiagen (QT00095501)
RUNX1	Runt related transcription factor	Qiagen (QT00026712)
SCL/TAL1	Stem cell protein (SCL/TAL1, T-cell acute lymphocytic leukemia protein 1)	Qiagen (QT00012530)

Adipogenic Phenotype

C/EBPα	CCAAT/enhancer-binding protein α	Qiagen (QT00203357)
C/EBPβ	CCAAT/enhancer-binding protein β	Qiagen (QT00237580)
C/EBPδ	CCAAT/enhancer-binding protein δ	Qiagen (QT00224357)
PPARγ	Peroxisome proliferator activated receptor γ	Qiagen (QT00029841)
FABP4	Fatty acid binding protein 4	Qiagen (QT01667694)
LEP	Leptin	Qiagen (QT00030261)

Housekeeping Gene

β-actin	Housekeeping gene, beta-actin	Qiagen (QT01680476)

2.3 Immunofluorescent cell staining

All cells were plated at 20,000 cells/cm^2 density on 4- or 8-chambered slides for 48 hours prior to staining to allow for adherence. Cells were fixed with methanol and then stained for hematopoietic and progenitor cell markers using primary antibodies for one hour at room temperature, with the exception of CD45 (Table 2.2). Primary antibodies were diluted in phosphate-buffered saline (PBS) with 1% bovine serum albumin. Following primary incubation, cells were incubated with Fluorescein-conjugated secondary antibody (Vector Laboratories, Burlingame, CA) for one hour at room temperature. Slides were then counterstained and mounted with ProLong® Diamond Antifade Mountant with DAPI (Life Technologies). Images were taken using Olympus BX-51 fluorescent microscope (Olympus Canada Inc., Richmond Hill, ON) and SPOT Basic Image Capture & SPOT Advanced Microscope Imaging Software (SPOT Imaging Solutions, Sterling Heights, MA). Images were acquired at the same exposure for each antigen. Staining intensity was measured by NIH Image J software (https://imagej.nih.gov/ij/). Measurements were double-normalized, first to background levels (areas on respective slides without cells), and then to negative control slides (no primary antibody).

Table 2.2 Primary antibodies used for immunofluorescence staining

Antigen	Host	Source (Catalogue #)	Dilution
GATA2	Rabbit	Santa Cruz (sc9008)	1:200
GPR56	Rabbit	Invitrogen (720373)	1:200
SCL/TAL1	Mouse	eBioscience (E17791-101)	1:200
CD45	Rabbit	Abcam (ab10558)	1:200
CD150	Rabbit	Abcam (ab156288)	1:200
RUNX1	Rabbit	Abcam (ab189153)	1:200

2.4 IH specimens and immunostaining

All studies were conducted following approval by the Research Ethics Board at Western University, London, Ontario, Canada. Paraffin-embedded IH specimens were obtained from the Department of Pathology Tissue Archives at the London Health Sciences Centre (LHSC, London Ontario, Canada). The proliferating phase of IH specimens was confirmed through medical history and histological analysis of densely packed capillaries. In addition, all IH sections were immunostained with GLUT1 to confirm diagnosis. Tissue blocks were sectioned at 5 µm thickness. Sections were deparaffinized in xylene, hydrated in ethanol gradient, and subjected to antigen retrieval using Tris/EDTA buffer (10 mM Trizma-base, 1 mM EDTA, 0.05% Tween-20, pH 9.0) in 2100 Retriever (Electron Microscopy Sciences, Hatfield, PA).

I used two separate cases of proliferating IH for my immunostaining experiments. Slides were blocked with 5% horse serum for 30 minutes. Without washing, primary antibodies outlined in Table 2.2 were added at 1:100 dilution for 1 hour at room temperature. Following primary antibody incubation, fluorescein- or Alexa Fluor 488-conjugated secondary antibodies (Vector Laboratories, Burlington, ON) were used for detection. Lectin from *Ulex europaeus*-Atto 594 conjugate (Ulex, Sigma-Aldrich) was used to stain for ECs. Ulex europaeus I is a lectin specific for some alpha-L-fucose-containing glycocompounds which are highly expressed on ECs.[228] Slides were counterstained with DAPI (Vector Laboratories). Images were taken using the Olympus BX-51 microscope (Olympus Canada Inc.).

Control specimens for staining comprised of early gestation human placenta (22-25 weeks) specimens, pyogenic granuloma specimens (highly vascular tumour commonly used in IH studies), and human skin specimens. Only one case for each control specimen was used. Negative controls for staining experiments were performed without the primary antibody added.

2.5 Differentiation assays

To assess adipogenic differentiation potential, HemSCs, BM-MPCs and FLSCs were first expanded in culture. Cells were then seeded into 24-well plates at a density of 50,000 cells/cm^2 in EBM2/20% growth media. Adipocyte differentiation assays typically involve plating cells at sub-confluent densities. However, this assay incorporates both early mitotic burst and then differentiation.[229] To specifically assess adipogenic differentiation in HemSCs and normal counterparts, I elected to plate cells at confluent densities to minimize the contribution of different growth kinetics. After plating and allowing cells to adhere for 24 hours, growth media was replaced with StemPro Adipogenesis Differentiation Media (Adipo media; Life Technologies) or control media, which consisted of Adipogenesis Basal Media supplemented with 10% FBS and 1x PSF. Both differentiation and control media was changed every other day. After 7 days, cells were stained with LipidTOX (Thermo Fisher) for the presence of lipid droplets. RNA was also isolated at this time to perform RT-PCR for transcription factors involved in adipogenesis (see Table 2.1).

Hematopoiesis was induced in HemSCs, BM-MPCs and FLSCs using MethoCult media (StemCell Technologies). Optimal cell seeding density was determined by testing seeding densities at 10K, 25K, 50K, and 100K cells/mL of Methocult media. Cells were mixed with MethoCult media using a 1 mL syringe/16-gauge blunt end needle and dispensed into 35 mm plates in duplicate cultures. After 14 days cultures were assessed for the presence of blast colony-forming units (BFUs) under phase-contrast microscopy.

2.6 Statistical analysis

Most of the studies are qualitative and focused on the detection of phenotype-associated transcripts and antigen immunoreactivity in cells and tissues. For these studies, appropriate positive and negative controls were included to validate the results. Statistical analysis was performed on quantitative mRNA data from the differentiation assay. In this

case, a students' t-test was performed to compare induction media to respective control media. A p value of less than 0.05 was considered significant.

Chapter 3

3 Results

3.1 Transcript profiling reveals multilineage priming of IH-derived stem cells

My first objective was to assess the expression of genes associated with various lineages in CD133-selected cultures of HemSCs. For these studies, I utilized normal BM-MPCs and CD133-selected FLSCs cultured under identical conditions for comparisons. First, I used RT-PCR to confirm the expression of stem and pluripotency genes in HemSC cultures from three patient samples (Hem106, Hem127, and Hem115), alongside BM-MPCs and FLSCs. Analysis of CD133 transcripts showed undetectable levels in all cell types studied (Figure 3.1). This is not surprising as CD133 is readily lost as soon as cells adhere to culture dishes following isolation from tissues. However, both HemSCs and FLSCs were confirmed to be positive for CD133 mRNA following isolation (data not shown). My results also show that all IH cell preparations expressed genes which exclusively mark pluripotent cells, including the Yamanaka transcription factors OCT4, SOX2 and NANOG.[230,231] In addition, PODXL and DPPA4 were also expressed in HemSCs. It was interesting to find undetectable levels of TGF-β family members GDF3 and LEFTY1 in HemSCs, perhaps indicating some level of differentiation in cultured cells.

Next, I investigated the expression of mesodermal lineage genes in HemSCs. Mesoderm gives rise to muscle, connective tissue, dermis and subcutaneous layer of the skin, bone and cartilage, endothelium of blood vessels, hematopoietic cells, as well as the kidneys and the adrenal cortex. I expected multiple mesodermal genes to be expressed in HemSCs as well as the positive controls BM-MPCs and FLSCs. I utilized the same experimental platform to detect transcripts of various mesodermal lineages to determine whether HemSCs show priming towards a specific lineage. I observed expression of most mesodermal markers including ALCAM, ENG, CD73/NT5E, and THY1 in HemSCs (Figure 3.2A). NGFR expression was inconsistent, being present in only two out of three IH cell preparations, and in FLSCs but not BM-MPCs. Analysis of endothelial cell genes

showed that HemSCs express CD31, CD34, and KDR/VEGFR2 (Figure 3.2B). CD34 was detected in all three patient samples of HemSCs and FLSCs but not BM-MPCs, whereas CD31 was positive in HemSCs and BM-MPCs but not FLSCs. Only VEGFR2 expression was similar among all cell types. However, there was no detection of fully mature and functional ECs, otherwise indicated by VE-CADH or vWF expression, in HemSCs. Previous studies from our laboratory have shown that HemSCs only acquire markers of fully differentiated ECs upon implantation in mice.[54] This suggests that the signal for proper endothelial differentiation are missing *in vitro* and may explain the lack of detectable VE-cadherin and vWF transcripts.

Given that my HemSC samples expressed genes indicating a stem cell phenotype, I wanted to explore the possibility that they exhibit hematopoietic and EHT-related genes. Interestingly, I found that ETV6, GATA2, and RUNX1 were present at similar levels between all cell types (Figure 3.3). GPR56 was present in all three patient samples of HemSCs and in FLSCs but not in BM-MPCs. Furthermore, there was no detection of SCL/TAL1 mRNA in any of the cell lines tested. HemSCs clearly lacked the expression of GATA3, which was robustly expressed in FLSCs and BM-MPCs. GATA3 has been shown to be expressed in long-term hematopoietic stem cells (LT-HSCs), which are deeply quiescent HSCs capable of reconstituting all hematopoietic cell lineages indefinitely in irradiated mice.[232] In the study by Frelin et al (2013), GATA3 induces LT-HSCs to exit from cell cycle quiescence and decreases their long-term reconstitution ability. When GATA3 was deleted however, LT-HSCs exhibited enhanced regenerative activity and self-renewal.[232] Hence the absence of GATA3 expression in HemSCs may point to a mechanism underlying the self-renewing and undifferentiated nature of these IH-initiating cells.

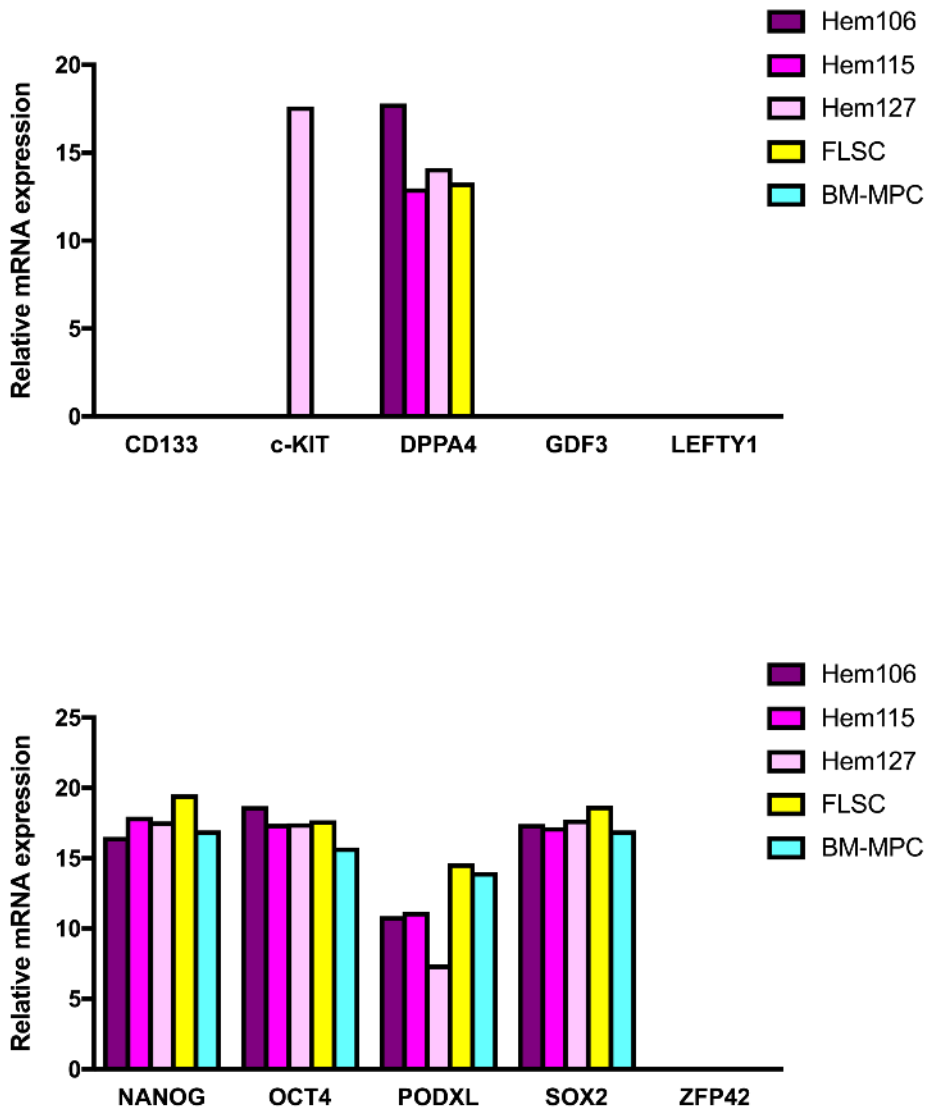


Figure 3.1 Expression of pluripotency-associated genes in IH stem cells.

RT-PCR analysis was done on CD133-selected cultures of HemSCs from three different patients (Hem106, Hem115, Hem127), BM-MPCs and FLSCs at cell passages 8-10. The specificity of the amplification was determined by melting curve analysis. Data is normalized to β-actin expression. Data expressed as means. Three biological replicates were used for HemSCs and one biological replicate for BM-MPCs and FLSCs. The experiment was repeated three times using one technical replicate. The figure represents typical results.

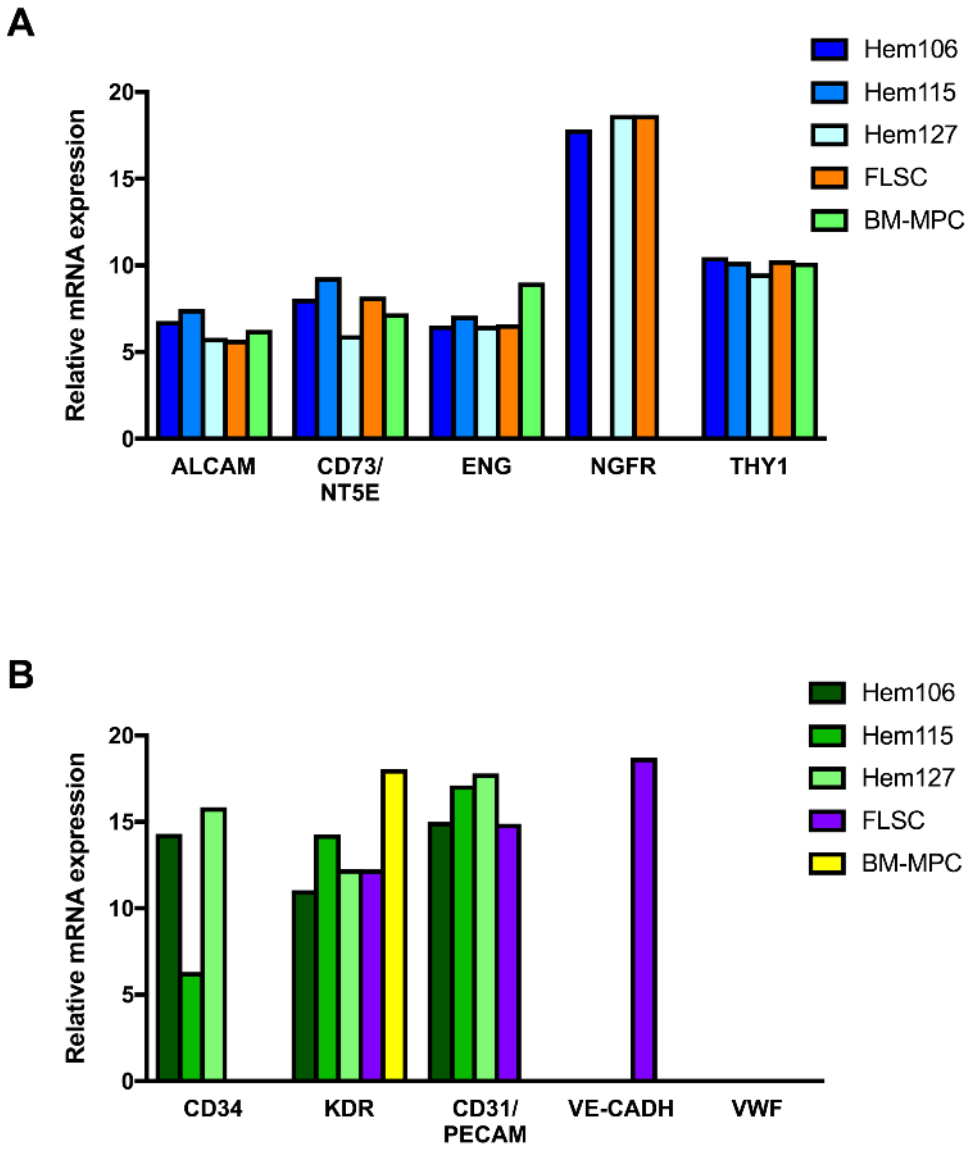


Figure 3.2 Detection of mesenchymal- and endothelial cell-selective genes in HemSCs.

RT-PCR analysis was done on CD133-selected cultures of HemSCs from three different patients (Hem106, Hem115, Hem127), BM-MPCs and FLSCs at cell passages 8-10. The specificity of the amplification was determined by melting curve analysis. Data is normalized to β-actin expression. Data expressed as means. Three biological replicates were used for HemSCs and one biological replicate for BM-MPCs and FLSCs. The

experiment was repeated three times using one technical replicate. The figure represents typical results.

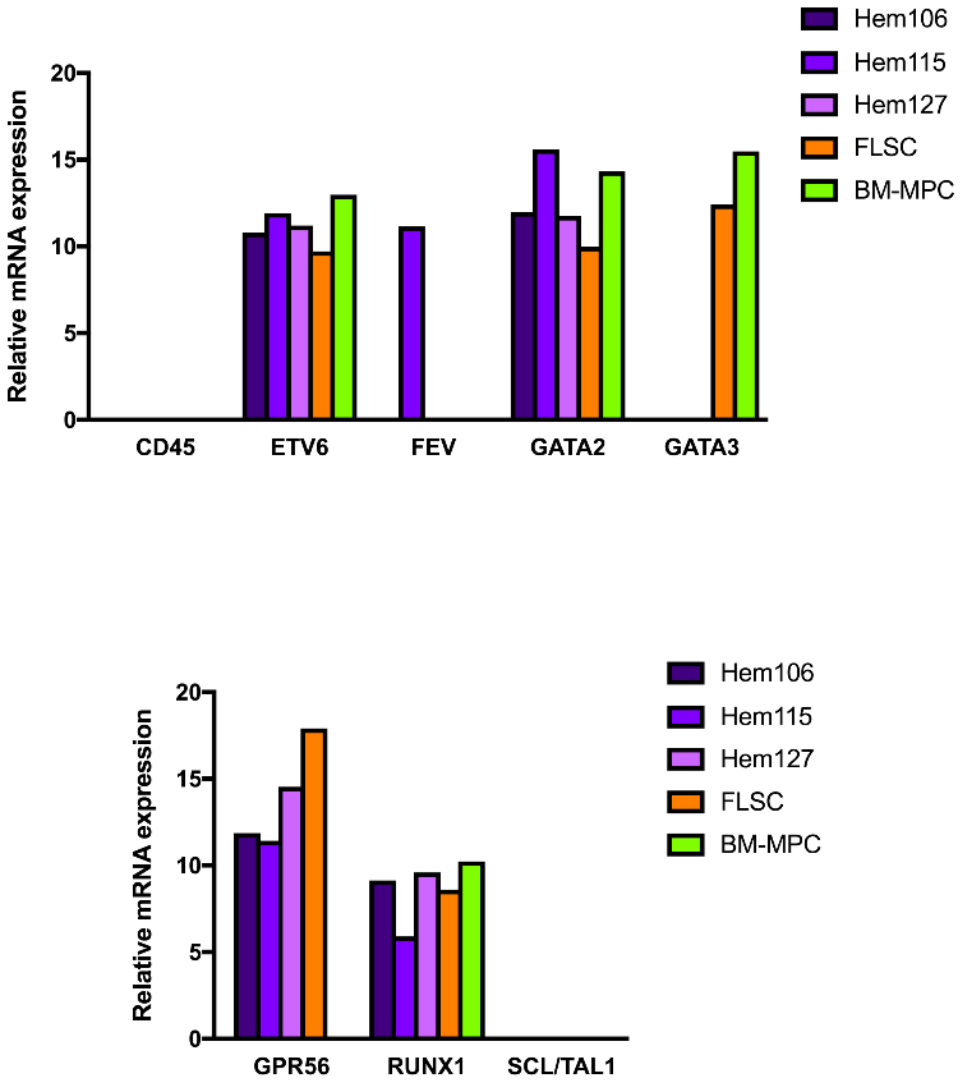


Figure 3.3 Expression of genes associated with hematopoiesis and EHT.

RT-PCR analysis was done on CD133-selected cultures of HemSCs from three different patients (Hem106, Hem115, Hem127), BM-MPCs and FLSCs at cell passages 8-10. The specificity of the amplification was determined by melting curve analysis. Data is normalized to β-actin expression. Data expressed as means. Three biological replicates were used for HemSCs and one biological replicate for BM-MPCs and FLSCs. The experiment was repeated three times using one technical replicate. The figure represents typical results.

3.2 IH stem cells express hematopoietic-lineage proteins

Based on my gene expression findings, I stained HemSC, FLSC and BM-MPC for hematopoietic and EHT antigen expression. Current literature indicates that RUNX1, GATA2, GPR56, and SCL/TAL1 are master regulators of hematopoiesis,[233] and CD150 is a marker found on all HSCs.[195] My results show that HemSCs are immunopositive for RUNX1, GPR56, and CD150 (Figure 3.4 and Figure 3.5). Consistent with my gene analysis study, SCL/TAL1 was absent in all cell types. RUNX1 showed nuclear localization and CD150 was localized to the cell plasma membrane, as expected**.** Quantification of staining intensity confirmed these findings, reaffirming RUNX1, GPR56 and CD150 expression but not GATA2 and SCL/TAL1 in all cells investigated (Figure 3.5). Surprisingly, I observed GPR56 immunopositivity in cell nuclei rather than membranes, which was unanticipated given that GPR56 is an adhesion receptor and thus contains a particularly elongated extracellular domain.[234] Furthermore, GPR56 is a G protein–coupled receptor (GPCR) and the traditional model of GPCRs entails a cell membrane localization where these proteins activate heterotrimeric G proteins and their intracellular signaling pathways. However, it should be noted that this model is not able to account for GPCRs, G proteins, and their downstream effectors that are found on the nuclear membrane or in the nucleus. Nuclear localization of GPR56 in these stem cells may be a readout of increased cycling and proliferation in culture.

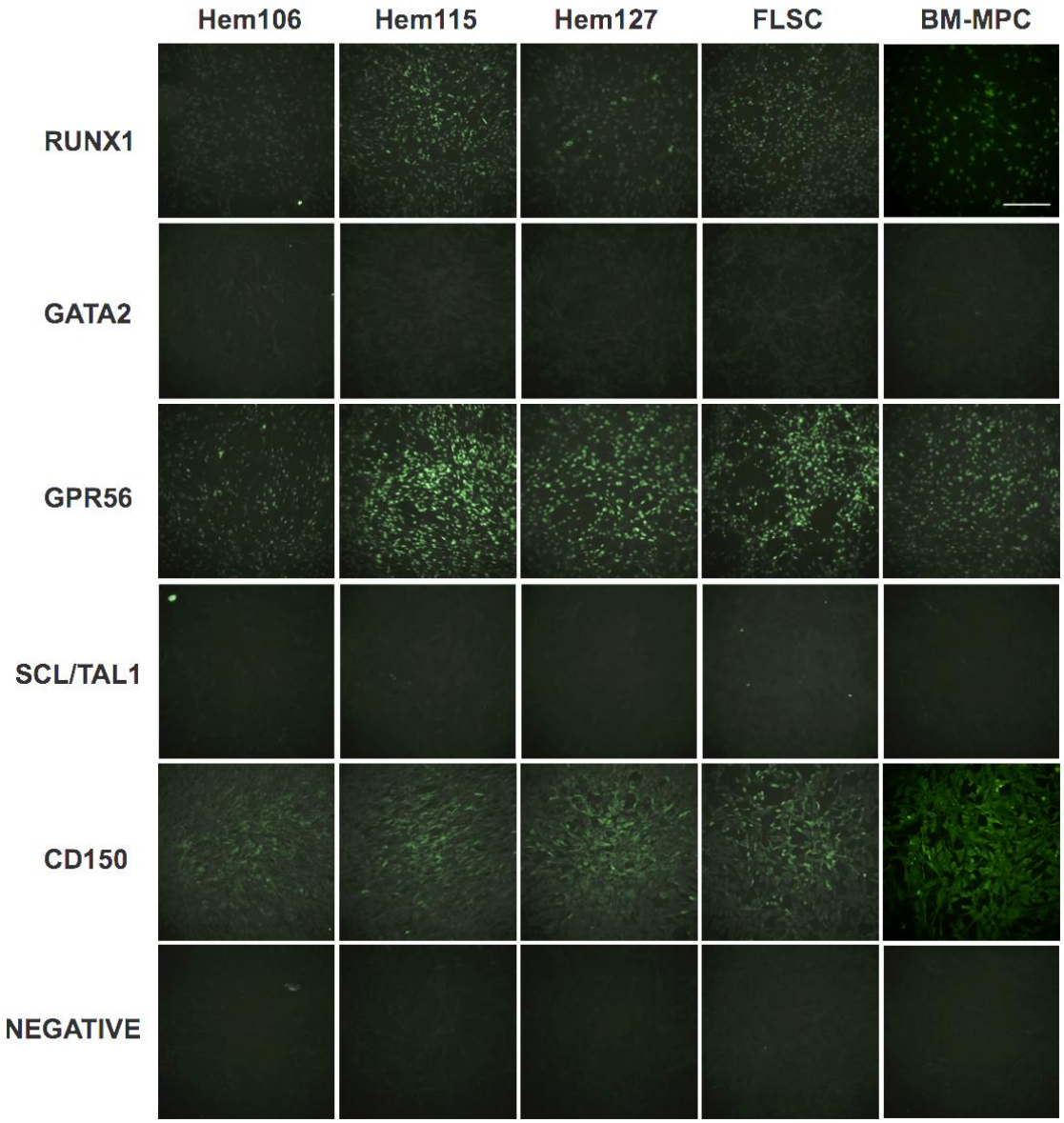


Figure 3.4 Immunofluorescence staining for markers associated with hematopoiesis and EHT in HemSC.

Three IH samples (Hem106, Hem115, Hem127), FLSCs and BM-MPCs were seeded on glass slides and stained using primary antibodies against RUNX1, GATA2, GPR56, SCL/TAL1 and CD150 (green). All cells were seeded at a density of 20,00 cells/cm^2 and cultured for 48 hours prior to staining. Experiments were carried out with cells at passages 8-10. Images were taken at 20x magnification. Scale bar represents 200μM. Figure is representative of images captured at multiple fields of view.

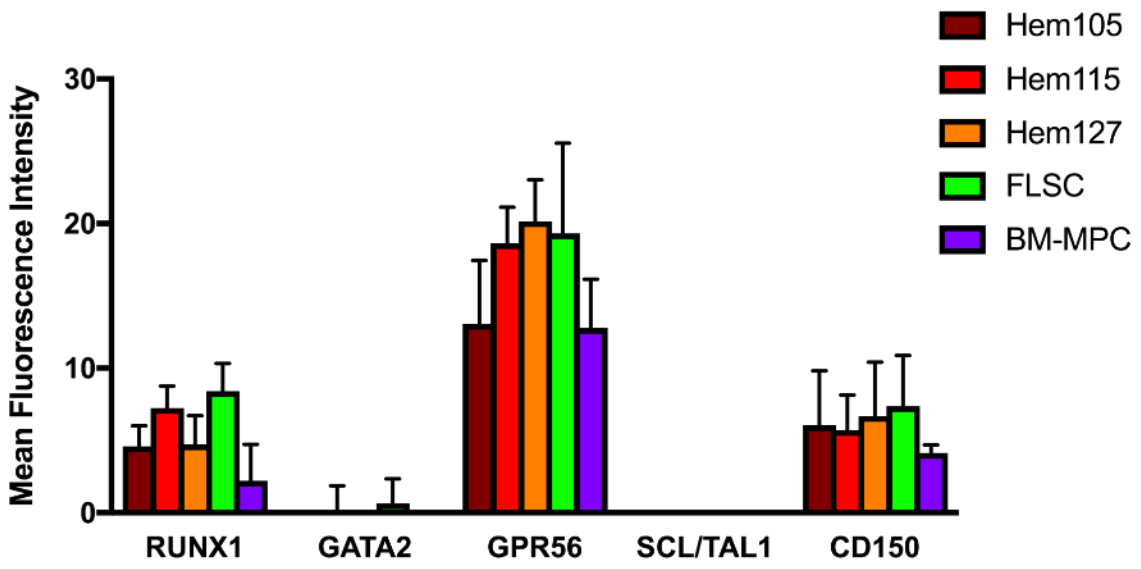


Figure 3.5 Fluorescence intensity analysis of hematopoietic and EHT antigens.

Immunofluroescence staining intensity of hematopoiesis- and EHT-antigens was measured using Image J. RUNX1, GPR56 and CD150 were expressed in all cell types with no discernable difference. GPR56 displaying strong immunoreactivity in all cell types. GATA2 and SCL/TAL1 expression was absent upon normalization to negative control (no primary antibody). Data expressed as mean ± SD.

3.3 IH blood vessels express hematopoietic stem cell markers

Previous work in our lab has shown that IH originates from HemSCs, which give rise to GLUT1-positive microvessels.[54] Whether IH vessels express proteins associated with hematopoietic lineage is not currently known. However, my *in vitro* data showed that some hematopoiesis- and EHT-associated genes are expressed in HemSCs. Therefore, I stained proliferating IH tissues for HSC markers CD45 and CD150. Surprisingly, samples obtained from 2 different IH patients demonstrated CD45 and CD150 reactivity localized to ECs (shown in yellow; ECs stained with Ulex) (Figure 3.6). Both CD45 and CD150 almost exclusively marked ECs in IH specimens. Intrigued by this finding, I speculated whether expression of HSC markers in IH endothelium was indicative of EHT. Thus, I also stained for EHT markers RUNX1, GATA2, SCL/TAL1 and GPR56. I observed RUNX1 and GATA2 positivity in ECs lining IH vessels as well as in perivascular cells (Figure 3.7A, B). Both GATA2 and RUNX1 showed typical nuclear localization. Furthermore, both IH samples were immunoreactive to GPR56, and expression was found in ECs, similar to CD45 and CD150 (Figure 3.7C). SCL/TAL1 was not detected in either patient samples (Figure 3.7D), confirming my cell staining and gene analysis findings.

Since GLUT1, the diagnostic marker for IH, has also been shown to be expressed in placenta,[235] I wanted to compare my IH sample findings to placental tissue (22-25 weeks). Staining of placenta specimens showed CD45 and CD150 reactivity primarily in the synctiotrophoblasts (Figure 3.8). Rarely, CD150 was observed in ECs present in the mesenchyme. RUNX1 and GATA2 stained scattered cells in the mesenchyme but showed no reactivity in ECs marked by Ulex labelling. Similar results were obtained for GPR56 showing robust positivity in the mesenchyme.

To bolster my findings, I also stained pyogenic granuloma tissues, which is a vascular lesion similar to IHs (often referred to as ‘eruptive’ or ‘lobular’ IH) and commonly used in IH research for comparisons. Recent molecular studies have shown that pyogenic granuloma exhibits high enrichment for gene ontology corresponding to vasculature development.[236] Furthermore, ECs in pyogenic granuloma also express OCT4, SOX2, and NANOG.[237] Therefore, I reasoned that pyogenic granuloma ECs will share the

expression of key hematopoietic and EHT-related proteins with IHs. As expected, I found antigen staining of pyogenic granuloma to parallel results obtained from IH specimens (Figure 3.9). CD45, CD150, and GPR56 displayed co-localization to the ECs in pyogenic granuloma. In addition, RUNX1 and GATA2, although expressed, did not display immunopositivity in the endothelium. These transcription factors were found primarily in the interstitium.

For my immunostaining studies, I used human adult skin tissues as a negative control for hematopoietic markers. None of the hematopoietic cell markers were seen in ECs (Figure 3.10). Although CD45 expression in human skin (both fetal and postnatal) has been reported in the literature,[238,239] no discernable staining was seen in Ulex-marked ECs. RUNX1 and GATA2 showed reactivity in the epidermal layer. However, the pattern of staining (non-nuclear) suggested background signal rather than specific staining. In addition, GPR56 was found in the dermal layer, but again no reactivity was seen to co-localize with Ulex marking.

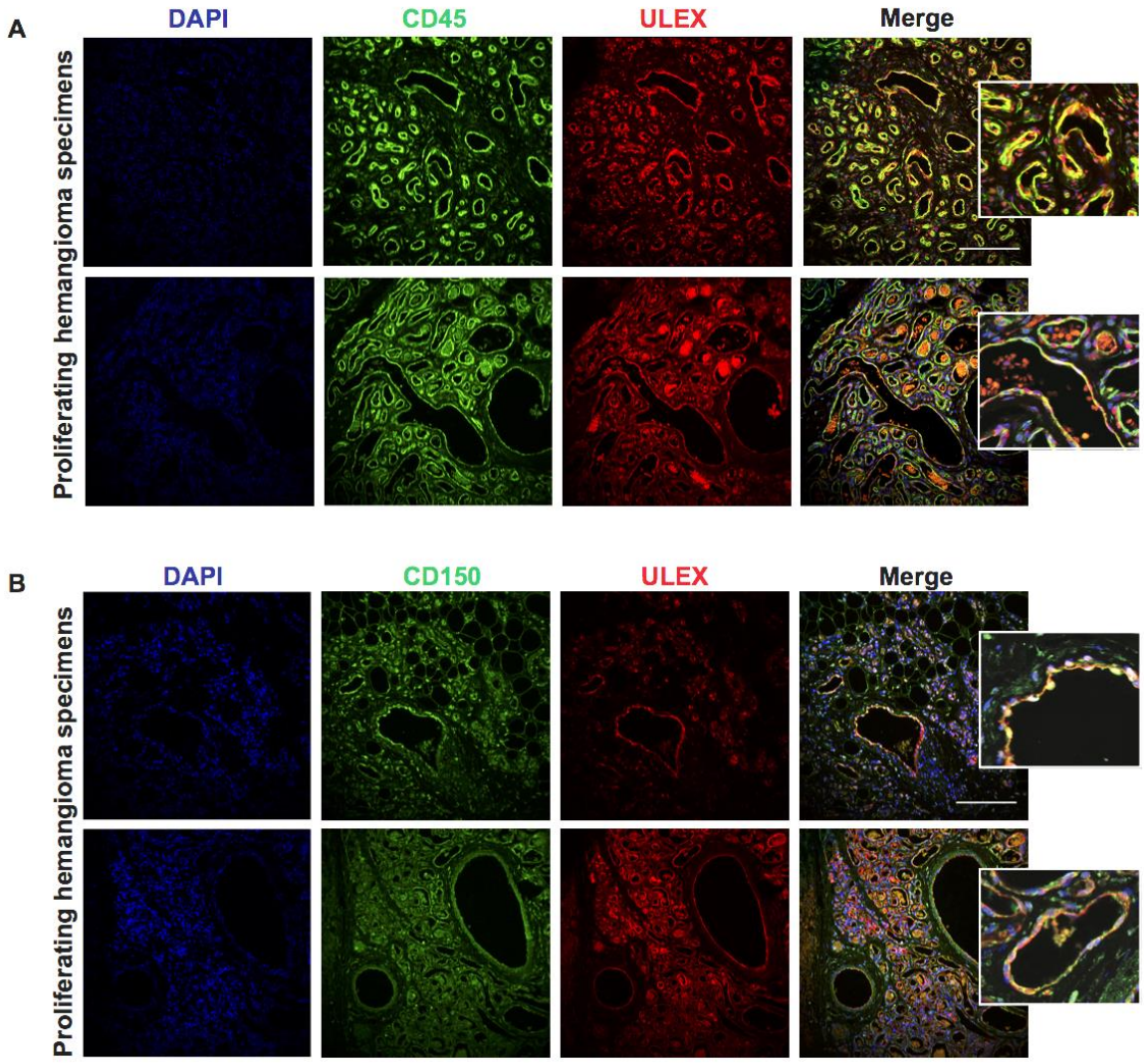
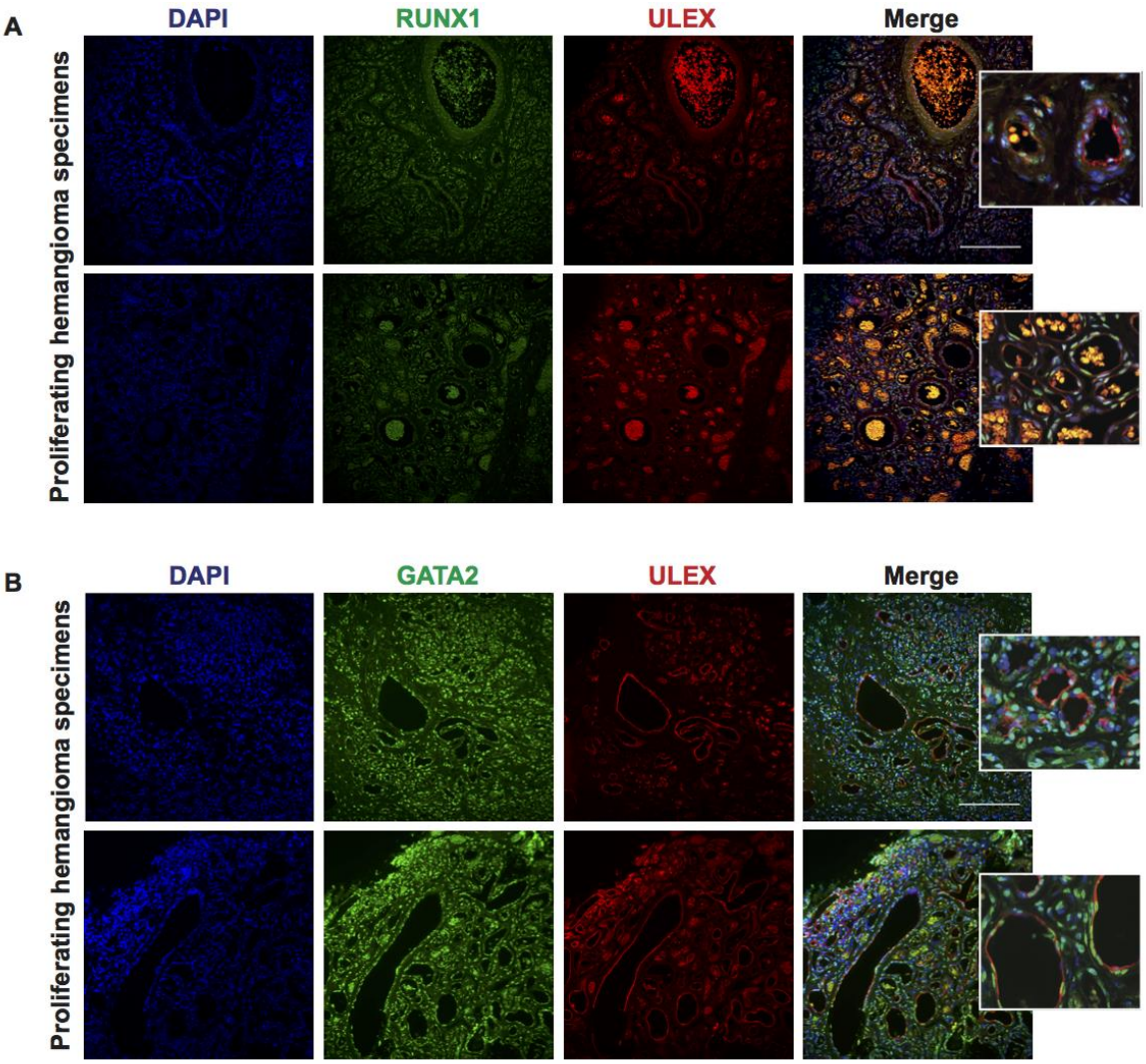


Figure 3.6 ECs in IH specimens express HSC markers CD45 and CD150.

IH sections were labeled with antibodies against (A) CD45 and (B) CD150 (green), Ulex for endothelial cells (red), and DAPI for nuclei (blue). Primary antibody and Ulex co-localization is shown as yellow. Images were captured at multiple fields of view at 20x magnification. Scale bar represents 200μM. Figure represents typical results for two cases of proliferating IH.


A
DAPI
RUNX1
ULEX
Merge
Proliferating hemangioma specimens
B
DAPI
GATA2
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Merge
Proliferating hemangioma specimens

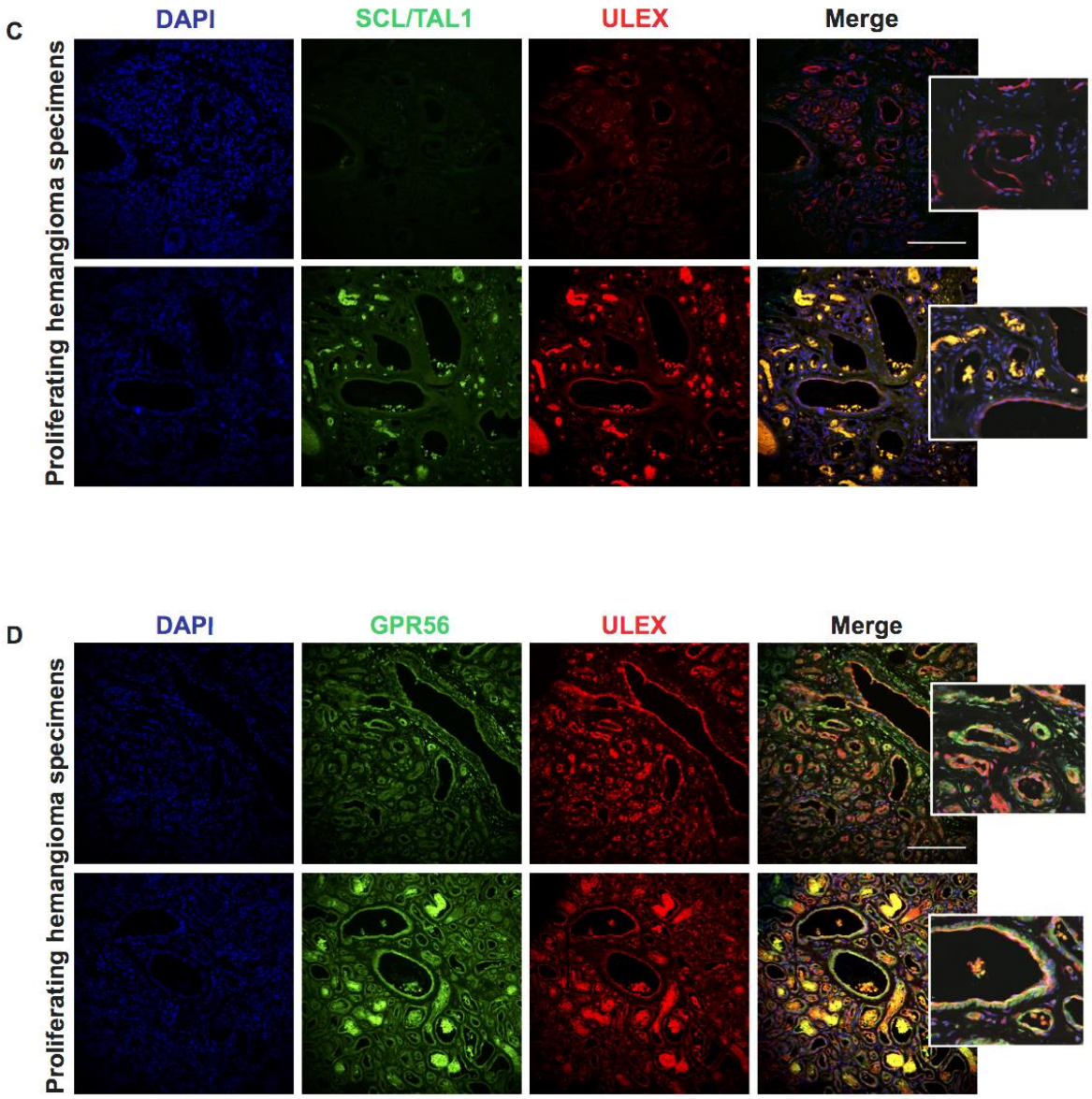

Figure 3.7 RUNX1, GATA2 and GPR56 expression in IH specimens.

IH sections were labeled with antibodies against (A) RUNX1, (B) GATA2, (C) SCL/TAL1 and (D) GPR56 (green), Ulex for ECs (red), and DAPI for nuclei (blue). Primary antibody and Ulex co-localization is shown as yellow. Images were captured at multiple fields of view at 20x magnification. Scale bar represents 200μM. Figure represents typical results for two cases of proliferating IH.

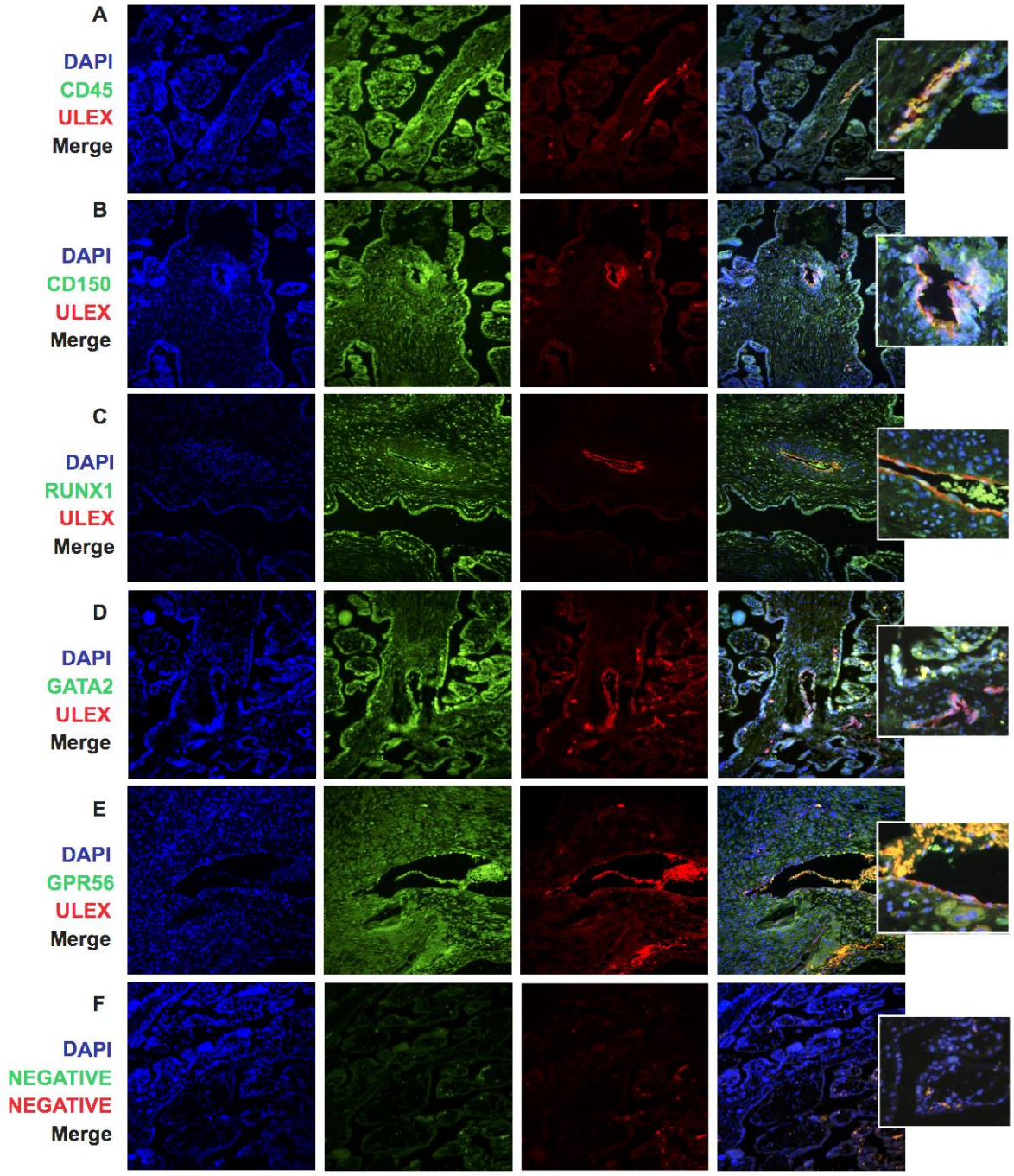

Figure 3.8 HSC and EHT immunofluorescence staining of placenta specimens.

Placenta tissue (22-25 weeks) was labeled with antibodies against (A) CD45 (B) CD150 (C) RUNX1, (D) GATA2, (E) GPR56 (green), Ulex for ECs (red), and DAPI for nuclei (blue). Primary antibody and Ulex co-localization is shown as yellow. Images were

captured at multiple fields of view at 20x magnification. Scale bar represents 200μM. Figure represents typical results for one case of placenta tissue (22-25 weeks).

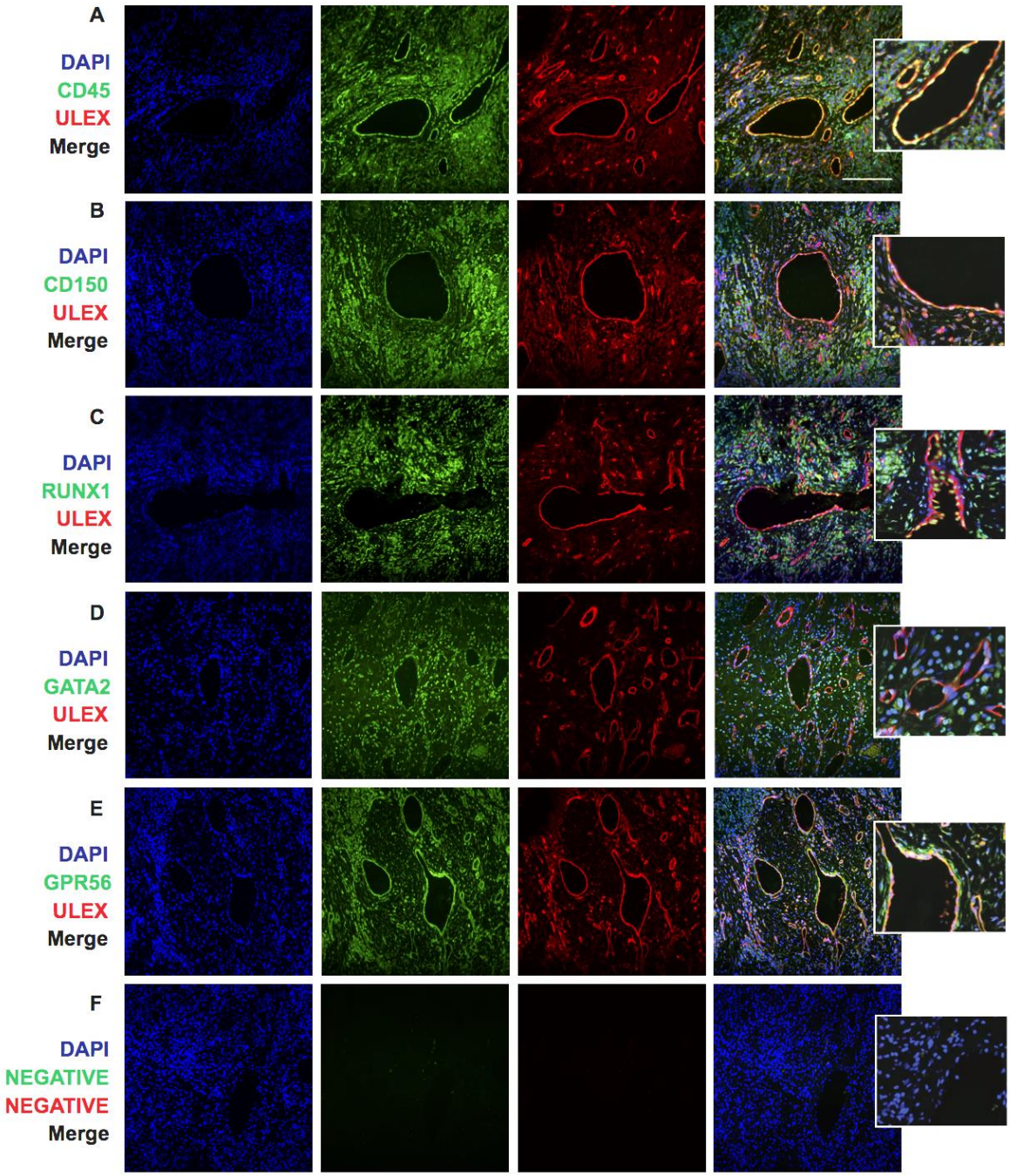

Figure 3.9 HSC and EHT immunofluorescence staining of pyogenic granuloma.

Pyogenic granuloma samples were labeled with antibodies against (A) CD45 (B) CD150 (C) RUNX1, (D) GATA2, (E) GPR56 (green), Ulex for ECs (red), and DAPI for nuclei (blue). Primary antibody and Ulex co-localization is shown as yellow. Images were

captured at multiple fields of view at 20x magnification. Scale bar represents 200μM. Figure represents typical results for one case of pyogenic granuloma.

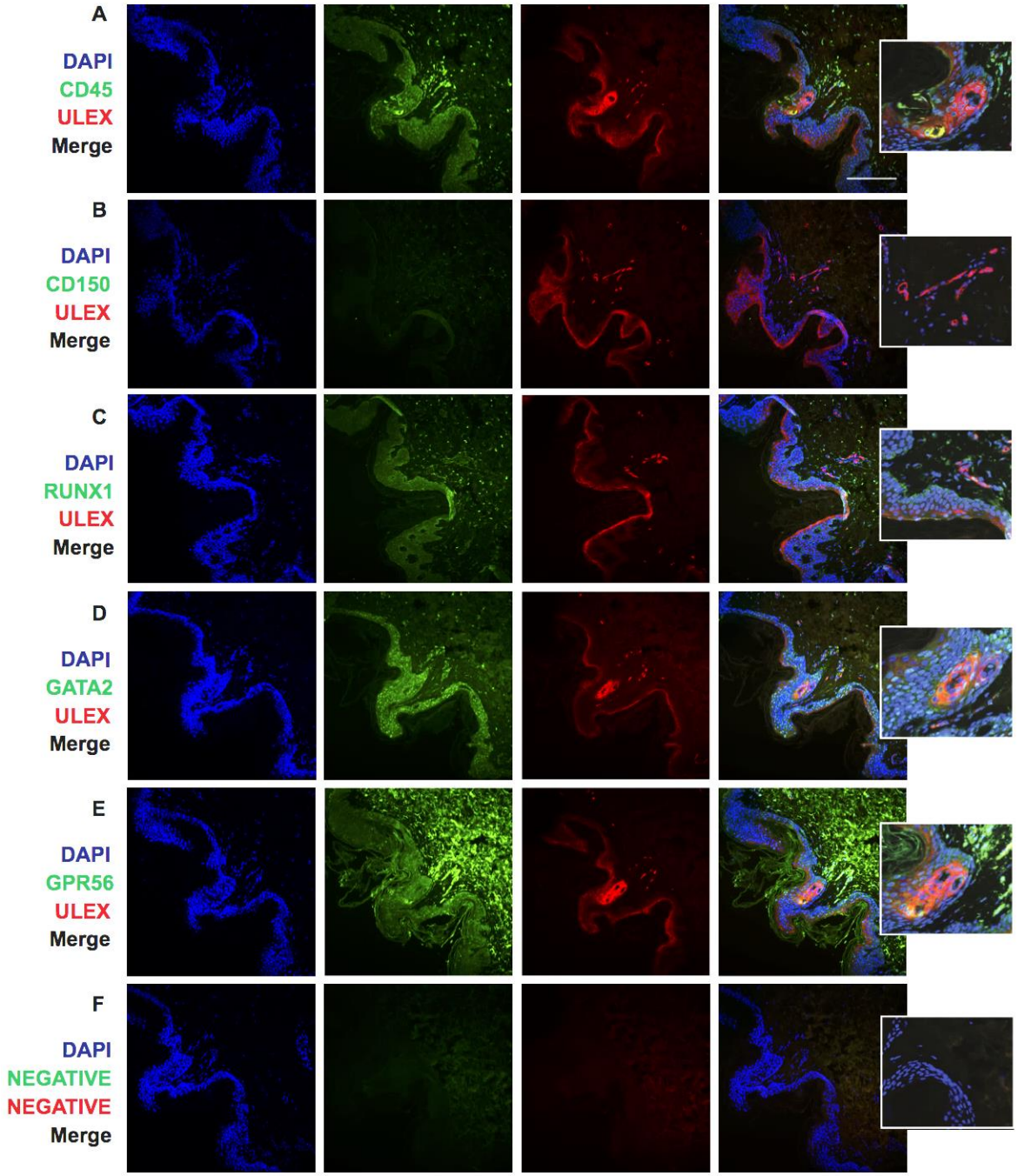

Figure 3.10 HSC and EHT immunofluorescence staining of human adult skin.

Human adult skin samples were labeled with antibodies against (A) CD45 (B) CD150 (C) RUNX1, (D) GATA2, (E) GPR56 (green), Ulex for ECs (red), and DAPI for nuclei (blue). Primary antibody and Ulex co-localization is shown as yellow. Images were

captured at multiple fields of view at 20x magnification. Scale bar represents 200μM. Figure represents typical results for one case of human adult skin.

3.4 IH stem cells lack hematopoietic differentiation ability *in vitro*

Our laboratory has previously shown that HemSCs are able to differentiate into mesenchymal cells (adipocytes, osteocytes, chondrocytes) and ECs, but cannot undergo hematopoietic differentiation as assessed by traditional *in vitro* assays.[54,240] Based on my findings that HSC and EHT markers are expressed in HemSCs and in proliferating IH tissues, I wanted to confirm the selective differentiation potential of HemSCs.

First, I induced adipogenesis in HemSC cultures and analyzed for lipid droplet formation and adipocyte gene expression, using BM-MPCs as control. I found that HemSCs displayed robust lipid formation, similar to BM-MPCs, whereas FLSCs did not (Figure 3.11). I then utilized RT-PCR to confirm adipogenic differentiation in HemSCs by measuring CCAAT/enhancer-binding proteins (C/EBP)-α, -β, and -δ, peroxisome proliferator-activated receptor gamma (PPARγ), Leptin, and fatty acid binding protein 4 (FABP4) mRNA levels. C/EBPs (α, β, δ) and PPARγ are transcriptional factors required for adipogenic differentiation. Once cells fully differentiate into adipocytes, late markers such as Leptin and FABP4 are induced. As expected from the LipidTOX staining results, HemSCs showed upregulation of C/EBPδ and PPARγ compared to the control/non-inducing media. Interestingly, however, I also observed increased adipocyte marker expression in FLSCs. Though no studies have investigated the adipogenic potential of CD133-selected FLSCs, a recent study by Wang et al (2016) showed that mesenchymal stem cells (MSCs) isolated from fetal liver are unable to differentiate into adipocytes.[241] Hence my studies may perhaps suggest that FLSCs respond to external pro-adipogenic stimuli, but their ability to accumulate lipid droplets (as seen by the complete absence of LipidTOX positivity) is inhibited.

I then sought to determine whether HemSCs can differentiate into hematopoietic lineages. We have previously shown that this may not be the case.[54] However, one key piece of information was missing previously. My gene expression analyses showed variable expression of select genes which may point to differential ability of HemSCs for hematopoietic differentiation. For example, Hem127 expressed NGFR (Figure 3.2) but lacked detectable mRNA for FEV (Figure 3.3). Whereas, Hem115 lacked NGFR but

expressed FEV. NGFR has been shown to be expressed on non-hematopoietic stem cells in the marrow.[242] Furthermore, FEV is designated as a key fetal hematopoiesis regulator.[243] Therefore, I tested Hem115 and Hem127 for hematopoietic differentiation ability and used bone marrow-derived mononuclear cells (BM-MNCs) as my positive control. My results show that after 14 days of induction in Methocult media, no blast colony-forming units (BFUs) are produced in either HemSC cultures (Figure 3.13). Similarly, no hematopoietic activity was observed in FLSCs. These results suggest that *in vitro* culture of HemSCs possibly strips the cells of hematopoietic activity or that hematopoietic activity may be inhibited in HemSCs.

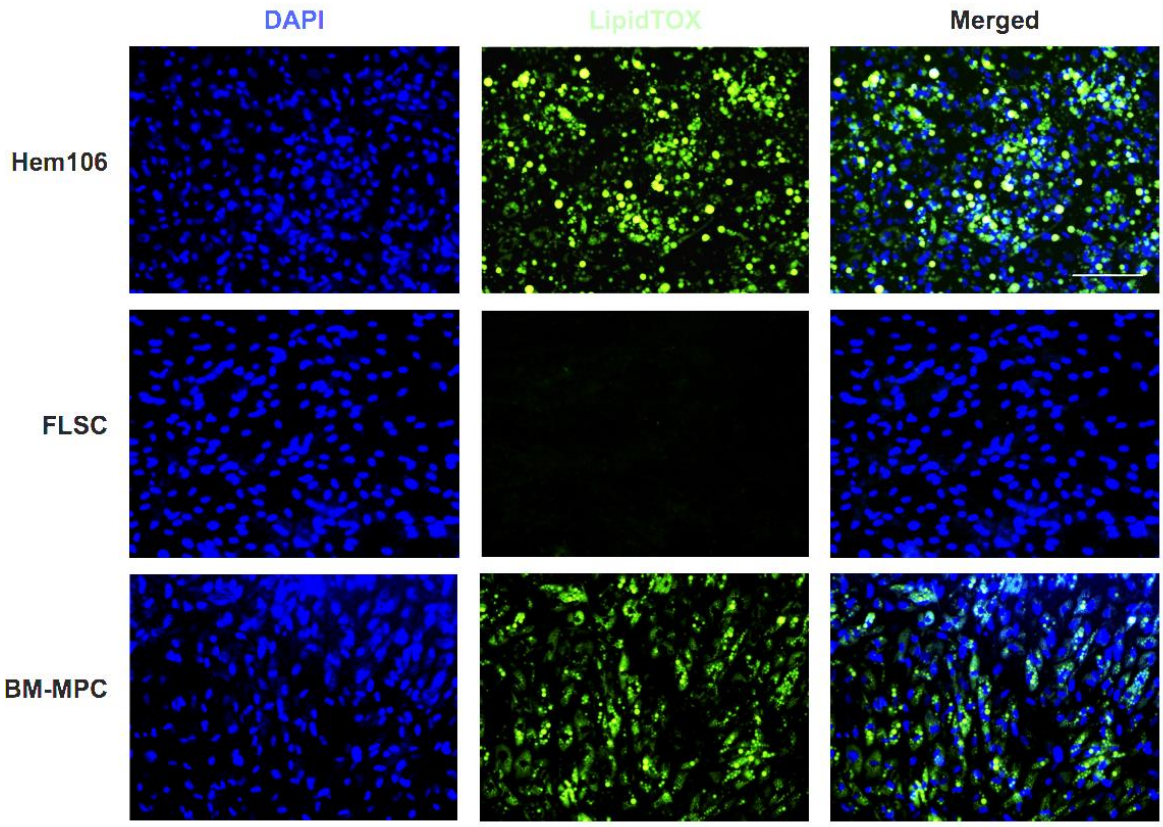

Figure 3.11 HemSCs are able to differentiate into adipocytes similar to BM-MPCs.

HemSCs (Hem106), FLSCs and BM-MPCs were cultured in adipocyte induction media for 7 days before staining for lipid droplets with LipidTOX (green). BM-MPCs were used as a positive control. Cells were at passages 6-8 at the time of induction. Images were taken at multiple fields of view at 4x magnification. Scale bar represents 100μM. Figure represents typical results.

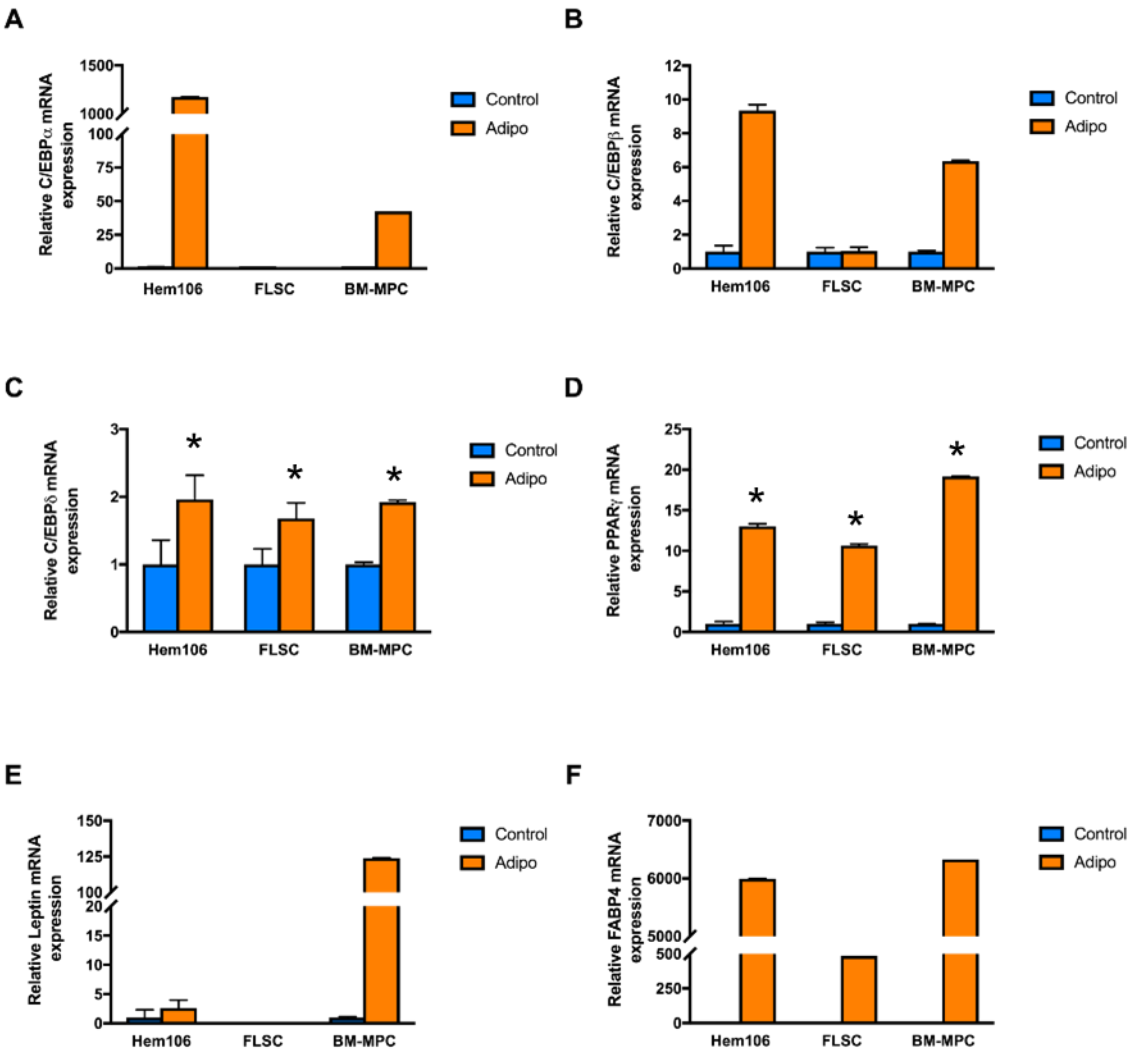

Figure 3.12 Induction of adipogenesis-specific transcription factors in HemSCs upon differentiation.

HemSCs (Hem106), FLSCs and BM-MPCs were cultured in adipocyte induction media and control media (basal adipogenic media without differentiation factors) for 7 days prior to gene analysis. Markers specific for adipocytes were assessed: (A) C/EBPα, (B) C/EBPβ, (C) C/EBPδ, (D) PPARγ, (E) Leptin, and (F) FABP4. Values represent fold-change expression relative to control media. BM-MPCs were used as a positive control. Cells were at passages 6-8 at the time of induction. Data expressed as mean ± SD. *$p<0.05$ compared to respective control media.

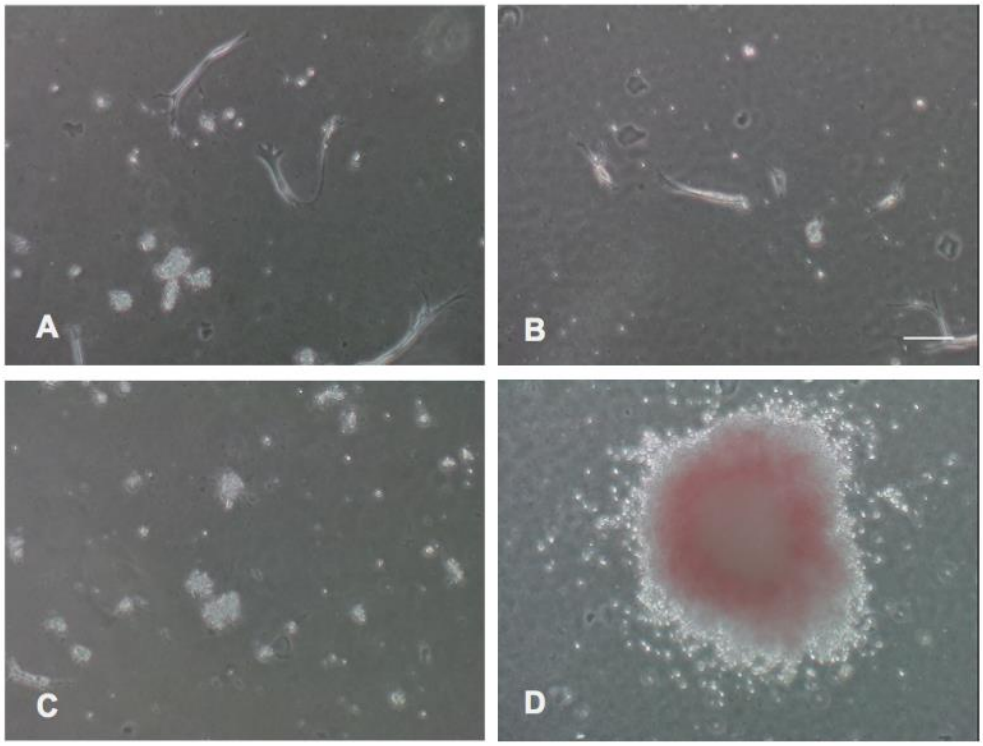

Figure 3.13 HemSCs are unable to form hematopoietic colonies *in vitro*.

(A) Hem127, (B) Hem115, (C) FLSCs and (D) BM-MNCs were plated at a seeding density of 10,000 cells/mL of Methocult induction media. After 14 days, cultures were analyzed for the formation of blast colony-forming units (BFUs), such as seen in BM-MNCs (D). Cells were at passages 6-8 at the time of induction, except for BM-MNCs, which were at passage 0. Images were taken at multiple fields of view at 4x magnification. Scale bar represents 100μM. Figure represents typical results.

Chapter 4

4 Conclusions

4.1 Discussion

One of the key findings of my studies is the expression of hematopoiesis-related genes in IH-derived stem cells (HemSCs). I show that HemSCs express genes of mesenchymal, endothelial, and hematopoietic lineages in culture, as expected of multipotential stem cells. Surprisingly, my immunostaining experiments showed co-localization of CD45, CD150, GATA2, and RUNX1 in ECs lining IH vessels in proliferating IH tissue samples. However, HemSCs lack hematopoietic activity when assessed in culture. These findings have provided greater insight into the phenotype of IH-initiating cells and may lead to the discovery of the cellular origin of IHs.

I first set out to investigate the transcript profile of HemSCs. I screened for genes characteristic of pluripotent stem, mesenchymal, and endothelial cells. As expected, HemSCs expressed NANOG, OCT4, and SOX2, which are transcription factors known to maintain pluripotency and have been implicated in various malignancies.[244-246] In a seminal study, SOX2 in conjunction with OCT4, c-MYC, and KLF4 was found to be sufficient for producing induced pluripotent stem cells from mouse cells.[247] Furthermore, hypermethylation of binding sites and downregulation of SOX2 and OCT4 strips pluripotency in germ cells.[248] In my studies, I also show that HemSCs expressed the pluripotency markers PODXL and DPPA4, which reaffirms their multipotential capabilities.

Despite being selected for CD133, relative expression of this gene was undetectable in all cell types. However, previous studies have reported that CD133+ progenitor cells no longer express CD133 antigens once they adhere in culture.[249-251] I also found that HemSCs expressed markers typically associated with mesenchymal stem cells (MSCs). Given the ability of IH-derived cells to differentiate into adipocytes during the involuting phase, this finding is not surprising. When cultured with respective induction media, HemSCs demonstrate the ability to differentiate into all mesenchymal cell types; namely,

adipocytes, chondrocytes, and osteocytes.[54] Indeed, my adipogenic differentiation studies showed that HemSCs formed lipid droplets after several days in induction media, and expressed transcription factors essential for adipogenic differentiation.[252]

Interestingly, despite possessing a stem cell phenotype, HemSCs expressed endothelial genes including CD34, VEGFR2/KDR and CD31. This may be indicative of EC priming in HemSCs, as reinforced by their ability to generate blood vessels *in vivo.*[54] As co-expression of stem and endothelial markers has been exhibited in cells undergoing EHT, I wanted to explore this further by detecting the expression of genes associated with hematopoiesis. My RT-PCR data indicates that HemSCs express some factors of EHT. Namely, ETV6, RUNX1, GATA2, and GPR56 were consistently expressed in all three HemSC cultures derived from separate patients. The presence of hematopoietic factors was further reinforced by my cell staining experiments in which HemSCs showed immunopositivity for RUNX1, GPR56 and CD150. Collectively, this suggests that HemSCs express markers characteristic of hematopoietic lineages *in vitro.*

I then sought to determine whether my findings in culture paralleled those in IH tissue. I discovered that cells in proliferating IH samples express markers of HSCs. Being transcription factors, RUNX1 and GATA2 showed nuclear localization in IH specimens marking both vessel-lining ECs and interstitial cells. However, GPR56, CD45 and CD150 exclusively co-localized with IH endothelium. As CD45 is a well-established, HSC-specific marker, my findings could suggest that HemECs are undergoing an EHT-like mechanism or at a minimum, have the signaling machinery intact. Moreover, the co-expression of CD45 and CD150 implies a mature HSC phenotype.[177] The likely event taking place here is that ECs lining IH vessels have an atypical, immature, and stem cell-like phenotype. There also is the possibility that ECs in IHs are dedifferentiating in an EHT-like manner. The ability of ECs to transition into another cell type is reminiscent of endothelial-to-mesenchymal transition (EndMT) seen in cancer,[253-255] and numerous studies have reported the interconversion of non-cancer cells into cancer stem cells (CSCs).[256-258] This switch can be due to genetic manipulation,[246] changes in the tumour microenvironment,[257] hypoxia,[259] or upon therapeutic stress, such as pharmacological

therapy.[260] Considering that hypoxia and CVS have been linked to IH,[88] these extrinsic factors may induce cell-fate changes in IH cells.

The co-expression of HSC markers in IH endothelium has never been shown before; however, the lack of hematopoietic potential in these HemSCs demands further exploration. Despite expressing markers of mature HSCs and EHT, my studies show that HemSCs are unable to form BFUs in hematopoietic induction media. There are two possible explanations. First, *in vitro* culture of cells may have affected them. This could be in the form of ageing, differentiation, or lack of signals which are normally experienced *in vivo*. The fate of HSCs is known to be regulated by cytokines, adhesion molecules, and interaction with stromal cells in their respective niches.[147,185,261] For example, IFN-γ and TNF-α have been shown to inhibit hematopoiesis *in vitro*.[262] Additionally, activation of NOTCH1 inhibits HSC differentiation into granulocytes and erythrocytes by stimulating GATA2 activity.[263] Hence modulation of these pathways and many others may explain the lack of hematopoietic activity observed in IH-derived cells. The second possibility, of course, is that hematopoietic activity in HemSCs may be inhibited (ie. these cells are primed to differentiate into ECs and adipocytes but not hematopoietic cells). As our knowledge of which pathways regulate hemangiogenesis is still very limited, I believe that future studies investigating changes in the IH microenvironment as it progresses through each stage will be of great significance. One avenue to pursue is to examine the role of GATA3. GATA3 was expressed in both FLSCs and BM-MPCs but not in any of the IH cell preparations. GATA3 is a zinc-finger transcription factor that is essential for differentiation and function throughout the hematopoietic cell hierarchy and is shown to be expressed by LT-HSCs.[264-266] Increased GATA3 expression was also associated with acquisition of a HSC gene signature in a lymphoblastic leukemia cell line.[267] It would be interesting to determine whether hematopoietic activity can be unmasked in HemSCs upon induced expression of GATA3.

Though much of my work suggests an atypical precursor-like phenotype of ECs in IHs, alternatively, one can speculate that HemSCs expressing HSC markers may be homing to vessels of the developing IH, where interaction with cells and secreted factors propels the HemSCs to endothelial commitment.[78,268] Prior studies done in both our laboratory and in

others have repeatedly demonstrated the robust vasculogenic power of HemSCs *in vivo*.[54,269,270] High-throughput phenotypic and genotypic analyses also indicate that HemSCs highly express markers for vasculogenesis and angiogenesis.[98] Given that stem/progenitor cell trafficking is mediated by hypoxia and hypoxia-induced factors are upregulated in proliferating IH, it is not unlikely that the developing IH microenvironment is hypoxic and thus attracts circulating HemSCs.[83,271] Once localized, nearby cells may promote endothelial differentiation. Vitiani et al (2010) have shown this effect in glioblastoma stem-like cells (GSCs).[272] In their study, 20-90% of ECs in glioblastoma had identical gene expression profiles as tumour cells, suggesting that most of the tumour endothelium was of stem cell origin. Furthermore, injection of GSCs into immunodeficient mice produced glioblastomas, which is reminiscent of the study done by Khan et al (2008).[54] The ability of stem cells to differentiate into ECs and form vascular networks has been described for neural stem cells,[273] melanoma,[274] breast cancer[275] and prostate cancer,[276] and has been coined the term 'vasculogenic mimicry'.[274] In addition, one study showed that melanoma cells challenged to an ischemic microenvironment *in vivo* were able to transdifferentiate into ECs and revascularize the ischemic area.[277] Hence, HemSCs may possibly exhibit transendothelial capabilities.

The emergence of these results then begs the question: which came first, the HemSC or the HemEC? Did HemSCs with hematopoietic potential migrate to a site propitious to EC differentiation, or do changes in the microenvironment after vasculogenesis (due to stress, inherent mutations and/or paracrine factors) induce HemECs to differentiate into HemSCs, in a process reminiscent to EHT? The complexity of this question is exacerbated by the fact that many pathways and factors involved in EHT and HSC emergence, such as VEGF, hypoxia, Wnt and NOTCH, are also heavily implicated in EC differentiation.[100,278-283] This suggests that these pathways are inherently plastic, behaving accordingly to external signals from the microenvironment. Perhaps then, HemSCs may give rise to a population of hemogenic ECs that later, under the right conditions, are able to revert to a stem cell-like phenotype in order to help propagate the growth of the developing IH. Regardless of whether this insight bears any truth, it is clear that there is still much to learn regarding the origin of IH and how it develops. My studies show that IH is a complex and dynamic disease, potentially involving mechanisms that have never

been considered before. Understanding the effects of various pathways, the IH microenvironment, and cell plasticity will not only have implications in IH management, but also in prevention, perhaps by manipulating forces involved in HemSC emergence and therefore IH development.

4.2 Limitations

All studies have limitations. In my cell culture experiments, CD133 selection was used to isolate putative stem cells from IHs and FL. My RT-PCR experiments revealed that CD133 mRNA was absent in all cell types, even those that were purified based on CD133 expression. Although the loss of CD133 expression once CD133+ cells have adhered in culture has been published in the literature,[249-251] cells utilized in my experiments were of higher passage number (6 to 10), and thus may not demonstrate true stem or progenitor behaviour. Furthermore, CD133-selected BM cells were not used. CD133-selected cells from human BM samples were cultured but did not yield sufficient cell numbers required to perform all gene expression and staining experiments. This may be due to the fact that most CD133-expressing cells in the adult marrow are HSCs which do not adhere to tissue culture plastic. In addition, my FLSC and BM-MPC cultures did not contain an appropriate tissue representation in my *in situ* immunohistochemical analyses, due to limited FL and BM tissue samples. As a result, my marker expression studies comparing HemSCs, FLSCs and BM-MPCs was limited to gene analysis and differentiation potential experiments.

Lastly, not utilizing the animal model of IH which was developed in our laboratory is a limitation. It would be beneficial to compare the differential expression of hematopoietic genes in HemSCs to the *in vivo* ability of cells to home to the marrow, produce IH lesions, and determine whether this correlates to the involutive process in IH.

4.3 Future Directions

The discovery of hematopoietic marker expression in HemSCs provides novel insight as to the potential mechanisms at play in IH pathogenesis. Given that IHs are vascular lesions and that HemSCs can differentiate into endothelial but not hematopoietic cells, it would be valuable to investigate the process by which hematopoiesis is suppressed in HemSCs. Future studies can be divided into two series: studies which immediately build on the findings of my study, and studies which provide a more broader perspective. Studies that should immediately build on the results presented here include determining whether freshly isolated and purified HemSCs exhibit hematopoietic activity. This study will address the concern of prolonged *in vitro* culture of IH cells. Next, I propose that knockdown experiments be utilized for genes involved in EHT. As I have shown above, certain markers of EHT are expressed in HemSCs and proliferating IH tissue. Using RNA interference or CRISPR/Cas9-mediated gene knockout, the effects of depleting RUNX1, GATA2, and GPR56 on HemSC growth and differentiation can be elucidated.

The strong immunopositivity exhibited by GPR56 demands further investigation into its role in hematopoiesis and EHT. Although numerous reports state that GPR56 is an essential regulator of HSC generation, a recent study by Rao et al (2015) shows that it is actually dispensable for the development and maintenance of HSCs.[168] Therefore more studies are needed in order to elucidate the role of GPR56 in hematopoiesis and ultimately IH.

Other studies which highlight a broader perspective and bring together other discoveries in our laboratory include examining the role of T-box transcription factor 2 (TBX2) in hematopoiesis. Our laboratory has shown that knockdown of TBX2 upregulates expression of hematopoietic marker CD45 and downregulates endothelial marker CD34,[240] which is reminiscent of EHT. Furthermore, TBX2 overexpression enhanced adipogenesis in HemSCs.[284] Future studies may explore the relationship between TBX2 and hematopoiesis by looking at TBX2 and EHT marker co-expression in involuting and involuted IH specimens.

Another pathway that is involved in IH and that has also been heavily implicated in EHT is the NOTCH signaling pathway. NOTCH plays an important role in vascular development and tumour angiogenesis.[70,285-287] Both proliferating and involuting IHs have been shown to express NOTCH1, NOTCH3, NOTCH4, and its ligands JAGGED1 and DLL4.[288,289] Furthermore, NOTCH expression changes based on the stage of IH as well as cell type. A study by Wu et al (2010) reported NOTCH3 upregulation in HemSCs, while HemECs expressed NOTCH1 and NOTCH4.[288] Notably, NOTCH1 plays a pivotal role in HSC emergence and self-renewal,[290,291] and mediates endothelial and hematopoietic lineage specification in mesodermal progenitor cells.[280] One of the major regulators of NOTCH signaling is the VEGF pathway, which is a well-established mediator of IH pathogenesis.[292] Based on this data, I anticipate that altering NOTCH signaling in HemSCs may have significant implications on their ability to differentiate into various cell lineages, especially hematopoietic.

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Curriculum Vitae

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EDUCATION

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RELATED WORK EXPERIENCE

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ABSTRACTS & PROFESSIONAL PRESENTATIONS

1. **Montwill NM** and Khan ZK. Defining the cellular origin of IH. Presented at Pathology and Laboratory Medicine Research Day, London ON, March 2017.
2. **Montwill NM** and Khan ZK. Defining the cellular origin of IH. Presented at London Health Research Day, London ON, March 2017.
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4. **Montwill NM**, Pavlosky A, Kwok C, Su Y, Huang X, Zhang ZX and Jevnikar AM. Double-stranded RNA mediates microvascular endothelial cell death through toll-like receptor 3 following cardiac allograft transplantation. Presented at Pathology and Laboratory Medicine Research Day, London ON, April 2016.
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