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Mechanisms regulating stem cell phenotype in infantile hemangioma

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ABSTRACT

Infantile hemangiomas (IHs) are benign vascular neoplasms characterized by the differentiation of multipotential stem cells (hemSCs) into endothelial cells during the early proliferative phase, and later into adipocytes during spontaneous involution. Transforming growth factor-β (TGFβ) has been shown to be significantly elevated upon IH involution and this coincides with repression of a developmentally-regulated transcription factor T-box 2 (TBX2). These findings implicate both TGFβ and TBX2 in mediating hemSC differentiation during IH evolution. The aim of my study is to understand the role of TGFβ and TBX2 in hemSC differentiation.

I performed immunofluorescence staining to localize TBX2 protein in sectioned IH tissues. I then characterized hemSCs isolated from IH patient specimens for TBX2 expression through mRNA analysis and cell staining. To determine the role of TBX2 in hemSC differentiation, I cultured the cells in mesenchymal, endothelial, neuroglial, and hematopoietic induction media following TBX2 knockdown. Next, I studied the potential downstream targets by conducting expression analysis for cell cycle regulators, pluripotency factors, and T-box family members. To investigate potential upstream regulators of TBX2, I cultured hemSCs with the addition of exogenous TGFβ, and an inhibitor of TGFβ signaling pathway. To confirm functional response of hemSCs to the treatments, expression of TGFβ-responsive genes collagen I and IV was assessed.

My results show that TBX2 is expressed and primarily localized to the nuclei of hemSCs; the level of expression varied between cultures of different patient-derived hemangiomas. HemSCs showed morphological changes upon differentiation towards mesenchymal, endothelial, and neuroglial lineages. TBX2 knockdown caused upregulation of hematopoietic lineage markers CD34, CD45, and ckit. Pluripotency factor and cell cycle regulator p16 expression was also upregulated upon TBX2 knockdown. Lastly, my studies show that TGFβ represses TBX2 expression, which correlates with upregulated pluripotency factor expression.

My studies suggest that TBX2 modulates the differentiation-competence of hemSCs. TBX2 may in turn be regulated by TGFβ during IH involution. Understanding the signaling mechanisms regulated by TBX2 may provide a novel target pathway to promote premature involution of IH as a therapeutic option for patients.

Keywords: Infantile hemangioma, T-box 2, TGFβ, stem cells, differentiation, pluripotency

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bm-MNCs	Bone marrow mononuclear cells
bm-MPCs	Bone marrow mesenchymal progenitor cells
C/EBP	CCAAT/enhancer binding protein
CD133	Cluster of differentiation-133; human stem cell antigen
CD31	Cluster of differentiation-31; human endothelial cell antigen (PECAM-1)
CD34	Cluster of differentiation-34; human endothelial/stem cell origin
CD45	Cluster of differentiation-45; human hematopoietic antigen
cDNA	Complementary deoxyribonucleic acid
DAPI	4’,6-diamidino-2-phenylindole
DMEM	Dulbecco’s modified eagle’s medium
EBM2	Endothelial Basal Medium-2
ECs	Endothelial cells
FBS	Fetal bovine serum
FLCs	Fetal liver cells
GFAP	Glial fibrillary acidic protein
Glut1	Glucose transporter-1
HDMECs	Human dermal microvascular endothelial cells
HemECs	Hemangioma endothelial cells
HemSCs	Hemangioma-derived stem cells
IH	Infantile hemangioma
IL-6	Interleukin 6
MCP-1	Monocyte chemoattractant protein-1

mRNA	Messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NANOG	STranscription factor involved in embryonic stem cell renewal
NKX3.2	NK3 Homeobox 2
OCT4/POUF5	Octamer-binding transcription factor 4
PBS	Phosphate-buffered saline
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PPARγ2	Peroxisome proliferator-activated receptor gamma 2
PDGF	Platelet-derived growth factor
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
siRNA	Small interfering-RNA (silencing RNA)
SOX2	SRY (Sex Determining Region Y)-Box 2
SOX9	SRY (Sex Determining Region Y)-Box 9
SP7	Osterix
TBX2	T-box 2
TGF-β	Transforming growth factor-beta
TUBB3	Tubulin beta-3 chain
uPAR	urokinase-type plasminogen activator receptor
VE-cadherin	Vascular endothelial-cadherin
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor
α-SMA	Alpha-smooth muscle actin

Chapter 1

1 INTRODUCTION

1.1 Infantile Hemangioma

Infantile hemangioma, a benign tumour of vascular endothelial cells, is the most common type of childhood tumour appearing in 1 out of every 100 newborns.[1, 2] Hemangiomas exhibit a strong predilection for the head and neck regions, which account for at least 60% of all lesions.[3-5] They present more frequently in females than males, and are more prevalent in premature and Caucasian babies.[6, 7] Uniquely, hemangiomas are characterized by distinct phases of proliferation and regression.[8-10] Typically, a red patch arises within the first few weeks of life. Following formation of the initial lesion, the tumour progresses rapidly into the first phase: the proliferative phase. On average, proliferation perseveres for a period of 1 year during which masses of endothelial cells (EC) without defined vascular architecture is observed.[11] At the age of approximately 1 year, the tumour enters the slow involuting phase, which may last between 1 and 5 years. During this stage, changes are observed solely at the cellular level where EC hyperplasia is reduced and mature vessels become distinct.[8] The final stage of this tumour is known as the involuted phase. This stage is typically reached by the age of 5–8, and is characterized by regression of the tumour due to the replacement of blood vessels by fibrofatty tissue.[10, 12] No other vascular tumours demonstrate this type of life-cycle pattern, and as such this natural progression is utilized clinically to classify and diagnose IH.

1.1.1 Clinical features of hemangioma

Hemangiomas are classified based on their anatomical location and histological properties. Slightly elevated lesions situated on the epidermis that appear red or purple in color are classified as superficial hemangiomas.[13] Lesions located beneath the skin and within internal organs have often been referred to as deep hemangiomas. [8, 13] Categorizations of the vascular tumours as localized, segmental, indeterminate and multifocal lesions have also been proposed. [3] Further vocabulary used to describe morphological features of hemangiomas include strawberry, capillary, port-wine, cavernous, capillary-cavernous, and lymphangiohemangioma. [11, 14, 15] Due to the variability in nomenclature that is used to describe hemangiomas, misdiagnoses of

vascular tumours as malformation and vice versa is quite common.[14] A landmark study by Mulliken and Glowacki[11] established proper classification of IH based on the cellular and the histological features. Their work demonstrated hyperplasia in hemangiomas but not vascular malformations.

Currently, the most accurate diagnostic confirmation of hemangiomas is achieved by staining biopsy specimens for Glucose transporter-1 (Glut-1).[5, 16] Glut-1 is a member of a large transporter protein family, which is most commonly associated with erythrocytes.[17, 18] In ECs, Glut-1 is noted to be expressed at the blood–brain/retinal-barrier and in the placenta [17-19] A large-scale study conducted by North et al. (2001) [20]established the unique immunoreactivity of endothelial Glut-1 in hemangioma specimens, which was not observed in normal skin or other vascular anomalies including malformations.

1.1.2 Current therapies for hemangioma

Approximately 20% of hemangiomas develop in areas that may cause serious and life-threatening complications.[21] These lesions frequently result in vision and airway obstruction in the infant. Internal bleeding, ulceration, and congestive heart failure are also complications of hemangiomas obstructing internal organs.[5] Most commonly, corticosteroids are administered to treat hemangiomas, however only 30% exhibit accelerated regression with this treatment.[9] In addition, corticosteroid therapy has been noted to cause severe adverse effects in children, including growth retardation, immunosuppression and edema.[22]

Recently, propranolol was serendipitously found to be an effective treatment for IH with higher efficacy when compared to corticosteroid use.[23, 24] Propranolol is a non-selective β-adrenergic receptor antagonist, yet its mechanism of therapeutic effect is currently unknown. It is suggested to act on the pericytes surrounding capillaries that cause vasoconstriction (reviewed in[25]). Despite remarkable efficacy of propranolol,[24] there are some adverse effects, which include sleep disturbances, acrocyanosis, hypotension, and hypoglycemia.[24, 26, 27] Another concerning finding from a number studies is that some IHs regrow upon cessation of propranolol treatment, in as many as 20% of the cases.[28, 29] While current therapies offer effective elimination of the tumour in some cases, the plausible side effects from their use cause great concerns for the health and

wellbeing of the children affected. Further understanding of the cellular and molecular basis of hemangiomas is necessary to prevent complications and properly treat the tumour.

1.1.3 Etiology of hemangiomas

Several hypotheses exist on the etiology of hemangiomas. Two main ones include the extrinsic and the intrinsic hypotheses. The extrinsic hypothesis suggests that the tumour microenvironment regulates hemangioma-genesis, whereby a signal imbalance in the tissue adjacent to the lesion contributes to the proliferation and progression of the tumour.[30] For example, increased angiogenic factor expression (basic fibroblast growth factor, vascular endothelial growth factor) and hyperplasia in the epidermis overlaying proliferating-phase hemangioma have been reported.[30] However, whether the primary signal imbalance originates from the microenvironment or the tumour itself is not fully clear.

Conversely, the intrinsic theory entails that a somatic mutation in one or more genes controlling EC proliferation is responsible for the tumour formation. Examination of molecular markers expressed *in situ* and in cultured IH cells has granted insight to the possible origin and mechanism of IH pathogenesis. Studies have shown that IH specimens express immature cell markers in addition to fully differentiated EC markers.[31, 32] Further study of expression patterns of cultured IH-derived endothelial cells (hemEC) has revealed upregulated GLUT-1, and vascular endothelial growth factor (VEGF).[31, 32] As previously mentioned, GLUT-1 is a particularly useful cell surface marker for the diagnosis of these lesions. The similar pattern of Glut-1 expression observed throughout various stages of hemangiomas and placenta insinuates the possibility of a connection between the two, especially when it comes to the origin of the tumour.[18] Cumulatively, the intrinsic hypothesis suggests that the ECs in hemangiomas originate from a single stem/progenitor cell and therefore implies that the cells are clonal in nature. The potential placental origin of these stem/progenitor cells is strengthened by the evidence showing that disturbances in the microenvironment, such as transcervical chorionic villus sampling (CVS), increases the incidence of infantile hemangioma.[33, 34] It is supposed that the invasive procedure causes intravascular dislodgment of placental cells, which enter the circulation and embolize to the developing fetus. [35-37]

The working hypothesis of our laboratory is a combination of the intrinsic and extrinsic hypotheses. We have shown IH to arise from multipotential stem cells termed hemSCs.[16] HemSCs, isolated based on expression of stem cell antigen CD133, form GLUT-1 positive microvessels in immunodeficient mice. These GLUT-1 positive vessels are later replaced by human adipocytes, thus recapitulating the natural stages of human IH. A schematic depicting our working model on the origin of hemangiomas is provided in **Figure 1**.

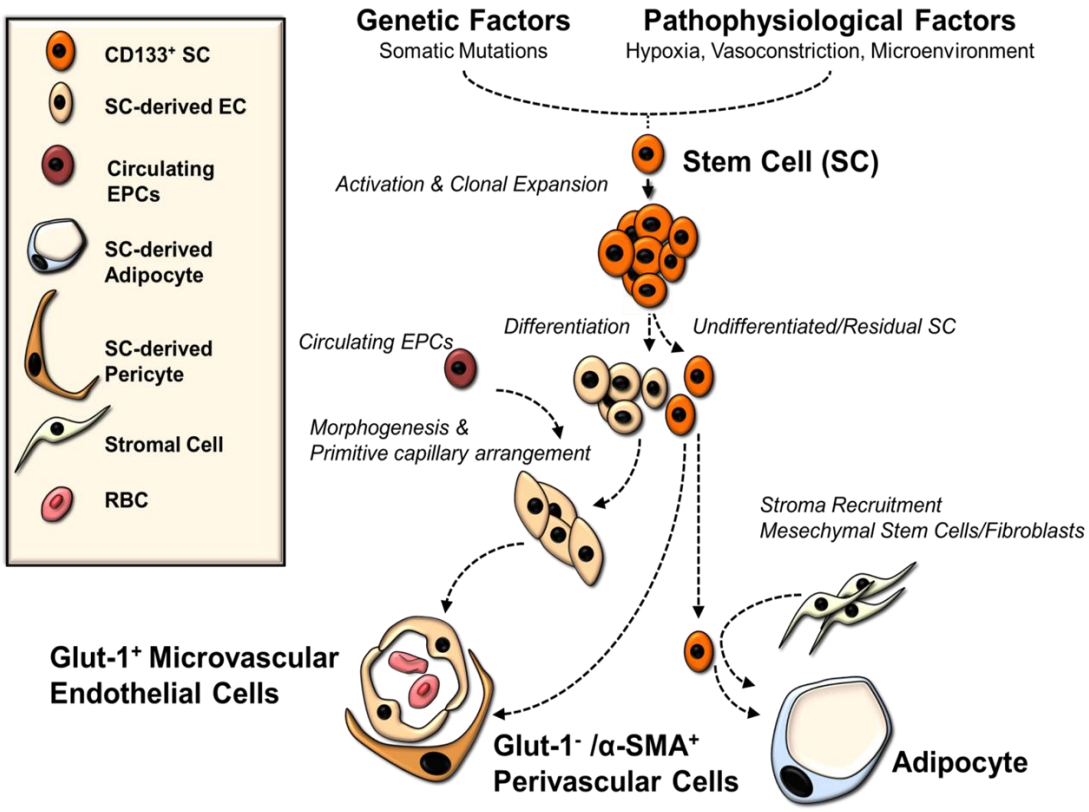

Figure 1: Current theories of hemangioma etiology. Schematic diagram illustrating the theory behind the evolution of infantile hemangioma. A combination of genetic and environmental factors cause CD133+ cells to undergo clonal expansion and to give rise to all of the cellular constituents within the tumour. (EC: endothelial cell; EPC: endothelial progenitor cell; SC: stem cell; SMA: smooth muscle actin; RBC: red blood cell)

1.1.4 Aberrant signaling pathways in hemangioma

Several signaling pathways have been implicated in IH pathogenesis. Studies have focused particularly on the VEGF (vascular endothelial growth factor A) pathway. VEGF-A is a regulator of angiogenesis and vasculogenesis.[38] VEGF-A has been shown to be present at higher levels during the proliferating phase of hemangioma growth as compared to the involuting phase.[32, 39-42] Serum levels of VEGF-A also decrease following systemic steroid therapy.[43] The high expression of VEGF might be related to hypoxia, as increased hypoxia inducible factor-1α (HIF-1α) stabilization was reported in patients with proliferating IH. [40] A recent study demonstrated that silencing the expression of VEGF-A or VEGF receptor 1 (VEGFR-1) in hemSCs by short hairpin RNA (shRNA) was sufficient to block blood vessel formation when the cells were injected in immunodeficient mice. [41, 44]

The angiopoietin-Tie2 pathway has also been suggested to contribute to IH pathogenesis. Angiopoietin-1 (ANGPT1) and angiopoietin-2 (ANGPT2) signal through TIE1/2 to regulate distinct steps in vascular remodeling, vessel maturation and vascular inflammation.[45, 46] Tie2 mRNA and protein have been shown to be up-regulated in hemECs relative to normal endothelial cells.[47] Calicchio and colleagues also found ANGP2 mRNA significantly increased in hemangioma endothelium relative to normal placental vessels.[48] Although robust levels of mRNA and protein have been noted in hemangiomas and hemangioma-derived cells, confirmatory studies in animal models as well as mechanistic studies are needed to clearly define the role of angiopoietins and TIE2 in the growth and involution of IH.

The Notch pathway has also been recently implicated in hemangioma-genesis. This evolutionarily conserved signaling system regulates cell-fate determination during development and in stem cells. In the vascular system, interaction of Notch receptors (Notch1 to Notch4) with their ligands (Delta-like 1, Delta-like 3, Delta-like 4, Jagged-1and Jagged-2) regulates the specification of endothelial cells into arterial and venous phenotypes during development.[49] JAGGED1 has been reported to be expressed in IH endothelium.[48, 50] In addition, Notch3 is expressed by hemSCs and becomes prominently expressed in the perivascular cells in the involuting phase.[50] Thus, it seems that signaling between hemangioma endothelial cells and pericytes is mediated by the Notch pathway.

More recently, the mammalian target of rapamycin (mTOR) signaling pathway was shown to be involved in hemangioma pathogenesis. mTOR is a major signal convergence protein that transduces signals from the extracellular matrix to corresponding changes in basic intracellular processes including proliferation, protein synthesis and autophagy.[51] Recent studies have found that rapamycin (inhibitor of mTOR) inhibits the proliferation and the self-renewal activity of the hemSCs. It prevented hemSCs, either alone or combined with endothelial cells, from forming blood vessels *in vivo*. [52] Beside its anti-vasculogenic effect on hemSCs, rapamycin has anti-angiogenic effect on the hemECs, suppressing their proliferation.

The renin-angiotensin system has recently been suggested to play a role in IH pathogenesis as well as in the response to propranolol.[53] Specifically, it was suggested that angiotensin II could drive proliferation of endothelial progenitor cells into mitotically active cells that characterize IH.[54] However, this notion is based mostly on immunohistochemical staining of IH specimens and support for this theory from *in vivo* studies is lacking. Poor effect of the ACE inhibitor captopril on IH also discounts this signaling pathway as being involved in hemangioma pathogenesis.[55]

Various other factors have also been found to be differentially expressed in the proliferating phases of IH but their role is less defined. These include monocyte chemoattractant protein-1 (MCP-1),[56] interleukin 6 (IL-6) and urokinase-type plasminogen activator receptor (uPAR) [42] and insulin-like growth factor 2 (IGF-2).[57, 58] The cell adhesion molecule E-selectin, normally only expressed in inflamed endothelium, is strongly expressed on vessels in proliferating phase IH.[59] E-selectin is also expressed by proliferating phase hemECs and may mediate interactions with hemSCs. [60] While many pathways have been shown to be aberrantly expressed/active in IH, no single defining signaling mechanism regulating hemSC differentiation or hemEC proliferation has yet been established.

1.1.5 Stem cells in hemangioma

We and others have found IH specimens to be immunoreactive to embryonic stem cell (ES) markers, such as OCT4, Nanog, Sox2, c-myc, SALL4. [31, 61-63] These markers are associated with pluripotency due to their expression and activity coinciding with the ability of ES to differentiate

into all three germ layers.[64-68] Interestingly, the expression of ES markers in IH is localized to both the interstitium and the endothelium, whereas CD133 expression is restricted to the endothelium. Cells isolated based on CD133-expression (hemSCs) exhibit competence to differentiate into cells of multiple lineages *in vitro*, and repeated endothelial differentiation upon serial implantation in mice.[16] HemSCs exhibit fibroblastic morphology in culture and share cell surface marker expression with bone marrow-derived mesodermal/mesenchymal stem cells including CD90, neuropilin-1 and VEGF receptors. Since hemSCs were isolated based on the expression of CD133 (a marker restricted to the endothelium by immunostaining), it is plausible that upon culture, these cells revert to their parental mesodermal stem cell state.

Recently, Bischoff et al. (2015) have demonstrated the GLUT1-positive endothelial cells lining the blood vessels of IH, to have properties of mesenchymal/mesodermal stem cells.[69] Upon isolation and *in vitro* culture, these cells reverted to a mesenchymal stem cell phenotype by upregulating CD90, platelet-derived growth factor receptor-β (PDGFRβ), and neural glial antigen-2 (NG2). Notably, the GLUT1-negative population retained a stable endothelial phenotype in culture. GLUT1-positive cells also displayed robust clonogenic capacity, suggesting that these cells possibly derive from a mesenchymal/mesodermal stem cell. It is important to note that hemSC population demonstrated to recapitulate IH upon transplant in mice were isolated purely on the basis of being CD133-positive, and therefore likely included both GLUT1 positive and negative populations.

The perivascular cells surrounding the nascent vessels in the proliferative phase hemangiomas express the pericyte markers α-smooth muscle actin (α-SMA), NG2, PDGFRβ, calponin, and smooth muscle myosin heavy chain.[70] Pericytes are abundant in the proliferating phase and appear to undergo a maturation process concurrently with the endothelial cells. [71] Recently, Yuan et al. isolated PDGFRβ-positive cells and showed expression of CD105, CD90, vimentin, and CD133. These cells showcased multi-lineage differentiation *in vitro* into adipocytes, osteocytes, and chondrocytes. Upon transplant, these PDGFR-B+ pericytes underwent adipogenic differentiation. Thus, it is possible that both GLUT1-positive ECs and PDGFRβ-positive pericytes are derived from CD133+ population isolated in our previous study.

We have shown that hemSCs differentiate into mesenchymal (adipocyte, osteoblast, and chondrocyte), endothelial, and neuroglial lineages in culture.[16] Notably, one lineage for which

that hemSCs have not been fully characterized is the hematopoietic lineage. This is noteworthy as studies have shown IH lesions to be comprised of a significant proportion of cells with myeloid lineage markers. Ritter et al. comprehensively demonstrated IH specimens to stain positive for CD45, CD14, CD15 and CD83.[72] Significantly, the dendritic cell marker CD83 and the myeloid marker CD15 were found to be expressed in IH endothelium, and decreased during involution of the tumour. [72] In addition, Glut1 and CD32 (which are both present in the placenta as well as on myeloid cells) was shown to be co-expressed with CD83 and CD14 positive endothelium, respectively. This suggests that the former two markers may indeed be indicative of a myeloid cell lineage as opposed to a placental origin.

Additionally, mast cells are present in IH.[73, 74] Their number predominates in the early to middle involuting phase, whereas lower numbers are seen in the proliferative and the involuted phases.[32] This difference has led to the hypothesis that mast cells play a role in the regression of IH. Moreover, it has recently been demonstrated that mast cells are present in comparable numbers in various skin tumours, including basal cell carcinoma, squamous cell carcinoma, melanomas and nevi.[75] Thus, the unique role of mast cells in IH requires further study.

One group has observed IH biopsies to generate enucleated erythrocytes *in vitro*, however they failed to characterize which cellular constituents from the explants were responsible for the demonstrated erythropoiesis. [76] Yet, their work proposes an interesting link between the endothelial cell derivation seen by Boscolo et al.[44] and the myeloid markers seen by Ritter et al.[72] Historically, a common precursor to endothelial and hematopoietic lineages has been debated for many years. The concept of a hemangioblast, a mesodermal precursor for the hematopoietic and endothelial lineages, was first suggested in the early 20th century by Sabin and Murray (reviewed in [77]), due to the proximity of the hematopoietic and endothelial lineage development within the blood islands. Evidence for the hemangioblast has since been found *in vitro*, where groups have identified embryoid bodies developed from human ES contain a unique population of precursor cells for both lineages. These precursor cells formed blast-like colonies which were shown to have similar expression patterns for genes common to both hematopoietic and endothelial cells, including CD34, Tal1/SCL, and flk-1 (VEGFR1).[78] These blast colony-forming cells (BL-CFCs) generated both hematopoietic and endothelial cell precursors, while cultured in growth factors supporting the differentiation of both lineages.[77]

Similarly, evidence for haemogenic endothelium during embryonic development has been demonstrated at the aorta-gonad-mesophrenos (AGM) stage of hematopoietic activity. It is interesting that IH typically emerges at a temporally determined stage. Its post-natal appearance may correspond to the hematopoietic transition from the fetal liver to the bone marrow. IH has been reported in the infant liver. In fact, with respect to visceral hemangioma, liver is the most commonly involved internal organ, followed by lungs, brain, and intestine.[79] If we contemplate the possibility that hemSCs in IH originate from a signaling alteration or mutation at the hemangioblast level, or a switch in mesoderm signaling to haemogenic endothelium, this may explain the mesenchymal, endothelial, and hematopoietic potential of hemSCs in IH.

1.2 Transcription factor T-box 2

Recent gene expression profile has revealed a number of genes altered in hemangioma specimens which may be responsible for the underlying pathogenesis. One gene identified was transcription factor TBX2 which is significantly downregulated upon IH involution.[57] Our laboratory has shown that knockdown of TBX2 in hemSCs impedes adipogenic differentiation.[80] This suggests that TBX2 may have a role in the progression of the tumour. However, the precise role and underlying mechanisms are not known.

T-box 2 belongs to the T-box family of transcription factors that play a critical role during development. Only a few mutations in T-box genes are known, but all have drastic effects on development, including a targeted mutation in mice causing an embryonic lethal phenotype, and two human T-box gene mutations that results in developmental syndromes (reviewed in [81]). T-box family members are characterized by a highly conserved region of approximately 200 amino acid residues corresponding to the DNA binding domain known as the T-box. The prototype of the T-box family, Brachyury or T, is the most extensively studied and has been used to elucidate the structure and function of other family members. All members characterized to date have been shown to bind to the core sequence GGTGTGA, referred to as the T-element, as a monomer.[82] Although all T-box proteins recognize similar DNA sequences, evidence suggests they regulate different target genes which may be dependent on the promoter context and co-factors.[83-85] Tbx2 belongs to the Tbx2 subfamily of T-box transcription factors, which include Tbx3, Tbx4, and Tbx5.[86, 87] Whereas TBX2 is most closely related to TBX3, TBX4 and TBX5

are more closely related to each other. In the mouse, Tbx2 is located on chromosome 11[87] while in humans, TBX2 has been mapped to chromosome 17q23.[88] The T-box domain of the human TBX2 shares 90% DNA and 96% peptide sequence homology with its mouse counterpart.[89]

Unlike most members of the T-box family that function as transcription activators, Tbx2 is a potent repressor of its target genes. [90, 91] Paxton et al.[92] identified two separate transcription repression domains; a novel amino-terminal repression domain and confirmed the presence of a carboxy-terminal repression domain identified in an earlier study. [92, 93] A weak activation domain was also located within the T-box and indeed Tbx2 was capable of activating a promoter containing multiple T-elements. [92] The presence of both activation and repression domains within the Tbx2 protein suggests multiple but specific roles for this transcription factor, depending on the cellular context or species.

1.2.1 Known functional roles of T-box 2 in development

Tbx2 is expressed in the mouse heart, lung, kidney, ovary and in cells of melanocyte lineage.[87, 90] In humans, it is expressed in a wide variety of tissues including kidney, lung, placenta, ovary, prostate, spleen, testis, breast, heart, thymus, intestine, and polymorphonucleocytes. [88, 89] Targeted mutagenesis has shown that heterozygous mutants are normal while homozygous mutants die of cardiac insufficiency between 10.5 and 14.5 days post coitum (reviewed in [94]). Human TBX2 has not yet been linked to any known genetic syndrome but has been implicated in several developmental processes, such as coordinating cell fate, patterning, and morphogenesis of a wide range of tissues and organs including limbs, kidneys, lungs, mammary glands, heart, and craniofacial structures (see reviews, [94, 95]).

During cardiogenesis, Tbx2 is important in proliferation and patterning of the developing heart.[96] At around 8 and 10 days post coitum, Tbx2 is detected in the nonchamber myocardium of the developing mouse heart, which is consistent with Tbx2 expression patterns during chick heart development.[97, 98] *In vitro* reporter assays and transgenic mice studies have shown that during non-chamber formation, Tbx2 represses the transcription of natriuretic peptide A, and connexins (Cx) 40 and 43 thus regulating the formation of the multi-chambered heart.[97, 98]

Tbx2 has been shown to regulate expression of the genes encoding collagen type 1a and Cx43, which are both crucial in bone formation. Gap junctions, composed of Cx protein subunits connect the cytoplasm of adjacent osteocytes and are, therefore, important in cell–cell communication. Cx43 is the predominant gap junction protein in bone and its tightly regulated temporal expression pattern plays a critical role in normal ossification and osteoblast function.[99] Tbx2 and Cx43 are co-expressed in osteogenic progenitors and osteoblasts, suggesting that Cx43 may be a potential Tbx2 target gene in bone.[100] Osteosarcoma cell lines transfected with anti-sense Tbx2 have demonstrated that inhibition of Tbx2 results in a marked increase in Cx43 expression.[100, 101]

Tbx2, Tbx3, Tbx4, and Tbx5 are expressed in developing mouse limbs either at the time of limb field specification, during limb bud outgrowth, or both.[102, 103] A study by Suzuki et al.,[104] suggests that Tbx2 and Tbx3 play an important role as a positive regulator of Shh expression within the posterior limb bud. A recent study also suggests Tbx2 is responsible for limiting Shh expression to the margins of the limb bud.[105]

Both TBX2/Tbx2 and TBX3/Tbx3 have been implicated in mammary gland development in mice and humans. Both factors are expressed in the developing mouse mammary gland, where they are expressed in specific spatiotemporal patterns in the mesodermal and epithelial cell layers (reviewed in [106]). During the emergence of the mouse mammary gland, Tbx2 is expressed in the underlying mesodermal cells, while Tbx3 is expressed only in the epithelial cells. Interactions between the epithelial and mesodermal cell layers are known to govern induction and development of the mammary glands (reviewed in [107, 108]).

Finally, Tbx2 expression has been observed in several melanocyte and melanoma cell lines but not in premelanoblast cells.[90] Studies have also implicated Tbx2 in the patterning and development of the hypothalamus,[109] and the mammalian embryonic eye (reviewed in [110]).

1.2.2 Known regulators of T-box 2

While the mechanisms directly regulating TBX2 are not comprehensively characterized, there is evidence suggesting a number of pathways involved in cellular processes contributing to embryonic development and tumourigenesis, are modulators of TBX2 expression.

Wnt signaling has been suggested to activate TBX2 during embryonic development and cancer. TBX2 is activated by bone morphogenetic protein (Bmp) 2 and bone morphogenetic protein 4 during heart patterning, downstream of the Wnt/ β-catenin pathway.[111] Additionally, inhibition of β-catenin degradation induced TBX2 mRNA and protein levels, in pancreatic cancer cells.[112]

Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signaling has been suggested as a modulator of TBX2, as adrenal carcinoma cells treated with a PI3K inhibitor demonstrated decreased TBX2 mRNA levels.[113] PI3K is associated with upregulated VEGFR-2 signaling in tumourigenic cells,[114] and can play a crucial role in cancer progression by co-operating with cell proliferation signals.[115] However, a mechanistic detail as to how is may interact with the TBX2 promoter has not yet been described.

TBX2 is also up-regulated by the protein kinase C (PKC) signaling pathway.[116] The latter was upregulated in normal human fibroblasts by the tumour promoting agent TPA. In response, PKC signaling phosphorylates histone H3 leading to chromatin remodelling and recruitment of transcription factor specificity protein 1 (Sp1) to the TBX2 promoter and subsequent activation of TBX2 mRNA expression.

The transforming growth factor β1 (TGF-β1) pathway is critical for a number of cellular processes including cell differentiation, proliferation, apoptosis, and during the immune response (reviewed in [117]). This multi-functional cytokine mainly exerts its effects by binding and activating the TGFβ receptors I and II, which initiate a cascade of signaling events frequently involving Smad proteins and co-factors (the canonical pathway) but which can also be Smad-independent (non-canonical).[118] Recently, TBX2 mRNA was found to be upregulated following TGF-β treatment in mammary epithelial cells, and furthermore shown to be a driver of epithelial to mesenchymal transition (EMT) in breast cancer.[119] Contrarily, a positive relationship between TGFβ and TBX2 has been suggested, whereby the anti-proliferative effect of TGFβ on B16 melanoma cells was rescued by Tbx2 overexpression.[120] TGF-β is also known to be upstream of TBX2 in cardiac development[121] and melanoma cell development.[122] Furthermore, it is upregulated upon involution in IH.[39]

1.2.3 Known functional roles of T-box 2 in tumourigenesis

In addition to its key function in development, evidence suggests Tbx2 plays a role in carcinogenesis. For example, Tbx2 and the closely related factor Tbx3, have been implicated in cell cycle regulation and their expression is upregulated in a number of cancers, including breast,[123, 124] pancreatic,[125, 126] melanoma,[127, 128] liver,[129] and bladder:[130]

Tbx2 and TBX3/Tbx3, as mentioned earlier, are involved in normal breast development and studies have shown that their altered expression may be important in the pathogenesis of breast cancer. TBX2 was shown to be amplified and overexpressed in a subset of breast cancer cell lines and primary tumours in BRCA1- and BRCA2-related breast tumours.[123, 131] Both TBX2 and TBX3 have also been associated with pancreatic cancer. While TBX2 was found to be overexpressed in 50% of 31 pancreatic cancer cell lines tested,[125] TBX3 was shown to be upregulated in metastatic pancreatic endocrine neoplasms.[126] Furthermore, both TBX2/Tbx2 and TBX3 are expressed in normal melanocytes and have been found to be strongly upregulated in a subset of melanoma cell lines.[90, 127, 128]

A possible mechanism for how TBX2 and TBX3 may contribute toward the oncogenic process is suggested by studies which have shown that they are both able to function as immortalizing genes that enable the cells to bypass senescence.[131] The main mediators of senescence are the cyclin dependent kinase inhibitors (Cdkis), p21WAF1/CIP1/SDII (referred to as p21) and p16INK4a, with p21 thought to be necessary for initiating the senescence-like growth arrest while p16INK4a is required for maintenance of this state.[132] The induction of p21 appears to be largely p53- dependent and the Cdki, p19ARF, has also been shown to initiate a senescent-like growth arrest by stabilizing p53 protein levels.[133] Using two different cell culture models of senescence, investigators have shown that both Tbx2 and Tbx3 can behave as immortalizing genes by preventing senescence.[131, 134, 135] In these studies, TBX2 and TBX3 were shown to prevent senescence by a mechanism involving their ability to transcriptionally repress p19ARF (mouse homologue of the human p14ARF) and p21. Whether TBX2 and TBX3 also contribute to tumour progression via other mechanism(s) is poorly understood.

In summary, Tbx2 has been implicated in several developmental processes, is emerging as a key regulator of the cell cycle, and has been implicated in several cancers. However, very little is

known about the biochemical pathways regulating the expression and activity of Tbx2; there is limited information regarding its target genes. Based on its demonstrated roles coordinating cell fate during development and promoting oncogenesis, we believe it presents as a strong candidate for contributing towards hemangioma pathogenesis.

Chapter 2

2 PURPOSE AND OBJECTIVES OF THESIS

2.1 Purpose of thesis

While the precise role of TBX2 in hemangioma remains elusive, up-regulated expression of this transcription factor in the proliferative phase suggests a potential role in tumour progression. Increased expression of TBX2 in hemangiomas has been identified by Ritter and colleagues in the proliferative stage.[57] However, the level decreases during the involution phase, suggesting that TBX2 is a regulator of initiation and/or growth of hemangioma. The purpose of my study is to investigate the role of transcription factor T-box 2 in modulating the differentiation competence of hemSCs (**Figure 2.1**). I hypothesize that *high TBX2 maintains a differentiation competent state in hemangioma-derived stem cells by modulating cell pluripotency and proliferation*.

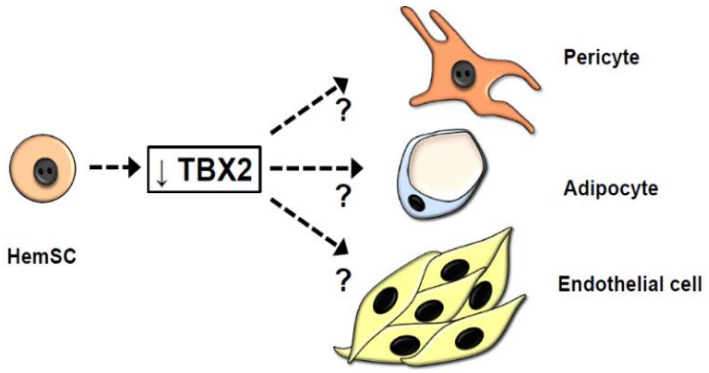

Figure 2.1: Stem cell derived cellular constituents of infantile hemangioma.
Schematic diagram illustrating the potential role of transcription factor T-box 2(TBX2) in modulating the differentiation of hemSCs in infantile hemangioma to give rise to the cellular constituents seen within the tumour.

2.2 Objectives of thesis

My studies focused on the characterization of TBX2 expressing cells in hemangiomas. I sought to study the effect of TBX2 in hemangioma-derived cells and investigate the TBX2 signaling pathway (**Figure 2.2**). My specific objectives were:

I. Localization of TBX2 expressing cells in hemangioma specimens and characterization of T-box family expression in hemangioma cell culture.
II. Determination if inhibition of TBX2 leads to loss of differentiation ability in hemSCs.
III. Investigation of the signaling network regulated by TBX2 in hemSCs through identifying upstream modulators and downstream targets of TBX2.

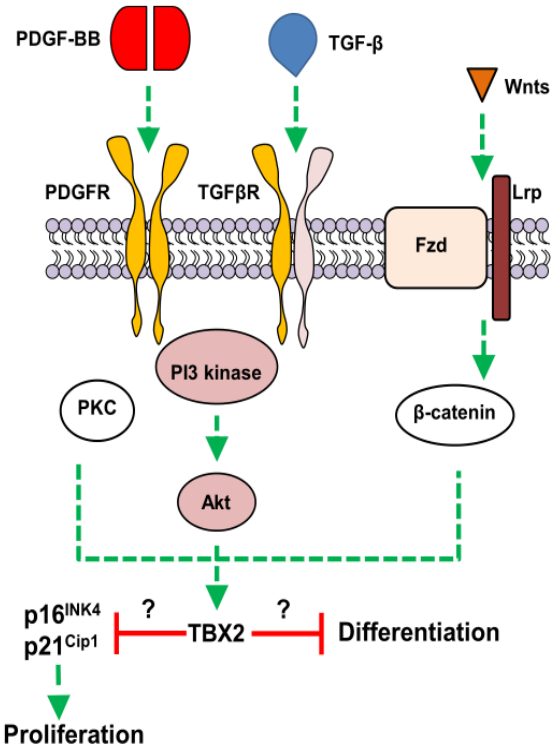

Figure 2.2: Mechanisms regulating differentiation-competence of hemSCs.
Schematic diagram illustrating the signaling pathways of interest within this study that may regulate the differentiation-competence of hemSCs via transcription factor TBX2.

Chapter 3

3 MATERIALS AND METHODS

3.1 Infantile hemangioma 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 Archives at the London Health Sciences Centre (LHSC, London Ontario, Canada). The proliferating phase was confirmed through medical history, physical examination, and histological analysis of densely packed capillaries. In addition, all hemangioma sections were immunostained with Glut1 to confirm diagnosis. Tissue sections were deparaffinized, hydrated, 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). Slides were incubated with rabbit anti-TBX2 antibody (1:50, HPA008586, Sigma-Aldrich) and mouse anti-human CD31 (1:100, M0823, Dako Canada, Mississauga, ON); rabbit alpha-smooth muscle actin (1:200, ab5694, Abcam Plc); mouse anti-OCT4 (1:200, MAB17531, R&D Systems) for 1 hour at room temperature; Fluorescein- or texas red-conjugated secondary antibodies (Vector Laboratories, Burlington, ON) were used for detection. Slides were counterstained with DAPI (Vector Laboratories). Images were taken using the Olympus BX-51 microscope (Olympus Canada In., Richmond Hill, ON) equipped with a Spot Pursuit digital camera (SPOT Imaging Solutions, Sterling Heights, MI). Positive controls for staining experiments comprised of human early gestation placenta (20-25 weeks) specimens; malignant melanoma specimens; lung carcinoma specimens; renal cell carcinoma specimens. Negative controls for staining experiments were conducted with human hemangioblastoma specimens; incubations of hemangioma specimens without the primary antibody added to the sample (to indicate if any non-specific binding or false positives may be due to non-specific binding of the secondary antibody).

3.2 Infantile hemangioma cell culture

Proliferating IH-derived CD133+ cells (hemSCs) were provided by Dr. Joyce Bischoff (Children's Hospital Boston, Boston, MA). We have previously characterized these cells through qRT-PCR, immunostaining, and cellular activity assays.[16] Freshly isolated human bone marrow-mesenchymal progenitor cells (bm-MPCs; isolated from bone marrow mononuclear preparations; 2M-125B, Lonza Inc., Walkersville, MD) were used as normal stem/progenitor controls. CD133-positive human fetal liver cells were obtained from DV Biologics (Yorba Linda, CA). All cells were cultured in complete EBM2 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). Cells were cultured under identical conditions and experiments were performed with a minimum of 2 biological replicates and 3 technical replicates. Throughout the results sections, biological replicates (hemSC preparations isolated from different patient tumours) are represented as "N"; experimental replicates (repetitions of the same experiment on different dates) are represented as "n". Within each cell culture experimental set, a minimum of 3 technical replicates were included (ie. 3 technical replicates per "n"). Furthermore, analysis via RT-PCR (**Section 3.6**) included 3 technical replicates per RNA sample.

3.3 Differentiation assays

To study multilineage differentiation, cells were treated with induction media for 14 days. Mesenchymal differentiation of the cells was carried out with adipogenic media (StemPro Adipogenesis Differentiation Kit, ThermoFischer Scientific, A1007001), osteogenic media (StemPro Osteogenesis Differentiation Kit, ThermoFischer Scientific, A1007201), and chondrogenic media (StemPro Chondrogenesis Differentiation Kit, ThermoFischer Scientific, A1007101). Endothelial cell differentiation was carried out in serum-free medium containing VEGF-B (EBM-2, 1× insulin-transferrin-selenium, 1:5000 linoleic acid–albumin, 1 μM dexamethasone, 60 μM ascorbic acid–2–phosphate, 10 ng/mL VEGF-B). For neuroglial differentiation, cells were exposed to initiating medium (DMEM/10% FBS, 1 mM β-mercaptoethanol) for 24 hours. Induction medium (DMEM, 0.1 μM dexamethasone, 0.25 μg/mL

insulin, 5 μM cAMP, 0.5 mM isobutylmethylxanthine, 5 μM trans-retinoic acid, 0.25 mM l-glutamine, and 25 ng/mL nerve growth factor) was then used for 14 days. Hematopoietic activity was assessed in MethoCult medium (StemCell Technologies) at 10^3, 10^4, 10^5, and 10^6 cells per 35-mm dish. Mesenchymal differentiation was assessed by upregulation of *CEBPα* and *PPARγ* gene expression for adipogenesis, *SP7/Osterix* for osteogenesis, *NKX3.2* and *SOX9* for chondrogenesis, *TUBB3* and *GFAP* for neuroglial; *CD31*, *VE-cadherin* and *VWF* for endothelial differentiation; *CD45*, *CD34* and *c-kit* for hematopoietic lineage differentiation. Morphological changes were also observed and recorded. Images were taken with an Olympus CK2 using SPOT Advanced 3.5.9 software (Diagnostic Instruments Inc.) and a ×20/0.45 objective lens.

3.4 Cellular Transfection with siRNA

Cells were grown in complete EBM2 media. The day prior to the transfections, the media was replaced with antibiotic/antimycotic-free media. Twenty-four hours later, the cells were trypsinized and then transfected with either TBX2 siRNA (sc-38469) or control siRNA (sc-37007) using electroporation (Neon® Transfection System, Life Technologies). For siRNA transfections, we used the following protocol: 1400v, 20 ms, 2 pulses. Effective concentration of siRNA (both control and TBX2 siRNA) utilized was 50 μM (50 x 10^{-6} mol/L). Knockdown efficiency was determined at 24 h, 48 h post-transfection or at day 7 using real time RT-PCR. This protocol was used to transfect hemSCs.

3.5 Cell viability assays

Post-transfection, the growth of hemSCs was assayed by measuring total live cell number with Scepter 2.0 Automated Cell Counter (Millipore) for both control and TBX2 knockdown cultures. Viability was measured at 24 h, 48 h, 72 h and 7 days. Transfection efficiency was confirmed at the time of cell count (following lysis and RNA isolation).

3.6 RNA isolation and qRT-PCR

RNA was isolated using either RNeasy Micro Plus Kit (Qiagen, Mississauga, CA), or SingleShot Cell Lysis Kit (172-5080, Bio-Rad Laboratories, Hercules, CA). RNA was 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). All cellular differentiation and TBX2 pathway component gene expression was assessed by qRT-PCR (**Table 1**). Target gene mRNA data was normalized to *β-actin* (QT01680476, Qiagen). Pluripotency factor expression was also analyzed utilizing a human iPSC colony qBiomarker screening PCR array (337221 IPHS-001A, Qiagen), for which data was normalized to *GAPDH*. All qRT-PCR reactions consisted of 10 μL RT² SYBR Green FAST Mastermix (330602, Qiagen), 2 μL of both forward and reverse primers (at a 10 μM concentration), ~2 μL cDNA (volume adjusted for individual cDNA samples to constitute 300ng per PCR reaction), and 6 μL of H2O. All reactions were performed for 40 cycles using the following temperature profiles: 95°C for 2 minutes (initial denaturation); and 60°C for 30 seconds (annealing and extension). Data was analyzed by CFX Manager Software using normalized relative quantity (ΔCT; ΔΔCT) method with two housekeeping genes (*β-actin* and *GAPDH*).

Table 1: List of primers used

Gene	Source (Catalogue ID)
B-actin	Qiagen (QT01680476)
CD31	Qiagen (QT00081172)
CD34	Qiagen (QT00056497)
CD45	Qiagen (QT02306717)
CD133	Qiagen (QT00075586)
C/EBPα	Qiagen (QT00203357)
CKIT	Qiagen (QT00080409)
COL1	Qiagen (QT00037793)
COL4	Qiagen (QT00005250)

E-caderin	Qiagen (QT00080143)
DNMT3A	Qiagen (QT00090835)
DNMT3B	Qiagen (QT00032067)
DPPA4	Qiagen (QT00046515)
GLUT1	Qiagen (QT00068957)
GATA2	Qiagen (QT00045381)
GATA3	Qiagen (QT00095501)
GFAP	Qiagen (QT00081151)
LEFTY	Qiagen (QT00037373)
NANOG	Qiagen (QT01025850)
NKX3.2	Qiagen (QT01079582)
OCT4/POUF5	Qiagen (QT00210840)
p16ARF/p19/CDKN2A	Qiagen (QT00089964)
p21/CDKN1A	Qiagen (QT00062090)
PPARγ	Qiagen (QT00029841)
RUNX1	Qiagen (QT00026712)
SCL/TAL	Qiagen (QT00012530)
SOX2	Qiagen (QT00237601)
SOX9	Qiagen (QT00001498)
SP7/Osterix	Qiagen (QT00213514)
TBX2	Qiagen (QT00091266)
TBX3	Qiagen (QT00022484)
TBX4	Qiagen (QT00024052)
TBX5	Qiagen (QT00056574)
TUBB3	Qiagen (QT00083713)
VE-cadherin	Qiagen (QT00013244)
vWF	Qiagen (QT00051975)
ZFP42	Qiagen (QT00051009)

3.7 Signaling network assays

To investigate potential upstream regulators of TBX2, I cultured hemSCs with the addition of exogenous pathway component agonists/antagonist to control media in a dose-dependent manner. The investigated pathways were selected based on known regulators of TBX2.[136] Reagents and concentrations used are listed in **Table 2**. The effective concentrations of these signaling pathway modulators were determined empirically in our laboratory. Reagent concentrations were optimized for cell reponse, ie. TGFβ1(240-B-010, R&D Systems) was added at concentrations 5ng/mL, 10ng/mL, 15ng/mL. To confirm functional response of hemSCs to the treatments, we examined up-regulation of known downstream targets of treatments post-culture (ie. upregulation of collagen I and IV mRNA expression for TGFβ1 treatment).

Expression analysis for *TBX2* mRNA was conducted at 24 h, 48 h, 72 h, and day 7 timepoints, following RNA isolation.

Table 2: List of pathway component treatments used

Reagent	Target pathway	Effective concentration	Source
Calmodulin dependent kinase inhibitor KN-93	Microtubule-dependent cell cycle events; calcium signal transduction	500 nM	Tocris Biosciences (1278)
Chelerythrine chloride	PKC translocation; apoptosis	700 nM	Cayman Chemicals (11314)
GW788388	TGF-β signalling	10 nM	R&D Systems (3264/10)
IWR-Endo	Wnt signalling	10 μM	Cayman Chemicals (13659-10)
PI-828	PI3K signalling	2 μM	R&D Systems (2814/1)
PNU74654	Wnt-β catenin pathway	20 μM	R&D Systems (3534/10)

Rho Kinase Inhibitor VII	Phosphorylation, dephosphorylation	10 nM	EMD Millipore (555556)
Rac1 Inhibitor II	Phosphorylation, dephosphorylation	25 µM	EMD Millipore (553512)
TGF-β1	TGF-β signalling	10 ng/mL	R&D Systems (240-B)
TGF-β3	TGF-β signalling	10 ng/mL	R&D Systems (302-B2)
Wnt Agonist	Wnt signalling	1 µM	Calbiochem EMD Millipore (853220-52-7)

3.8 Statistical Analysis

Where appropriate, student's unpaired t-tests or analysis of variance (ANOVA) were performed. P values <0.05 were considered statistically significant. The data were expressed as means ± standard deviation (SD) where student's unpaired t-tests was performed, as a measure of data variability around the sample population mean; ± standard error of the mean (SEM) where ANOVA was performed, as a measure of precision for an estimated population mean. Following ANOVA, post-hoc analysis was conducted utilizing the Bonferroni procedure.

Biological replicates (hemSC preparations isolated from different patient tumours) are represented as "N"; experimental replicates (repetitions of the same experiment on different dates) are represented as "n". Within each cell culture experimental set, a minimum of 3 technical replicates were included (ie. 3 technical replicates per "n"). Furthermore, analysis via RT-PCR (**Section 3.6**) included 3 technical replicates per RNA sample.

Chapter 4

4 RESULTS

4.1 TBX2 localization in hemangioma

I first screened for the expression of TBX2 in IH specimens by immunohistochemistry. I confirmed that the specimens were hemangiomas by staining for Glut-1 (**Figure 1-App**). I observed that proliferating hemangioma specimens were positive for TBX2, which was determined to be mostly nuclear as shown in merged images of TBX2 and DAPI (**Figure 4.1A**). TBX2 also co-localized with CD31 positivity, suggesting hemangioma endothelial cells expressed TBX2. However, TBX2 expression was not restricted to the endothelium and showed positivity in both interstitial and perivascular cells. To mark the perivascular cells, I performed staining for α-smooth muscle actin on serial sections (**Figure 4.1B**). These results confirm that TBX2 localizes to the perivascular cells as well the endothelium. However, as both TBX2 and α-smooth muscle actin were from the same host and hence, definitive co-expression could not be determined on the same sections.

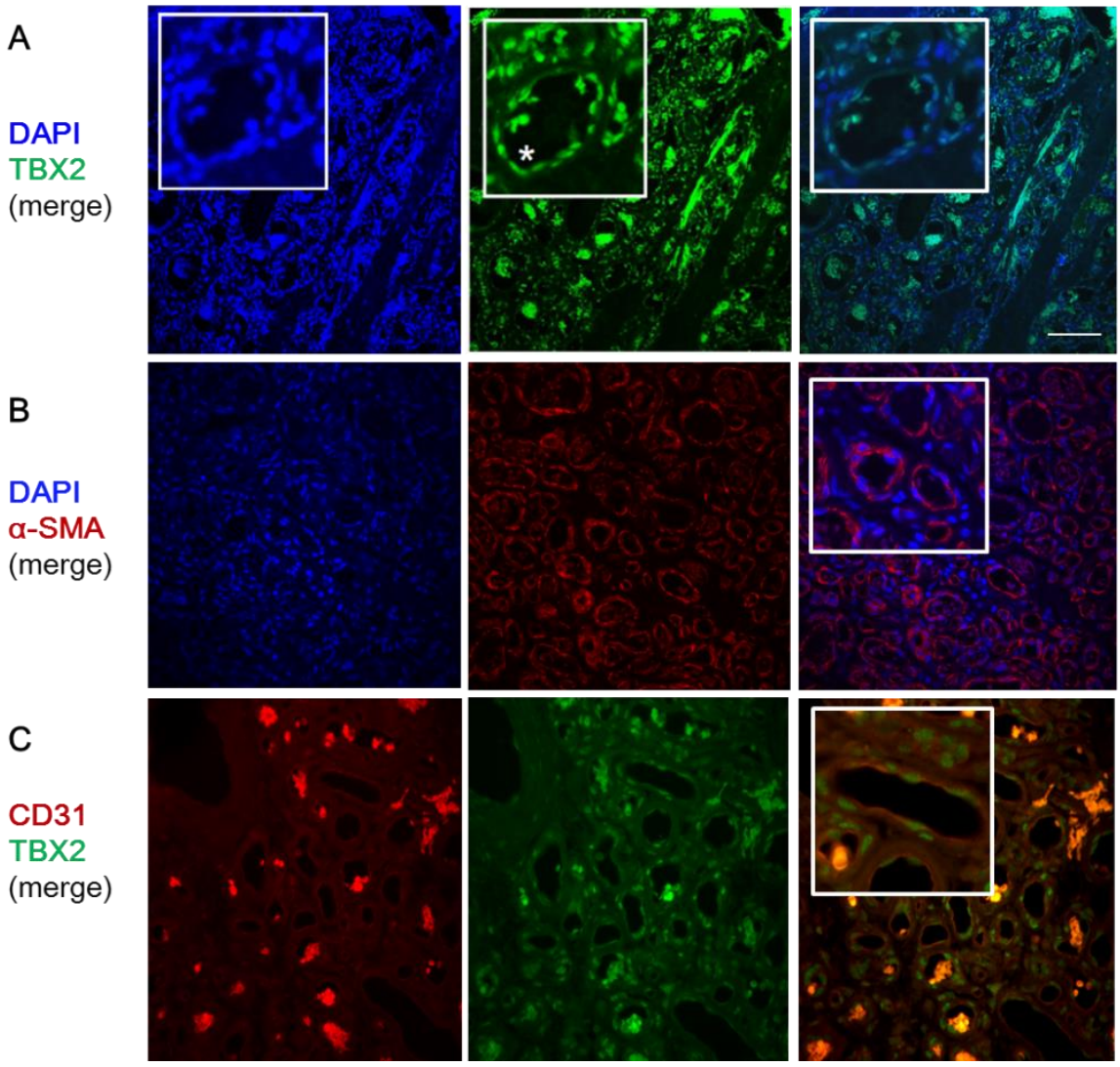

Figure 4.1: TBX2 is expressed in the nucleus of endothelial cells in infantile hemangioma.
(A) Immunofluorescence (IF) staining Tbx2 (green).
(B) IF staining alpha-smooth muscle actin (red).
(C) IF imaging for endothelial marker CD31 (red) and TBX2 (green)
[Scale bar represents 100µM; DAPI nuclear stain = blue].

4.2 Hemangioma stem cells express T-box transcription factors

I confirmed that TBX2 expression was retained in hemSCs, by immunofluorescent staining of CD133-positive hemangioma-derived cells in culture. TBX2 was found to have primarily nuclear localization, however some cytoplasmic localization was also observed. HemSC cultures of different primary patient origin appeared to exhibit unique nuclear/cytoplasmic TBX2 localization as expected (**Figure 4.2.1**). *TBX2* mRNA analysis also showed robust expression in cultured primary hemSCs (**Figure 4.2.2**). Cell preparations which exhibited higher levels of TBX2 mRNA expression tended to show mostly nuclear TBX2 localization. However, because of limited hemangioma cell preparations available, this was not pursued further with detailed analysis.

I furthermore characterized *TBX2* in hemSCs relative to bone marrow-derived mesenchymal progenitor cells (bm-MPCs) throughout serial passaging, to determine whether *TBX2* expression was specific to hemSCs. As TBX2 is known to commonly interact with other members of the T-box family, in particular TBX3,[122] I also investigated whether hemSCs and bm-MPCs co-expressed additional T-box transcription factors. My results show that *TBX2* mRNA levels increased in bm-MPC cultures over early subculture (**Figure 4.2.3**). However, at comparable subculture level, *TBX2* mRNA were lower in normal bm-MPCs compared to hemSCs (**Figure 4.2.4**). Interestingly, TBX3 mRNA was also expressed in hemSC cultures. When *TBX2* levels were found to be higher in a primary cell culture, *TBX3* levels were intrinsically lower, and vice-versa (**Figure 4.2.4**).

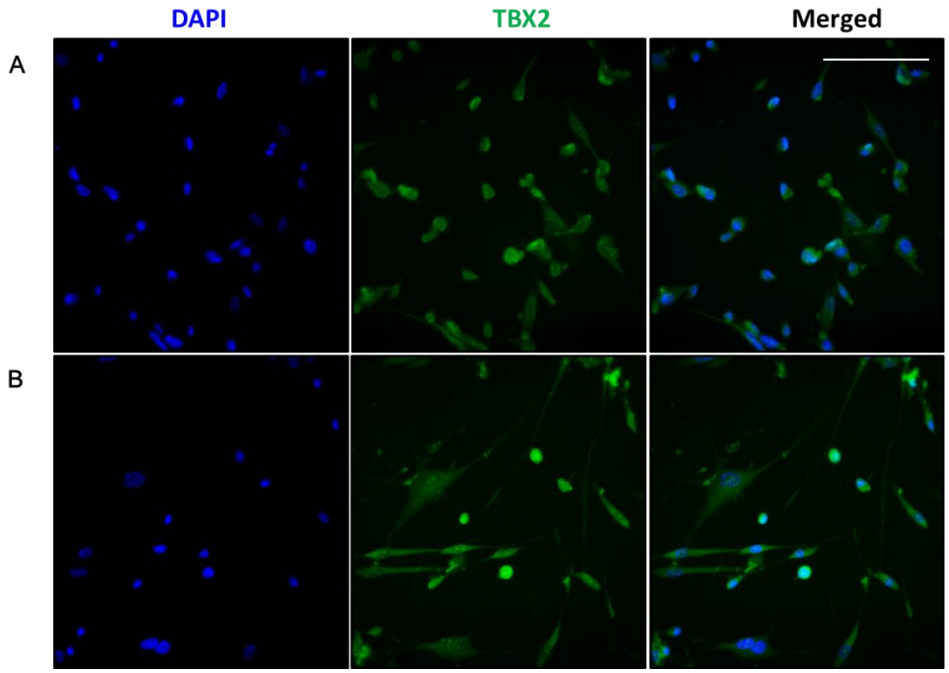

Figure 4.2.1: TBX2 is expressed in the nucleus and the cytoplasm of primary hemSC cultures. (A) Immunofluorescence staining for TBX2 (green) in hem115 primary cell culture (high *TBX2* mRNA; see **Figure 4.2.2**) [DAPI nuclear stain = blue] (B) Immunofluorescence staining for TBX2 (green) in hem120 primary cell culture (low *TBX2* mRNA) [scale bare represents 100µM]. The number in the various cell preparations represents the de-identified patient sample number.

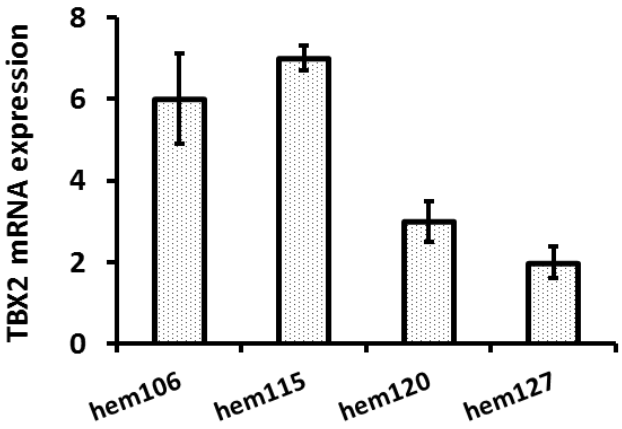

Figure 4.2.2: TBX2 expression is variable between hemSC cultures of different patient origin. CD133-selected hemSC cultures from four different IH patients were assessed for TBX2 mRNA expression. Relative RNA normalized to β-actin; N=4; n=3. Data expressed as mean ± SD.

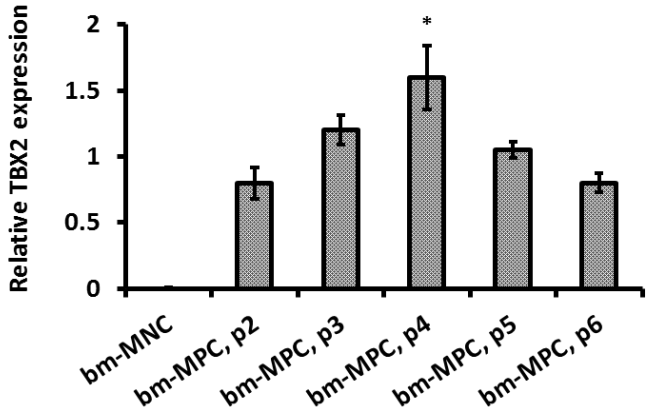

Figure 4.2.3: TBX2 expression increases through passage culture of bm-MPCs.
Bone marrow mononuclear cells (bm-MNC) were cultured and assessed for TBX2 mRNA levels at different time points. Upon adherence and culture, bm-MNCs are referred to as bone marrow derived mesenchymal progenitor cells (bm-MPCs). *TBX2* expression increased at early passage numbers (passage 3-4), and furthermore decreased throughout later passages. Relative mRNA normalized to β-actin; N=1; n=3; two-tailed student's t-tests were performed; *=$p<0.05$. Data expressed as mean ± SD.

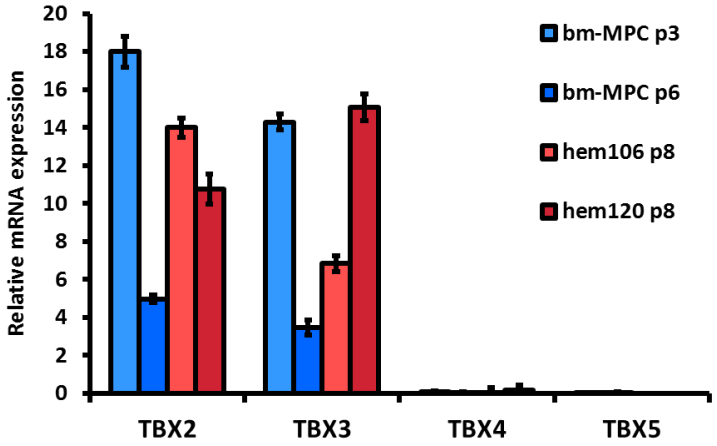

Figure 4.2.4: T-box family expression profile in hemSCs and bm-MPCs
bm-MPCs exhibit mRNA expression of TBX2 and TBX3 in early culture passages, however hemSC retain high levels of expression at later passage numbers. Relative mRNA normalized to β-actin; bmMPC N=1; hemSC N=2; n=3. Data expressed as mean ± SD.

4.3 TBX2 modulation of hemSC cell cycle regulation

To investigate the potentially tumourigenic role of TBX2 in hemangioma, I sought to determine its effect on cell cycle regulators p16 and p21, as studies have found TBX2 to repress p19[131], the alternate product of the Ink4a/Arf locus transcribing p16,[137] and p21.[135] HemSCs with intrinsically higher levels of *TBX2* mRNA exhibited lower levels of cell cycle regulator expression, while lower levels of *TBX2* corresponded with increased cell cycle regulator mRNA levels (**Figure 4.3.1**; **4.2.2**). Knockdown of *TBX2* via siRNA resulted in the significant upregulation of cell cycle regulator p16 mRNA levels (**Figure 4.3.2**). Additionally, *TBX2* knockdown resulted in significantly reduced total live cell counts in hemSC cultures 24, 48, and 72 hours after transfection (**Figure 4.3.3**). At day 7, total live cell counts in hemSC cultures with *TBX2* siRNA were comparable to those with control siRNA.

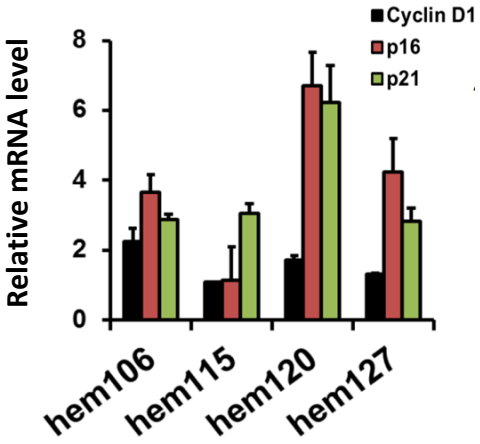

Figure 4.3.1: Cell cycle regulator expression in different primary hemSC cultures. HemSC cultures isolated from four different IH patients were assessed for p16, p21, and cyclin D1 mRNA levels. Cultures with intrinsically higher levels of TBX2 mRNA (**Figure 4.2.2**) have decreased mRNA expression of p16 and p21 compared to cultures with lower TBX2 mRNA. Relative mRNA normalized to β-actin; N=4; n=3. Data expressed as mean ± SD.

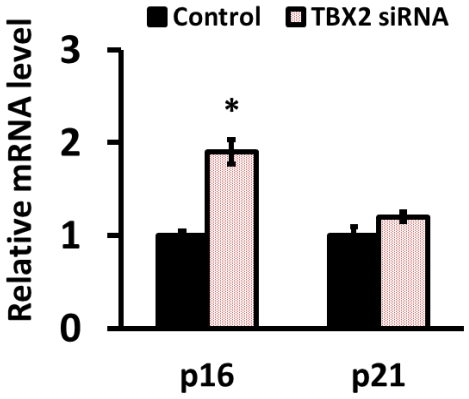

Figure 4.3.2: Cell cycle regulator p16 expression is upregulated upon TBX2 knockdown in hemSC. HemSC were transfected with TBX2 siRNA and cultured in EBM2. Expression analysis for cell cycle regulators p16 and p21 was performed at 48 hours post-transfection. Only experimental replicates which exhibited 40% or greater TBX2 knockdown were included in analysis. Relative mRNA normalized to β-actin; N=2; n=2, two-tailed student's t-tests were performed; *=$p<0.05$. Data expressed as mean ± SD.

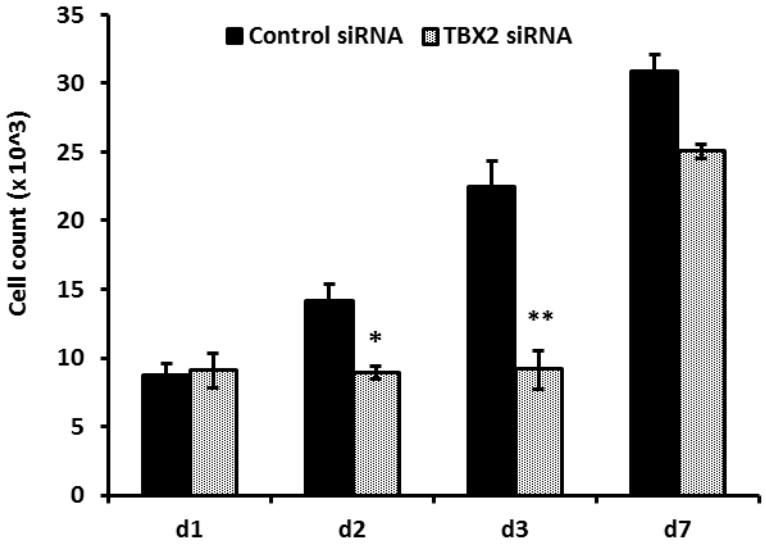

Figure 4.3.3: Reduced live HemSC numbers upon TBX2 knockdown. HemSC were transfected with TBX2 siRNA and cultured in EBM2. Total live cell counts were conducted at 24 hours (d1), 48 hours (d2), 72 hours (d3) and one week (d7) post-transfection. Only experimental replicates which exhibited 40% or greater TBX2 knockdown were included in analysis for d1, d2, and d3 (at d7 all replicates showed *TBX2* mRNA levels comparable to controls). Cells were originally transfected at a density of 15, 000 cells per well (96-well plate). N=2; n=3; analysis of variance (ANOVA) followed by Bonferroni post-hoc analysis; *=$p<0.05$, **=$p>0.01$. Data expressed as mean ± SEM.

4.4 TBX2 modulation of hemSC pluripotency

Hemangioma specimens are immunoreactive to pluripotency factors SOX2, OCT4 and Nanog.[31] Furthermore, TBX2 modulation has been show to alter adipogenic differentiation of hemSCs.[80] As the latter may represent a change in hemSC differentiation state, I investigated the effect of TBX2 on hemSC pluripotency. HemSCs were found to express a number of pluripotency factors (**Figure 4.4.1**), and upon *TBX2* knockdown pluripotency factors *OCT4/POU5F, SOX2*, and *NANOG* were significantly upregulated (**Figure 4.4.3**). These findings suggest that TBX2 may regulate the stem cell phenotype of hemSCs. In support of this notion, my studies also show that TBX2 co-localizes with OCT4 in the nuclei of cells lining vessels and interstitial cells in IH specimens (**Figure 4.4.2)**.

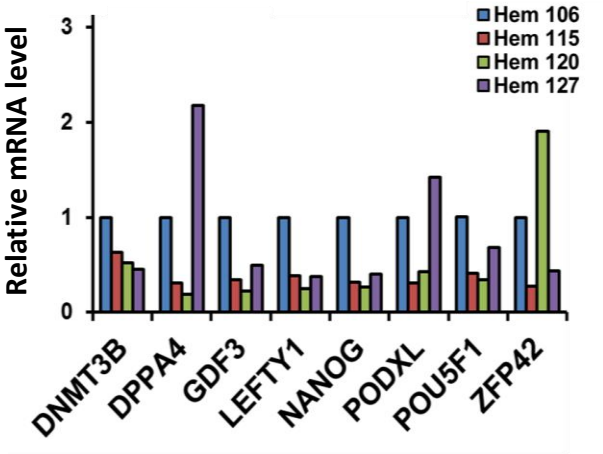

Figure 4.4.1: Pluripotency factor expression in different primary hemSC cultures. HemSC cultures isolated from four different IH patients were assessed for pluripotency factor mRNA expression utilizing a qBiomarker screening PCR array (IPHS-001A, Qiagen). Relative mRNA normalized to GAPDH; N=4; n=3.

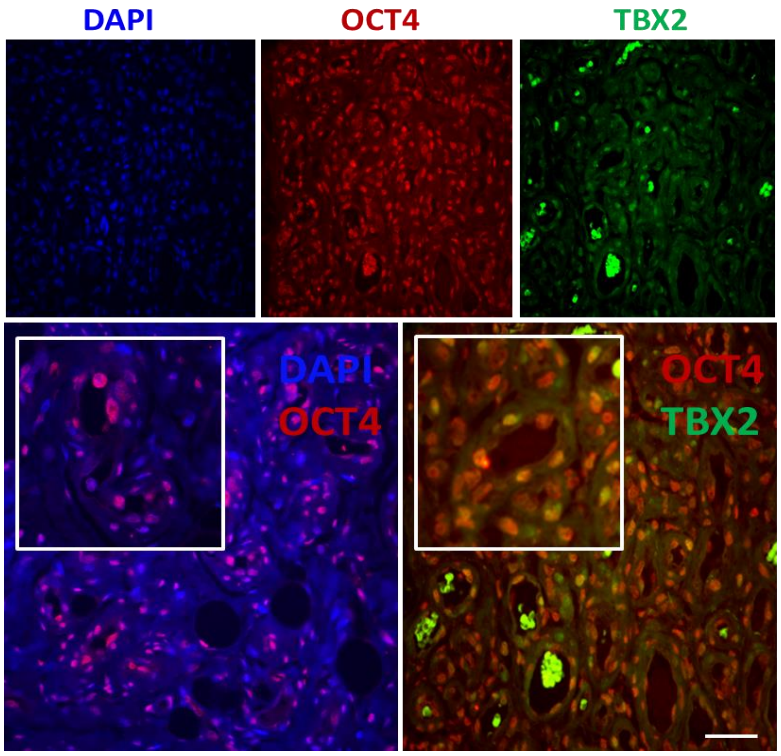

Figure 4.4.2: IH tissue sections stain positive for pluripotency marker OCT4. Immunofluorescence (IF) staining for Oct4 (red) and Tbx2 (green) in fixed human infantile hemangioma specimens [DAPI nuclear stain = bue]. TBX2 co-localized with OCT4 expression in the nuclei of cells lining IH vessels and interstitial cells [scale bar represents 100µM].

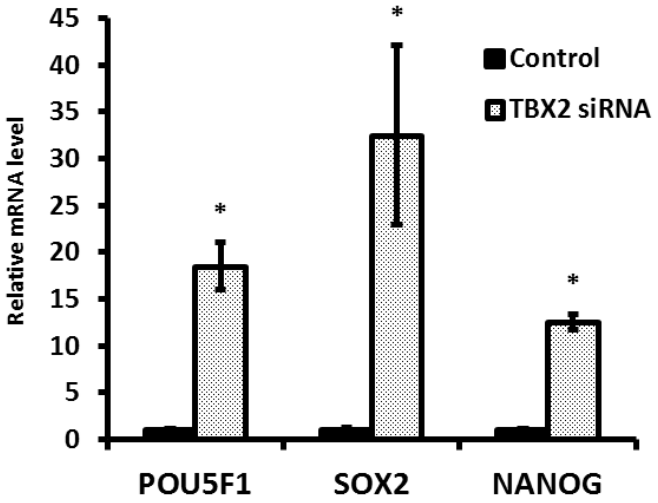

Figure 4.4.3: Pluripotency factors are upregulated upon TBX2 knockdown in hemSCs. HemSCs were transfected with TBX2 siRNA and cultured in EBM2. Gene expression analysis for pluripotency factors *OCT4*, *SOX2*, and *NANOG* was performed at 48 hours post-transfection. Only experimental replicates which exhibited 40% or greater TBX2 knockdown were included in analysis. Relative mRNA normalized to β-actin; N=2; n=2; two-tailed student's t-tests were performed; *=$p<0.05$. Data expressed as mean ± SD.

4.5 Hemangioma stem cells exhibit multi-lineage differentiation potential

HemSCs have previously been shown to exhibit multi-lineage differentiation potential in culture[16] towards mesenchymal, neuroglial and endothelial lineages. I confirmed the upregulation of lineage specific gene expression in respective induction media (**Figure 4.5.1**), as well as morphological observations during differentiation (**Figure 4.5.2**). I additionally investigated hemSC differentiation-competence towards the hematopoietic lineage. I compared CFUs in hemSCs to bm-MPCs. bm-MNCs exhibited CFU formation in MethoCult medium at multiple seeding densities (**Figure 4.5.3**). Intrinsically, hemSCs did not exhibit any hematopoietic activity in MethoCult medium (**Figure 4.5.4**).

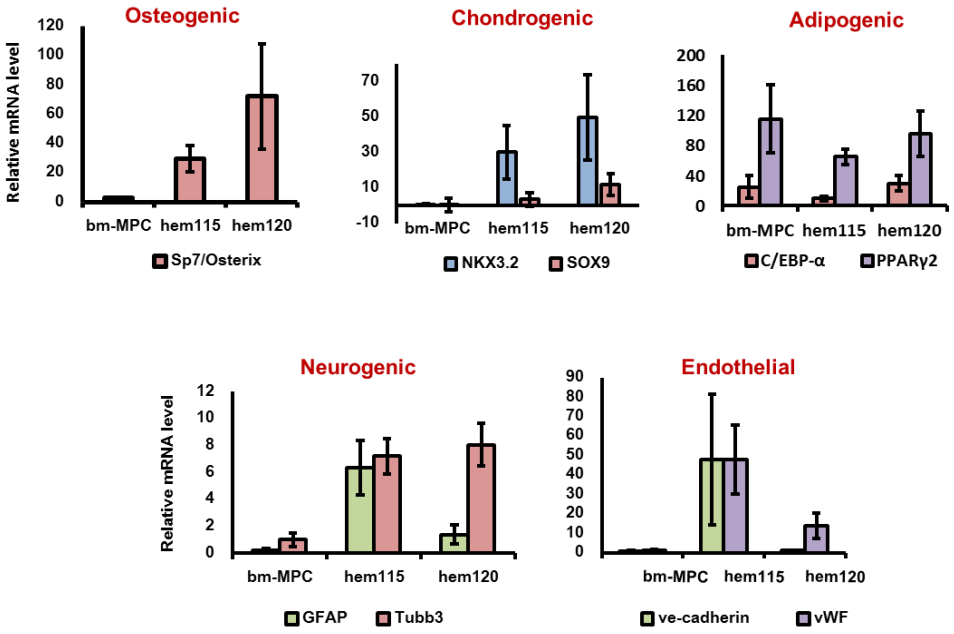

Figure 4.5.1: HemSC demonstrate differentiation-competence towards endothelial, neuroglial, and mesenchymal lineages in respective induction media. HemSCs and bmMPCs were cultured in endothelial, neuroglial, osteogenic, chondrogenic, and adipogenic induction media. Marker expression for each induction lineage (CD31, VE-cadherin; TUBB3, GFAP; SP7; NKX3.2, SOX9; C/EBP-α, PPARγ2 respectively) were assessed at d7 of induction, comparative to control (EBM2) cultures. Relative mRNA normalized to β-actin; bmMPC N=1; hemSC N=2; n=3. Data expressed as mean ± SD.

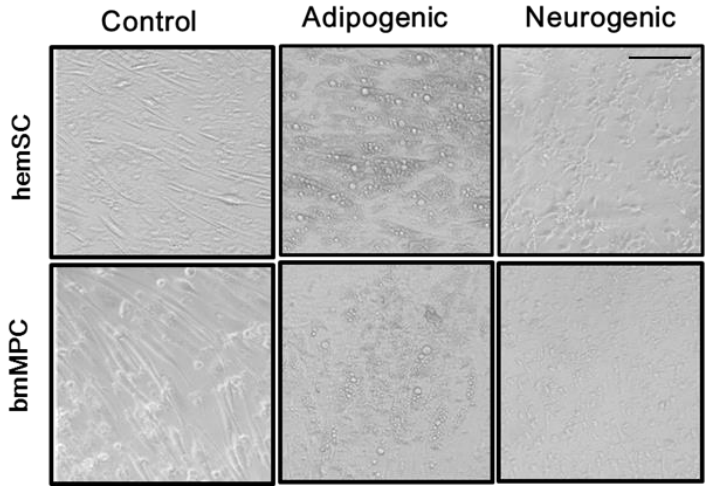

Figure 4.5.2: Morphological differences observed in hemSC cultures with control, neuroglial, and adipogenic induction media. HemSCs cultured in control (EBM2) media exhibited spindle-shaped morphology, where neuronal and adipogenic cultures demonstrated ramification and lipid-droplet accumulation, respectively. Bm-MPCs did not show equivalent morphological changes in neuronal media. [Scale bar represents 100μM].

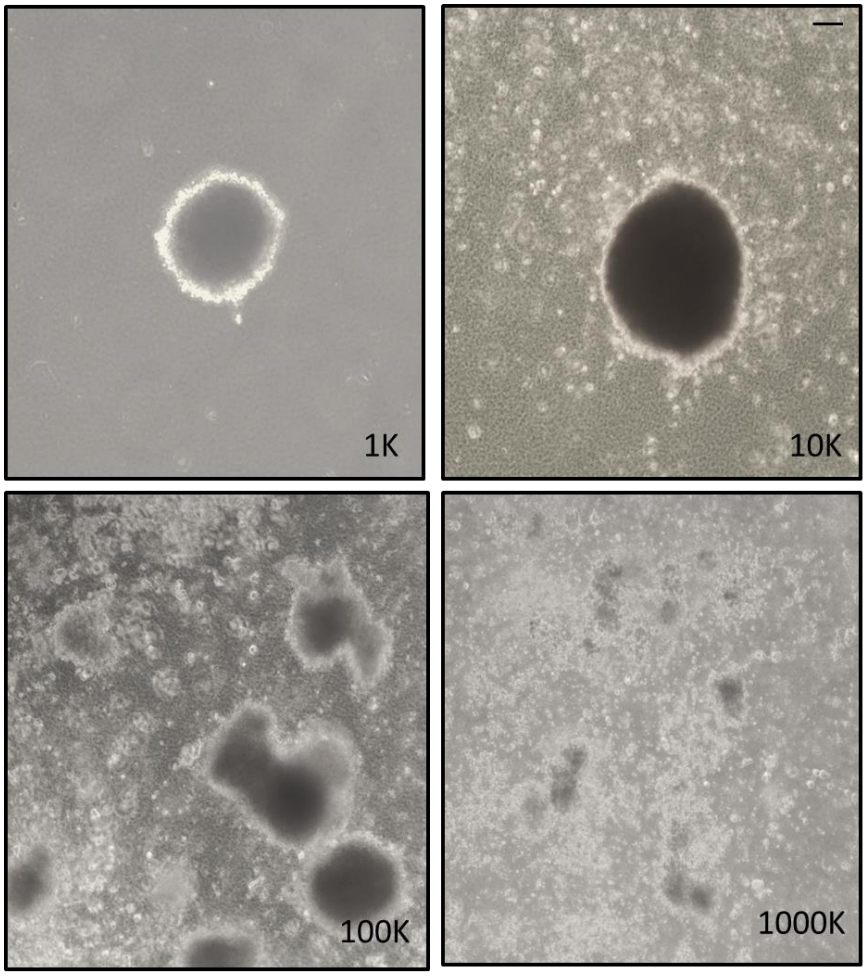

Figure 4.5.3: Assessing plating density for optimal hematopoietic activity in MethoCult. bm-MNC were plated at density of 1x10e^3; 1x10e^4; 1x10e^5; 1x10e^6 cells per mL methylcellulose (MethoCult) media. The maximal BFU formation observed was used to evaluate optimal plating density. [Scale bar represents 100μM].

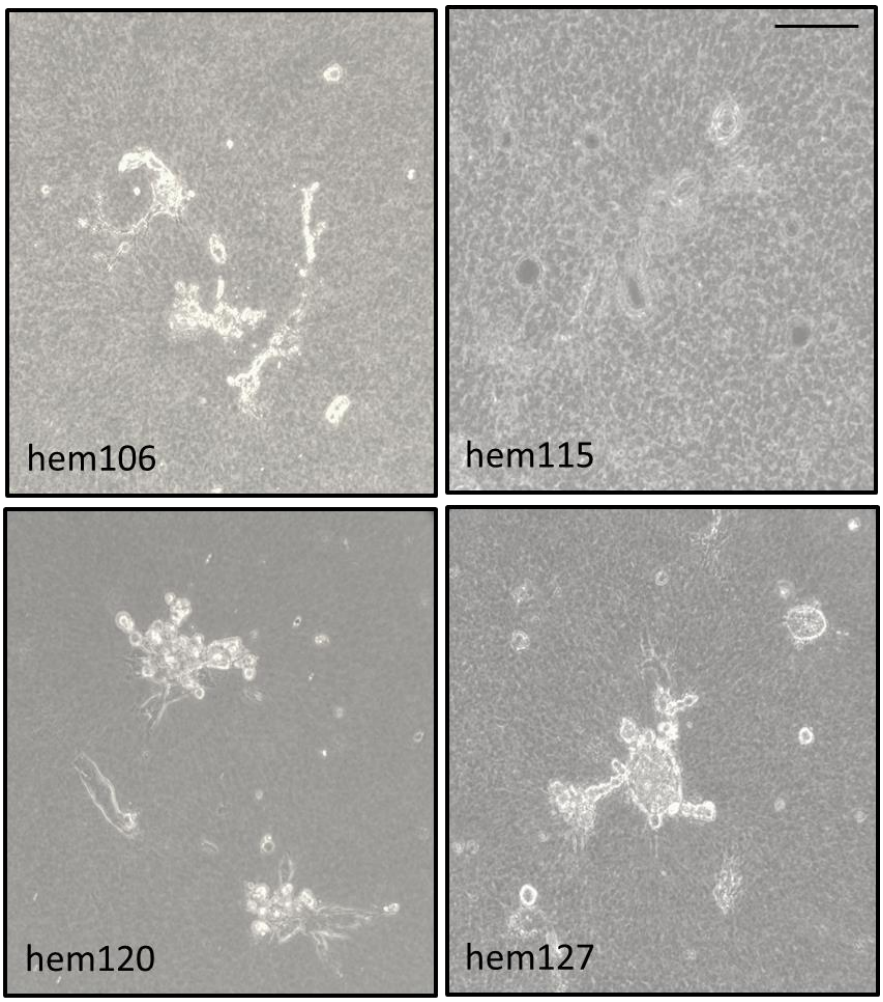

Figure 4.5.4: HemSCs do not exhibit intrinsic hematopoietic activity in MethoCult media. Four different hemSC primary cultures were plated in methylcellulose media for BFU colony observation. Relative to bm-MPCs, no BFU formation was observed, indication the lack of intrinsic hematopoietic activity. [Scale bar represents 100 μM].

4.6 TBX2 modulation of hemSC differentiation-competence

To investigate the potential effect of TBX2 on differentiation competence, I performed hemSC differentiation following *TBX2* knockdown. My results show that *TBX2* knockdown in hemSCs delays the upregulation of endothelial cell marker *CD31* in respective induction medium, relative to control siRNA transfected hemSC cultures (**Figure 4.6.1**). Morphologically, there were no obvious differences between the normal growth (control, EBM2) medium and endothelial differentiation medium with *TBX2* knockdown at day 7 of differentiation. Cells transfected with control siRNA did exhibit morphological changes in endothelial induction medium, however full endothelial cell differentiation was not conclusively observed. This lack of full endothelial differentiation in culture is consistent with our previous study. Adversely, *TBX2* knockdown did not appear to affect neuroglial cell differentiation as assessed by *GFAP* and *TUBB3* mRNA expression, as well as morphological assessment. Interestingly, *TBX2* knockdown significantly upregulated hematopoietic lineage specific cell surface marker *CD45*, as well as hematopoietic progenitor cell marker *CD34* mRNA in control medium conditions.

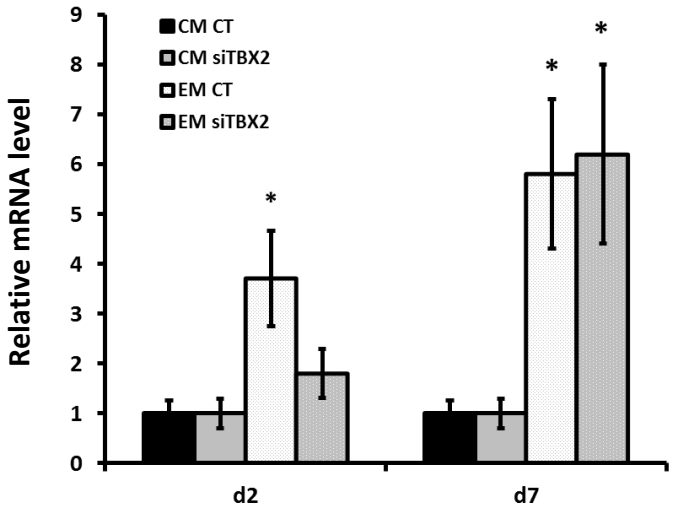

Figure 4.6.1: TBX2 knockdown delays CD31 expression upon culture of hemSCs in endothelial induction media. HemSCs were transfected with TBX2 siRNA and cultured in endothelial induction media (EM = endothelial media). Control replicates were cultured in EBM2 (CM = control media); transfected with control siRNA (CT). Knockdown efficiencies were assessed at 48 hours post-transfection; CD31 expression was assessed at day 2 (d2) and day 7 (d7) of culture with induction media. Only experimental replicates which exhibited 40% or greater TBX2 knockdown were included in CD31 expression analysis at d2. Relative mRNA normalized to β-actin; N=2; n=2; analysis of variance (ANOVA) followed by Bonferroni post-hoc analysis; *=$p<0.05$. Data expressed as mean ± SEM.

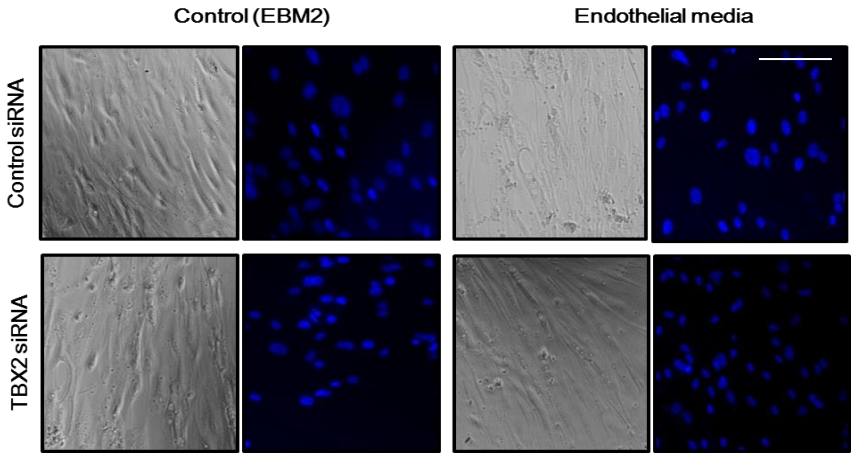

Figure 4.6.2: Morphological observations of hemSC cultures after transfection with either control or TBX2 siRNA transfection in EBM2 and endothelial induction media.
HemSCs cultured in endothelial induction media at day 7 after transfection with TBX2 siRNA retain spindle-shaped morphology. Nuclear staining with DAPI indicates culture viability. *Note: percent TBX2 knockdown efficiency was not assessed for imaged cultures. [Scale bar represents 100 μM].

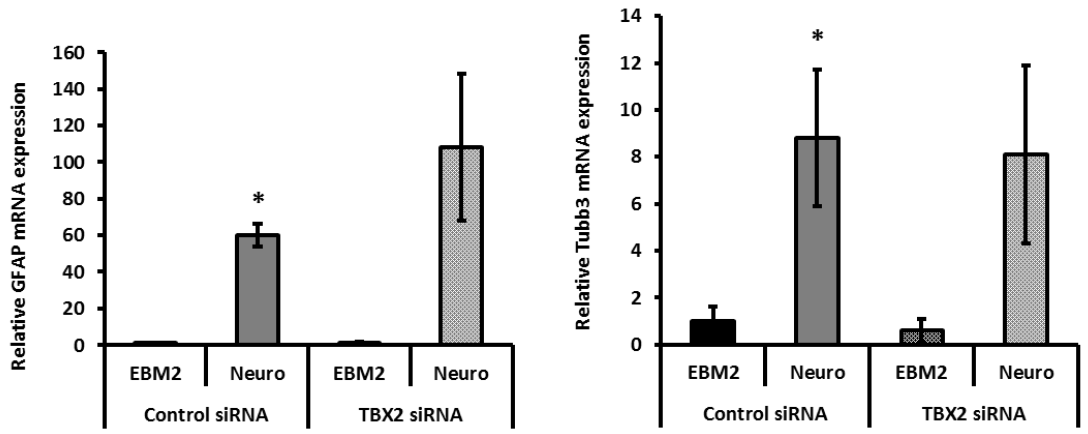

Figure 4.6.3: TBX2 knockdown does not alter TUBB3 or GFAP expression upon culture of hemSCs in neuroglial induction media.

HemSC were transfected with TBX2 siRNA and cultured in neuronal induction media. Control replicates were cultured in EBM2. Knockdown efficiencies were assessed at 48 hr post-transfection; TUBB3 and GFAP expression was assessed at d7 of differentiation. Only technical replicates which exhibited 40% or greater TBX2 knockdown were included in marker expression analysis. Relative mRNA normalized to β-actin; N=2; n=3; analysis of variance (ANOVA) followed by Bonferroni post-hoc analysis; *=$p<0.05$. Data expressed as mean ± SD.

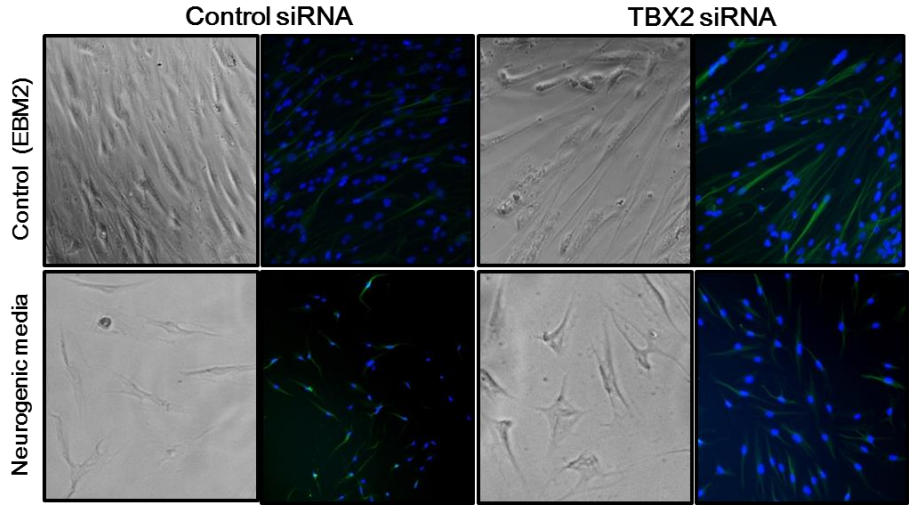

Figure 4.6.4: Morphological observations of hemSC cultures after transfection with either control or TBX2 siRNA transfection in EBM2 and neuroglial induction media.

HemSCs cultured in neuronal induction media at day 7 after transfection with TBX2 siRNA demonstrate obvious star-shaped morphological changes. Nuclear staining with DAPI (blue) indicates culture viability. All cultures stain positive for TUBB3 (green). *Note: percent TBX2 knockdown efficiency was not assessed for imaged cultures. [Scale bar represents 100 μM].

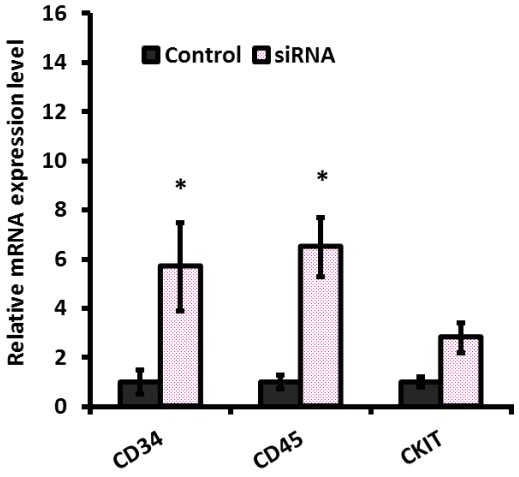

Figure 4.6.5: HemSCs exhibit upregulated hematopoietic lineage marker expression upon TBX2 knockdown.

HemSC were transfected with TBX2 siRNA and cultured in EBM2. Knockdown efficiencies were assessed at 48 hours post-transfection. Only experimental replicates which exhibited 40% or greater TBX2 knockdown were included in expression analysis. Cell surface markers CD34, CD45, and ckit mRNA are upregulated with TBX2 knockdown at 48 hours post-transfection in control media conditions. Relative mRNA normalized to β-actin; N=2, n=3; two-tailed student's t-tests were performed; *=$p<0.05$. Data expressed as mean ± SD.

4.7 TBX2 potentially labels hemangioma cell of origin

As TBX2 knockdown upregulated hematopoietic lineage markers in control conditions, I investigated potential upregulation of other associated hematopoietic genes. I found *TBX2* knockdown to upregulate *GLUT1* mRNA (**Figure 4.7.1**). As previously mentioned, GLUT1 is a diagnostic marker of IH, however it is also used as a marker of the hematopoietic lineage-derived myeloid cells, monocytes.[72, 138] Furthermore, given that hemangioma presents itself shortly after birth and has been diagnosed in the infant liver, I considered the possibility that hemSCs may arise from aberrant signaling during the transition of active hematopoiesis signaling from the fetal liver to the bone marrow. Interestingly, I found both heterogenous fetal liver cells (FLC; unsorted) cultures and CD133-selected FLC cultures to express both *TBX2* and *GLUT1* (**Figure 4.7.2**).

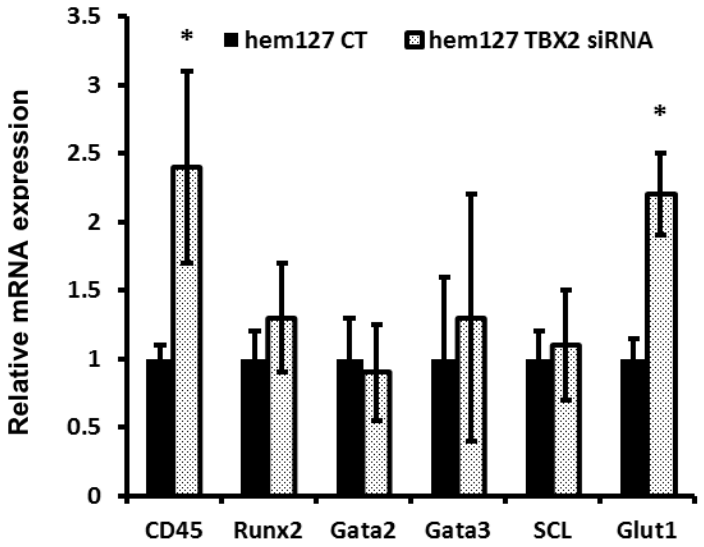

Figure 4.7.1: GLUT1 mRNA is upregulated in hemSC upon TBX2 knockdown.
Known hematopoietic lineage transcription factors Runx2, Gata2/3, and SCL do not show significant changes in expression levels upon TBX2 knockdown, where GLUT1 is significantly upregulated (in hemSC cultures from **Figure 4.9**). Relative mRNA normalized to β-actin; N=1, n=3, two-tailed student's t-tests were performed; *=$p<0.05$. Data expressed as mean ± SD.

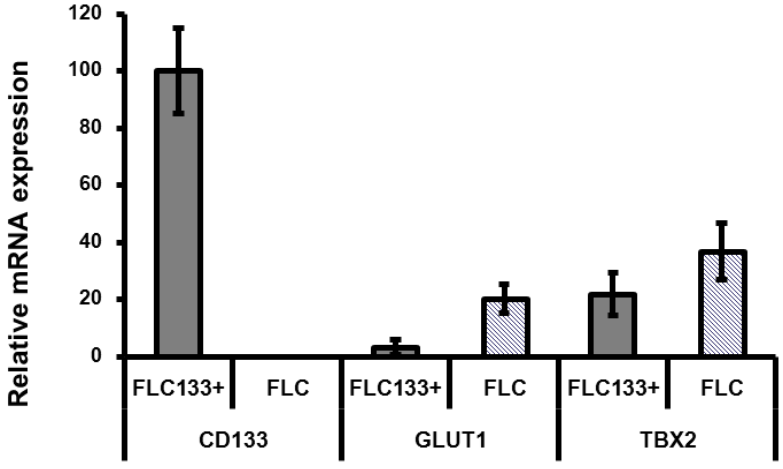

Figure 4.7.2: Fetal liver cells express TBX2 and GLUT1 in culture.
CD133-selected and unsorted population of FLCs express both TBX2 and GLUT1 mRNA in culture similar to hemSCs [FLC = unsorted fetal liver cells' FLC133+ = sorted CD133-positive fetal liver cells]. Relative mRNA normalized to β-actin; N=1, n=3. Data expressed as mean ± SD.

4.8 TGF-β modulates TBX2 expression

To investigate potential upstream modulators of TBX2, I treated hemSCs with agonists/antagonists of target pathways as listed in **Table 2**. The Wnt pathway has been shown to be upstream of TBX2 in cardiac development[111]; the PI3K/Akt pathway is associated with upregulated VEGFR-2 signaling in tumourigenic cells [114]; TGF-β is upstream of TBX2 in cardiac development[121] and melanoma cells[122], and is also upregulated upon involution in IH.[39] I identified TGF-β treatment of hemSCs to significantly suppress *TBX2* mRNA levels (**Figure 4.8.1**). I confirmed the efficacy of treatment by identifying that the addition of TGF-β pathway inhibitor GW788388, normalized *TBX2* expression levels (**Figure 4.8.2**); TGF-β treatment also induced the significant upregulation of known downstream targets collagen I and IV mRNA (**Figure 4.8.3**). Interestingly, the suppression of *TBX2* by TGF-β treatment also induced the significant upregulation of pluripotency factors *OCT4/POUF5*, *SOX2*, and *NANOG* (**Figure 4.8.3**), as seen with *TBX2* knockdown (**Figure 4.4.3**). Addition of PI3K pathway inhibitor PI828 also seemed to depress TBX2 expression, however the results were not significant at $p<0.05$ (**Figure 4.8.1**). Additional cell preparations are needed to rule out PI3K. Wnt agonist did not appear to affect *TBX2* mRNA expression levels (**Figure 4.8.1**).

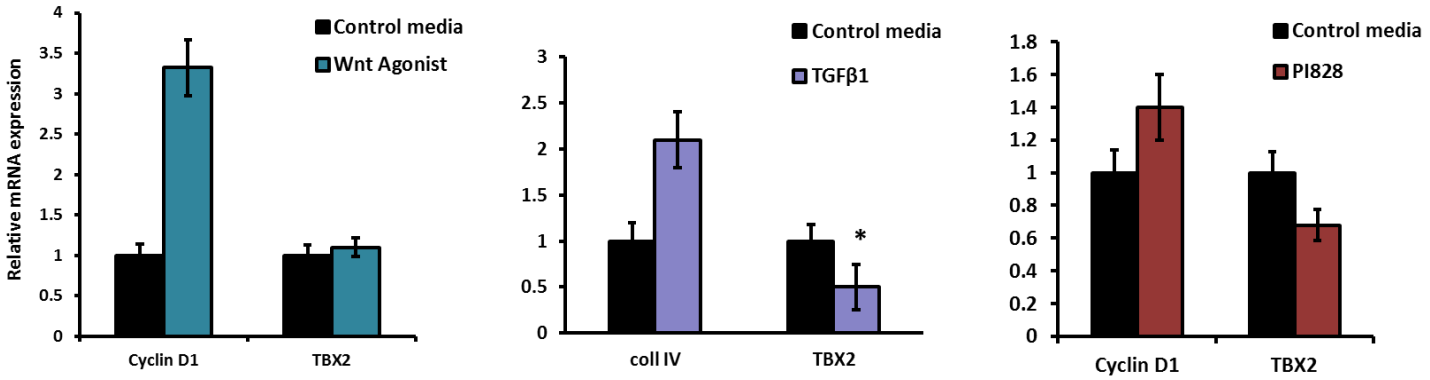

Figure 4.8.1: Screening for upstream modulators of TBX2 reveals TGFβ to significantly repress TBX2 mRNA expression. HemSCs were cultured in EBM2 with the addition of Wnt agonist (1 μM, 681665, EMD Millipore); TGFβ1 (10 ng/mL, 100-B, R&D Systems); and PI 3-kinase inhibitor (2 μM, PI 828, 2814, R&D Systems). TBX2 mRNA expression was assessed at day 3 and day 7 of culture (combined time point data is represented above). Treatment with TGFβ1 ensued significant repression of TBX2 mRNA levels. Relative mRNA normalized to β-actin; N=2; n=3; two-tailed student's t-tests were performed; *=p<0.05. Data expressed as mean ± SD.

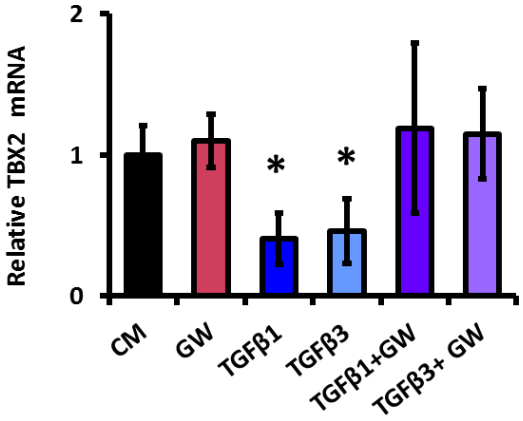

Figure 4.8.2: TGFβ repression of TBX2 mRNA expression is rescued by the addition of TGFβ receptor inhibitor. HemSCs were cultured in EBM2 with the addition of TGFβ1 (10 ng/mL, 100-B, R&D Systems); TGFβ3 (10 ng/mL, 243-B3, R&D Systems), and an inhibitor of TGF-beta type I and II receptor kinases (GW) (100 nM, GW788388, R&D Systems). TBX2 mRNA expression was assessed at day 3 and day 7 of culture (combined time point data is represented above). Treatment with both TGFβ1 and TGFβ3 ensued significant repression of TBX2 mRNA expression. The addition of TGFβ receptor inhibitor rescued TBX2 mRNA to control expression levels. Relative mRNA normalized to β-actin; N=2; n=3; two-tailed student's t-tests were performed; *=p<0.05. Data expressed as mean ± SD.

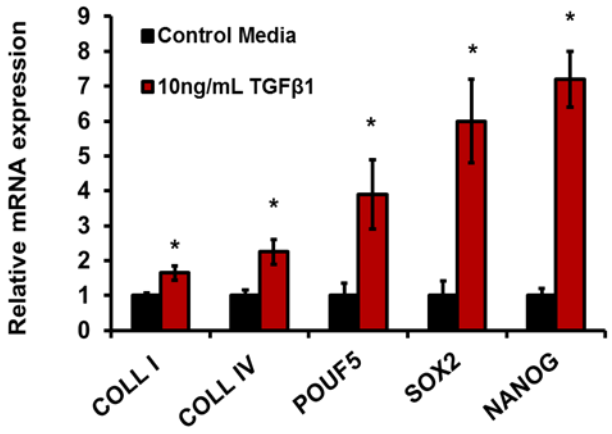

Figure 4.8.3: TGFβ repression of TBX2 mRNA expression is ensued by upregulation of pluripotency factor expression. HemSCs were cultured in EBM2 with the addition of TGFβ1 (10 ng/mL, 100-B, R&D Systems). Pluripotency factor (OCT4/POUF5; SOX2; NANOG), collagen I (COLL I), and collagen IV (COL IV) mRNA expression was assessed at day 3 of culture conditions. Treatment with TGFβ1 resulted in the upregulation of downstream TGFβ pathway targets COLL I and IV, as well as pluripotency factor mRNA expression. Relative mRNA normalized to β-actin; N=2; n=3; two-tailed student's t-tests were performed; *=$p<0.05$. Data expressed as mean ± SD.

4.9 TGF-β modulates hemSC differentiation

To confirm whether TBX2 suppression by TGF-β may also affect hemSC differentiation-competence, I performed cell differentiation experiments with the addition of TGF-β to induction media. I focused my studies on the endothelial lineage as this lineage is the most critical during the proliferative phase of IH. Furthermore, my studies with *TBX2* knockdown showed delayed CD31 induction in cells exposed to endothelial differentiation media. Therefore, endothelial cell differentiation of hemSCs provides an excellent readout of any potential effects of TGF-β. I confirmed that TGF-β suppressed TBX2 expression in endothelial induction media (**Figure 4.9.1**). I also found that treatment of hemSC with TGF-β in endothelial induction media significantly suppresses CD31 expression at day 7 of differentiation relative to control endothelial media (**Figure 4.9.2**). This corroborated my findings of *TBX2* knockdown via siRNA during endothelial differentiation (**Figure 4.6.1**)

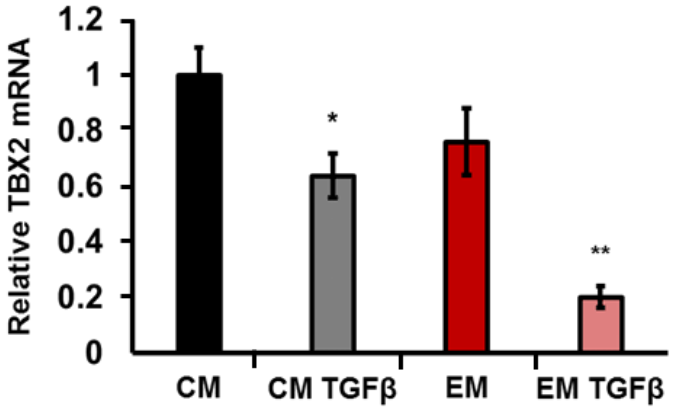

Figure 4.9.1: Addition of TGFβ to endothelial induction media significantly represses TBX2 expression. HemSCs were cultured in EBM2 media (CM) and endothelial induction media (EM) with the addition of TGFβ1 (10 ng/mL, 100-B, R&D Systems). TBX2 expression was assessed at day 3 and day 7 of culture (combined time point data is represented above). Treatment with TGFβ1 suppressed TBX2 mRNA in both control and endothelial induction mediums. Relative mRNA normalized to β-actin; N=2; n=3; analysis of variance (ANOVA) followed by Bonferroni post-hoc analysis; *=p<0.05. Data expressed as mean ± SEM.

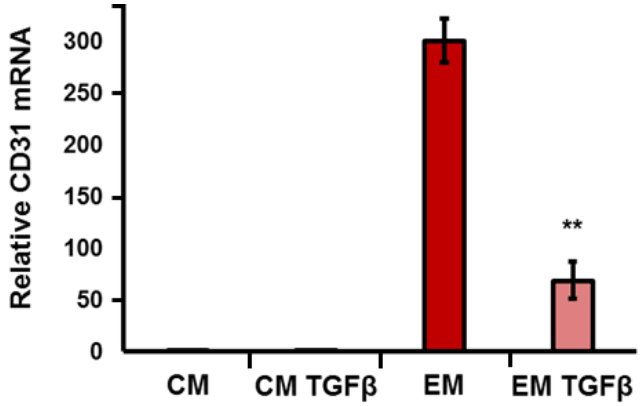

Figure 4.9.2: Addition of TGFβ to endothelial induction media significantly represses CD31 expression of hemSC. HemSCs were cultured in EBM2 media (CM) and endothelial induction media (EM) with the addition of TGFβ1 (10 ng/mL, 100-B, R&D Systems). Endothelial lineage cell surface marker CD31 expression was assessed at day 3 and day 7 of culture (combined time point data is represented above). Treatment with TGFβ1 significantly suppressed CD31 expression in endothelial induction media. Relative mRNA normalized to β-actin; N=2; n=3; analysis of variance (ANOVA) followed by Bonferroni post-hoc analysis; *=p<0.05, **=p.0.01. Data expressed as mean ± SD.

Chapter 5

5 DISCUSSION

5.1 Discussion of experimental findings

My findings suggest that transcription factor T-box may contribute to hemangioma pathogenesis by regulating both the growth and differentiation of hemSCs.

My studies demonstrated TBX2 to have a nuclear localization in IH specimens, displaying co-expression with endothelial cell marker CD31 (**Figure 4.1**). This indicates TBX2 is expressed in the endothelium of IH vessels. Previous work in our lab has shown these CD31 positive endothelial cells to also co-express CD133, suggesting they are stem cells. Isolated CD133-expressing cells also recapitulate human hemangioma in mice. In conjunction with the co-expression of TBX2 and OCT4 in cells lining the lumen of IH vessels (**Figure 4.4.2**), I believe TBX2 expression to be associated with a stem-cell like phenotype in hemangioma. The presence of TBX2 positivity in interstitial and perivascular cells, which appear to have the same localization as α-SMA positive cells (**Figure 4.1**), may suggest that cells other than those of the endothelial lineage demonstrate stem cell-like properties in hemangioma. As the current stem cell of origin for hemangioma is unknown, it could be insightful to conduct a lineage analysis to determine whether 1) perivascular and interstitial cells in hemangioma are also derived from endothelial cell-forming CD133-positive cells, and 2) TBX2 expression and localization alters through different cell types and/or tissue layers throughout the life-cycle of the tumour. Based on the work of our laboratory as well as others, I would expect that TBX2-labeled cells will give rise to hemangioma vessel endothelial cells as well as other cellular constituents in the tumour. Furthermore, based on the findings of my study, I also expect the levels of TBX2 to change upon cell differentiation and hemangioma involution.

In culture, my studies have shown TBX2 to be localized primarily in the nucleus of hemSCs, however one patient-derived hemSC primary cell culture also exhibited cytoplasmic as well as nuclear TBX2 localization (**Figure 4.2.1**). Being a transcription factor, is it expected to have a nuclear localization when in an active state. TBX2 has no known nuclear localization signals (NLS), and to date has only been found to have a cytoplasmic localization in *C. elegans*.[139] This might suggest that TBX2 has a novel NLS sequences or that it requires binding to a NLS-

containing protein(s) to chaperone it to the nucleus. The differences seen in TBX2 protein localization as well as in relative mRNA expression (**Figure 4.2.2**) may reflect differences from the patient tumour from which they were derived. Depending on the exact stage of tumour life-cycle upon when the hemSCs were isolated from the patient, it is possible for different primary cultures to retain intrinsically different TBX2 expression profiles. If a hemSC population was isolated from a more proliferative tumour, this may reflect altered levels of TBX2 expression and localization. Additionally, it is possible there are variable levels of TBX2 expression based on the clonal expansion of a particular subset of hemSCs throughout the culture expansion. However, it is important to note that TBX2 mRNA expression was retained at high levels relative to bm-MPC cultures throughout passages (**Figures 4.2.3 and 4.2.4**). Additionally, prior studies in our lab have shown hemSCs to exhibit much higher TBX2 mRNA expression in culture than compared to normal endothelial cells such as cord-blood derived endothelial progenitors, and human microvascular endothelial cells.[80] This suggests that TBX2 expression in hemSC may be associated with their pathogenicity.

Interestingly, TBX3 was also found to be expressed in hemSC cultures. While TBX3 expression in IH specimens has not been identified, it is known to be co-expressed with TBX2 in a number of developmental and tumourigenic processes as previously described. It has been suggested that while TBX2 promotes tumour cell proliferation, TBX3 promotes endothelial-to-mesenchymal (EMT) cell migration.[140] Is it interesting that for my expression profile studies, when TBX2 mRNA was elevated in a particular hemSC culture TBX3 mRNA was decreased and vice-versa, levels in bm-MPCs simply dropped evenly over subculture (**Figure 4.2.4**). This may suggest that TBX2 and TBX3 are both active in hemSC, although further analysis of TBX3 expression in IH lesions would be necessary to confirm clinical significance of this finding.

What may hold potential clinical significance however, are my findings from investigating the effect of TBX2 expression level on cell cycle regulation. My data suggests that TBX2 may repress cell cycle regulator p16, as TBX2 knockdown via siRNA significantly upregulated p16 mRNA expression (**Figure 4.3.2**), while simultaneously altering the number of total live cells in culture. As previously mentioned, p16 is a major target in carcinogenesis, rivaled in frequency only by the p53 tumour-suppressor gene. Its mechanism of action as a CDKI involves binding to and inactivating the cyclin D-cyclin-dependent kinase 4 (or 6) complex, rendering the

retinoblastoma protein inactive. This effect blocks the transcription of important cell-cycle regulatory proteins and results in cell-cycle arrest.[141] As prior literature has found TBX2 to repress cell cycle regulators, it is a probable that the high levels of TBX2 expression in hemangioma may be contributing to unregulated cell growth during the proliferative phase of the tumour life cycle. Supporting this, is the data by Ritter et al. which demonstrates that the significant decrease in TBX2 expression in IH coincides with the time of involution.[57] However, direct binding of TBX2 to p16 has not been investigated in this study, and would be required to support p16 as a downstream target of TBX2. Quantification of p16 protein would also be necessary to definitively state whether the observed changes have a significant effect on hemSC proliferation due to p16. Additionally, while I observed total live cell counts to significantly decrease in hemSC cultures with TBX2 knockdown relative to those with control siRNA, this effect was lost after 7 days (**Figure 4.3.3**). I believe this is likely due to the effect of the siRNA knockdown lasting typically 5-7 days.

Interestingly, as p16 mRNA was being upregulated during TBX2 knockdown, pluripotency factors OCT4, SOX2, and NANOG mRNA expression were upregulated in hemSC (**Figure 4.4.3**). This may suggest that TBX2 has an effect on the stem cell-like phenotype of hemSCs, where decreased levels of TBX2 reverts the hemSC to an undifferentiated state. The concept of dedifferentiation is well known in the stem cell field, whereby cells regress from a specialized function to a simpler state reminiscent of stem cells.[142] Subsequently, it may be deduced that elevated TBX2 promotes hemSC differentiation. Or perhaps more correctly, their differentiation-competence, or potential to differentiate when supplied with the appropriate factors or signaling mechanisms. Of note, the effects of TBX2 knockdown on hemSC pluripotency factor expression was variable depending on percent knockdown, and time in culture (**Figure 2-App and Figure 3-App**), suggesting this may be a temporary cell state, or even an *in vitro* artifact.

However, my studies investigating hemSC differentiation-competence with TBX2 modulation cumulatively suggest the transcription factor may be involved in hemSC differentiation to some extent. TBX2 knockdown resulted in the upregulation of hematopoietic lineage cell surface marker CD45 mRNA (**Figure 4.6.5**), and delayed the expression of endothelial cell surface marker CD31 mRNA when compared to control siRNA in respective induction media (**Figure**

4.6.1). While this is potentially reminiscent of endothelial to hematopoietic transition (EHT), extensive further hemSC differentiation analysis is necessary to propose this as a plausible mechanism during IH involution. Challenges encountered during these lineage analyses include the lack of convincing endothelial cell differentiation of hemSCs in culture with control TBX2 levels. Ideally, endothelial cell differentiation would be confirmed functionally by tube formation assay, and phenotypically by FACs sorting for endothelial cell specific lineage markers. However, my assessment was restricted to the upregulation of CD31 mRNA expression. Similarly, hematopoietic activity would be confirmed through function assessment via the formation of BFUs upon TBX2 knockdown. While attempted, these experiments did not convincingly demonstrate full hematopoietic or endothelial cell differentiation. Therefore, optimizing lineage induction protocols would be required for a comprehensive analysis of the effect of TBX2 modulation on differentiation-competence. Nonetheless, contemplating EHT as a potential mechanism during upon the reduction of TBX2 during IH involution may be one explanation for the cell metaplasia/cell replacement observed during this transition from vascular to fibro-adipose tissue. Additionally, if we consider GLUT1 a monocyte marker as suggested by Ritter et al.,[72] my data suggests this is also upregulated upon TBX2 knockdown in conjunction with other hematopoietic lineage markers (**Figure 4.7.1**). While this appears to be in contradiction to a study which found GLUT1-positive cells to significantly decrease upon IH involution,[69] if EHT is indeed occurring during IH involution it is plausible the loss of GLUT1 expression observed during the Bischoff study is recompensed by an increase in circulating GLUT1-positive hematopoietic cells.

To better understand the potential signaling pathway of TBX2 in IH, I tested a number of candidate pathway agonists/antagonists as listed in **Table 2**. I identified TGF-β to significantly repress TBX2 expression in hemSCs (**Figures 4.8.1, 4.8.2**), upon which similar downstream effects as seen with TBX2 knockdown via siRNA were observed. These effects included upregulation of pluripotency factor expression, and inhibition of CD31 mRNA expression in endothelial cell induction medium (**Figures 4.8.3 and 4.9.2**). Interestingly, TGF-β has previously been shown to suppress TBX2 via TBX3 in human breast cancer cells.[122] Furthermore, TGF-β signaling has been associated with the suppression of endothelial cell differentiation. [143, 144] This supports our evidence that the TGF-β-TBX2 axis may be contributing towards hemSC lineage specification, as depicted in **Figure 5.1**:

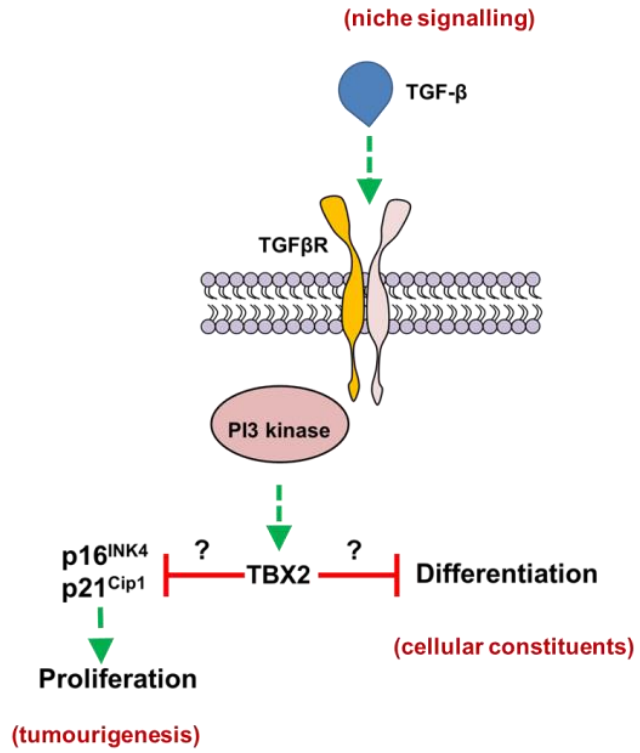

Figure 5.1: Schematic diagram illustrating the hypothesis and acquired findings. Circulating niche factor TGF-β suppresses TBX2 expression to repress hemSC proliferation via cell cycle regulator inhibition, as well as inhibiting endothelial lineage differentiation. This signaling pathway may be mediated by PI3 kinase.

While my evidence that PI3 kinase inhibition suppresses TBX2 expression in hemSCs is preliminary (**Figure 4.8.1**), it is possible there may be cross-talk between PI3 kinase and TGF-β pathways. The transforming growth factor (TGF)-β and phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling pathways are used in cells to control numerous responses, including proliferation, apoptosis, and migration. Previous literature has alluded to both modulating human embryonic stem cell differentiation, and contributing to cancer progression.[145, 146] In the former studies, PI3K signaling was shown to antagonize definitive endoderm differentiation of human ES by attenuating the duration of Smad2/3 activation via the mTOR complex 2 (mTORC2). In addition, TGF-β is known for its cytostatic effects in premalignant states and its pro-oncogenic activity in advanced cancers, where PI3K/AKT activation antagonizes the TGF-β/SMAD-induced cytostatic response. Further studies are required to demonstrate direct cross-talk between the pathways in hemSCs.

Identifying the molecular and biochemical mechanisms regulating hemSC differentiation would be a major advancement in the field, and may provide new treatment targets for IH. In addition to providing insight into the etiology and pathogenesis of the tumour, this project aimed to characterize pathways which regulate the viability of IH stem cells. The ability to pinpoint an origin of the hemSCs is ultimately restricted by the current definitions of stem cells. Based on current definitions for cell phenotype and respective cell lineage markers, hemSCs could be scientifically supported to be of mesodermal/mesenchymal stem cell origin, its pathogenesis originating possibly from an aberrant step in differentiation. However, the term 'mesenchymal' seems restrictive to the mesenchymal lineage and does not truly reflect the demonstrated ability of hemSCs. As the field furthers its understanding of what markers and signaling mechanisms are true identifiers of stem cell prospective differentiation lineages, it will advance IH research one step further to the origin and development of the IH pathology. As current therapies for this tumour are non-specific, recognizing drugs which specifically target cells initiating the tumour would have far-reaching implications for clinical treatment options. As IH affects infants at a critical stage during development, it is imperative to find therapies with known mechanisms of action, and minimal side effects. In addition, the potential role of TBX2 as a regulator of stem cell differentiation-competence may have implications for research outside of IH. If the function of TBX2 is conserved in normal stem cells, it may be targeted to upregulate hematopoietic lineage differentiation. In summary, the potential role of TBX2 as a regulator of differentiation competence may have implications for the development of stem cell research, providing new insight into the field of regenerative medicine.

5.2 Clinical implications of study

Identifying candidate drugs which target hemSCs may revolutionize the current treatment of infantile hemangioma and other stem-cell derived tumours, changing policy for the clinical approaches to these diseases. As previously mentioned, the most common treatment for IH is currently propranolol. I have performed pilot studies to examine the effect of propranolol treatment on TBX2 mRNA expression in hemSCs (**Figure 5-App**). Although limited in scope, these studies show that propranolol upregulates TBX2 mRNA. Therefore, it is possible that treatment with propranolol may in fact allow for hemSC survival in lesions. This may be one

explanation for the observed recurrence of IH in certain case studies post-propranolol therapy cessation. Furthermore, if TBX2 knockdown truly inhibits endothelial cell differentiation, it may be possible that overexpression of TBX2 promotes hemSC differentiation as well as proliferation. Thus, targeting TBX2 *in vivo* may alter the progression of IH. Further studies investigation the effects of both TBX2 knockdown and overexpression *in vivo* would be essential for identifying the true effects of modulating TBX2 on IH life-cycle.

Additionally, the potential role of TBX2 as a regulator of stem cell differentiation may have implications for many branches of stem cell research. In particular, fields utilizing a directed differentiation approach to generate cells of a particular lineage, and those developing induced pluripotent stem cells, could benefit from targeting a transcription factor which regulates differentiation-competence. Specifically, the up-regulation of hematopoietic activity observed with TBX2 knockdown may have implications for patients requiring HSC therapies. Beyond the clinical impacts, characterizing the function of TBX2 within hemSC and normal stem cells would provide insight into the role of this multi-faceted transcription factor in the development and origin of infantile hemangioma.

5.3 Future directions

To conclusively characterize the TBX2 signaling pathway in hemSCs, I propose utilizing chromatin immunoprecipitation followed by microarray hybridization (ChIP-Chip), which enables genome-wide analysis of transcription factor binding.[147] This would allow for the identification of upstream and downstream targets of TBX2 within hemSCs, thus delineating the signaling mechanism by which this transcription factor influences cell fate decisions. Understanding the latter will allow us to target specific regulators of the proliferation and involution of IH.

I also believe it imperative to identify candidate drug therapies which target TBX2 and viability of hemSCs, altering the progression of IH *in-vivo*. As discussed, propranolol treatment studies in patients have found that some IHs regrow upon cessation of propranolol treatment.[28, 29] This has been attributed to early treatment withdrawal and/or a long proliferating phase of IH. Recent work in our lab has concluded that while propranolol induces apoptosis in hemangioma

endothelial cells, hemSCs are resistant to caspase-3 activation by propanolol.[148] A number of recent studies have also shown that propranolol treatment of normal endothelial cells as well as endothelial cells derived from IH specimens causes activation of caspase-3.[149, 150] Interestingly, IH-derived endothelial cells are unable to recapitulate IH *in vivo*, as our lab has demonstrated with hemSCs.[16] We have previously shown that hemSCs treated with propranolol show induction of anti-apoptotic pathways, including Akt (also known as protein kinase B), and insulin-like growth factor receptor-1 (IGFR-1).[148] Therefore it is plausible that hemSCs may be responsible for the recurrence of IH upon cessation of propranolol treatment, owing to their ability to resist propranolol-induced apoptosis. Interestingly, preliminary data suggests that hemSCs treated only with a very high dose of propranolol (100 μmol/L) show up-regulation of TBX2 mRNA expression (**Figure 5-App**). Based on my data, I anticipate that compounds which induce expression of TBX2 will promote differentiation of hemSCs reflective of the involution process during IH. Thus, drugs which target both the hemSC viability and the TBX2 signaling pathway, may prompt the most rapid regression of the tumour.

Chapter 6

6 REFERENCES

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APPENDIX: Supplementary Data

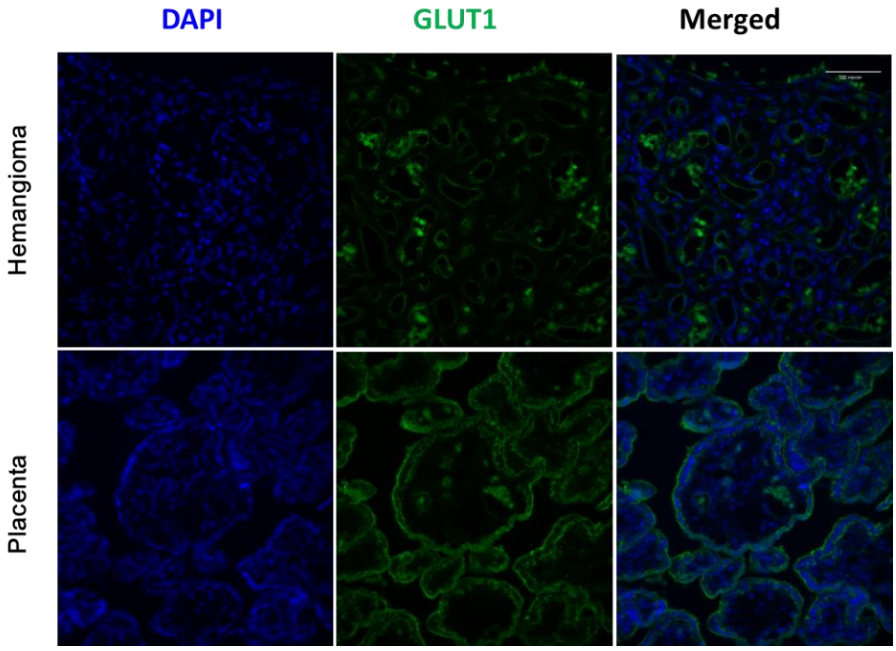

Figure 1-App: Confirmation of GLUT1-positive hemangioma specimens.
Proliferating hemangioma and early gestation placenta (<25 weeks) specimens stain positive for the diagnostic marker GLUT1 (green), distinguishing the IH specimens from other types of vascular anomalies. Nuclear staining is seen with DAPI (blue). [40x mag; scale bar represents 100μM; DAPI nuclear stain = blue].

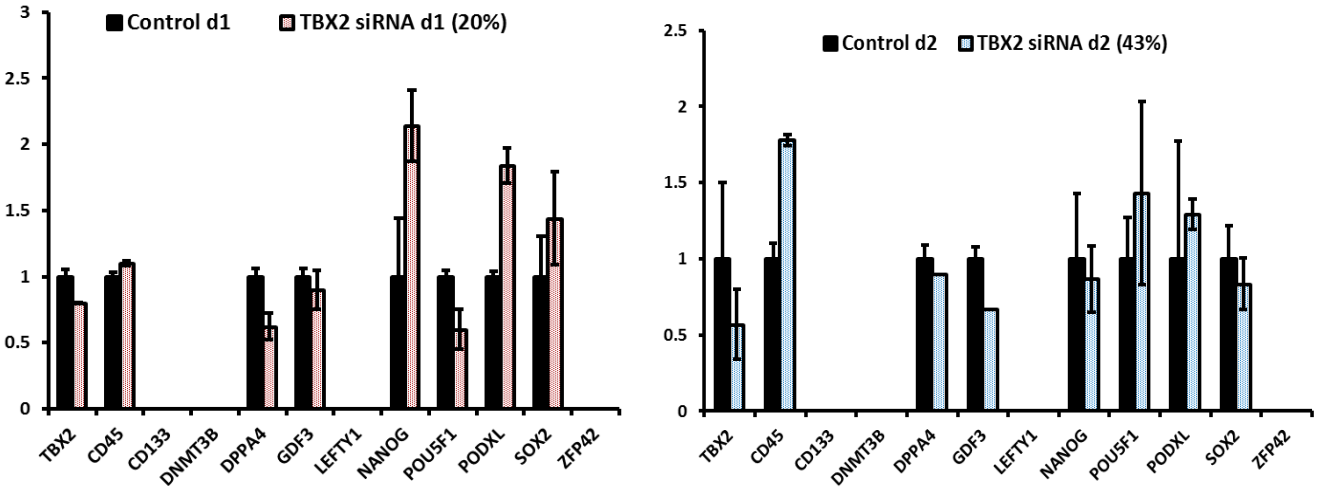

Figure 2-App: Pluripotency factor and CD45 expression is dependent on the degree of TBX2 knockdown. An assessment was made for variability in induction of pluripotency factor and CD45 mRNA expression analysis with different degrees of TBX2 knockdown and time of assessment. Hem127 cultures were transfected with TBX2 siRNA and cultured in EBM2. Expression analysis was assessed at 24 hours (d1) with 20% knockdown efficiency and 48 (d2) hours post-transfection with 43% knockdown efficiency. It appears that pluripotency factors may be induced prior to cell surface marker mRNA expression. Relative mRNA normalized to β-actin; N=1; n=2.

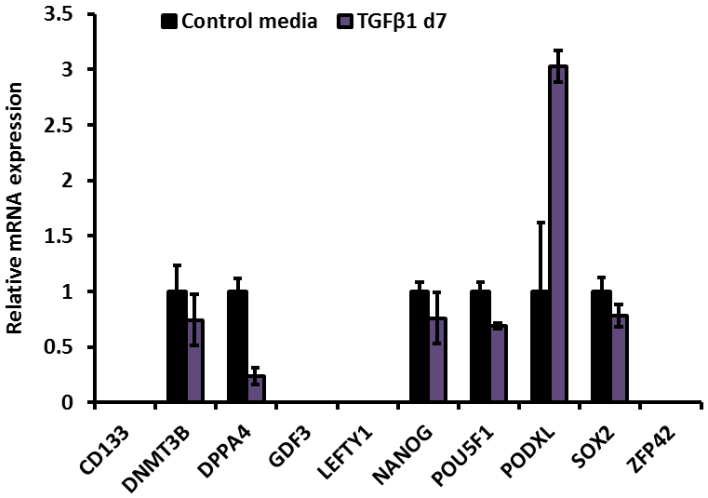

Figure 3-App: Pluripotency factor upregulation by TGFβ treatment.
An assessment was made for variability in induction of pluripotency factor mRNA expression analysis with TGFβ time of assessment. Hem127 were cultured in EBM2 with the addition of TGFβ1 (10 ng/mL, 100-B, R&D Systems). Expression analysis was assessed at day 7 of treatment with TGFβ1. It appears that induction of pluripotency factors may be temporally dependent. Relative mRNA normalized to β-actin; N=1; n=2.

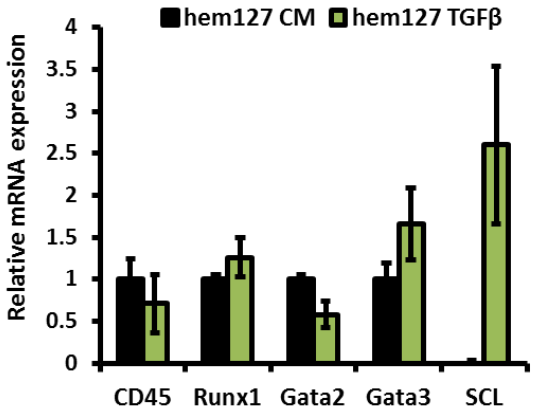

Figure 4-App: Hematopoietic lineage induction of hemSCs upon TGFβ treatment.
An assessment was made for possible induction of hematopoietic lineage induction with the addition of TGFβ to hemSC cultures. Hem127 were cultured in EBM2 with the addition of TGFβ1 (10 ng/mL, 100-B, R&D Systems). Expression analysis was assessed at day 3 of treatment with TGFβ1. While lineage specific cell surface marker CD45 was not upregulated, as seen with repression of TBX2 via siRNA in **Figure 4.9**, hematopoietic transcription factor SCL is upregulated. This may also suggest a temporally dependent mechanism. Relative mRNA normalized to β-actin; N=1; n=2.

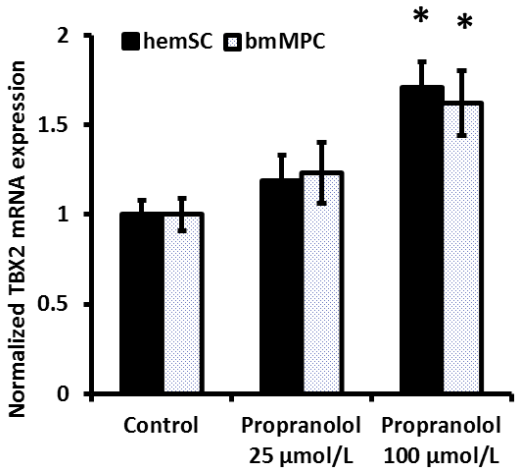

Figure 5-App: Clinical perspectives – Effect of propranolol on TBX2 expression in hemSC.
RNA isolated from hemSC cultures with the addition of current therapeutic drug compound propranolol (25 µM/L; 100 µM/L), kindly provided by lab member Jina Kum, was converted to cDNA and assessed for TBX2 mRNA expression. High concentrations of propranolol resulted in significant upregulation of TBX2 expression in hemSC. Relative mRNA normalized to β-actin; N=2; n=3; two-tailed student's t-tests were performed; *=$p<0.05$.

CURRICULUM VITAE

EDUCATION

2014 – present **University of Western Ontario**, London, ON, Canada
- Master of Science, Pathology
- Collaborative Graduate Program in Developmental Biology

2008 – 2013 **University of Waterloo**, Waterloo, ON, Canada
- Bachelor of Science, Honours Biology Major
- Specialization in Animal Physiology

PRESENTED RESEARCH

06/2016 **Developmental Biology Annual Research Day**, London, ON, Canada
- Abstract: *Transforming growth factor-β regulates differentiation potential of hemangioma stem cells by modulating T-box 2.*
- Authors: Richmond, N., Khan, Z. Department of Pathology and Laboratory Medicine, UWO

04/2016 **London Health Research Day**, London, ON, Canada
- Abstract: *Transforming growth factor-β regulates differentiation potential of hemangioma stem cells by modulating T-box 2.*
- Authors: Richmond, N., Khan, Z. Department of Pathology and Laboratory Medicine, UWO

03/2016 **Pathology and Laboratory Medicine Research Day**, London, ON, Canada
- Abstract: *Transforming growth factor-β induces pluripotency genes in hemangioma stem cells through repressing T-box 2.*
- Authors: Richmond, N., Khan, Z. Department of Pathology and Laboratory Medicine, UWO

11/2015 **Diabetes Research Day**, London, ON, Canada
- Abstract: *Diabetic marrow adipogenesis alters the composition of the stem cell niche and impairs CD133-positive stem cell survival.*
- Authors: Piccinin, M., Richmond, N., Khan, Z. Department of Pathology and Laboratory Medicine, UWO

10/2015 **Till & McCulloch Meetings**, Toronto, ON, Canada
- Abstract: *Transforming growth factor-β induces pluripotency genes in hemangioma stem cells through repressing T-box 2*.
- Authors: Richmond, N., Khan, Z. Department of Pathology and Laboratory Medicine, UWO

06/2015 **Developmental Biology Annual Research Day**, London, ON, Canada
- Abstract: *T-box 2 regulates hematopoietic competency of infantile hemangioma stem cells.*

- Authors: Richmond, N., Khan, Z. Department of Pathology and Laboratory Medicine, UWO

04/2015 **London Health Research Day**, London, ON, Canada
- Abstract: *T-box 2 regulates hematopoietic competency of infantile hemangioma stem cells.*
- Authors: Richmond, N., Khan, Z. Department of Pathology and Laboratory Medicine, UWO

03/2015 **Pathology and Laboratory Medicine Research Day**, London, ON, Canada
- Abstract: *Hematopoietic progenitor phenotype induced by T-box 2 knockdown in infantile hemangioma cells.*
- Authors: Richmond, N., Khan, Z. Department of Pathology and Laboratory Medicine, UWO

10/2014 **Till & McCulloch Meetings**, Ottawa, ON, Canada
- Abstract: *T-box 2 knockdown induces hematopoietic progenitor phenotype in infantile hemangioma cells*.
- Authors: Richmond, N., Khan, Z. Department of Pathology and Laboratory Medicine, UWO

03/2014 **American Society of Animal Science**, Des Moines, Iowa, USA
- Abstract: *Effect of nursery diet protein quality and fish oil supplementation on growth performance, immune response, and gut morphology in starter pigs*.
- Authors: Hooda, S., Richmond, N., Karrow, N. A., de Lange, C. F.M. Department of Animal and Poultry Science, University of Guelph

RESEARCH EMPLOYMENT

09/2014-present **Graduate Research Assistant,** Department of Pathology and Laboratory Medicine, University of Western Ontario

Supervisor: Dr. Zia A. Khan
- Studied the pathological properties of the disease model infantile hemangioma (IH)
- Performed experimental and analytical studies on the differentiation potential of stem cells
- Utilized cell culture and molecular biology techniques to isolate nucleic acids; perform quantitative real-time PCR; tissue and cell immunofluorescence staining
- Performed statistical analysis on raw data to interpret biological significance of studies; relayed such findings via PowerPoint and poster presentations to peers

04/2013-09/2013 **Research Assistant,** Department of Animal and Poultry Science, University of Guelph

Supervisor: Dr. Kees de Lange
- Assisted with organizing and conducting an 8 week immunologic research trial
- Responsible for the health and maintenance of 160 Yorkshire pigs
- Obtained swine biological samples through ethical means
- Performed immunizations; skin-fold testing; dissections; dietary challenges
- Analyzed blood, microbiota, and tissue samples in-laboratory for statistical analysis

TEACHING AND MENTORING ACTIVITIES

2012-present **Teaching Assistant,** Department of Medical Sciences, The University of Western Ontario, ON
Department of Biology, University of Waterloo, ON
Course codes: MEDSCI 4100 (UWO) BIOL 309 (UW); BIOL 373L (UW); BIOL 1001B/1201B (UWO)

- Developed and presented lectures to students on animal model design and use in research
- Assisted with midterm, final exam design and testable content development
- Prepared and lectured tutorial sessions for third year molecular biology students
- Demonstrated rodent animal handling and laboratory techniques to students
- Marked and graded students work including midterms, essays and lab reports

2014-current **Let's Talk Science Educator**, Western University, London, ON

- Supervised grades 2-8 students engaging in science-related group activities during visits to rural community elementary and middle schools
- Presented scientific topics to young students in a fun and interactive manner through live demonstrations and PowerPoint to encourage interest in science

01/2013 **Science Student Help Team Member,** University of Waterloo, ON

- Provided guidance and support to students with challenges regarding academics, degree planning, or stress management

2006-2007 **Peer Tutor**, Cameron Heights Collegiate Institute, Kitchener, ON

- Assisted secondary school students with chemistry, biology, and math problems
- Developed homework study modules for students enrolled in science subjects

DEPARTMENTAL AND COMMUNITY ACTIVITIES

09/2015-current **Ontario Institute for Regenerative Medicine (OIRM) Graduate Education Committee**

- Student representative for Western University, directing the interests of graduate student training in stem cell and regenerative medicine in Ontario

08/2015-current **Western Stem Cell Research Group: Co-founder and co-director**

- Student representative for Western University, uniting 12 principal investigators across the London region in an effort to promote collaboration and excellence in stem cell research

2015-current **Pathology Research Day Committee Member**

- Assisted with organizing the annual Department of Pathology Research Day

2015-current **Stem Cell Talks**, Western University, London, ON

- Executive Member, Content Committee
- Developed content and presented topics in stem cell research to highschool students through laboratory demonstrations and PowerPoint to encourage interest in research

AWARDS AND HONOURS

04/2016 **The Cameron Wallace Graduate Student Award in Pathology**, UWO

04/2016 **Dr. M. Daria Haust Award for the Best Basic Science Presentation**, UWO

09/2015 **Dutkevich Travel Award,** UWO

08/2015 **Ontario Institute for Regenerative Medicine Travel Award,** OIRM

08/2015 **Stem Cell Network Travel Award,** SCN

2012/13 **University of Waterloo Science Dean's Honours List**

2007/08 **University of Waterloo County Entrance Scholarship**