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β-adrenergic receptor-dependent and -independent effects of propranolol in infantile hemangioma

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Graduate Program in Pathology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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β-adrenergic receptor-dependent and -independent effects of propranolol in infantile hemangioma

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by

Jina J.Y. Kum

Graduate Program in Pathology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
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London, Ontario, Canada

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Abstract

Infantile hemangioma (IH) is the most common tumour of infancy. The recommended treatment for IH is a non-selective β-adrenergic receptor antagonist, propranolol. Although propranolol is effective in regressing hemangiomas, the mechanism of its action is poorly understood. Moreover, some hemangiomas regrow following cessation of treatment. We have recently shown that IH arise from multi-potent stem cells. Whether IH stem cells are responsive to propranolol is unknown and is the focus of this study. Hemangioma-derived stem cells and vascular endothelial cells were exposed to propranolol and were assayed for cellular and molecular alterations. Our studies show that propranolol inhibits the growth of hemangioma stem cells but does not cause apoptosis. We further show that the mechanism may involve serotonin receptors in hemangioma stem cells. These findings are in contrast to endothelial cells, which exhibit apoptosis potentially through the action of propranolol on β-adrenergic receptors. This study reveals that propranolol’s therapeutic effect is β-adrenergic receptor-independent in hemangioma-derived stem cells.

Keywords:
Infantile hemangioma, propranolol, β-adrenergic receptors, 5-HT (serotonin) receptors, antagonists
Co-Authorship Statement

Manuscript: Mechanism of propranolol action in infantile hemangioma

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Jina J.Y. Kum Drafted the manuscript.

Zia A. Khan Supervisor; edited and finalized the manuscript.

Manuscript: Propranolol inhibits growth of hemangioma-initiating cells but does not induce apoptosis


Jina J.Y. Kum Designed and conducted experiments.

Zia A. Khan Supervisor; assisted in all aspects of the experiments; all experiments conducted under his grant approval.
Dedication

To my mom, who has taught me to always smile and not to forget to work hard to achieve my aspirations.
Acknowledgments

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<td>α-adrenergic receptors</td>
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<td>β-ADRs</td>
<td>β-adrenergic receptors</td>
</tr>
<tr>
<td>5-HTRs</td>
<td>5-hydroxytryptamine receptors</td>
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<tr>
<td>AC</td>
<td>Adenylate cyclase</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>Bcl2</td>
<td>B-cell CLL/lymphoma 2</td>
</tr>
<tr>
<td>bm-MPCs</td>
<td>Bone marrow mesenchymal progenitor cells</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>CCAAT/enhancer binding protein alpha</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>EBM2</td>
<td>Endothelial Basal Medium-2</td>
</tr>
<tr>
<td>ECs</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbant assay</td>
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<td>EPAC</td>
<td>Exchange protein activated by adenylate cyclase</td>
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<td>GDP</td>
<td>Guanosine diphosphate</td>
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<td>Glut1</td>
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<tr>
<td>GPCRs</td>
<td>G protein-coupled receptors</td>
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<tr>
<td>GTP</td>
<td>Guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>HDMECs</td>
<td>Human dermal microvascular endothelial cells</td>
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</table>
HemECs  Hemangioma-derived endothelial cells
HemPericytes  Hemangioma-derived pericytes
HemSCs  Hemangioma-derived stem cells
IGF1  Insulin-like growth factor 1
IGFR1  Insulin-like growth factor receptor 1
IH  Infantile hemangioma
IL  Interleukin
MAPK  Mitogen activated protein kinase
MelSCs  Melanocyte stem cells
MMPs  Matrix metalloproteinases
mTOR  Mammalian target of rapamycin
PI3 kinase  phosphatidylinositol-4,5-bisphosphate 3-kinase
PKA  Protein kinase A
PPARγ2  Peroxisome proliferator-activated receptor gamma 2
qRT-PCR  Quantitative reverse transcription polymerase chain reaction
siRNA  Silencing RNA (Small interfering-RNA)
VEGF  Vascular endothelial growth factor
VEGFR  Vascular endothelial growth factor receptor
Chapter 1

1 Introduction\textsuperscript{1,2}

1.1 Infantile hemangioma

Infantile hemangioma (IH) is the most common vascular tumour of infancy, affecting one of every 100 newborns\textsuperscript{1, 2}. For reasons unknown, IH affects more females than males, and is also more prevalent in premature and Caucasian babies\textsuperscript{3, 4}. IH is often noticed soon after birth where approximately 80\% of these lesions are found in the head and neck regions, but they can be located elsewhere in the body\textsuperscript{5-7}. It has been well-established that IH follows three developmental phases\textsuperscript{8, 9}. The first phase entails expansion of undifferentiated tumour cells\textsuperscript{10, 11}. This proliferating stage is completed by 8 months of age in most cases\textsuperscript{11}. In the following involuting phase, these tumour cells differentiate into atypical vascular endothelial cells (ECs)\textsuperscript{12, 13}. Uniquely, IH endothelium exhibits robust expression of glucose transporter-1 (Glut1)\textsuperscript{14, 15}. The involuting phase, like the proliferating phase, is a continuum of cellular and molecular changes with the end result being appearance of adipocytes and fibrofatty residuum\textsuperscript{8, 9, 13}. Most IHs resolve spontaneously and do not require treatment\textsuperscript{16}. However, therapeutic intervention is necessary in cases where the lesion grows in certain locations and to sizes that could result in life-threatening complications. An example of such a situation is the growth of IH in the airway to obstruct the respiratory system\textsuperscript{17}. 
1 Parts of this chapter have been published: Kum JJY, Khan ZA. Mechanisms of propranolol action in infantile hemangioma. Dermato-Endocrinology. 2014; 6:1, e979699, DOI: 10.4161/19381980.2014.979699. Dermato-Endocrinology applies the Creative Commons Attribution License (CC-BY Attribution 2.0) to works. Under this license, authors retain ownership of the copyright for their content. No permission is required from the publishers. 2 Parts of this chapter have been published: Kum JJY, Khan ZA. Propranolol inhibits growth of hemangioma-initiating cells but does not induce apoptosis. Pediatric Research. 2014;75:381-8. DOI:10.1038/pr.2013.231. Pediatric Research applies the Nature Publishing Group (NPG) author licence policy to works. Under the license, authors retain ownership of the copyright for their published contributions. No permission is required from the publisher of the journal.

1.2 Treatment options for hemangioma

Although many attempts have been made, the treatment guidelines for IH are not fully established due to differential effects of diverse therapeutic options, the differences in the location, stage, and size of the tumour, and the age of patients [16]. Typically, treatment is initiated as soon as possible to avoid unnecessary disfigurement as hemangioma growth is highly unpredictable. Hence, many treatments are introduced during the early proliferative phase of IH. Currently, laser treatments, β-blockers, surgery and corticosteroids are available for hemangioma patients [18-21]. Although many treatment options are available, the therapeutic mechanism of action of the pharmacological treatment options is poorly understood. Many studies suggest that treatments target the anti-angiogenic pathway to reduce the rapid growth of blood vessels during the early proliferative phase of hemangiomas.

Corticosteroids were once considered to be a standard treatment for hemangiomas. Unfortunately, high doses of systemic corticosteroid treatment
over extended periods of time have produced severe side effects including severe growth retardation, immunosuppression, and inhibition of wound healing [22, 23].

Propranolol is a synthetic non-selective β-adrenergic receptor (β-ADR) antagonist, commonly referred to as a β-blocker. It is a commonly used drug for cardiac complications, such as hypertension and myocardial infarctions. In 2008, propranolol was accidentally found to be an effective pharmacological alternative for hemangiomas in two infants [24]. These infants, while receiving corticosteroids for hemangioma, were also given propranolol to treat cardiac complications. Upon propranolol treatment, hemangiomas regressed rapidly in these patients. Since then, propranolol has been used world-wide as the first-line treatment option for hemangioma patients. Many studies have compared corticosteroids and propranolol use for hemangiomas, and it has been shown that propranolol is more effective with minimal side effects when compared to corticosteroid use [24, 25].

Despite remarkable efficacy of propranolol [26], there are some adverse effects, which include sleep disturbances, acrocyanosis, hypotension, and hypoglycemia [25, 27, 28]. There are also reports of IH regrowth following cessation of treatment in as many as 20% of the cases [29, 30]. Therefore, greater understanding of the potential mechanisms underlying the therapeutic effect is needed to develop better and safer treatment options. Many mechanisms have been proposed, though only tested in culture studies, to explain the therapeutic mechanism of propranolol in treating IH. Theories involving vasoconstriction
EC apoptosis via β-ADR signaling [32, 33] and caspase activation [34, 35], and inhibition of angiogenesis via the modulation of vascular growth factors [33, 36, 37] have been suggested.

1.3 β-adrenergic receptors (β-ADRs)

Propranolol has been known to bind to β-ADRs. β-ADRs are a family of G protein-coupled receptors (GPCRs) that mediate physiological responses to adrenaline and noradrenaline. It has been previously shown that the transmembrane region of β-ADRs play a critical role in determining the binding of selective agonists/antagonists [38]. To date, three subtypes of β-ADRs have been identified: β1-3 ADRs. There is limited information available on β1-3 ADR distribution at the cellular and tissue levels. Highest levels of β1-ADR are thought to be observed in the heart and brain [39]. β2-ADR shows a wide distribution pattern and β3-ADR is believed to be predominantly expressed in adipose tissue [40, 41]. In blood vessels, studies have utilized β-ADR antagonist binding to show sites in all cellular layers of vessels [42, 43]. Predominant ADR subtypes in vessels include β1- and β2- as confirmed by β-ADR subtype knockout studies [42]. Using immunohistochemistry, β1- and β2-ADR proteins have been localized to IH endothelium (co-localized to CD31-positive cells) as well as perivascular cells (co-localized to α-smooth muscle actin-positive cells) [44, 45]. In addition, β3-ADR has also been reported in all phases of IH [37]. Given that β-ADRs are present in normal vessels and IH vessels, the question
arises as to the role of β-ADRs in vessel function and the effect of β-ADR blockade in IH resolution.

1.3.1 β-ADR signaling events

β-ADRs associate with downstream signaling molecules upon activation (Figure 1). β1 and β3 generally couple with Gs (stimulatory) proteins, whereas β2 may couple with Gs or Gi (inhibitory). In the unstimulated state, the trimeric G protein is bound to GDP. Activation of ADRs promotes exchange of GDP for GTP. The G protein α subunit with bound GTP then dissociates from the β and γ subunits to activate adenylyl cyclase (AC) and increase intracellular cyclic adenosine monophosphate (cAMP) levels. Gi may counteract this increase by inhibiting AC. Intracellular cAMP activates cAMP-dependent protein kinase A (PKA) which may have multiple cellular consequences [46]. For example, PKA has been shown to be involved in elaboration of angiogenic factors through cAMP response element-binding protein (CREB) [47]. In addition to PKA-mediated signaling, activated AC may also activate mitogen-activated protein kinase (MAPK) pathway through exchange protein activated by adenylate cyclase (EPAC) [48, 49]. Dissociated Gβγ may also lead to activation of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3 kinase) and Akt/protein kinase B. Interestingly, it has been shown that Akt activation can ultimately increase vessel size and angiogenesis [50]. In addition, ADR signaling may entail a G protein independent signaling pathway. A well-characterized example is the β-arrestin-mediated activation of MAPK pathways [51, 52]. Involvement of these pathways
indicates that inhibiting β-ADR through propranolol in IH may, indeed, have beneficial effects by reducing cell survival as well as inhibiting angiogenesis.

Figure 1: Schematic illustrating β-ADR signaling.

Ligand binding to β-ADRs results in Gs-mediated activation of adenylate cyclase (AC) and conversion of ATP into cAMP. Intracellular cAMP activates PKA to phosphorylate target proteins. cAMP may also activate exchange protein activated by adenylate cyclase (EPAC) leading to mitogen-activated protein kinase signaling pathway and downstream effects on cellular processes. Another pathway activated by β-ADRs is the PI3 kinase and protein kinase B (Akt) pathway. In addition to G protein-mediated signaling, β-ADRs may also participate in G protein-independent signaling through β-arrestin and MAPK.

Figure reproduced from Kum JJY, Khan ZA. Mechanisms of propranolol action in infantile hemangioma. Dermato-Endocrinology. 2015; 6:1, e979699.
1.4 Hemangioma-derived endothelial cells (HemECs)

Exposure of endothelial cells (ECs) isolated from hemangioma specimens to propranolol has been shown to induce apoptosis [53]. This is evident upon exposure to 100 µM propranolol in the culture media [35, 53]. Previous literature has found significant increase in caspase-3 and -9 cleavage products, but not caspase-8 cleavage following propranolol exposure, suggestive of an intrinsic apoptotic pathway mediated by propranolol [53]. However, other studies have found an increase in protein and mRNA levels of caspase-8 [35], indicative of both intrinsic and extrinsic involvement of the apoptotic pathway. At the mRNA level, it was reported that propranolol induces expression of apoptotic genes, such as Bax, p53, caspase-8, and cytochrome c in hemangioma ECs that may be responsible for its apoptotic effect [35, 53]. These studies have essentially examined the effect of propranolol without the addition of β-ADR stimulation. This may suggest a constitutively active β-ADR pathway. A caveat worth noting here is that these ECs are considered to be hemangioma tumour-derived (hemECs). Because there is no specific marker or cellular activity of hemangioma tumour ECs, it is possible that these cultures may represent a heterogeneous population of tumoural and non-tumoural ECs (recruited or angiogenic cells). Interestingly, propranolol’s effect is not specific to hemECs, as it has been shown to cause apoptosis in a similar manner in other EC types as well including normal human dermal ECs [32, 54].

In addition to caspase-mediated apoptosis, propranolol may block phosphorylation of vascular endothelial growth factor receptor 2 (VEGFR-2) [55].
It was found that when hemECs were challenged with higher concentrations of propranolol (50 and 100 µM), the expression of vascular endothelial growth factor (VEGF) at the protein level was reduced in a dose-dependent manner [53, 56]. This reduction in the level of activated VEGFR-2 and VEGF protein upon propranolol exposure was a critical element that affected the survivability of these hemECs [55, 57]. In addition, decrease in key cyclin levels and an increase in cell cycle inhibitor levels were observed [55]. This suggested that cell cycle regulation is also another mechanism involved in mediating propranolol's therapeutic effect. HemECs show a greater proportion of cells in the G1 phase than the S/G2 phase when treated with propranolol [32, 55]. This was further confirmed with decreased expression of cyclin proteins such as cyclins A1, A2, B2, D1, D2, D3 [32, 55], while cell cycle inhibitor proteins p15, p21, p27 [55], were up-regulated.

Many studies have gone in-depth with analyzing the expression levels of the different β-ADR subtypes. It has been shown that hemECs and other EC types express both β1- and β2-ADRs at very similar levels, but not β3 [55, 58]. Despite the various β-ADRs expressed, it is believed that the main mechanism of action of propranolol in hemECs may involve β1 and/or β2-ADR pathway. A recent report has shown that ICI-118551 (selective β2-ADR antagonist) was more effective than metaprolol (selective β1-ADR antagonist) in inhibiting hemECs proliferation [59].
1.5 Hemangioma-derived pericytes (HemPericytes)

Pericytes are cells that control EC proliferation and survival by stabilizing the vascular wall and releasing pro-survival signals [60]. In addition, pericytes promote survival of ECs through vascular endothelial growth factor A (VEGF-A) signaling and Bcl-w [61]. It is known that addition of β-ADR agonists and cAMP analogues can induce relaxation of pericytes [62]. Recently, pericytes isolated from hemangiomas (hemPericytes) have also been tested for a potential role in propranolol-mediated vascular regression. When hemPericytes were exposed to propranolol, epinephrine-induced relaxation was prevented [63]. Furthermore, the proliferative capacity of hemPericytes was reduced. These pericytes also expressed β2-ADRs on their cell surface [63]. With knockdown of β2-ADR, hemPericytes lost epinephrine-induced relaxation and propranolol had no effect [63]. This suggested that β2-ADR is involved with relaxation and contractility of hemPericytes in response to propranolol [63]. In addition, when hemPericytes co-implanted with hemECs were exposed to propranolol, propranolol decreased the vascular volume indicative of increased vasoconstriction [63]. This may be suggestive of a possible mechanism by which propranolol causes increased constriction of the vasculature in IH to reduce the blood flow to the tumor, limiting its growth.
1.6 Hemangioma-derived stem cells (HemSCs)
We have shown that IHs are derived from multipotential stem cells termed hemangioma stem cells (hemSCs) [10]. Clonally expanded hemSCs differentiate into ECs and produce Glut1-positive microvessels in immunodeficient mice. Interestingly, hemECs are unable to produce microvessels in nude mice showing that hemangioma initiating cells are hemSCs. Binding of VEGF-A and vascular endothelial growth factor B (VEGF-B) to vascular endothelial growth factor receptor 1 (VEGFR-1) expressed on the surface of hemSCs has been shown to be required for the induction of hemSCs to EC differentiation, and for blood vessel formation [64]. Immunostaining of IH specimens shows co-labelling of EC markers and stem cell markers indicative of an immature EC phenotype in IH [13]. Therefore, these cells represent the true cellular target to understand the mechanism of propranolol action in hemangiomas.

1.7 Effect of propranolol in other neoplasms
The progression of various cancers has been associated with alteration of β-ADR signaling pathways. Hence, β-blockers have been proposed as therapeutic agents for various cancers. Pediatric melanoma is a rare disease but its incidence has increased in the young population. Melanoma is accountable for up to 3% of all pediatric malignancies [65]. Similar to IH, melanoma cases are more commonly diagnosed in Caucasian and female patients [66], and almost 20% of malignant melanoma occurs in the head and neck region [67]. Melanocyte stem cells (MelSCs) generate melanocytes that produce melanin-
pigment throughout adult life. The pathogenesis of melanoma is still subject to debate, but many have suggested mutations in MelSCs involving the cell cycle and apoptosis pathways, such as tumour protein p53 pathways, and stressors that increase catecholamines are involved in tumour progression [68, 69].

The increase in norepinephrine and epinephrine primarily modulates the β-ADR pathways through PKA and MAPK signaling mechanisms, ultimately affecting the growth and progression of melanoma [70]. Additionally, increase in the expression of VEGF, interleukin (IL) -6 and IL-8 after catecholamine stimulation correlates with the aggressiveness of the tumour [70, 71]. Melanoma cells express both β1- and β2-receptors with β1-ADR expression being weaker relative to β2-ADR [68, 70]. Recently, β3-ADRs have been proposed to be involved in melanoma growth and vascularization [72], and the use of β-blockers in malignant melanoma decreased the risk of progression [73]. Although the exact mechanism underlying the effectiveness of these medications in reducing tumour progression is unknown, it has been suggested that β-blockers reduce angiogenic factors and metastatic progression [73]. β-blockers may inhibit angiogenesis by reducing VEGF activity via MAPK signaling. In addition, β-blockers modulate matrix metalloproteinases (MMPs) that can alter the tumour microenvironment involved with angiogenesis [74]. Recently, specific inhibition of β3-ADRs in melanoma cells was found to impair cell growth and induce apoptosis [72].

β-ADR has also been implicated in breast cancer. Breast cancer cells express both β1- and β2-ADRs [75, 76], and the polymorphisms of β-ADR subtypes may
be associated with breast cancer susceptibility [77]. Breast cancer patients who received propranolol for hypertension displayed reduced metastasis and cancer recurrence [78]. This may be due β-ADR involvement in gene expression within the primary tumour [79]. Further investigation of β-ADR signaling provided evidence that the β-ADR pathway controls the stimulation of the arachidonic acid cascade [80]. In breast cancer development, arachidonic acid is a critical molecule that has been shown to activate mTOR (mammalian target of rapamycin) and increase the activity of VEGF [81]. mTOR and VEGF seem to be a common pathway in breast cancer and in hemECs, involving enhanced angiogenesis.
1.8 Rationale

A number of studies have investigated the effect of propranolol on IH-derived endothelial cells to offer insight into the mechanisms of therapeutic effect of propranolol [53, 55, 58]. These studies show that propranolol causes apoptosis in IH endothelial cells by activating caspase-3 and also blocks other cellular activities including migration and tubule formation [53, 55]. This effect of propranolol is also exhibited by normal endothelial cells [32, 55]. We have shown that IH arises from multipotent stem cells [10]. Utilizing stem cell antigen CD133, we isolated stem cells from hemangiomas and showed that these cells produce hemangioma lesions in mice. Interestingly, IH-derived endothelial cells (exhibiting mature endothelial phenotype as assessed by endothelial cell markers) fail to produce Glut1-positive microvessels [10]. This suggests that the cell of interest, at least in proliferating phase IH when treatments are required, is the hemSCs.

It has been observed that all hemangiomas do not respond the same way to propranolol, as up to 20% of cases of hemangiomas regrow upon cessation of propranolol treatment [82]. This puzzling finding has been attributed to early treatment withdrawal and/or long proliferating phase of IH. Since IHs regrow in a significant proportion of patients that discontinue propranolol treatment [29], it is possible that hemSCs, unlike hemECs and normal vascular ECs, are not susceptible to propranolol-induced apoptosis.
1.9 **Hypothesis**

We hypothesize that propranolol does not cause apoptosis in hemangioma stem cells. If true, this may explain why recurrence of these tumours often occurs following cessation of treatment.
Chapter 2

2 Materials and Methods

2.1 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 slides with mouse anti-human CD31 (1:50; M0823, Dako Canada, Mississauga, ON) and rabbit anti-human CD133 antibody (1:100; ab19898, Abcam, Cambridge, MA) 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).
2.2 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 [83]. 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. Neonatal human dermal microvascular endothelial cells (HDMECs; CC-2516, Lonza Inc.) were also used as controls. All cells were cultured on fibronectin-coated (FN; 1 µg/cm², FC010-10, Millipore, Temecula, CA) plates in complete EBM2 media (Lonza) 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 (different IH cell preparations) and 3 technical replicates.

2.2.1 Cell growth assay

To determine the effect of propranolol on cellular growth and survival, we plated each cell type at 5000 cell/cm² in complete EBM2 growth media (described above). After 24 hours, media was removed and cells were exposed to the following treatments: (RS)-1-[(1-Methylethyl)amino]-3-(1-naphthalenyl)oxy)-2-propanol hydrochloride (25, 50, or 100 µM Propranolol; 0624, R&D Systems, Minneapolis, MN); CGP 20712 dihydrochloride (10 nM CGP; 1024, Tocris Bioscience); ICI 118,551 hydrochloride (10 nM ICI; 0821, Tocris Bioscience);
nadolol (25, 50, or 100 µM; 253175, Santa Cruz); 5-carboxamidotryptamine maleate (200 nM or 1 µM 5-CT; 0458, Tocris Bioscience, Bristol, UK); SDZ 21009 (200 nM or 1 µM SDZ; 1516, Tocris Bioscience); (S)-(6-Methoxyquinolin-4-yl)((2R,4S,8R)-8-vinylquinuclidin-2-yl) methanol hydrochloride (1, 25, 50, or 100 µM Quinidine; 4108, Tocris Bioscience) in fresh EBM2 media (Table 1). Number of live cells was determined at 24 or 72 hours using Scepter 2.0 Automated Cell Counter (Millipore) with appropriate histogram gating setup [84, 85]. Data were normalized to each respective control groups as multiple experiments were performed in various plate sizes.
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2.2.2 Cellular transfection

To transfect the cells with silencing RNA, we coated 12-well plates with basal EBM2 media (Lonza) supplemented with 20% fetal bovine serum (Lonza), EGM-2 SingleQuots (CC-4176, Lonza Inc.) without GA-1000 (gentamicin sulfate; CC-4381a, Lonza) and 1X antibiotic antymycotic media (PSF; Life Technologies) (EBM2 antibiotic/antimycotic free media) for 30 minutes on the day of transfection. The cells were transfected with either control siRNA-A (sc-37007, Santa Cruz, Santa Cruz, CA), β1-adrenergic receptor siRNA (sc-29580, Santa Cruz), or β2-adrenergic receptor siRNA (sc-39866, Santa Cruz) at a concentration of 200 nM. Cells were transfected with siRNA using an electroporation device (Neon® Transfection System, MPK5000S, Life Technologies). Transfected cells were incubated for 24 hours in antibiotic/antimycotic-free EBM2 media. After 24 hours, complete EBM2 media was added. After 24 or 72 hours of culture, cells were collected for total number of live cells and for RNA isolation. β1- or β2-ADR knockdown was confirmed by qRT-PCR. Identical protocol was used to transfct both hemSCs and HDMECs.

2.3 RNA isolation, mRNA profiling & quantitative RT-PCR

RNA was isolated using Aurum Total RNA isolation kit (Bio-Rad) or RNeasy Micro Plus Kit (Qiagen, Mississauga, ON). 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). We performed gene expression analyses using RT² Human Cell Death
Pathway Finder PCR arrays (PAHS-212Z; Qiagen) (Table 2). Data was analyzed by CFX Manager Software using normalized ($\Delta\Delta C_T$) method with two housekeeping genes ($\beta$-actin and GAPDH were both used for normalization after empirically determining the expression for stability in our treatment groups).

Various receptor levels and downstream signaling gene expression was assessed by qRT-PCR (Table 2,3). Reactions consisted of 10 µL ssoFast Evagreen (1725200, Bio-Rad), 2 µL of both forward and reverse primers (at a 10 µM concentration), 2 µL cDNA, and 6 µL of H$_2$O. Target gene mRNA data was normalized to $\beta$-actin (QT01680476, Qiagen). 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 using normalized ($\Delta\Delta C_T$) method or relative quantity ($\Delta C_T$).

Adipogenesis-specific gene expression was also assessed by qRT-PCR (Table 2). Adipogenesis was assessed by C/EBPα (QT00203357, Qiagen) and PPARγ2 (sequence shown in [86]). Target gene mRNA data was normalized to $\beta$-actin (QT01680476, Qiagen). All reactions were performed for 40 cycles using the following temperature profiles: 95°C for 2 minutes (initial denaturation); and 55°C for 12 seconds (annealing and extension). Data was analyzed using relative quantity ($\Delta C_T$).
### Table 2: List of primers used

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<td>Actin, beta (β actin)</td>
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<td>Cyclin-D1 (CCND1)</td>
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<td>Qiagen (QT00203357)</td>
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**Plate**

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v-akt murine thymoma viral oncogene homolog 3 (AKT3)
B-cell CLL/lymphoma 2 (BCL2)
Cyclin-D1 (CCND1)
cAMP responsive element binding protein 1 (CREB1)
protein kinase, cAMP-dependent, catalytic, alpha (PRKACA)
protein kinase, cAMP-dependent, catalytic, beta (PRKACB)
protein kinase, cAMP-dependent, catalytic, gamma (PRKACG)

5-hydroxytryptamine (Serotonin) receptors
5-hydroxytryptamine (serotonin) receptor 1A (HTR1A)
5-hydroxytryptamine (serotonin) receptor 1B (HTR1B)
5-hydroxytryptamine (serotonin) receptor 1D (HTR1D)
5-hydroxytryptamine (serotonin) receptor 1E (HTR1E)
5-hydroxytryptamine (serotonin) receptor 1F (HTR1F)
5-hydroxytryptamine (serotonin) receptor 2A (HTR2A)
5-hydroxytryptamine (serotonin) receptor 2B (HTR2B)
5-hydroxytryptamine (serotonin) receptor 2C (HTR2C)
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2.4 **Protein quantification**

Total proteins from the cultured cells were extracted using Cell Extraction Buffer (Life Technologies) with complete protease inhibitor cocktail (Roche Diagnostics, Laval, Quebec). Proteins were measured by BCA Protein Assay Reagent (Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, IL) and equal amounts were used for various protein measurements.

2.4.1 **Caspase-3 and Cyclin-D1 measurements**

To measure activated caspase-3, caspase-3 (active) Human ELISA kit (Life Technologies) was used. Data were collected using Thermo Scientific Multiskan FC Microplate Photometer (Thermo Scientific), measuring absorbance at 450nm. Cyclin-D1 level was measured similarly using PathScan Total Cyclin-D1 Sandwich ELISA kit (Cell Signaling Technology Inc., Danvers, MA).

2.4.2 **Protein Kinase A Activity measurement**

To quantify Protein kinase A (PKA) activity, PKA Kinase Activity kit (Abcam) was used. Data were collected using Thermo Scientific Multiskan FC Microplate Photometer (Thermo Scientific), measuring absorbance at 450nm.
2.5 Adipogenic Differentiation

To induce adipogenic differentiation, hemSCs were seeded at a density of 40,000 cells/cm\(^2\) in StemPro Adipogenesis differentiation media (Adipo media; Life Technologies). Control media consisted of Dulbecco’s Modified Eagle Medium supplemented with 10% FBS. Media was changed every other day. RNA was isolated from cells after 7 days to perform qRT-PCR for β-adrenergic receptor expression. To determine whether cell growth/proliferation may alter adipogenesis, we pretreated hemSCs with 10 μg/mL mitomycin C (MitoC; Sigma Aldrich, Oakville, ON) for 2 hours. Cells were then washed, resuspended, and plated at 40,000 cells/cm\(^2\) in adipogenesis media. RNA was isolated at day 4 to assay for C/EBPα and PPARγ2 levels (transcription factors essential for adipogenic differentiation).

2.6 Statistical Analysis

The data were expressed as means ± SEM. Where appropriate, student’s unpaired t-tests or analysis of variance (ANOVA) were performed. P values < 0.05 were considered statistically significant.
Chapter 3

3 Results

3.1 Atypical phenotype of hemangioma endothelium

Previous studies have investigated the effect of propranolol on IH-derived endothelial cells to offer insight into its mechanism of therapeutic effect [53, 55, 58]. However, we have shown that CD133-selected cells from human IH initiate hemangioma lesions in mice, producing Glut1-positive microvessels [10]. This suggests that the mechanism of therapeutic effect of propranolol needs to be investigated on hemSCs. Therefore, we performed immunostaining for CD133 to probe for microvessels that are lined by CD133-expressing cells (hemangioma vessels). Results from two proliferating IH specimens show that all microvessels within IH tissues are immunoreactive to CD133 (Figure 2). This suggested that for understanding the effect of propranolol, CD133-selected hemSCs are essential.
Figure 2: Hemangioma vessels are lined with CD133-positive cells.

Proliferating hemangioma specimens were characterized through immunostaining. Hemangioma specimens were double labeled for CD31 (endothelial cell marker; red) and CD133 (stem cell antigen; green). 4′,6-Diamidino-2-phenylindole (blue) was used as counterstain. Staining illustrates complete co-localization of CD31 and CD133 in both proliferating hemangioma specimens (images were taken at magnification of ×20; insets illustrate high magnification; bar = 200 μm). Human skin and placenta were used as negative and positive controls respectively for CD133 (Data not shown).
3.2 Propranolol inhibits hemSCs growth

Our next objective was to determine the effect of propranolol on the growth of hemSCs. We cultured the cells with 25, 50, or 100 µM propranolol and assayed for live cell number at both 24 and 72 hours. We chose these concentrations based on previous studies that have observed significant differences in IH endothelial cells [32, 55]. Furthermore, propranolol has been shown to have immediate effects in inhibiting cell viability at 24 hours [53]. Interestingly, no significant changes to cell number were observed in hemSCs at 24 hours (Figure 3A). At 72 hours, bm-MPCs and HDMECs showed significant reductions in cell number at all concentrations of propranolol when compared to control (Figure 3B). In contrast, a proliferative effect was observed when hemSCs were treated with 25 µM propranolol and a reduction in live cell number with 100 µM propranolol (Figure 3B).
Figure 3: 100 μM propranolol reduces hemSCs growth after 72 hours.

Live cell number after (A) 24 hours and (B) 72 hours of treatment with different concentrations of propranolol. Propranolol treatment at 100 μM reduced number of cells compared with control after 72 hours. (*P < 0.05 compared with respective control).
3.3 Reduced cell number following propranolol treatment is not due to apoptosis in hemSCs

As the cell number decreased significantly for all cell types when cultured in 100 µM propranolol (at 72 hours), we examined whether this was due to apoptosis. Therefore, cells treated with propranolol were assayed for active caspase-3. Caspase-3 is the most frequently activated death protease and has been shown to play a role in inducing endothelial cell apoptosis upon propranolol treatment [53, 55]. Unexpectedly, hemSCs treated with 100 µM propranolol showed a significant reduction in the level of active caspase-3. This suggested that in hemSCs, the reduction in cell number might be due to inhibited cell growth and not apoptosis. bm-MPCs did not show a significant difference upon propranolol treatment as caspase-3 levels remained unchanged (Figure 4A). On the other side of the spectrum, propranolol-treated HDMECs showed a significant increase in the level of activated caspase-3 as expected. These data demonstrate that propranolol treatment induces apoptosis in HDMECs, whereas the decrease in cell number in hemSCs and possibly bm-MPCs is mediated by a reduction in cell growth.
3.4 Propranolol halts cell cycle progression but does not induce apoptosis in hemSCs

We examined the effect of propranolol on cyclin-D1 level. Cyclin-D1 is a key regulator in the progression from G1/S phase and has recently been shown to be maintained in G2 phase [87]. Recent studies have shown that propranolol reduces cyclin-D1 in a time-dependent manner in endothelial cells [32]. Surprised by previous experimental findings, we wanted to determine if propranolol reduces cyclin-D1 in hemSCs. Our results do show significantly reduced cyclin-D1 in hemSCs upon propranolol treatment (Figure 4B). Similarly, bm-MPCs and HDMECs cyclin-D1 levels were also significantly reduced (Figure 4B). The greatest change in cyclin-D1 was seen in bm-MPCs which may explain the reduced cell number seen earlier. These results suggest that propranolol inhibits cell cycle progression in all cell types by decreasing level of cyclin-D1.
Figure 4: Propranolol inhibits caspase-3-mediated apoptosis in hemSCs.

Levels of (A) active caspase-3 and (B) cyclin-D1 in hemSCs, bm-MPCs, and HDMECs following 100 μM propranolol treatment for 72 hours by ELISA. (*P < 0.05 compared with respective control).
3.5 Propranolol induces anti-apoptotic pathways in hemSCs

In order to understand the possible mechanism by which propranolol induces apoptosis in endothelial cells but not hemSCs, we used quantitative RT-PCR to profile for genes important in the central mechanisms of cellular death. We used a Human Cell Death Pathway Finder PCR Array (Qiagen; see Methods for details) which comprises 84 key genes important for cell survival and apoptosis. HemSCs, bm-MPCs, and HDMECs were cultured in normal growth media or in media containing different concentrations of propranolol for 72 hours. Using this PCR-based array, we found that propranolol significantly induces various anti-apoptotic pathways in hemSCs and normal bm-MPCs (Figure 5). These included Akt (also known as protein kinase B; induced 31.2x in hemSCs, 7.58x bm-MPC), Bcl2 (295.9 x in hemSCs, 143.2x bm-MPC)/Bcl2A (23.4x in hemSCs, 10.9x in bm-MPC), and insulin-like growth factor receptor-1 (IGFR1, 53.6x in hemSCs, 9.2x in bm-MPC). In contrast, we did not observe any alteration of these anti-apoptotic pathways in HDMECs (Figure 5).
Figure 5: Propranolol induces anti-apoptotic signaling pathways in hemSCs.

Propranolol induced the expression of anti-apoptotic genes in hemSCs and bm-MPCs but not in HDMECs. (mRNA levels were measured using RT² Cell Death Pathway Finder (Qiagen, Mississauga, ON) and normalized to β-actin and glyceraldehyde 3-phosphate dehydrogenase levels; red dashed lines highlight 100 μM propranolol groups; graph shown are representative of multiple PCR arrays).
3.6 β2 and β3 are the predominant β-ADRs in hemSCs

We next assayed for β-ADR expression in hemSCs and compared the levels to mature endothelial cells. Our results show that hemSCs express both β2- and β3-ADRs (Figure 6). β1-ADR mRNA levels, although detectable, were significantly lower. Interestingly, we found that bm-MPCs share β-ADR profile with hemSCs. Mature endothelial cells (human microvascular endothelial cells; HDMECs) on the other hand exhibited higher level of β1-ADR expression (Figure 6). No significant differences were found in the level of β2- or β3-ADRs between endothelial cells, hemSCs, and bm-MPCs.
Figure 6: β2/3-ADRs are predominantly expressed in hemSCs.

qRT-PCR analysis of β-ADRs in hemSCs, bm-MPCs, and HDMECs were determined by real-time quantitative reverse-transcriptase PCR. (Data normalized to β-actin; *P < 0.05 compared with β1-ADR mRNA levels in hemSCs and bm-MPCs; †P < 0.05 compared with β2- and β3-ADR mRNA levels). RNA was isolated from cells cultured in Endothelial Basal Media-2 (EBM2) under identical conditions. The specificity of the amplification was determined by melting curve analysis (Appendix A).
3.7 β-ADR downstream signaling molecules are detectable, but too low to quantify in hemSCs

To ensure the effects we observed upon propranolol exposure were through β-ADR inhibition, we investigated the change in key downstream signaling molecules upon propranolol exposure. PKA is an important mediator for cellular processes where its function depends on cAMP. Although we have observed a linear standard curve for active PKA standards in our study (Figure 7, Table 4), PKA activity upon 25 and 100 µM propranolol was below the detection limit in both hemSCs and HDMECs. Even with the addition of 20 ng of exogenous active PKA in hemSC and HDMEC protein samples, the amount of active PKA remained below the quantitative limit (Table 5).

![Linear standard curve for active PKA standards](image)
Table 4: PKA activity in purified PKA standards

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<td>18.70</td>
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Table 5: PKA activity in hemSCs and HDMECs

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</table>
3.8 Specific β-ADR antagonist does not affect hemSCs

As β1-ADR was predominantly expressed in the well-differentiated endothelial cells, we questioned whether specific β1-ADR antagonists can mimic propranolol. We cultured the cells with 10 nM CGP (β1-selective blocker) and/or ICI (β2-selective blocker) and assayed for live cell number after 72 hours. If propranolol is acting through β1-receptor that is predominantly expressed in endothelial cells, then we would expect decreased growth in this particular cell type with CGP. Indeed, our results show that 10 nM of CGP, but not ICI, significantly decrease cell number when compared with control (Figure 8). When CGP and ICI are combined to represent propranolol's non-selective binding characteristic, HDMEC number is also significantly reduced (Figure 8). In contrast, no significant changes to live cell number were observed in hemSCs with all treatments using specific β-ADR antagonists (Figure 8). This suggests that β-ADR signaling pathway may not be involved in hemSCs.
Figure 8: Specific β1- and β2-ADR antagonist does not affect growth of hemSCs.

Live cell number after 72 hours of treatment with selective β-ADR antagonists. β1- and β2-selective blockers did not affect the cell number compared to control in hemSCs. (*P < 0.05 compared to respective control).
3.9 Specific β-ADR silencing does not affect hemSCs growth

To rule out propranolol’s β-ADR-dependent mechanism of action in hemSCs, we transfected hemSCs with specific β-ADR silencing RNA (siRNA) in complete growth media with or without 100 µM propranolol. Similar to specific β-ADR antagonist use, the growth of hemSCs was not affected upon knockdown of β1- and β2-ADR (Figure 9A). However, control and β1 siRNA-transfected hemSCs exposed to propranolol significantly reduced cell growth. This was not seen with β2 siRNA-transfected hemSCs cultured in propranolol media (Figure 9A).

In contrast, β-ADR knockdown significantly decreased HDMECs cellular growth (Figure 9A). Specific β1 siRNA-transfected HDMECs cultured with propranolol significantly reduced cell number compared to β1 knockdown in complete growth media (Figure 9A). Interestingly, β1-ADR knockdown HDMECs cultured with propranolol significantly increased growth when compared to control transfection with propranolol (Figure 9A). Our findings indicate live cell number is affected in HDMECs with β-ADR knockdown, but not hemSCs.

Transfection efficiency was confirmed by qRT-PCR. We were able to achieve greater than 80% suppression of β2-ADR in hemSCs and HDMECs (Figure 9B,C), and greater than 70% suppression of β1-ADR in HDMECs (Figure 9C).
**Figure 9: Specific β1- and β2-ADR siRNA does not affect growth of hemSCs.**

Transfecting hemSCs with β1- and β2-ADR siRNA (A) did not affect growth, unlike HDMECs. (*P < 0.05 as compared to respective control; †P < 0.05 as compared to respective β-siRNA; γP < 0.05 as compared to respective control siRNA with propranolol). (B,C) Knockdown efficiency was measured through β-ADR mRNA levels. β2-ADR knockdown efficiency was greater than 80% in both cell types. β1-ADR knockdown efficiency was greater than 70% in HDMECs. (*P < 0.05 as compared to respective control).
3.10 5-HTR7 levels are significantly high in hemSCs

To investigate β-ADR-independent mechanism of propranolol, we have profiled various ADRs and downstream signaling molecules. We also measured the expression of 5-HTRs to which propranolol has been shown to bind [88]. Interestingly, all of α- and β-ADR subtypes were significantly lower in hemSCs when compared to HDMECs (Figure 10A). As well, majority of downstream signaling molecules were lower in level in hemSCs, except Bcl2 (Figure 10B). Similarly, many 5-HTRs were significantly lower in hemSCs, except 5-HTR7 (Figure 11). This hinted to us that propranolol may mediate its effects in hemSCs through 5-HTR7.
Figure 10: Low expression of α-ADR, β-ADR, and downstream signaling molecules in hemSCs.

Gene profiling assay was performed to observe the mRNA levels of (A) α- and β-ADRs and (B) downstream signaling molecules. (*P < 0.05 compared to HDMECs).
Figure 11: 5-HTR7 is highly expressed in hemSCs.

Gene profiling assay was performed to observe the mRNA levels of 5-HTRs. (*P < 0.05 compared to HDMECs).
3.11 Serotonin agonist decreases hemSCs growth

Previous literature has reported that propranolol can interact with 5-HTRs (serotonin receptors) [88]. Therefore, we explored the possibility that the reduction in cellular growth upon propranolol exposure in hemSCs is mediated through 5-HTR pathway. Since it is not known whether binding of propranolol to 5-HTRs leads to activation or inhibition, we first tested hemSCs with a selective 5-HTR1/7 agonist, 5-CT, at various concentrations. Interestingly, both concentrations of 5-CT significantly reduced live cell number after 72 hours, similar to propranolol. If propranolol affected the hemSCs through 5-HTR pathway, then we would expect no significant difference when we combine propranolol with 5-HTR antagonist to eliminate propranolol’s effect. Indeed, when we combine SDZ with propranolol, the cell number is normalized to control levels (Figure 12A). Surprisingly, 5-CT at 200 nM had no significant effect on HDMECs, unlike hemSCs (Figure 12B). This suggests that the two cell types may exhibit different cellular signaling pathways upon propranolol exposure.
Figure 12: 5-CT mimics the effect of propanolol in hemSCs growth.

Live cell number after 72 hours of treatment with (A) 5-CT (5-HTR agonist) and combination of propanolol with SDZ (5-HTR antagonist) in hemSCs and (B) 5-CT in hemSCs and HDMECs. (*P < 0.05 compared to respective control).
3.12 Propranolol-induced altered differentiation in hemSCs

While our studies were underway, a report showed that presence of propranolol in adipogenic differentiation media increased the differentiation level in hemSCs as compared to cells in adipogenic media alone [34]. Higher levels of C/EBPβ and δ were found at day 4. Interestingly, when the cells were maintained in the differentiation media supplemented with propranolol for 7 days, significant cell death was observed. This is in contrast to our observations in normal growth media where a significant reduction in cell number is evident without apoptosis. Therefore, we explored the possibility that this enhanced adipogenic differentiation with propranolol is mediated through cell growth regulation. To test this idea, we treated hemSCs with mitomycin C to inhibit proliferation and tested for C/EBP expression. Our results show that C/EBPα was significantly higher when mitomycin C-treated hemSCs were exposed to adipogenic differentiation media for 4 days (Figure 13A). No change was observed in PPARγ2 levels. C/EBPα is a critical transcription factor in adipogenesis and enhanced levels suggest that inhibition of cell proliferation increases the differentiation capacity of hemSCs and this may be the mechanism underlying propranolol’s effect. We then assayed for β-ADR expression and show here that adipogenesis is associated with significantly higher levels of all three ADRs (Figure 13B). Therefore, cell death in adipogenic media supplemented with propranolol [34] might be due to increased expression of β-ADR, which accompanies hemSCs differentiation.
Figure 13: Growth inhibition enhances adipogenesis.

Effect of mitomycin C treatment on adipogenic differentiation (A) in hemSCs at day 4. (*P < 0.05 compared with control media; †P < 0.05 compared with adipogenic differentiation media without mitomycin C treatment). (B) Induction of β-ADRs following adipogenic differentiation in hemSCs at day 7. (*P < 0.05 compared with control media).
3.13 β-ADR levels change with propranolol exposure

Changes in β-ADR expression upon hemSC differentiation prompted us to assess the changes in receptor profile in the presence of propranolol. Therefore, we measured the level of β-ADR mRNA upon 100 µM propranolol exposure after 72 hours. In contrast to what we had expected based on Figure 13 (increase in β-ADR to increase susceptibility of hemSCs for cell death/cell cycle inhibition), hemSCs significantly decreased β2-receptor level after propranolol exposure (Figure 14A). Furthermore, only β1-ADR level significantly increased upon propranolol challenge in HDMECs, whereas β2-ADR level did not significantly differ (Figure 14B). Our findings indicate that the apoptosis of HDMECs with propranolol media may be due to the increase in β1-ADR (Figure 14B). In addition, our observations indicate that propranolol changes the receptor profile of both cells differently, which may explain why a difference in response is observed.
Figure 14: Propranolol alters level of β-ADRs.

Effect of propranolol exposure on β-ADR mRNA levels (A) in hemSCs and (B) HDMECs after 72 hours. (*P < 0.05 compared to respective control).
3.14 Nadolol inhibits hemSCs growth
There have been indications that propranolol can cause central nervous system-related side effects due to its lipophilic properties [89]. Therefore, we utilized nadolol, a non-selective β-ADR antagonist that is hydrophilic. It has also been suggested that due to its inability to cross blood brain barrier, use of nadolol may be a safer treatment alternative. We cultured the cells with 25, 50, or 100 μM nadolol and measured cell number at 72 hours. Interestingly, 25 and 100 μM nadolol significantly reduced hemSC number when compared to control (Figure 15). In HDMECs, only at 100 μM was there a significant decrease in cell number, suggesting that nadolol may have its effect in our cell types through an alternative pathway, different from propranolol’s mechanism.
Figure 15: Nadolol decreases growth of hemSCs.

Live cell number after 72 hours of treatment of hemSCs and HDMECs with different concentrations of nadolol. Nadolol at 25 μM reduced hemSC number, while 100 μM reduced both hemSCs and HDMECs compared to respective controls. (*P < 0.05 compared to respective control).
3.15 Quinidine decreases hemSCs growth
Propranolol has been observed to have membrane stabilizing effects [90]. Hence, we wanted to examine whether this property mediates the effect of propranolol. To investigate whether propranolol’s effect in hemSCs is through stabilizing the membrane, we exposed hemSCs to 1, 25, 50, or 100 μM quinidine, a membrane stabilizing agent. Surprisingly, at the lowest concentration of quinidine we used, 1 μM, there was a significant increase in hemSCs growth; however, at high concentration of 100 μM, significant reduction of hemSCs growth was observed (Figure 16). Surprisingly, HDMECs were unaffected across the various concentrations of quinidine. Our findings indicate that propranolol may mediate its effects in hemSCs through stabilizing the membrane.
Figure 16: Quinidine affects hemSCs growth.

Live cell number after 72 hours of treatment with different concentrations of quinidine. Quinidine treatment at 100 μM reduced number of hemSCs compared to control. (*P < 0.05 compared to respective control).
Chapter 4

4 Discussion & Future direction

4.1 Discussion

Propranolol is a widely used treatment for IH; however, the mechanism of therapeutic effect is still unknown. In addition, some IH regrow after stopping propranolol treatment [29, 30, 91]. In the present study, we have demonstrated that proliferating hemangioma specimens are atypical in nature as IH microvessels express endothelial (CD31) and stem cell (CD133) markers (Figure 2). We have also shown that propranolol does not induce apoptosis in CD133-expressing hemSCs, as seen in mature/differentiated endothelial cells (Figure 4A). This suggests that the direct effect of propranolol in IHs may be through modulating mature endothelial cells and angiogenesis. The mechanism by which hemSCs and possibly normal progenitor cells (modeled here by bm-MPCs) escape apoptosis may include induction of anti-apoptotic pathways, a novel mechanism we have observed (Figure 5). We found Akt, Bcl2/Bcl2A, and IGFR1 to be significantly induced in hemSCs and bm-MPCs. Akt induction is of particular importance here as this pro-survival kinase counteracts caspase-3 activity [92-94]. Bcl2 downregulation has been shown to increase caspase-3 in breast cancer cells [95]. Furthermore, Bcl2A mediates anti-apoptotic effects of fibroblast growth factor in chondrogenic progenitor-like cell line [96]. Therefore, these pathways may be involved in reducing/counteracting caspase-3 activity in hemSCs that is not evident in mature endothelial cells.
Another interesting finding of this study is that hemSCs predominantly express β2- and β3-ADRs (Figure 6). β1-ADR levels are almost ten-fold lower. Vascular endothelial cells, on the other hand, express significantly high level of β1-receptor compared to β2/3. These findings suggest that the differential effect of propranolol in hemSCs and endothelial cells may be due to distinct roles of β-ADR subtypes. Studies have shown that β1- and β2-ADR have opposing effects on regulating apoptosis [97-99]. For example, Communal et al. have shown that activation of β1-receptors on cardiac myocytes induces apoptosis, whereas β2-ADR activation opposes cell death [97]. Although this study involved activation of β-ADR and not antagonism, the concept of a distinct, receptor subtype-specific role is pertinent here. Moreover, Panjala et al. have demonstrated that β1-ADR knockout mice exhibit increased formation of degenerate capillaries in retina [100]. These are interesting findings because retinal endothelial cells express β1-ADR but not β2 [101]. Also associated with acellular capillaries in the retina in knockout mice were increased level of cleaved caspase-3. Based on our data, we suggest that antagonizing β1-receptor in IH endothelial cells is associated with cell death while β2 may be involved in cell cycle regulation (Figure 17). We know that hemSCs predominantly express β2- and β3-receptors and show almost 10-fold lower β1-level (Figure 6). bm-MPCs showed a similar response to propranolol in cellular activity and molecular alterations, and share the β-ADR profile with hemSCs (Figure 5,6).
Figure 17: Proposed mechanism of therapeutic effect of propranolol.

Schematic illustrating the proposed mechanism of propranolol action on hemSCs and mature endothelial cells. (A) Diagram illustrating the penetrance of apoptotic effect of propranolol in hemangioma regression. (B) Propranolol leads to apoptosis in endothelial cells and other mature cell types, including adipocytes, through alteration of β1-ADR signaling. Engagement of β2- (and possibly β3-) ADRs in ECs leads to cell cycle arrest and growth inhibition (potentially through decreasing cyclin-D1). Propranolol may also activate of 5-HTRs in hemSCs. ( denotes unclear mechanism through 5-HTR pathway).

Figure modified from Kum JJY, Khan ZA. Propranolol inhibits growth of hemangioma-initiating cells but does not induce apoptosis. Pediatric Research. 75:381-8.
A recent report by Ji et al. have shown that ICI-118551 (a selective β2-ADR antagonist) was more effective than metaprolol (selective β1-antagonist) in inhibiting hemECs proliferation [59]. However, our data suggests the opposite. β1-antagonist decreases cell growth, whereas β2-antagonist had no effect in HDMECs (Figure 8). When β1 and β2 specific antagonists were given simultaneously, it had less of an impact on the growth compared with β1-antagonist alone (Figure 8). As mentioned earlier, a similar phenomenon has been reported in cardiac myocytes where β1-receptors induce apoptosis and β2-ADR activation opposes cell death [102]. Our findings indicate that the key mediator in promoting apoptosis in mature endothelial cells is β1-ADRs.

To ensure the inhibition of β-ADR with propranolol in our model system, we measured for the downstream signaling molecule, PKA. Previous studies have shown that pre-treatment with β-agonist followed by β-antagonist can decrease cAMP levels in a dose-dependent manner [58]. However, our study suggests that without exogenously activating the β-ADR system, the cells do not exhibit sufficient β-ADR downstream signaling (Figure 8). We transfected our cells with β-ADR siRNA to determine whether propranolol mediates its effects directly through modulating β-ADR pathway. Interestingly, knocking down β-ADRs in HDMECs significantly reduced cell growth that was not evident in hemSCs (Figure 9A). These findings indicate that propranolol inhibits β-ADRs in HDMECs. Another unique finding is that there was an increase in cell number with β1-siRNA transfection under propranolol exposure when compared with control siRNA with propranolol (Figure 9A). When β1-ADR is knocked down in HDMECs,
there may not be sufficient levels of β1-ADR for propranolol to inhibit, relieving the apoptotic effect observed in control transfection with propranolol. This highlights β-ADR-dependent mechanism of propranolol in HDMECs. In contrast, hemSCs are not affected upon transfection with β-ADR siRNA alone (Figure 9A). Our findings indicate that propranolol may mediate its effect through a β-ADR-independent pathway in hemSCs.

To investigate β-ADR-independent mechanism of propranolol in hemSCs, we have performed a receptor profiling assay. All α- and β-ADRs and majority of 5-HTRs in hemSCs were significantly lower compared to HDMECs (Figure 10A, Figure 11). However, 5-HTR7 expression was almost 40 times more abundant (Figure 11). This was a unique finding in our study as it suggests that propranolol may be working through this receptor pathway. Previous studies have suggested that propranolol may bind to 5-HTRs with substantial affinity [102, 103]. There is also experimental evidence that propranolol acts as a 5-HT1A antagonist and a 5-HT1B agonist in the rat cortex [104]. Treatment with propranolol also inhibited basal cAMP and steroidogenesis in rat leydig cells, with effects evident at 0.1 μM [103]. Based on the data from previous studies and our receptor profile data, we examined whether activating or inhibiting 5-HTRs would mimic propranolol’s effect. We found that 5-HTR 1/7 agonist, 5-CT, can significantly reduce hemSCs growth (Figure 12A,B). Since 5-CT mimicked propranolol, we tested hemSCs growth in the presence of propranolol and 5-HT antagonist, SDZ. If propranolol’s mechanism of action is through activating 5-HTRs, exposing the cells with SDZ simultaneously will normalize the effect of
propranolol. Indeed, this is what we have observed. With the same concentration of 5-CT used for SDZ and simultaneously exposing hemSCs to propranolol, we were able to bring hemSCs growth to control levels (Figure 12A). Therefore, 5-HT pathway may in part be involved with propranolol’s effect in hemSCs.

Wong et al. have shown that propranolol enhances adipogenesis in hemSCs [34]. Specifically, presence of propranolol in adipogenic media initially caused differentiation of hemSCs but significant cell death at day 7 [34], which is the typical in vitro time for full functional adipocyte differentiation. We reasoned that the initial effect of propranolol might be mediated through cell cycle disruption. To test this possibility, we treated hemSCs with mitomycin-C before exposing the cells the adipogenic differentiation media. We noted that mitomycin-C treated cells had significantly higher levels of C/EBPα induction (Figure 13A), suggesting that enhanced adipogenesis in hemSCs may be related to a change in the differentiation timeline (reduced growth and earlier differentiation). We also tested whether adipogenesis itself alters β-ADR expression in hemSCs, thereby making cells more sensitive to propranolol’s direct effect through β-ADR. Indeed, differentiation of hemSCs significantly increased the expression of all β-ADR subtypes including β1 (29.47-fold increase as reported in our study) (Figure 13B).

IH ends its continuous developmental phase when adipocytes replace majority of the tumour lesion. Yu et al. first reported presence of cells with adipogenic differentiation potential in proliferating phase IH [105]. Culturing hemSCs in the presence of propranolol enhances adipogenesis and this may offer another
possible mechanism of the beneficial effects of propranolol in IH resolution. Furthermore, continuous culture of hemSCs in adipogenic differentiation media supplemented with propranolol causes cell death [34]. These results suggested that propranolol treatment accelerated the dysregulated differentiation process in hemSCs that ultimately resulted in increased apoptosis of adipocytes derived from hemSCs [34]. It is possible that differentiation of hemSCs causes a shift in β-ADR expression profile and an increase in β1-ADR which may induce apoptosis.

It has been noted that propranolol causes more central nervous system-related side effects than hydrophilic β-blockers as it can cross the blood-brain barrier [89]. Therefore, we wanted to determine whether nadolol, a hydrophilic non-selective β-blocker, can have similar effects as propranolol in hemSCs. Nadolol may be used as a safer alternative as it does not cross the blood-brain barrier and have little myocardial effect, unlike propranolol [106, 107]. Interestingly, hemSCs growth was significantly reduced at 25 and 100 µM nadolol. A significant reduction of HDMECs was also evident but only at 100 µM nadolol treatment (Figure 15). Clinically, it has been shown that nadolol effects are more favorable compared to propranolol-use after 24 weeks with similar doses administered [108]. These findings suggest that other β-blockers can offer another therapeutic option; however, further studies need to be performed to understand the mechanism of how these various β-blockers may be regressing IH.

Previous studies have observed that propranolol can act as a membrane stabilizing agent [90, 109]. To determine whether propranolol’s effect was
mediated through receptors expressed on the cell surface or indirectly by stabilizing the cell membrane, we have treated the cells with quinidine, a membrane stabilizing agent that can block sodium and potassium channels. Interestingly, it has been noted that 100 µM quinidine can inhibit proliferation and induce apoptosis by increasing caspase-3 and -9 in human glioma U86-MG cells [110]. Surprisingly, at 1 µM quinidine, there was a significant increase in live hemSCs number and only at 100 µM quinidine did it significantly decrease hemSCs growth compared with control (Figure 16). This profile is identical to the one seen with propranolol at 25 and 100 µM after 72 hours (Figure 3B). Surprisingly, HDMECs were unaffected at all concentrations of quinidine used when compared with control (Figure 16). Our data suggest that the cellular components of hemSCs and HDMECs are quite different, and it would be important to investigate whether the decrease in hemSCs upon 100 µM quinidine is due to apoptosis and/or cell cycle arrest. Furthermore, our findings highlight the importance of understanding the cellular receptor components to target hemSCs. Our study demonstrates that although hemSCs are responsive to propranolol by inhibiting cellular growth and inducing anti-apoptotic genes, the mechanism by which propranolol mediates its effects in hemSCs are through β-ADR-independent effects.
4.1.1 Concluding remarks

Propranolol has shown promising effects in IH resolution and many studies have sought to understand the mechanism of propranolol’s effective treatment. Although the mechanism of action is not fully understood, our study represents an important step towards understanding propranolol's action. Our findings provide novel insight into the possibility that propranolol may affect hemangioma-initiating cells (hemSCs) through a β-adrenergic receptor-independent pathway, potentially involving 5-HTRs and membrane stabilization. Identification of cytoplasmic regulatory proteins in hemSCs that interact with β-ADR and 5-HTRs may represent an attractive future research area for the development of cell-type specific therapies.
4.2 Future Direction

Although propranolol is efficacious, there is quite a bit of knowledge gap. There are possibilities that need to be explored: 1) involvement of receptor dimerization and α-ADR signaling, 2) involvement of 5-HT signaling, and 3) role of membrane stabilizing effects.

Although data is limited, propranolol does stereoselectively bind and inhibit α-ADR in the heart [111]. Immunoprecipitation studies also show that β1-ADR and α2-ADR heterodimerize when co-expressed [112]. This interaction changes the pharmacological properties of β1-ADR as shown by ligand binding assays. β1- and β2- ADR have also been shown to heterodimerize [113, 114]. These findings suggest that the profile of ADR receptors may have functional cellular consequence and represents an area of significant future research interest. In addition, our data suggest that β1-ADR is the key receptor that mediates apoptosis in HDMECs, and since hemSCs β1-ADR level is hardly detectable, apoptosis is not evident in this cell type. Therefore, it would be interesting to investigate whether overexpression of β1-ADR in hemSCs would induce apoptosis upon propranolol exposure.

Moreover, a number of pressing questions remain. For example, is propranolol selective to IH vessels? Based on recent findings in endothelial cells (normal vs IH-derived) and our own findings in hemSCs, this seems unlikely. Alternatively, the effectiveness of propranolol in IHs may be a function of increased levels that are sustained in the capillary mass of IHs.
Lastly, are there better alternatives to propranolol? Further studies need to be conducted to determine whether activation of 5-HTRs can induce apoptosis and/or reduce anti-apoptotic genes. This would allow for alternative treatments to target 5-HTRs rather than β-ADRs as it is highly expressed in hemSCs. Also, based on our data, nadolol has similar growth effect in hemangioma-derived stem cells to propranolol. Therefore, further studies are needed to determine whether similar anti-apoptotic gene profile is induced, and whether it can induce apoptosis in hemSCs to prevent recurrence of hemangiomas. Moreover, it would be interesting to observe the receptor profile after nadolol exposure and compare to the changes of propranolol’s receptor profile.

These studies are underway in our laboratory and the findings may enhance our understanding of the mechanism of therapeutic action of propranolol and may provide more effective treatment options.
Chapter 5

5 References


Appendices

Appendix A: Representation of β1-ADR melting curve analysis in hemSCs and HDMECs.
Curriculum Vitae

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Revised: April 2015

EDUCATION

2013- M.Sc  Western University, London ON
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Thesis title: Propranolol inhibits growth of hemangioma-
initiating cells but does not induce apoptosis
Thesis advisor: Zia A. Khan, PhD

HONORS and AWARDS

2015  Dr. Cameron Wallace Graduate Student Award
Western University, London ON

2014  Till & McCulloch Meeting 2014 Top Abstract Trainee Award
Till & McCulloch Meetings 2014, Ottawa ON

2014-2015  Ontario Graduate Scholarship (OGS)
Western University, London ON

2013-2015  Western Graduate Research Scholarship
Western University, London ON

2014  Dutkevich Memorial Foundation Travel Award
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2015  Pathology and Laboratory Medicine Research Day
Committee, Department of Pathology and Laboratory
Medicine, Western University, London ON

2014-2015  Chair, Western Pathology Association
Western University, London ON
**TEACHING and MENTORING ACTIVITIES**

2015
Graduate Teaching Assistant, Biology 1002B and 1202B
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